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Combining fetal sonography with genetic and allele pathogenicity studies to secure a neonatal diagnosis of Bardet–Biedl syndrome

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Abstract

Bardet–Biedl syndrome (BBS) is a rare pediatric ciliopathy characterized by marked clinical variability and extensive genetic heterogeneity. Typical diagnosis of BBS is secured at a median of 9 years of age, and sometimes well into adolescence. Here, we report a patient in whom prenatal detection of increased nuchal fold, enlarged echogenic kidneys, and polydactyly prompted us to screen the most commonly mutated genes in BBS and the phenotypically and genetically overlapping ciliopathy, Meckel–Gruber syndrome (MKS). We identified the common Met390Arg mutation in *BBS1* in compound heterozygosity with a novel intronic variant of unknown significance (VUS). Testing of mRNA harvested from primary foreskin fibroblasts obtained shortly after birth revealed the VUS to induce a cryptic splice site, which in turn led to a premature termination and mRNA degradation. To our knowledge, this is the earliest diagnosis of BBS in the absence of other affected individuals in the family, and exemplifies how combining clinical assessment with genetic and timely assays of variant pathogenicity can inform clinical diagnosis and assist with patient management in the prenatal and neonatal setting.

Keywords

Bardet–Biedl syndrome; ciliopathy; prenatal; ultrasound; variant functional analysis

The ciliopathies are comprised of multiple clinical entities that are characterized by defects of the cilium and its anchoring structure, the basal body (1). Inheritance is primarily autosomal recessive, but these frequently oligogenic disorders are subject to extensive inter- and intra-familial variability. They are characterized by a constellation of syndromic features that include retinal degeneration, skeletal and limb defects, central and peripheral nervous system defects, cysts and fibrosis of the kidney, liver and pancreas, cardiac

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Conflict of interest

The authors declare no conflict of interest.

abnormalities and left-right asymmetry defects (2). To date, ciliary disorders have been attributed to genetic lesions in more than 60 genes encoding proteins necessary for ciliogenesis, and ciliary maintenance and function (2). Extensive phenotypic, genetic, and allelic heterogeneity in this disease group, as well as a dearth of phenotype-genotype correlations at most primary causal ciliopathy loci, contribute to the current challenges of achieving a timely and accurate clinical and molecular diagnosis in affected individuals.

Two rare ciliary disorders, Bardet-Biedl syndrome (BBS; MIM 209900) and Meckel-Gruber syndrome (MKS; MIM 24900) have emerged as clinically distinct but phenotypically overlapping disorders that can both display hallmark syndromic phenotypes prenatally. Minimal diagnostic criteria for MKS include hepatic fibrosis, occipital encephalocele and cystic kidneys. Postaxial polydactyly accompanies this triad in nearly all cases (3), and the structural anomalies are evident on prenatal ultrasound. MKS is typically lethal in the first few hours of neonatal life because of the severity of the neural tube defects (3), and pregnancy termination is a reasonable option for many women with affected pregnancies.

In contrast, the multi-systemic features of BBS are not fully manifest until childhood. While some of the developmental abnormalities such as polydactyly may be evident on a prenatal ultrasound scan, the degenerative and homeostatic defects of the disorder do not become evident until later in childhood at which point either four primary criteria (rod-cone dystrophy, limb defects, obesity, learning difficulties, and renal tract abnormalities) or three primary criteria plus two secondary criteria (including speech disorder, cataracts or astigmatism, ataxia, diabetes mellitus, dental crowding, congenital heart disease, or hepatic fibrosis) are sufficient for a clinical diagnosis of BBS (4). Although parents usually observe multiple BBS features by 2–3 years of age, the average age of diagnosis for BBS is 9 years of age (4), with some affected individuals not diagnosed until adolescence (5).

Genetic causality has been assigned to >80% of BBS cases for patients of European descent. Seventeen BBS loci have been described (*BBS1–BBS17* (6–23)), two of which have also been shown to cause MKS (14). First, null mutations in *MKS1/BBS13* are a frequent genetic cause of MKS (24), however, hypomorphic alleles at the same locus have also been shown to cause BBS (14). Second, null mutations in *CEP290/BBS14* have been described both in multiple MKS families and also in one consanguineous BBS family (14, 25). Moreover, heterozygous changes in additional MKS-causing genes including *RPGRIP1L*, *TMEM67/MKS3*, and *TMEM237* have been detected in BBS cases and some alleles may modulate specific endophenotypes (14, 26, 27).

Reciprocal genetic relationships between causal BBS genes and MKS have also been reported. Meckel-like neonatal lethal ciliopathy cases have been shown to harbor null mutations in *BBS2*, *BBS4*, *BBS6*, and *BBS10* (28, 29). Taken together, these observations suggest that BBS and MKS are allelic variants of the same disorder and demonstrate the challenges of achieving diagnosis when the phenotypic and genetic demarcation between the two disorders is not always discrete.

For BBS cases, reports of confirmed prenatal diagnosis by ultrasound are sparse. In one study, retrospective molecular analysis of Meckel-like fetuses with renal and limb abnormalities demonstrated that these fetuses harbored molecular lesions in BBS genes (28). In addition, in a BBS family with two children diagnosed with the disorder based on postnatal diagnostic criteria, second trimester targeted sonographic anatomy scanning was able to identify a third affected pregnancy based on the detection of polydactyly at 16 weeks (30). Finally, prenatal diagnosis of BBS and the genetically and phenotypically overlapping McKusick-Kaufman syndrome (caused by mutations in *BBS6* (12)) have been achieved

through ultrasound detection of both polydactyly and hydrometrocolpos (31); however, these combined hallmark phenotypes occur only in a modest proportion of BBS cases (~2% (32)). Together, these examples demonstrate that for the majority of BBS cases, improved approaches are required to decrease the time to secure a molecular diagnosis in the absence of a family history of BBS. As a first step toward achieving this goal, we report the combined use of ultrasound imaging and prenatal molecular diagnostics to obtain a suspected prenatal diagnosis of BBS in a family not known to harbor BBS mutations, and the use of variant functional studies to confirm this diagnosis shortly after birth.

Materials and methods

Mutational analysis

Fetal DNA was extracted from cells in an amniotic fluid sample collected as patient standard of care at 21 weeks 4 days gestation according to standard procedures. Coding exon sequences and ~50 bp of intron boundary sequence flanking exons of *BBS1*, *BBS10*, *BBS2*, and *MKS1* were sequenced in a Clinical Laboratory Improvement Amendment (CLIA)-certified setting (Prevention Genetics). Subsequent to informed consent, we obtained either peripheral blood (parents) or residual clinical fetal DNA samples (proband) from pedigree DM037 for research studies. Following DNA extraction (Gentra Puregene Blood kit, Qiagen, Valencia, CA), *BBS1* exons 12 and 13 coding regions and intron/exon boundaries were (re)sequenced by PCR-amplification and BigDye Terminator v3.1 chemistry on an ABI 3730 automated sequencer (Applied Biosystems, Foster City, CA). Sequences were aligned with Sequencher v4.10.1 (Gene Codes, Ann Arbor, MI), and variants were confirmed by visual assessment of chromatograms.

Skin fibroblast cell culture

We obtained proband foreskin sample subsequent to circumcision at 3 days of age. Skin fibroblast cell culture was established according to standard procedures at the Duke University Health System Molecular Diagnostics Laboratory. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) and 10% fetal bovine serum. For expression analyses, cells were cultured in the presence or absence of Emetine (100 µg/ml) for 8 h.

BBS1 expression studies

We extracted total RNA from skin fibroblast cell pellets using Trizol (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Total RNA (1 µg) was DNase treated and reverse transcribed using oligodT priming according to the QuantiTect Reverse Transcription kit instructions (Qiagen). The resulting cDNA was amplified with primers spanning *BBS1* exons 11 through 15. The single PCR product was purified from a 2% agarose gel fragment (QIAquick Gel Extraction kit, Qiagen), cloned (TOPO-TA cloning, Invitrogen) and sequenced using BigDye Terminator v3.1 chemistry on an ABI 3730 automated sequencer (Applied Biosystems).

Results

We consulted a 31-year-old nulliparous patient of Northern European descent at 21 weeks 3 days estimated gestational age because of prior ultrasound findings of echogenic kidneys, thickened nuchal fold, and polydactyly of the left foot noted at 20 weeks gestation. Fetal echocardiogram showed normal structure and function of the heart. The family's history was non-contributory and consanguinity between the couple was denied. The fetal nuchal translucency measured 3.4 mm during routine aneuploidy screening at her referring institution, and the patient opted for chorionic villus sampling. The fetal karyotype was normal, 46, XY. An ultrasound evaluation in our unit confirmed the findings of the previous

study (Fig. 1a) and also documented polydactyly of the left hand. No encephalocele was noted.

The patient and her husband had genetic counseling, and she opted for amniocentesis at 21 weeks 4 days for the purpose of alpha-fetoprotein analysis; prenatal microarray; biochemical analyses for Smith–Lemli–Opitz syndrome (SLO; MIM 270400), an autosomal recessive cholesterol deficiency syndrome which manifests postaxial polydactyly (33); and targeted molecular testing for known MKS and BBS genes. A prenatal microarray through the Columbia University Prenatal Cytogenetic Diagnosis by Array-based Copy Number Analysis trial (IRB Protocol number: 0220090164) did not reveal a known clinically significant copy number variant. Gas chromatography/mass spectrometry for 7-dehydrocholesterol in amniotic fluid yielded 9 ng/ml, which is within normal range for this assay and virtually eliminated the possibility of an SLO diagnosis (34).

Fetal MRI was performed to further assess neuroanatomy and the study showed a normal appearing spine and intracranial anatomy. We opted to proceed with molecular testing of genes contributing to both MKS and BBS: one of the two most commonly mutated MKS genes, *MKS1*, contributing to ~8% of MKS cases (24, 35); and three genes harboring the greatest mutational burden in US BBS cohorts, *BBS1*, *BBS2*, and *BBS10* with ~23%, ~8%, and ~20% contribution to disease, respectively (36). While *MKS1*, *BBS2*, and *BBS10* sequencing did not detect any novel changes predicted to alter amino acid sequence or mRNA splicing, we identified a heterozygous pathogenic variant in *BBS1* and a second allele of unknown significance (Fig. 2a). First, we identified a heterozygous c.1169T>G change in exon 12 encoding p.Met390Arg, which segregated from the proband's mother (Fig. 2a,b). This variant is one of the two most commonly reported BBS mutations in populations of European descent (36), and has been shown in both mouse knock-in (37) and zebrafish *in vivo* complementation models (38) to be a hypomorphic allele. In addition, we identified a novel intronic variant of unknown significance (VUS) in the region upstream of the exon 13 splice acceptor site (c.1181-9C>G). This allele was paternally inherited, and is not present in 7020 European American control chromosomes publicly available on the NHLBI Exome Variant Server (<http://evs.gs.washington.edu/EVS/>) (Fig. 2a,b). This change is predicted *in silico* to create a weak acceptor splice site upstream of the canonical acceptor splice site according to Neural Network Splice Site Prediction algorithms (www.fruitfly.org/egi-bin/seq_tools/splice.pl (39)) (Fig. 2a,c).

The proband, with suspected BBS based on the molecular *BBS1* findings, was delivered at 40 weeks 2 days by normal spontaneous vaginal delivery and had a normal birth weight of 3885 g. The newborn was examined by a clinical geneticist after birth and was noted to have a mildly high arched palate, nuchal redundancy, and bilateral postaxial polydactyly of the hands and feet (Fig. 1b–d). An abdominal ultrasound showed mildly enlarged kidneys with no pyelectasis. An echocardiogram revealed a patent foramen ovale. The newborn hearing exam was normal.

To investigate the pathogenicity of the c.1181-9C>G variant and provide additional evidence toward a conclusive diagnosis, we monitored *BBS1* mRNA splicing (Fig. 3a–c). We established a primary skin fibroblast cell line using a foreskin biopsy from circumcision obtained shortly after birth of the proband and we cultured cells in the presence and absence of Emetine [to block nonsense-mediated decay (NMD)]. Reverse transcription-polymerase chain reaction (RT-PCR) analysis of cell lysates from the proband cell line yielded a single PCR product, which was sequenced. In the absence of Emetine, the patient presented molecularly as a functional hemizygote; 100% of clones (10/10) were derived from the maternal allele as indicated by arginine at amino acid position 390 suggesting the degradation of the paternal transcript (Fig. 3b). Subsequent to Emetine treatment to block

protein synthesis, we repeated the *BBS1* RT-PCR experiment. We detected the maternally derived transcript in 75% of clones (8/12) as indicated by 390Arg; 25% of clones (4/12) were transcribed from the paternal transcript as indicated by 390Met and also the retention of 8 bp of intron 12 sequence. Insertion of 8 bp results in the introduction of a premature termination codon, thus subjecting the transcript to NMD, an observation consistent with our data (Fig. 3c). Together, our studies suggest that c.1181-9C>G is a functional null, and *in trans* with p.Met390Arg. Thus we conclude that loss of function of *BBS1* is the primary cause of congenital anomalies in the proband, securing a molecular diagnosis of BBS at 3 months of age.

Discussion

This case report highlights how the combined clinical and research-based efforts to conduct careful prenatal phenotyping, genetic counseling, molecular screening, and variant functional analysis were successful in obtaining an early molecular diagnosis of BBS. This is a disorder that is historically difficult to diagnose in infants, particularly since the full spectrum of minimal diagnostic criteria are often not fully evident until later in childhood (4), which exposes patients and extended families to protracted medical investigations. Although no treatment paradigms currently exist for BBS, this early diagnosis will assist the family and caregivers with expectations about prognosis and help in guiding the management of the child since his medical issues can be anticipated. We hope that early diagnosis will eventually also be useful in therapeutic intervention. For example, photoreceptor loss in BBS starts at ~2 years of age (40): a diagnosis at 3 months offers a 21-month intervention window if and when such treatments become available.

Although this case had a successful mutational screening outcome with a targeted analysis of four of the most frequently mutated BBS and MKS genes, a similar strategy may not always return informative results. The extensive genetic (17 BBS genes and 10 MKS genes) and allelic (>150 alleles associated with BBS alone (38, 41)) heterogeneity pose significant economic and interpretive challenges to the medical geneticists and genetic counselors ordering prenatal testing. With the exception of two recurrent BBS mutations, *BBS1* Met390Arg (18) and *BBS10* Cys91LeufsX5 (22), the vast majority of molecular BBS gene lesions are private alleles, hampering the utility of targeted testing approaches described here, or the use of genotyping chips testing for known BBS gene mutations (42). Although next-generation sequencing subsequent to capture of coding regions of all BBS genes has been proposed to overcome these limitations (43, 44), we expect that the eroding cost of whole exome sequencing will supersede focused gene testing approaches.

Finally, this case represents a useful template for the future dissection of prenatal-onset human genetic disorders that display overt anatomical defects detectable by ultrasound imaging. With the accelerated implementation of whole exome sequencing as a clinical diagnostic test (45), we anticipate an increased need for not only the interface between clinical and research enterprises for the interpretation of VUS but also the further development of approaches to interpret allele pathogenicity through the use of physiologically relevant *in vitro* and *in vivo* functional tools.

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Fig. 1.

Prenatal ultrasound findings and neonatal phenotypes of proband DM037-003. **(a)** Initial sonographic imaging showed postaxial polydactyly of the left foot of the fetus at 21 weeks 3 days estimated gestational age. White arrows indicate each digit. **(b)** At birth, the proband presented with bilateral postaxial polydactyly of the feet with a complete extra digit on the left foot, and attachment of an extra digit by a stalk of soft tissue on the right foot (not shown). Black arrows indicate the sixth digit. **(c)** and **(d)** Bilateral postaxial polydactyly of the left hand **(c)** and right hand **(d)**, respectively. Black arrows indicate sixth digits of each hand attached by a soft tissue stalk.

Fig. 2.

Segregation of *BBS1* variants in pedigree DM037. **(a)** Schematic representation of the *Homo sapiens BBS1* locus. Coding exons, blue boxes (with exon numbers below); untranslated regions, white boxes; dashed lines, introns. *BBS1* variants detected in family DM037 are shown as red stars and are positioned in their approximate genomic location, p.Met390Arg in exon 12 and c. 1189-9C>G upstream of the splice acceptor site of exon 13. hg19 coordinates are shown; black arrows indicate reverse transcription-polymerase chain reaction primers. **(b)** Pedigree of BBS family DM037 indicating the inheritance of the two pathogenic *BBS1* alleles. **(c)** *BBS1* c.1189-9C>G model of pathogenicity. Transition of C>G (shown in red) 9 bp upstream of the canonical *BBS1* exon 13 splice acceptor site likely introduces a novel splice site. Exon sequence (shown in all caps), intron sequence (lower-case), splicing indicated with dashed lines.

Fig. 3.

Analysis of *BBS1* c.1181-9C>G effects on mRNA splicing. **(a)** Reverse transcription-polymerase chain reaction (RT-PCR) results of control fibroblasts. A 540 bp amplicon encompassing *BBS1* exons 11–15 (as indicated by blue boxes labeled 11–15; left) was amplified from oligo-dT primed cDNA generated from control skin fibroblasts. The single PCR fragment was TOPO-TA cloned and individual colonies were Sanger sequenced. As expected, 100% of control clones (5/5) had sequence at the exon 12/13 junction that matched the reference *BBS1* mRNA sequence (NM_024649). For each of panels a–c, chromatograms for 27 bp of sequence at the exon 12/13 junction are shown; gray boxes indicate exon 12 and exon 13 corresponding to the mRNA sequence. **(b)** RT-PCR results of DM037 fibroblasts. One hundred percent of clones (10/10) were transcribed from the maternal allele as indicated by Arg (shown in red) at amino acid position 390 suggesting the degradation of the paternal transcript. **(c)** RT-PCR results of DM037 fibroblasts treated with emetine. Subsequent to treatment with emetine to block protein synthesis, RT-PCR was repeated. Maternally derived transcript was present in 75% of clones (8/12; top) as indicated by 390Arg; and 25% of clones (4/12; bottom) were transcribed from the paternal transcript as indicated by 390Met and also the retention of 8 bp of intron 12 sequence (green rectangle). Insertion of 8 bp results in the introduction of a premature termination codon, likely subjecting the transcript to nonsense-mediated decay.