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Rare Variants in the Notch Signaling Pathway Describe a Novel Type of Autosomal Recessive Klippel–Feil Syndrome

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Abstract

Klippel–Feil syndrome is a rare disorder represented by a subgroup of segmentation defects of the vertebrae and characterized by fusion of the cervical vertebrae, low posterior hairline, and short neck with limited motion. Both autosomal dominant and recessive inheritance patterns were reported in families with Klippel–Feil. Mutated genes for both dominant (*GDF6* and *GDF3*) and recessive (*MEOX1*) forms of Klippel–Feil syndrome have been shown to be involved in somite development via transcription regulation and signaling pathways. Heterotaxy arises from defects in proteins that function in the development of left–right asymmetry of the developing embryo. We describe a consanguineous family with a male proband who presents with classical Klippel–Feil syndrome together with heterotaxy (situs inversus totalis). The present patient also had Sprengel's

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Conflicts of interest: J.R.L. has stock ownership in 23andMe and Lasergen, is a paid consultant for Regeneron and is a coinventor on multiple United States and European patents related to molecular diagnostics for inherited neuropathies, eye diseases, and bacterial genomic fingerprinting. The Baylor College of Medicine derives revenue from the chromosomal microarray analysis and clinical exome sequencing offered in the Baylor Miraca Genetics Laboratory (http://www.bmgl.com/BMGL/Default.aspx). R.A.G. has stock ownership in Lasergen. Other co-authors have no disclosures relevant to the article.

INTERNET RESOURCES

ExAC Browser: http://exac.broadinstitute.org/gene/ENSG00000203877

deformity, deformity of the sternum, and a solitary kidney. Using exome sequencing, we identified a homozygous frameshift mutation (c.299delT; p.L100fs) in *RIPPLY2*, a gene shown to play a crucial role in somitogenesis and participate in the Notch signaling pathway via negatively regulating *Tbx6*. Our data confirm *RIPPLY2* as a novel gene for autosomal recessive Klippel–Feil syndrome, and in addition—from a mechanistic standpoint—suggest the possibility that mutations in *RIPPLY2* could also lead to heterotaxy.

Keywords

Klippel–Feil syndrome; heterotaxy; *RIPPLY2*; notch signaling; *TBX6*; somitogenesis; right–left axis determination

INTRODUCTION

Klippel–Feil syndrome (KFS) is a specific type of segmentation defect of the vertebrae (SDV) and is clinically characterized by short neck, low posterior hairline, and limited neck movement resulting from fusion of the cervical spine [Tassabehji et al., 2008]. Normal development and segmentation of the vertebrae require successful completion of a highly organized, dynamic, and complex process signified by somitogenesis during early embryogenesis. Somites are paired metameric structures of paraxial mesoderm and are located at both sides of the neural tube occurring at regular spatial and temporal intervals, and their end formation gives rise to vertebrae and ribs, skeletal muscle, and dermis [Takahashi et al., 2013]. Mutations in *GDF6* (MIM#601147) and *GDF3* (MIM#606522) have been associated with autosomal dominant KFS [Tassabehji et al., 2008; Ye et al., 2010]. In several consanguineous families, homozygous truncating mutations have been identified in *MEOX1* (MIM#600147), which encodes a transcription factor with a well-established role in somite development [Bayrakli et al., 2013; Mohamed et al., 2013]. In addition, there are many other types of non-KFS cases of SDV that were also shown to be associated with variants in other genes including *DLL3*, *LFNG*, *MESP2*, *HES7*, and *TBX6* [McInerney-Leo et al., 2015, Wu et al., 2015]. While this study was in preparation, compound heterozygous mutations were identified in *RIPPLY2* in two siblings with SDV not restricted to the cervical spine [McInerney-Leo et al., 2015]. A convergence for these genes is that they are all components of the Notch signal transduction pathway; defects of which are associated with a variety of vertebral and rib abnormalities.

Situs inversus or heterotaxy is a clinically and genetically heterogeneous developmental condition characterized by randomization of the placement of visceral organs, including the heart, lungs, liver, spleen, and stomach. There are several clinical reports that describe patients with both KFS and situs inversus; however, the molecular etiology remains to be elucidated [Chacon-Camacho et al., 2012]. Here, we report a patient with autosomal recessive KFS and situs inversus totalis in which exome sequencing identified a homozygous frameshift mutation in *RIPPLY2*.

CLINICAL REPORT

The proband was a 13-year-and-9-months-old male with complaints of short stature, scoliosis, and an apparent webbed neck. Parents were first cousins and had two additional healthy boys. Antenatal follow up was not regular and delivery was at term, vaginal and uneventful. Head support was achieved at 4 months of age, while sitting without support and ambulation were noted at ages 1 year and 1.5 years, respectively. Height was 145 cm (third centile) and weight was 35 kg (3rd–10th centile). At the time of admission, he was a seventh grade student and school success was noted as moderate to good. Clinical evaluation showed that the patient also had a short neck with decreased motion capability, low posterior hairline, Sprengel's deformity, pectus excavatum (upper sternum), and pectus carinatum (lower sternum) (Fig. 1). Radiological evaluation detected fusion of the cervical vertebrae, scoliosis, dextrocardia, patent foramen ovale (PFO), and a solitary kidney (Fig. 1). Further radiological investigation by MRI and 3D computational tomography verified vertebral abnormalities and situs inversus totalis (Fig. 1).

MATERIALS AND METHODS

Informed consent that was approved by Institutional Review Board for Baylor College of Medicine was obtained from all participants prior to enrollment in this study. We applied exome sequencing (ES) to the proband at Baylor College of Medicine Human Genome Sequencing Center through the Baylor–Hopkins Center for Mendelian Genomics research initiative. During the analyses of candidate variants/mutations, we used external publicly available databases as described [Karaca et al., 2015]. All experiments and analyses were performed according to described methods [Bain-bridge et al., 2013]. Briefly, samples underwent exome capture using Human Genome Sequencing Center core design (52 Mb, Roche NimbleGen), followed by sequencing on the HiSeq platform, Illumina, Inc. (San Diego, CA) with an average depth-of-coverage of 115 × and 90.03% of bases covered at >20×. Sequence data were aligned and mapped to the human genome reference sequence (hg19) using the Mercury in-house bioinformatics pipeline. Variants were called by using tools as described [Karaca et al., 2015]. To confirm the mutation detected by exome sequencing and to perform segregation analysis, standard PCR was carried out as described [Pehlivan et al., 2012], by using the RIPPLY2F1: 5′-TGTTTGATCCAATTTACAGCTTG-3′ and RIPPLY2R1: 5′-TTGTACACTAAATTTAAACCCCACAA-3′ primer pair. Amplification products were separated on 0.7% agarose gels. The PCR products were purified using ExoSAP-IT (Affymetrix) and analyzed by standard Sanger dideoxynucleotide sequencing (DNA Sequencing Core Facility at Baylor College of Medicine, Houston, TX).

RESULTS

Exome capture followed by massively parallel next generation sequencing of the personal genome by ES of the proband identified an apparently homozygous c.299delT:p.L100fs, (RefSeq: NM_001009994; chr6:g. 84567019_CT>C [hg19]) frameshift substitution in the *RIPPLY2* gene located at 6q14.1. This variant has not been reported in the homozygous state in either our large-scale in-house-generated or public databases. There were only two loss of function (LOF) variants reported in the ExAC browser with very low frequencies (0.8×10^{-5}

and 1.2×10^{-4}, respectively) and neither of them were homozygous. Segregation studies showed that both parents and one available unaffected sibling had this variant in the heterozygous state, consistent with Mendelian expectations for recessive inheritance (Fig. 1A and B). B-allele frequencies calculated from ES data showed that there were 54 absence of heterozygosity (AOH) regions (≥0.5 Mb), covering 296,076,413 bases (~8.9% of the genome). The median length of AOH regions is ~1.409 Mb (range 0.508–61.156 Mb). The *RIPPLY2* gene was surrounded by one of these AOH regions (chr6:62390916-87722604) with a length of 25,331,688 bp. Given the observed clinical phenotype, we examined filtered ES variant call data for any potential causative variants in genes associated with KFS (*GDF6*, *GDF3*, and *MEOX1*), disorders of primary ciliary dyskinesia (http://omim.org/phenotypicSeries/PS244400), and heterotaxy diseases (*CFC1*, *ACVR2B*, *NODAL*, and *HITX1*). After first pass analysis, we also examined for variants in other SDV-associated genes (*MESP2*, *TBX6*, *HES7*, *DLL3*, and *LFNG*); however, we could not find any potentially damaging variant.

Human Ripply transcriptional repressor 2 (*RIPPLY2*) encodes a protein that is 128 amino acids in length. It was shown to play a role in somitogenesis [Chan et al., 2007] and in negative regulation of transcriptional activity of T-box transcription factors [Hitachi et al., 2009] (Fig. 2). g.Chr6:84567019 CT->C predicts a single base deletion in p.Leu100, which leads to a frameshift in the conserved segment of the RIPPLY protein domain, and, thus, a likely impairment of *RIPPLY2*'s function (Fig. 1B).

DISCUSSION

We identified a homozygous frameshift mutation (c.299delT:p.L100fs, RefSeq: NM_001009994; chr6:g.84567019_CT>C [hg19]) in *RIPPLY2* in a consanguineous family with a male patient presenting both KFS and situs inversus totalis. In the personal genome of the proband, *RIPPLY2* was included within a 25,331,688 bp (chr6:62390916-87722604) AOH region, the largest AOH segment in this patient.

The role of *Ripply2* in somitogenesis has been well-established in multiple animal models [Takahashi et al., 2013]. *Ripply2*$^{-/-}$ mice have severe vertebral and rib malformations and die perinatally [Chan et al., 2007]. Recently, two siblings with compound heterozygous mutations in *RIPPLY2* have been shown to present with failure of formation of the posterior elements of C1 to C4 with descent of the occipital bone, and vertebral defects including hemivertebrae and butterfly vertebra in both the cervical and the thoracic spine [McInerney-Leo et al., 2015]. Nevertheless, neither sibling showed neck vertebral fusion, which is characteristic of KFS. *Ripply2* negatively regulates *Tbx6* (and other T-box proteins), the end result of which converts T-box proteins from transcriptional activators to repressors [Hitachi et al., 2009]. This is achieved by simultaneous binding of the Ripply proteins to the DNA binding domain of T-box proteins and to Groucho/TLE transcriptional corepressor proteins, via their Ripply homology domain, and the tetrapeptide WRPW motif at the amino terminus, respectively. *Ripply2* is a direct transcriptional target of both *Mesp2* and *Tbx6*, suggesting its negative feedback functions in the interaction loop (Fig. 2) [Hitachi et al., 2009].

Although the heterotaxy phenotype could be due to another alteration that we were not able to detect because of technical issues, we speculate that *RIPPLY2* mutation described here may be responsible for both the KFS and heterotaxy phenotypes in the present patient. To the best of our knowledge, there are no functional data regarding a potential role for *RIPPLY2* in left–right axis determination. However, in a *Tbx6* null mouse model by Hadjantonakis et al. [2008], laterality defects were noted. Down regulation of Nodal and disruption of some components of the Notch and WNT signaling pathways in *Tbx6* mutant embryos were also noted, leading to the conclusion that *Tbx6* is upstream of the Notch signaling pathway in the perinodal region and has substantial effects on the morphology and motility of nodal cilia that result in a disruption of asymmetric Ca^{2+} signaling around the node [Hadjantonakis et al., 2008]. *Ripply2* is a negative regulator of *Tbx6*, and its Ripply homology domain mediates its binding to *Tbx6*, repressing *Tbx6* function via protein elimination. In the presented case, a single base deletion in p.Leu100, predicts a frameshift in this conserved segment of the Ripply homology domain. Moreover, in a recent study a Xenopus orthologue of human RIPPLY3—one of the three members of Ripply family—was shown to act in embryonal axis development [Li et al., 2013].

In conclusion, our clinical and genomic findings, together with animal model data and clinical reports in the literature, suggest that a homozygous frameshift mutation is responsible for a new type of autosomal recessive KFS. Additional reports of individuals with a similar phenotype and animal studies will be crucial to clarify the potential role of *RIPPLY2* in the etiology of heterotaxy.

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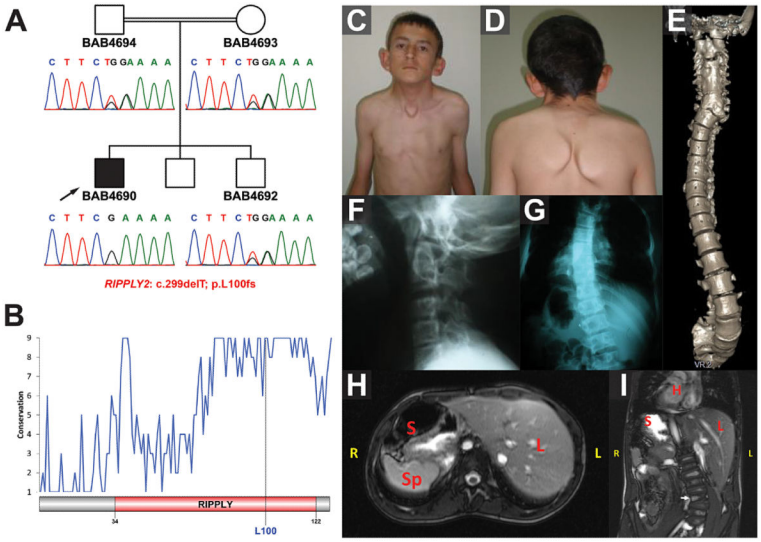

FIG. 1.

Pedigree of the family, segregation study, photographs of the proband, and radiologic images of the spine and body. A: Pedigree suggesting autosomal recessive inheritance. Sanger electropherograms of the sequencing for segregation analyses. Proband harbors chr6: g. 84567019_CT>C [hg19] variant at homozygous state, whereas unaffected individuals are heterozygous, which is consistent with Mendelian recessive expectations. B: Conservation profile of *RIPPLY2*. Conservation scores were computed using ConSurf [Ashkenazy et al., 2010] and protein domain profile was obtained from the Pfam database [Bateman et al., 2002]. C, D: Images of the proband show that he has a short webbed neck, pectus excavatum (upper sternum), and pectus carinatum (lower sternum). Posterior view shows that he has a low posterior hair line and Sprenger's deformity. E–G: Three-dimensional CT of the vertebrae and X-ray images of the neck and thorax. Fusion of the cervical vertebra and scoliosis is clearly demonstrated. Thorax X-ray also suggest situs inversus totalis. H, I: The MRI imaging of the body verifies situs inversus totalis in the patient. R (yellow), L (yellow), S (red), Sp (red), L (red) and H (red).

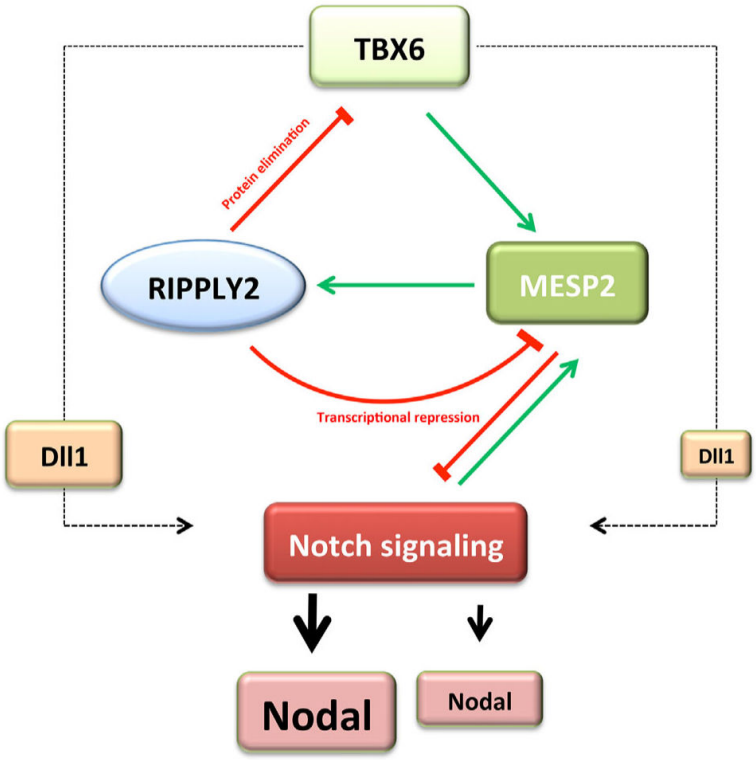

FIG. 2.

Characteristics of the interplay among *RIPPLY2*, *TBX6*, and *MESP2* and their relation in the notch signaling pathway and L/R axis determination. *RIPPLY2* is negatively regulating *TBX6* via transcriptional repression and protein elimination. *TBX6* is proposed to regulate asymmetric nodal expression through DII1 expression and notch signaling. This scheme was prepared based on the functional data provided by Hadjantonakis et al. [2008] and Takahashi et al. [2013]. Green arrows represent activation and red lines represent inhibition.