Published in final edited form as:
J Med Genet. 2014 February ; 51(2): 132–136. doi:10.1136/jmedgenet-2013-101785.

Exome sequencing of Bardet-Biedl syndrome patient identifies a null mutation in the BBSome subunit *BBIP1* (*BBS18*)

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Abstract

Background—Bardet-Biedl Syndrome (BBS) is a recessive and genetically heterogeneous ciliopathy characterized by retinitis pigmentosa, obesity, kidney dysfunction, post-axial polydactyly, behavioral dysfunction and hypogonadism. Seven of the 17 BBS gene products identified to date assemble together with the protein BBIP1/BBIP10 into the BBSome, a protein complex that ferries signaling receptors to and from cilia.

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Contributors

ES, SS provided clinical information. JM, KC, VG, CS analyzed the exome sequences. CS tested the mutation in the patient and screened controls. NWP designed and conducted the co-immunoprecipitation experiment. MVN, VM supervised the cell and molecular biology experiments. MVN, SS, CS, HD wrote the manuscript. HD supervised the whole project.

Competing interests

None.

Web Resources:

dbSNP: http://www.ncbi.nlm.nih.gov/projects/SNP/
Exome Variant Server: http://evs.gs.washington.edu/EVS/
Ensembl: http://www.ensembl.org/

Methods and results—Exome sequencing performed on a sporadic BBS case revealed for the first time a homozygous stop mutation (NM_001195306: c.173T>G, p.Leu58*) in the *BBIP1* gene. This mutation is pathogenic since no BBIP1 protein could be detected in fibroblasts from the patient and BBIP1[Leu58*] is unable to associate with the BBSome subunit BBS4.

Conclusions—These findings identify *BBIP1* as the eighteenth BBS gene (*BBS18*) and suggest that BBSome assembly may represent a unifying pathomechanism for BBS.

Keywords

Cilia; BBS; ciliopathy; exome sequencing; BBSome; BBIP1

INTRODUCTION

Bardet-Biedl Syndrome (BBS; MIM 209900) is a canonical ciliopathy characterized by: retinitis pigmentosa, obesity, kidney alteration, dystrophic extremities, behavioral dysfunction and hypogonadism.[1] Rapidly evolving strategies ranging from traditional homozygosity mapping to next generation sequencing (NGS) approaches have uncovered 17 BBS genes.

Primary cilia dysfunction underlies the pathogenesis of BBS; indeed eight proteins (BBS1, BBS2, BBS4, BBS5, BBS7, BBS8, BBS9 and BBIP1/BBIP10) have been shown to form the BBSome, [2,3] a stable complex involved in signaling receptor trafficking to and from cilia. BBS6, BBS10 and BBS12 assemble into a chaperonin complex that mediates BBSome assembly. BBS3/ARL6 recruits the BBSome to membranes and BBS17/LZTFL1 regulates BBSome entry into cilia. BBSome cargoes include Smoothened, a component of the Sonic Hedgehog (Shh) signaling pathway[4] and Somatostatin Receptor 3, a G protein coupled receptor.[5]

Mutations found in the seventeen known BBS genes are found in 80% of BBS patients while 20% of them still lack molecular diagnosis.[6] Through exome sequencing, we report the first BBS patient carrying a mutation in the *BBIP1* gene (also known as *BBIP10* standing for the BBSome Interacting Protein 1/of 10 kDa) encoding the eighth subunit of the BBSome. [2] Given our prior characterization of BBIP1 as a BBSome subunit essential for BBSome assembly[2] and given the severely reduced levels of BBIP1 in the patient's fibroblasts, we propose that *BBIP1* as the eighteenth BBS gene.

METHODS

Family selection

Among 450 BBS families screened, about 15% were devoid of mutation in known BBS genes. We report herein one of our BBS families, analyzed by exome sequencing.

Informed consent and ethical approval of the patient and his representative were obtained according to the French legislation. The objectives and the aim of the study were clearly explained to the patient.

Whole exome sequencing and SNP calling

Whole exome sequencing was performed by IntegraGen. Exons of DNA samples were captured using the in-solution SureSelect Target Enrichment System (Agilent, Human All Exon Kits v2), followed by a paired-end high-throughput sequencing on reads of 75bp using the Illumina HiSeq 2000. Image analysis and base calling were performed with default parameters of Illumina RTA v1.14 pipeline. The alignment of clean reads on the human

reference genome (hg19/GRCh37) and SNP calling were performed with CASAVA 1.8 (Illumina).

Variant Annotation and Ranking with VaRank

VaRank is an in-house pipeline (manuscript in preparation), using Alamut-HT (Interactive Biosoftware) to collect genomic annotations and effect predictions at both nucleotide and protein levels. VaRank gathers variant-specific information such as potential functional effects of amino acid changes on the protein (SIFT, [7] PolyPhen2[8]) and splicing effects (Human Splicing Finder, [9] MaxEntScan, [10] NNSplice[11]). Known mutations with reported SNPs flagged as "probably-pathogenic"/"pathogenic" in the "Clinical significance" field of dbSNP137 are highlighted. From all these information, a score is computed for each SNV/indel. Potential mutations are ranked according to potential pathogenicity. Initial filtering included the removal of variants with <15% of the total coverage or the variants not supported by at least 10X, variants present in dbSNP137, validated by at least two methods or with a minor allele frequency >2%.

Western blotting

Proteins from patient's fibroblasts obtained by skin biopsy (Supplementary Method) were extracted by trichloroacetic acid precipitation and immunoblot analyses performed as previously described.[12]

Immunoprecipitation

The p.Leu58* mutation was introduced by PCR to generate pCS2-6myc-*BBIP1*[Leu58*]. For co-immunoprecipitation, HEK293FT cells were transfected with plasmid DNA using X-tremeGENE 9 (Roche). After 48 hours, cells were lysed in buffer (50 mM Tris pH7.4, 150 mM NaCl, 1% Triton X-100) containing protease inhibitors (Leupeptin, Bestatin, Chymostatin, E-64, Aprotinin, AEBSF). After centrifugation, lysates were incubated for 1 hour with 9E10 anti-myc monoclonal antibody, then 1 hour with Protein-G-sepharose. After three washes in lysis buffer, beads were eluted in SDS sample buffer and western blotted with anti-BBS4 (as previously described[3]) or 9E10 antibodies.

Morpholino microinjections in zebrafish

Injections were performed as described previously.[13] Morpholinos (Gene Tools, LLC)[14] were injected at 12nl of 0.5 mM stock solution. Phenol red was added to the samples before injection (0.1% final concentration). For detecting *situs inversus*, morpholinos were injected into the *unc45b:GFP* stable line driving GFP expression in cardiac muscles.[15] (Supplementary Method)

Bbip1 PCR amplification

For rescue experiments a *bbip1* morpholino resistant fragment was amplified by PCR with primers bbip5′ and bbip3′ (Supplementary Table 1) and cloned into pCs2+GFP.

Immunohistochemistry

Embryos were fixed in 4% paraformaldehyde/PBS for 1 hour, washed with 1% PBS, blocked with BDP (5% BSA, 1% DMSO, 1% PBS), incubated over night at 4°C with monoclonal antibody against acetylated-tubulin (1:1000, Sigma), washed 5 times, and incubated for 1 hour with fluorescent secondary antibody (Anti-mouse IgG Cy3-conjugated, 1:1000, Sigma). 5μm sections of the eyes[16] were cut with a Leica microtome and stained with toluidine blue.

RESULTS

Clinical studies

Based on four major features (retinitis pigmentosa, obesity, kidney failure, cognitive disability) and one minor feature (brachydactyly), the patient was diagnosed as affected with BBS at 49 years old. He presented an end-stage renal failure 4 years after the diagnosis. He was the only one affected among 4 siblings born from consanguineous Italian parents.

Clinical examination in our Center for Rare Genetic Ophthalmologic Diseases (CARGO) in Strasbourg showed severe visual impairment (light perception, dense cataracts, retinal dystrophy), obesity (BMI: 37.7), behavioral dysfunction, learning difficulties (understood simple orders but never learned to read or write) and brachydactyly (Figure 1A).

Molecular analysis of known *BBS* genes and exome sequencing

Prior Sanger sequencing and a preliminary examination of the exome data identified no mutation in any of the 17 known BBS genes. Initial filtering of the exome sequencing data by the VaRank program revealed 7889 variants out of 50569 (Supplementary Table 2). The 3116 homozygous variants were further filtered against known BBS variations in our in-house database to identify unique homozygous variations. The final dataset consisted of 234 variants.

These novel homozygous variants included 1 nonsense, 1 frame-shift, 2 splice mutations, 9 missense with pathogenic predictions (SIFT[7] or PolyPhen2[8]) and 1 in-frame deletion. The 47 potentially pathogenic genes were manually screened, based on gene function and previous identification of a disease. We were able to focus the subsequent studies on a single homozygous nonsense mutation, c.173T>G, p.Leu58*, in the *BBIP1* gene encoding for the eighth BBSome subunit (not yet identified as a disease causing gene). This mutation was confirmed by Sanger sequencing and found at the heterozygous state in his father's sample (mother and sibling's DNA unavailable) (Figure 1B). This mutation was absent from the EVS database and among 160 exomes performed by IntegraGen.

Depletion of *BBIP1* leads to ciliopathy phenotypes in zebrafish

A search in Ensembl (Zv9) revealed in *Danio rerio* a single 72 amino acids protein (ENSDARP00000095567) exhibiting 69% identity with the human BBIP1. Injection of morpholino oligonucleotide (MO) targeting the start codon of *bbip1* (*bbip1-mo*)[2] resulted in bilateral cystic dilations of the pronephros, an equivalent structure to the kidney in zebrafish (Figure 2A,B). The effect is specific as it could be rescued by co-injection of a *bbip1* mRNA resistant to MO knock-down (Figure 2B). Examination of cilia morphology showed that pronephric cilia of morphants failed to maintain a parallel orientation to the antero-posterior axis of the duct and were shorter than in controls (Figure 2C–F). It is likely that cysts develop as a consequence of impaired pronephric flow. Kupffer's vesicle (analogous structure of human node) is a ciliated organ that initiates left-right asymmetry of the brain, heart and gut in zebrafish.[17] It was shown previously that *bbip1* morphants display abnormalities in Kupffer's vesicle.[2] 20% of morphants present with *situs inversus*, compared to 3% in the wild type population (Figure 2G, H). Bardet Biedl disease being characterized by retinitis pigmentosa, we also examined sections of eyes from morphant or control embryos at 3 dpf and found that the morphants showed abnormal retinal development. The control larvae developed normal retinal structures with a well-defined ganglion cell layer, inner nuclear and photoreceptor layers (Figure 2I, J). The *bbip1-mo* treated embryos exhibited a disorganized retina with a lack of separation between the retinal cell layers. All together the zebrafish data suggest a role of *Bbip1* in cilium function/or assembly.

BBIP1 is absent from the patient's fibroblasts

To assess the impact of p.Leu58* mutation on the expression of the *BBIP1* gene product, we probed patient's fibroblasts protein extracts with a previously characterized antibody against human BBIP1.[2] While BBIP1 can be readily detected in extracts from controls or BBS patients with unrelated mutations, it was not detected in the patient's extracts (Figure 3A). Although the c.173T>G mutation may lead to nonsense mediated mRNA decay, it is likely that the BBIP1[Leu58*] is subject to rapid turnover because of low folding efficiency or poor incorporation into the BBSome. Importantly, since we have previously shown that BBIP1 depletion dramatically affected BBSome assembly, it is likely that BBSome assembly is compromised in the BBIP1[Leu58*] patient.

Reduced incorporation of BBIP1[Leu58*] into the BBSome

The impact of p.Leu58* mutation on BBIP1 incorporation into the BBSome was assessed by testing the amounts of the BBS4 subunit that co-assembled with Myc-BBIP1 transiently expressed into HEK cells. While wild-type BBIP1 efficiently captured BBS4, little BBS4 was detected in a complex with BBIP1[Leu58*]. Since BBSome assembly is compromised in cells depleted of BBIP1[2] and since little BBIP1[Leu58*] becomes incorporated into the BBSome (Figure 3B), we conclude that BBSome assembly must be severely affected in the patient.

DISCUSSION

As other ciliopathies, BBS is extremely heterogeneous with 17 BBS genes reported to date. Identifying novel genes remains crucial for a better understanding of the cilia-related pathogenic mechanisms of BBS and to improve the molecular diagnosis and genetic counseling. Identification of novel BBS genes represents a significant challenge as the remaining unidentified genes reside mostly in small sized families. Herein, we describe a patient with an indisputable diagnosis of BBS. Exome sequencing revealed a nonsense mutation in *BBIP1,* a gene not yet known to be involved in BBS patient but that we had previously identified as the BBSome subunit playing a central role in the BBSome assembly.[2]

NGS has accelerated discovery of new genes that are very rarely mutated in cohorts of patients for a given disease with sometimes only one family initially identified.[12,18] Thus, functional analysis remains very important to ascertain the pathogenicity of mutations in a novel gene. Here, we were greatly aided by our prior functional characterization of the BBSome subunit BBIP1 and its importance in ciliogenesis and BBSome assembly. In addition, the functional consequences of BBIP1[Leu58*] mutation were assessed to validate its pathogenicity. First, as expected for such a nonsense mutation, no protein was detected by western blotting analysis. Second, co-immunoprecipation assays performed between transfected *Myc-BBIP1* and endogenous BBS4 showed a dramatic decrease in the amount of BBS4 captured by BBIP1[Leu58*] compared to BBIP1 thus pointing to a major effect of the mutation on BBSome assembly. Third, the zebrafish morpholino discloses a ciliopathy phenotype as described previously and moreover we show that rescue assays confirm the pathogenicity of the mutation in this model. These three functional assays confirm that this mutation has a major biological effect underlying the phenotype observed in the patient.

Importantly, our data suggest that BBSome assembly may represent a converging pathomechanism of BBS as the vast majority of BBS mutations so far tested result in aberrant BBSome assembly.[19] Alternatively, mutations in *BBS17/LZTFL1* or *BBS3/ARL6* appear to affect BBSome trafficking to cilia without impinging on BBSome assembly.[4]

It is interesting to note that BBIP1 has been proposed to influence microtubule acetylation independently of the BBSome[2] which suggests that mutations in *BBIP1* may lead to further symptoms beside the typical findings in BBS. However, the patient had no specific BBS features that could distinguish him from patients mutated in other BBS genes except for the absence of polydactyly, a feature from about 30% of the BBS patient population.[1] Thus, it is likely that BBIP1 functions exclusively within the BBSome.

Targeted high throughput diagnosis is currently being set for various conditions including ciliopathies[6] and we suggest that *BBIP1* be added to the screening panel as the eighteenth BBS gene. This patient's mutation adds to the BBS puzzle by pointing to *BBIP1* as a rarely mutated BBS gene but encoding for a protein with an important ciliary function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank the patient for his participation to the project and the patient's associations, Bardet-Biedl France, for their constant and strong support.

Funding

This work was funded by RETINA France, UNADEV, FORMICOEUR, BARDET-BIEDL France, the AVENIR INSERM program and the French Ministry of Health with the National PHRC program. Research in MVN lab was supported by NIH (GM089933) and March of Dimes (1-FY11-517). Research in US lab was supported by European IP ZF-Health and KlausTschira Stiftung.

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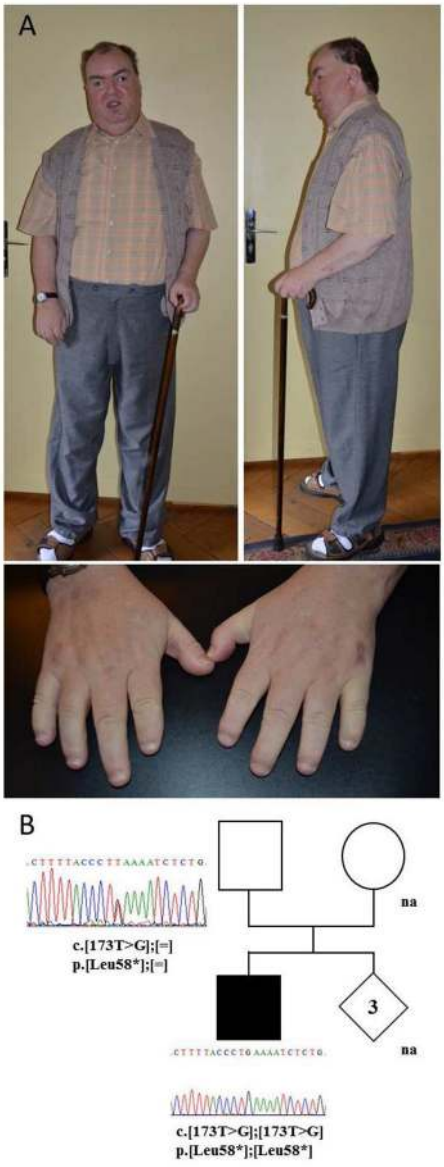

Figure 1.
Identification of the p.[Leu58*];[Leu58*] mutation in BBIP1 gene. (A) Pictures of the patient depicting an obese phenotype and overall enlarged hands with a shortened aspect of the fingers. (B) Electrophoregrams of a part of BBIP1 exon 3 showing the homozygous mutation in the patient and the heterozygous mutation in the patient's father (mother and siblings' DNA unavailable).

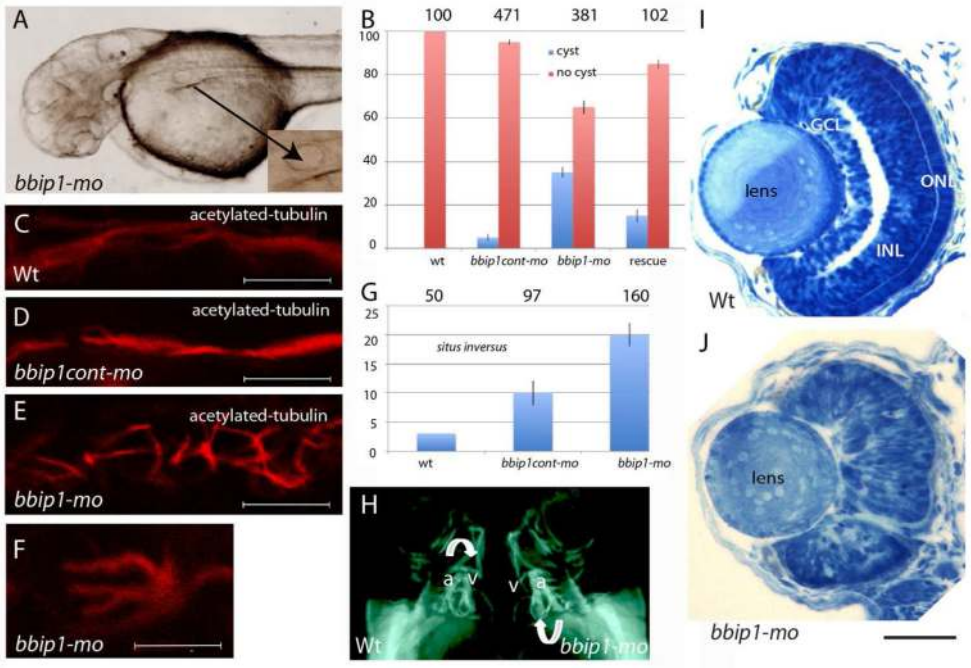

Figure 2.

Bbip1 morphants exhibit cilia abnormalities. (A) Kidney tubule dilatation observed in light microscopy in an embryo injected with 0.5 mM bbip1-mo. (B) Embryos were injected with 0.5 mM bbip1-mo or bbip1cont-mo: 37% of them show formation of kidney cysts. Rescue was obtained with 50ng/μl of bbip1 mRNA. (Numbers indicate the number of embryos examined). (C–F) Anti acetylated-tubulin staining of wild-type (C), control morphant (D), or bbip1 morphants (E and F (single cilia)). Morphants show shorter and misdirected cilia and pronephric dilation. (G) Graph showing the percentage of morphants exhibiting situs inversus compared to controls and uninjected embryos (Numbers indicate the number of fish examined). (H) Visualisation of situs inversus in morphant in unc45b:GFP reporter line (arrows indicate atrium(a)/ventricle(v) inversion). I–J: 5 μm section of uninjected (I) or bbip1-mo injected embryos stained with toluidine blue showing a disorganized retina. GCL; ganglion cell layer, INL internal nuclear layer, ONL: outer nuclear layer. Scale bars: 12 μm (C–E), 6 μm (F), 0.2 cm (I–J). Age of embryos: 48 hpf (A–F); 120 hpf (G–H); 72 hpf (I–J). Standard deviation is indicated in B and G. Each experiment was done at least 3 times.

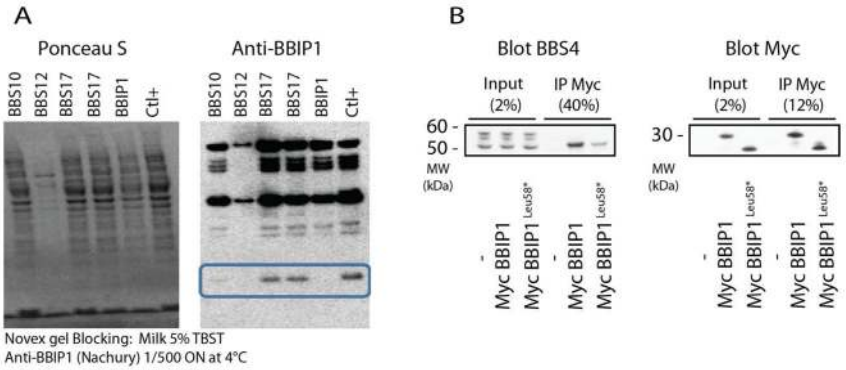

Figure 3.

The p.Leu58* mutation prevents BBIP1 assembly into the BBSome. (A) Immunodetection of BBIP1 in patient's dermal fibroblasts together with a Ponceau S staining of the membrane for protein loading control. (B) Extracts of HEK293 cells transfected with Myc-BBIP1 (WT or Leu58*) were subjected to immunoprecipitation with anti-Myc antibody. Extracts and elutes were immunoprobed for endogenous BBS4 and exogenous Myc-BBIP1.