Published in final edited form as:

Hum Mutat. 2015 June ; 36(6): 631–637. doi:10.1002/humu.22786.

Heterozygous Deletion of *FOXA2* Segregates with Disease in a Family with Heterotaxy, Panhypopituitarism, and Biliary Atresia

Ellen A. Tsai^{1,*}, Christopher M. Grochowski², Alexandra M. Falsey², Ramakrishnan Rajagopalan², Danielle Wendel³, Marcella Devoto^{4,5,6,7}, Ian D. Krantz^{4,5}, Kathleen M. Loomes^{3,5}, and Nancy B. Spinner^{2,8}

¹Genomics and Computational Biology Graduate Group, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA.

²Department of Pathology and Laboratory Medicine, Children's Hospital of Philadelphia, Philadelphia, PA.

³Division of Pediatric Gastroenterology, Hepatology, and Nutrition, Children's Hospital of Philadelphia, Philadelphia, PA.

⁴Division of Human Genetics, Children's Hospital of Philadelphia, Philadelphia, PA.

⁵Department of Pediatrics, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA.

⁶Department of Biostatistics and Epidemiology, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA.

⁷Department of Molecular Medicine, University La Sapienza, Rome, Italy.

⁸Department of Pathology and Laboratory Medicine, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA.

Abstract

Biliary atresia (BA) is a pediatric cholangiopathy with unknown etiology occurring in isolated and syndromic forms. Laterality defects affecting the cardiovascular and gastrointestinal systems are the most common features present in syndromic BA. Most cases are sporadic, although reports of familial cases have led to the hypothesis of genetic susceptibility in some patients. We identified a child with BA, malrotation, and interrupted inferior vena cava whose father presented with situs inversus, polysplenia, panhypopituitarism, and mildly dysmorphic facial features. Chromosomal microarray analysis demonstrated a 277kb heterozygous deletion on chromosome 20 which included a single gene, *FOXA2*, in the proband and her father. This deletion was confirmed to be *de novo* in the father. The proband and her father share a common diagnosis of heterotaxy, but they also each presented with a variety of other issues. Further genetic screening revealed that the proband carried an additional protein-altering polymorphism (rs1904589; p.His165Arg) in the *NODAL* gene that is not present in the father, and this variant has been shown to decrease expression of the gene. As *FOXA2* can be a regulator of *NODAL* expression, we propose that

*Corresponding Author: Ellen A. Tsai, Children's Hospital of Philadelphia, 3615 Civic Center Blvd, Philadelphia, Pennsylvania, 19104, United States, eatsai@mail.med.upenn.edu.

haploinsufficiency for *FOXA2* combined with a decreased expression of *NODAL* is the likely cause for syndromic BA in this proband.

Keywords

copy number variation; liver disease; variable expressivity; 20p11

Introduction

Biliary atresia (BA) is an uncommon, congenital cholangiopathy that presents within the first few months of life and results in progressive ascending destruction and obliteration of the extrahepatic biliary tree. BA is the most common indication for liver transplantation in the pediatric population and results in lifelong morbidity. Prompt surgical intervention with the Kasai portoenterostomy can re-establish bile drainage in about 50% of patients, but the majority will have progressive biliary cirrhosis due to ongoing inflammation, fibrosis, and destruction of the intrahepatic bile ducts [Shneider et al., 2006]. BA is thought to be multifactorial in etiology, with a genetic predisposition and likely environmental trigger, such as a viral or toxic exposure, that occurs early in life and can lead to an ongoing inflammatory process affecting previously formed bile ducts. Neither the putative genetic component nor the environmental component is well understood.

The majority of patients with BA have an isolated form of this disorder, with no additional clinical findings, but 10–15% of patients have additional abnormalities and fall into two groups. Ten percent of BA patients have a laterality-related defect (mediopositioned liver, intestinal malrotation, congenital heart defects, polysplenia or asplenia, and positional abnormalities of the major blood vessels), and another 5–6% have an abnormality (most commonly in the cardiovascular, gastrointestinal or genitourinary systems) that is not caused by disrupted left-right asymmetry [Carmi et al., 1993; Davenport et al., 2006; Schwarz et al., 2013]. A number of genes have been found to be associated with laterality defects such as *CFC1*, *FOXH1*, *NODAL*, and *ZIC3*, but few variants have been found in patients who also have BA. Genes with mutations identified in patients with laterality defects and BA include *CFC1* and *ZIC3*, although these findings are rare. *CFC1* variants include missense mutations, p.Asn21His and p.Arg47Glu, which were reported in two brothers with laterality defects and BA [Bamford et al., 2000; Jacquemin et al., 2002]. *ZIC3* variants include p.Ser43Ter, a nonsense mutation identified in two siblings with congenital heart disease and cholestatic liver disease, although only one of the siblings had asplenia [Ware et al., 2004].

In this study, we characterized a family with recurrent abdominal features that are found in association with BA, including intestinal malrotation, situs inversus, and polysplenia. Although both the father and proband presented with heterotaxy, only the proband was diagnosed with BA. We found that deletion of *FOXA2* (MIM# 600288) segregated with the abdominal anomalies and an additional polymorphism in *NODAL* (MIM# 601265), a gene involved in endoderm formation and determination of laterality, was present in the proband with a diagnosis of syndromic BA.

Materials and Methods

Patient Enrollment

The proband, unaffected sibling, parents, and paternal grandparents were enrolled into our study through a protocol approved by the Institutional Review Board (IRB) of the Children's Hospital of Philadelphia (CHOP). DNA samples from the proband, mother, and father were extracted from peripheral blood, and from the paternal grandparents and unaffected sibling were extracted from saliva.

In addition to this family, DNA samples from a cohort of BA patients were available to us as part of our ongoing work on this disease. These patients were enrolled through the Childhood Liver Disease Research Network (ChILDRen) funded by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) and used to follow up on candidate genes identified in this family. DNA samples from 46 syndromic BA patients with abdominal heterotaxy and exome sequencing data from 100 isolated BA patients were available for these followup studies.

Sanger Sequencing and Droplet Digital PCR (ddPCR)

PCR was performed using TaqGold polymerase under standard conditions and sequenced at the Nucleic Acid/Protein Research Core Facility (NAPCore) at CHOP. Sequences were aligned to the reference genome (hg19) and annotated for variants using the Sequencher software program [<http://www.genecodes.com>]. Primers for the two coding exons of *FOXA2* (ENST00000419308) as well as the *NODAL* polymorphism, rs1904589, were created using the Primer3 design tool [Untergasser et al., 2012] and are detailed in Supp. Table S1.

We assayed for copy number variations (CNVs) in *FOXA2* by ddPCR using the Bio-Rad QX100 system (Hercules, CA). The predesigned primers used to detect copy number include Hs00867271_cn (Life Technologies, Carlsbad, CA) located within the second coding exon of *FOXA2* and a TaqMan primer set containing a reference gene; *TERT* (cat#4403326). Droplets containing 40ng of genomic DNA, TaqMan primers containing *FOXA2* and *TERT* were multiplexed and ran in a standard thermocycler (95°C for 10 min, 42 cycles of 95°C for 30 sec and 58°C for 60 sec, 95°C for 10 min). The individual FAM and VIC signal from each droplet was quantified and interpreted with the QuantaSoft software from the manufacturer to make a precise copy number call.

SNP Array Analysis

Chromosome microarray analysis was carried out using single nucleotide polymorphism (SNP) genotyping arrays and the data was analyzed for deletions and duplications. Genotyping of the family was performed on the Illumina SNP array (San Diego, CA) from DNA derived from peripheral blood. The proband and parental samples were genotyped on the OmniExpress SNP Array, and the sibling and paternal grandparents were genotyped on the Omni 2.5M SNP Array at the Center for Applied Genomics (CAG) at CHOP. CNVs in each subject were identified using PennCNV software [Wang et al., 2007].

Exome Sequencing

Exome sequencing was carried out as part of an NIDDK-funded ancillary study to the ChiLDReN consortium on 100 isolated BA patients using Agilent SureSelect V4+UTR All Exon Kit (Santa Clara, CA) at 100X mean coverage (2×100bp PE; Illumina HiSeq 2500). High confidence variant calls from exome data were produced following the guidelines outlined in Genome Analysis ToolKit (GATK) Best Practices Pipeline [Van der Auwera et al., 2002]. Raw sequence reads were aligned using BWA [Li and Durbin, 2010], and the initial alignment was refined using GATK IndelRealigner. Base quality scores were recalibrated using GATK BaseQualityScoreRecalibrator. Variants were called using GATK HaplotypeCaller algorithm. Initial variant calls were filtered using a number of quality metrics to produce high-quality variant calls. Functional consequences of the variants identified were annotated using SnpEff [Cingolani et al., 2012]. Minor allele frequency (MAF) information was gathered from public databases including 1000 Genomes Project (1000G) [Abecasis et al., 2012] and NHLBI Exome Sequencing Project (ESP) [NHLBI Exome Sequencing Project, <http://evs.gs.washington.edu/EVS/>]. CNVs were assessed using XHMM with default parameters [Fromer et al., 2012].

Results

The female proband presented at two months of age with jaundice noted by family members, and she was referred to the Emergency Department for an urgent evaluation. Laboratory workup revealed conjugated hyperbilirubinemia and elevated liver enzymes with high levels of gamma-glutamyl transferase (479 U/L; normal range 17–126 U/L). At the time of presentation, total bilirubin was 8.5 mg/dL (0.2–0.8 mg/dL) and conjugated bilirubin was 3.3 mg/dL (0–0.3 mg/dL), consistent with neonatal cholestasis. A workup including blood and urine cultures, chest radiograph, EBV, hepatitis A and hepatitis B serologies, HCV and CMV PCR testing was done and the test results were negative. Chest X-ray was negative for butterfly vertebrae, no heart murmur was noted, and she did not have facial features consistent with Alagille syndrome, making this diagnosis unlikely. Abdominal ultrasound was remarkable for non-visualization of the gallbladder, and biliary obstruction was confirmed by a hepatobiliary iminodiacetic acid (HIDA) scan that revealed no excretion of radiotracer. In light of a high suspicion for biliary atresia with late presentation at 76 days of age, further laboratory workup was not pursued, and the patient was brought to the operating room for exploration on hospital day 6. The gallbladder remnant was fibrotic and therefore could not be cannulated, so the surgeon proceeded with liver biopsy and Kasai portoenterostomy. Histological examination of the liver biopsy and resected biliary remnant were both consistent with the diagnosis of BA. Other findings in the operating room included interrupted inferior vena cava (IVC), midline liver, and malrotation, consistent with a diagnosis of abdominal heterotaxy. Further discussion with the family revealed that the father's medical history included *situs inversus*, polysplenia, and panhypopituitarism. In addition, he had mildly dysmorphic facial features such as a wide nasal bridge, smooth philtrum, and broad chin, but these features were absent in the proband (Fig. 1). The pedigree includes five previous miscarriages including one fetus with congenital heart disease, one unaffected sibling, and two unaffected maternal half-siblings (Fig. 2). The paternal grandparents were asymptomatic.

A clinically available heterotaxy gene panel consisting of four genes, *CFC1* (MIM# 115150), *FOXH1* (MIM# 603621), *NODAL* (MIM# 601265), and *ZIC3* (MIM# 300265), was ordered and performed by the Heart Diagnostic Lab at Cincinnati Children's Hospital for the proband. No pathogenic variants were identified, but a heterozygous c.494A>G alteration at rs1904589 was identified, and this polymorphic site lies in the coding sequencing of *NODAL*. This polymorphism is a non-synonymous protein alteration (p.His165Arg), and it has been shown to be a *cis*-acting expression quantitative trait locus (eQTL) known to decrease *NODAL* gene expression [Roessler et al., 2009]. This variant has an allele frequency of 53% in the European population as reported by the 1000 Genomes Project [Abecasis et al., 2012].

Chromosome microarray analysis of the proband revealed 18 gene-containing CNVs ranging from 27 to 909 kb in size. We analyzed these variants to look for potentially pathogenic CNVs, either *de novo*, dominant (a CNV shared by the proband and her father), or recessive deletions and/or duplications (Table 1). CNVs found in the proband that did not follow the inheritance patterns above are listed in Supp. Table S2.

Among the 18 gene-containing CNVs, the only alteration that has not been observed in normal individuals reported in the Database of Genomic Variants (DGV) [Iafrate et al., 2004] was a 277kb heterozygous deletion within 20p11.21 (chr20:22,496,147–22,773,103 in hg19). This CNV includes a full gene deletion of the *FOXA2* gene. Parental analysis showed that this CNV was inherited from the proband's father. Analysis of ddPCR results also confirmed the full-gene deletion of *FOXA2*. Further analysis of other members within this pedigree revealed that this deletion was not present in either paternal grandparent and thus arose as a *de novo* event in the father. The proband's unaffected sibling did not have this deletion CNV, so the deletion of *FOXA2* appears to segregate with clinical symptoms in this pedigree (Fig. 3). We also investigated the inheritance of the *NODAL* functional variant (p.His165Arg) identified in the proband. This polymorphism was determined to be maternally-inherited, and the father did not carry this variant.

To further investigate a potential role of *FOXA2* in BA, we studied an additional 46 patients with clinical presentations similar to the proband who did not have a genetic cause of their disease previously identified. We looked for sequence variants in *FOXA2* by Sanger sequencing of its two coding exons and for larger copy number changes of the gene by ddPCR. A rare SNP, rs200459003, was detected in a single patient in this cohort (Table 2), but no CNVs containing *FOXA2* were found in these patients. We also had access to exome sequencing data for an additional cohort of 100 isolated BA patients, and we analyzed this data to look for potentially pathogenic loss-of-function and missense variants in *FOXA2*. We were unable to confidently confirm another pathogenic variant from the additional BA cohort sequenced as none of these patients carried CNVs or protein truncating mutations. However, we identified four missense variants not present in public genomic databases or rare (MAF < 0.01) in six patients, including the same rs200459003 SNP identified in the syndromic patient (Table 2). We validated these variants by Sanger sequencing and analyzed parental samples to determine inheritance. The two missense variants that have never been observed were submitted to LOVD, a publicly accessible locus-specific database [<http://>

www.lovd.nl/foxa2]. All *FOXA2* variants were inherited (Table 2), so it is difficult to assign pathogenicity or confirm a role in BA susceptibility.

Of the seven patients with a *FOXA2* missense mutation, six were heterozygous and one was homozygous for the G allele of rs1904589 known to reduce expression of *NODAL*. Because of its high frequency, we could determine parental origin (paternal or maternal) of the *NODAL* variant for only three of the six heterozygous samples. In the other three cases, the *NODAL* polymorphism was present in both parents making determination of parental origin not feasible. However it is interesting to note that in the three patients where we could determine the parental origin of the *NODAL* polymorphism, this originated from a different parent than their *FOXA2* missense mutation (Table 2).

Discussion

We report a novel 277kb deletion of 20p11.21 that encompasses *FOXA2* in a father and proband with variable features associated with syndromic BA and suggest that haploinsufficiency of this gene is related to clinically diagnosed heterotaxy. Clinical features in the proband include BA, interrupted inferior vena cava, and abdominal heterotaxy. Clinical features in her father include abdominal heterotaxy, panhypopituitarism, and mildly dysmorphic facial features. The father's parents were unaffected, and the paternal deletion was shown to be *de novo*.

Proximal deletions of 20p11 are rare. Four patients with 20p deletions of varying sizes, that include *FOXA2* have been previously reported in the literature [Dayem-Quere et al., 2013; Garcia-Heras et al., 2005; Kamath et al., 2009; Williams et al., 2011], and their reported symptoms include intellectual disability, developmental delay, seizures, panhypopituitarism, and facial dysmorphism [Dayem-Quere et al., 2013]. Three of the four patients had panhypopituitarism while the remaining patient had an empty sella and presented with growth hormone deficiency. Although none of these patients presented with BA, it is possible that some features associated with heterotaxy, such as vascular anomalies, could go unrecognized in the absence of comprehensive imaging studies. Interestingly, panhypopituitarism is a recognized cause of neonatal cholestasis, but these infants typically present with giant cell hepatitis on liver biopsy rather than signs of biliary obstruction; biliary atresia has not been reported in this setting. We hypothesize that *FOXA2* is associated with laterality defects in both our proband and her father, but additional factors, such as the *NODAL* polymorphism reported in the proband, are likely to have contributed to the development of BA.

FOXA2, or *HNF3 β* , is a transcription factor that regulates critical developmental processes, including the development of the liver bud and other endodermal tissues. Forkhead transcription factors, of which *FOXA2* is a critical member, have been well-characterized in the initiation and progression of liver development [Lee et al., 2006]. While mice bearing null mutations in *Foxa1* die within the first post-natal weeks of life [Kaestner et al., 1999] and mice carrying null mutations in *Foxa3* are phenotypically healthy [Shen et al., 2001], the mice with *Foxa2* null mutations die during gastrulation due to node and notochord defects [Ang and Rossant, 1994; Weinstein et al., 1994]. In addition, it has been observed

that mice heterozygous for the *Foxa2* null mutation appear to have reduced fitness. Fewer heterozygous females could carry embryos to term and 10% of heterozygotes born died within a day [Weinstein et al., 1994].

Foxa2 can act as an upstream activator of *Nodal* at the node during development [Brennan et al., 2002], and it has also been predicted to be a downstream target of *Nodal* [Hoodless et al., 2001]. Like *Nodal*, *Foxa2* is upregulated on the left side of the embryo from murine experiments on left-right axis formation [Collignon et al., 1996], an important developmental step that can be responsible for heterotaxy when disrupted. Collignon *et al.* investigated the roles of the *HNF3 β* and the *Nodal* pathway in left-right axis determination in a mouse model. In the *HNF3 β* (*Foxa2*) null animals, *Nodal* expression was not detected, indicating that *Foxa2* acts upstream of *Nodal*. Interestingly, mouse embryos doubly heterozygous for mutations in *Foxa2* and *Nodal* showed bilateral *Nodal* expression and demonstrated marked situs defects in the positioning of the heart and the abdominal organs. These results indicate a direct genetic interaction between the *Foxa2* and *Nodal* pathways that can result in abdominal situs anomalies [Collignon et al., 1996].

The presence of a functional *NODAL* polymorphism in the proband and the absence of the same polymorphism in the father may partly explain the discordant phenotypes between two individuals with the same *FOXA2* deletion. While both the proband and the father present with heterotaxy, the proband presents with a more severe clinical phenotype, having also been diagnosed with BA. This may arise from a severely downregulated *Nodal* pathway, due to two expression altering changes—a CNV deleting one copy of *FOXA2* and a polymorphism decreasing the expression of *NODAL*. The additional finding of BA in the proband may arise from the decreased expression of *NODAL* caused by the maternally-inherited polymorphism in combination with the haploinsufficiency of *FOXA2* resulting from the paternally-inherited deletion.

Although the level of *FOXA2* and *NODAL* expressions are likely to result in the clinical presentation in the proband, the bi-directional regulation of *NODAL* and *FOXA2* confound the underlying biological mechanism [Brennan et al., 2002; Collignon et al., 1996; Hoodless et al., 2001]. While the father who carries a deletion of *FOXA2* may only exhibit 50% of the gene expression, the proband, who carries both the deletion of *FOXA2* and a polymorphism that decreases *NODAL* expression, may have less than 50% gene expression of *FOXA2*. As *FOXA2* is an embryonic lethal gene, the level of gene expression at or below 50% of the normal dosage may have drastic phenotypic effects. The polymorphism at rs1904589 is benign in healthy individuals, but it may have a large effect on the proband in combination with the *FOXA2* deletion. Although no copy number or protein truncating changes in *FOXA2* were detected in our additional screening of 46 syndromic and 100 nonsyndromic BA patients, we did identify seven BA patients with rare or previously unobserved missense *FOXA2* sequence changes who also carried the A>G change (rs1904589) in the *NODAL* gene.

We present a unique family in which a *de novo* deletion involving *FOXA2* appears to be associated with heterotaxy in the proband and her father. As failure to carry embryos to term and reduced viability of pups are characteristics of mice heterozygous for the *Foxa2* null

allele, the miscarriages observed in this family may also be associated with the haploinsufficiency of *FOXA2* [Weinstein et al., 1994]. Comparison of this family with the very few other patients who present with chromosome 20p deletions suggests that panhypopituitarism may be the most highly penetrant feature associated with *FOXA2* deletion, while BA may require additional factors such as reduced *NODAL* expression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank the Data Coordinating Center of the ChiLDReN consortium as well as the principal investigators and clinical research coordinators at the ChiLDReN Centers for patient recruitment and acquisition of samples and data. The Fred and Suzanne Biesecker Liver Center at CHOP, R01-DK090045 to N.B.S and U01-DK062481 to K.M.L supported this work. This work was funded in part by NIH/NCATS (National Center for Advancing Translational Sciences), Grant UL1TR000003. We also thank Laura K. Conlin for her insightful comments.

References

- Abecasis GR, Auton A, Brooks LD, DePristo MA, Durbin RM, Handsaker RE, Kang HM, Marth GT, McVean GA. An integrated map of genetic variation from 1,092 human genomes. *Nature*. 2012; 491(7422):56–65. [PubMed: 23128226]
- Ang SL, Rossant J. HNF-3 beta is essential for node and notochord formation in mouse development. *Cell*. 1994; 78(4):561–574. [PubMed: 8069909]
- Bamford RN, Roessler E, Burdine RD, Saplako lu U, dela Cruz J, Splitt M, Goodship JA, Towbin J, Bowers P, Ferrero GB, et al. Loss-of-function mutations in the EGF-CFC gene CFC1 are associated with human left-right laterality defects. *Nature genetics*. 2000; 26(3):365–369. [PubMed: 11062482]
- Brennan J, Norris DP, Robertson EJ. Nodal activity in the node governs left-right asymmetry. *Genes Dev*. 2002; 16(18):2339–2344. [PubMed: 12231623]
- Carmi R, Magee CA, Neill CA, Karrer FM. Extrahepatic biliary atresia and associated anomalies: etiologic heterogeneity suggested by distinctive patterns of associations. *American journal of medical genetics*. 1993; 45(6):683–693. [PubMed: 8456846]
- Cingolani P, Platts A, Wang LL, Coon M, Nguyen T, Wang L, Land SJ, Lu X, Ruden DM. A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff: SNPs in the genome of *Drosophila melanogaster* strain w1118; iso-2; iso-3. *Fly*. 2012; 6(2):80–92. [PubMed: 22728672]
- Collignon J, Varlet I, Robertson EJ. Relationship between asymmetric nodal expression and the direction of embryonic turning. *Nature*. 1996; 381(6578):155–158. [PubMed: 8610012]
- Davenport M, Tizzard SA, Underhill J, Mieli-Vergani G, Portmann B, Hadzi N. The biliary atresia splenic malformation syndrome: a 28-year single-center retrospective study. *The Journal of pediatrics*. 2006; 149(3):393–400. [PubMed: 16939755]
- Dayem-Quere M, Giuliano F, Wagner-Mahler K, Massol C, Crouzet-Ozenda L, Lambert J-C, Karmous-Benailly H. Delineation of a region responsible for panhypopituitarism in 20p11.2. *American journal of medical genetics. Part A*. 2013; 161A(7):1547–1554. [PubMed: 23657910]
- Fromer M, Moran JL, Chambert K, Banks E, Bergen SE, Ruderfer DM, Handsaker RE, McCarroll SA, O'Donovan MC, Owen MJ, et al. Discovery and statistical genotyping of copy-number variation from whole-exome sequencing depth. *American journal of human genetics*. 2012; 91(4):597–607. [PubMed: 23040492]
- Garcia-Heras J, Kilani RA, Martin RA, Lamp S. A deletion of proximal 20p inherited from a normal mosaic carrier mother in a newborn with panhypopituitarism and craniofacial dysmorphism. *Clinical dysmorphology*. 2005; 14(3):137–140. [PubMed: 15930903]

- Hoodless PA, Pye M, Chazaud C, Labbe E, Attisano L, Rossant J, Wrana JL. FoxH1 (Fast) functions to specify the anterior primitive streak in the mouse. *Genes Dev.* 2001; 15(10):1257–1271. [PubMed: 11358869]
- Iafraite AJ, Feuk L, Rivera MN, Listewnik ML, Donahoe PK, Qi Y, Scherer SW, Lee C. Detection of large-scale variation in the human genome. *Nature genetics.* 2004; 36(9):949–951. [PubMed: 15286789]
- Jacquemin E, Cresteil D, Raynaud N, Hadchouel M. CFCI gene mutation and biliary atresia with polysplenia syndrome. *Journal of pediatric gastroenterology and nutrition.* 2002; 34(3):326–327. [PubMed: 11964968]
- Kaestner KH, Katz J, Liu Y, Drucker DJ, Schütz G. Inactivation of the winged helix transcription factor HNF3alpha affects glucose homeostasis and islet glucagon gene expression in vivo. *Genes & development.* 1999; 13(4):495–504. [PubMed: 10049364]
- Kamath BM, Thiel BD, Gai X, Conlin LK, Munoz PS, Glessner J, Clark D, Warthen DM, Shaikh TH, Mihci E, et al. SNP array mapping of chromosome 20p deletions: genotypes, phenotypes, and copy number variation. *Human mutation.* 2009; 30(3):371–378. [PubMed: 19058200]
- Lee AM, Clear AJ, Calaminici M, Davies AJ, Jordan S, MacDougall F, Matthews J, Norton AJ, Gribben JG, Lister TA, et al. Number of CD4+ cells and location of forkhead box protein P3-positive cells in diagnostic follicular lymphoma tissue microarrays correlates with outcome. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology.* 2006; 24(31):5052–5059. [PubMed: 17033038]
- Li H, Durbin R. Fast and accurate long-read alignment with Burrows-Wheeler transform. *Bioinformatics (Oxford, England).* 2010; 26(5):589–595.
- Roessler E, Pei W, Ouspenskaia MV, Karkera JD, Veléz JI, Banerjee-Basu S, Gibney G, Lupo PJ, Mitchell LE, Towbin JA, et al. Cumulative ligand activity of NODAL mutations and modifiers are linked to human heart defects and holoprosencephaly. *Molecular genetics and metabolism.* 2009; 98(1–2):225–234. [PubMed: 19553149]
- Schwarz KB, Haber BH, Rosenthal P, Mack CL, Moore J, Bove K, Bezerra JA, Karpen SJ, Kerkar N, Shneider BL, et al. Extrahepatic anomalies in infants with biliary atresia: results of a large prospective North American multicenter study. *Hepatology (Baltimore, Md.).* 2013; 58(5):1724–1731.
- Shen MM. Nodal signaling: developmental roles and regulation. *Development.* 2007; 134(6):1023–1034. [PubMed: 17287255]
- Shen W, Searce LM, Brestelli JE, Sund NJ, Kaestner KH. Foxa3 (hepatocyte nuclear factor 3gamma) is required for the regulation of hepatic GLUT2 expression and the maintenance of glucose homeostasis during a prolonged fast. *The Journal of biological chemistry.* 2001; 276(46):42812–42817. [PubMed: 11546810]
- Shneider BL, Brown MB, Haber B, Whittington PF, Schwarz K, Squires R, Bezerra J, Shepherd R, Rosenthal P, Hoofnagle JH, et al. A multicenter study of the outcome of biliary atresia in the United States, 1997 to 2000. *The Journal of pediatrics.* 2006; 148(4):467–474. [PubMed: 16647406]
- Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, Rozen SG. Primer3—new capabilities and interfaces. *Nucleic Acids Research.* 2012; 40(15):e115. [PubMed: 22730293]
- Van der Auwera, GA.; Carneiro, MO.; Hartl, C.; Poplin, R.; del Angel, G.; Levy-Moonshine, A.; Jordan, T.; Shakir, K.; Roazen, D.; Thibault, J., et al. *Current Protocols in Bioinformatics.* John Wiley & Sons, Inc.; 2002. From FastQ Data to High-Confidence Variant Calls: The Genome Analysis Toolkit Best Practices Pipeline.
- Wang K, Li M, Hadley D, Liu R, Glessner J, Grant SFA, Hakonarson H, Bucan M. PennCNV: an integrated hidden Markov model designed for high-resolution copy number variation detection in whole-genome SNP genotyping data. *Genome research.* 2007; 17(11):1665–1674. [PubMed: 17921354]
- Ware SM, Peng J, Zhu L, Fernbach S, Colicos S, Casey B, Towbin J, Belmont JW. Identification and functional analysis of ZIC3 mutations in heterotaxy and related congenital heart defects. *American journal of human genetics.* 2004; 74(1):93–105. [PubMed: 14681828]

- Weinstein DC, Ruiz i Altaba A, Chen WS, Hoodless P, Prezioso VR, Jessell TM, Darnell JE. The winged-helix transcription factor HNF-3 β is required for notochord development in the mouse embryo. *Cell*. 1994; 78(4):575–588. [PubMed: 8069910]
- Williams PG, Wetherbee JJ, Rosenfeld JA, Hersh JH. 20p11 deletion in a female child with panhypopituitarism, cleft lip and palate, dysmorphic facial features, global developmental delay and seizure disorder. *American journal of medical genetics. Part A*. 2011; 155A(1):186–191. [PubMed: 21204230]

Figure 1.

Facial features of father and proband. The mildly dysmorphic facial features in the father is captured in his (a) portrait and (b) profile photographs. He has a wide nasal bridge, smooth philtrum, and broad chin. The (c) portrait and (d) profile of the proband show no evidence of facial dysmorphism.

Figure 2.

Pedigree of family with heterotaxy and BA. The affected family had multiple spontaneous abortions, which could be an indication of a genetic defect causing reduced fitness or incompatibility with life. DNA samples were not available for the family members outlined in light gray. The proband inherited a 277kb 20p11.21 deletion spanning *FOXA2* from the father and a functional polymorphism (c.494A>G, p.His165Arg) in *NODAL* from the mother. The proband is the only member of the family carrying both variants. In addition, the paternal grandparents and the unaffected sibling did not carry the *FOXA2* deletion.

Figure 3.

Copy number analysis of *FOXA2*. The deletion of *FOXA2* was found in the proband and father in this pedigree. Analysis of the unaffected full sibling shows normal copy number. The unaffected grandparents also appear to carry two copies of *FOXA2*, so this deletion was determined to be *de novo* in the father.

Table 1

Analysis of CNVs identified in the proband

Region	# SNPs	Length (bp)	Copy Number	Genes in Region
A. De Novo CNV				
chr19:45050747-45059310	13	8,564	1	<i>CEACAM22P</i>
chrX:153660041-153738092	31	78,052	3	<i>ATP6AP1, FAM3A, FAM50A, GDII, LAGE3, PLXNA3, SLC10A3, UBL4A</i>
B. Paternally Inherited CNV				
chr2:228243905-228258288	23	14,384	3	<i>TM4SF20</i>
chr3:60745635-60809914	83	64,280	1	<i>FHIT</i>
chr4:942439-999255	31	56,817	3	<i>DGKQ, IDUA, SLC26A1, TMEM175</i>
chr7:35095894-35158850	10	62,957	3	<i>DPY19L2P1</i>
chr13:21728134-21746637	18	18,504	3	<i>SKA3</i>
chr15:24349069-24496990	74	147,922	3	<i>PWRN2</i>
chr20:22496147-22773103	269	276,957	1	<i>FOXA2, LINC00261</i>
C. CNV Inherited From Both Parents				
chr10:134587640-134610186	13	22,547	4	<i>INPP5A, NKX6-2</i>
chr19:1199676-1259079	23	59,404	4	<i>ATP5D, C19orf26, MIDN, STK11</i>
chr22:46448476-46465626	11	17,151	4	<i>C22orf26, LOC150381</i>

Analysis consisted of an investigation of three sets of gene-containing CNVs found in the proband: (a) *de novo* CNVs, (b) CNVs inherited from the father, and (c) CNVs inherited on both alleles from both parents. The first set corresponds to the hypothesis that the BA finding is unrelated to the clinical features of heterotaxy and is a spontaneous alteration. The second set corresponds to the hypothesis that BA is an associated finding of heterotaxy but with reduced penetrance. The third set corresponds to the hypothesis that BA is caused by a combination of CNVs on both parental alleles. From this list, the most suspicious variant was a heterozygous deletion of *FOXA2*, the only gene-altering CNV that is not observed in normal individuals.

Table 2

Variants in FOXA2 identified in additional BA patients

Patient No.	Disease Classification	FOXA2						rs1904589	
		dbSNP ID	Genomic Position on chr20	cDNA change	Protein Change	MAF (1000G)	Variant Origin	Genotype	G allele Origin
1	Syndromic BA	rs200459003	22562990	c.530C>T	p.Ala291Val	0.0008	Maternal	A/G	Paternal
2	Isolated BA	rs201902165	22563686	c.176G>A	p.Ser59Asn	0.0060	Paternal	A/G	Maternal
3	Isolated BA	-	22562889	c.991A>T	p.Ter325Ser	-	Maternal	A/G	N/A
4	Isolated BA	-	22563564	c.298G>A	p.Ala100Thr	-	Maternal	A/G	N/A
5	Isolated BA	rs200459003	22562990	c.530C>T	p.Ala291Val	0.0008	Maternal	A/G	N/A
6	Isolated BA	rs200459003	22562990	c.530C>T	p.Ala291Val	0.0008	Paternal	G/G	Both
7	Isolated BA	rs200459003	22562990	c.530C>T	p.Ala291Val	0.0008	Maternal	A/G	Paternal

We identified 4 *FOXA2* rare missense coding variants in 7 patients. None of these variants were observed in ESP; 2 were observed in the 1000 Genome Project (1000G) with a MAF<0.01, and 2 were not observed in 1000G. One of these variants appeared 3 times in exome sequencing of 100 patients with isolated BA and once through Sanger sequencing of 46 patients with syndromic BA with abdominal heterotaxy. The expression-altering *NODAL* polymorphism, rs1904589, is also shown with its corresponding inheritance pattern for each patient identified to have a rare, protein-altering *FOXA2* variant.