Published in final edited form as:
J Urol. 2012 January ; 187(1): 272–278. doi:10.1016/j.juro.2011.09.036.

Genetic Basis of Prune Belly Syndrome: Screening for *HNF1β* Gene

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Abstract

Purpose—Although the cause of prune belly syndrome is unknown, familial evidence suggests a genetic component. Recently 2 nonfamilial cases of prune belly syndrome with chromosome 17q12 deletions encompassing the *HNF1β* gene have made this a candidate gene for prune belly syndrome. To date, there has been no large-scale screening of patients with prune belly syndrome for *HNF1β* mutations. We assessed the role of *HNF1β* in prune belly syndrome by screening for genomic mutations with functional characterization of any detected mutations.

Materials and Methods—We studied patients with prune belly syndrome who were prospectively enrolled in our Pediatric Genitourinary DNA Repository since 2001. DNA from patient samples was amplified by polymerase chain reaction, sequenced for coding and splice regions of the *HNF1β* gene, and compared to control databases. We performed functional assay testing of the ability of mutant *HNF1β* to activate a luciferase construct with an *HNF1β* DNA binding site.

Results—From 32 prune belly syndrome probands (30 males, 2 females) *HNF1β* sequencing detected a missense mutation (V61G) in 1 child with prune belly syndrome. Absent in control databases, V61G was previously reported in 2 patients without prune belly syndrome who had congenital genitourinary anomalies. Functional testing showed similar luciferase activity compared to wild-type *HNF1β*, suggesting the V61G substitution does not disturb *HNF1β* function.

Conclusions—One genomic *HNF1β* mutation was detected in 3% of patients with prune belly syndrome but found to be functionally normal. Thus, functionally significant *HNF1β* mutations are uncommon in prune belly syndrome, despite case reports of *HNF1β* deletions. Further genetic study is necessary, as identification of the genetic basis of prune belly syndrome may ultimately lead to prevention and improved treatments for this rare but severe syndrome.

Keywords

genetics; medical; HNF1B protein; human; prune belly syndrome

Prune belly syndrome (Online Mendelian Inheritance in Man code 100100) is a severe multisystem congenital anomaly complex affecting 3.8 per 100,000 live male births.[1] By definition the syndrome includes 1) hypoplastic or absent abdominal wall musculature, 2) moderate to severe urinary tract dilatation and 3) bilateral undescended testes in males.[2]

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Prune belly syndrome is an extremely morbid condition, as 20% of patients are stillborn, 30% die during the initial hospitalization and the remaining 50% have degrees of urinary pathology during their lifetime, including renal failure in 67%. In addition, 43% of patients are born premature, 48% require respiratory intubation and mechanical ventilation, and 25% have congenital cardiovascular anomalies. Despite advances in perinatal care, the overall initial mortality rate does not seem to have improved since the middle of the last century. Abdominal wall weakness increases susceptibility to pulmonary infection, decreases physical mobility and causes psychological concerns due to poor cosmesis. Fertility is decreased and extensive surgery is required for reconstruction.

While the cause of PBS is unknown, several aspects of the syndrome, including a high concordance rate in twins (12.2 per 100,000 live births), monozygotic male twin case reports, familial case reports and a higher incidence in males, suggest that it is influenced by a sex-linked genetic factor, although some have suggested an autosomal recessive mode of inheritance.[3–6] A recent 9-year study from a national database found a 5:3:1:1 white-to-black-to-Hispanic-to-other racial distribution, with a twofold higher proportion of PBS in blacks than in the general population.[1] Thus, evidence suggests that PBS might be a genetic disorder. In a genetic disease the spectrum of DNA alterations ranges from point mutations and small deletions/insertions (detectable by direct DNA sequencing) to DNA rearrangements (detectable by karyotyping and fluorescence in situ hybridization) to single and multi-exon deletions and duplications (detectable by comparative genomic hybridization).[7] A panel that tests for multiple types of genomic alterations may be required to define a genetic disease.

Recently 2 case reports of PBS with interstitial deletions in chromosome 17q12 encompassing *HNF1β* (also known as *TCF2*) have made *HNF1β* a candidate PBS gene.[8,9] *HNF1β* is a transcription factor that regulates gene expression necessary for mesodermal and endodermal development and is expressed in numerous tissues, including mesonephric duct derivatives. Since *HNF1β* mutations and deletions have been postulated to contribute to urinary tract abnormalities,[10,11] we performed a large-scale screening of *HNF1β* mutations in patients with PBS.

MATERIALS AND METHODS

Sequencing

With institutional review board approval, we identified patients prospectively enrolled since 2001 in the University of Texas Southwestern Pediatric Urology DNA and Tissue Repository with a diagnosis of PBS. Genomic DNA was extracted from whole blood of PBS probands using standard procedures and screened for mutations by sequencing coding regions and intron-exon boundaries of *HNF1β* (www.polymorphicdna.com). Detected *HNF1β* mutations were cross-referenced to 2 large multiethnic databases, the NCBI dbSNP (www.ncbi.nih.gov/projects/SNP/) and 1000 Genomes Project (www.1000genomes.org/).

In Silico Functional Characterization

Interspecies comparison of *HNF1β* amino acid conservation was performed by using the NCBI protein-protein Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov/BLAST.cgi). To predict the structural and functional impact of detected *HNF1β* amino acid substitutions, the detected substitutions were analyzed using PolyPhen-2 (http://genetics.bwh.harvard.edu/) and SIFT (http://sift.jcvi.org/).

Functional Testing

Plasmid construction—The pcDNA 3.1 plasmid encoding the truncated form of mouse *HNF1β* lacking 236 amino acids at the C-terminal end (pcDNA3-HNF1βΔC) has been described previously,[12] as well as a luciferase reporter plasmid containing approximately 1.9 kb of the autosomal recessive polycystic kidney and hepatic disease 1 (*Pkhd1*) promoter linked to the coding region of firefly luciferase (pGL3-Pkhd1).[13] A plasmid (pRSVcat-N809) containing the human wild-type *HNF1β* cDNA was obtained from the CNRS Institut Pasteur, Paris. The *HNF1β* coding region was amplified from the pRSVcat-N809 with forward primer 5′-ATGGTGTCCAAGCTCACGTC and reverse primer 5′-CCAGGCTTGTAGAGGACACTG, and the 1.6kb band was cloned in the TA site of the pcDNA3.1/V5-HIS-TOPO vector to produce the pcDNA3.1-WT *HNF1β* vector. The V61G mutation was introduced in the pcDNA3.1-WT *HNF1β* plasmid using the forward primer 5′-GGGGCCGAGCCCGACACCAAGCCGGGCTTCCATACTCTCACCAACGGCCAC and the reverse primer 5′-GTGGCCGTTGGTGAGAGTATGGAAGCCCGGCTTGGTGTCGGGCTCGGCCCC to produce the pcDNA3.1-V61G-*HNF1β* plas-mid. Site directed mutagenesis was performed using the QuikChange® kit, as described previously,[12] and the presence of the desired mutation was verified by DNA sequencing.

Luciferase assay—mIMCD3 and HeLa cells were grown as described previously.[12] Cells were plated in 6-well dishes (1.2×10^5 cells per well) and transfected with 0.6 μg luciferase reporter plasmids using FuGENE® 6. Cells were cotransfected with 20 ng pRL plasmid encoding Renilla luciferase to control for differences in transfection efficiency. After growth for 48 hours the cells were lysed in 250 μl passive lysis buffer (Promega Corp., Madison, Wisconsin), freeze-thawed once and centrifuged. Supernatants (20 μl) were added to 96-well plates, and firefly and Renilla luciferase activities were measured using the Dual-Luciferase® Reporter Assay System according to the manufacturer directions. Luciferase assay reagent II (100 μl) was added, and light output was measured for 10 seconds using a VICTOR™ V multilabel counter. Firefly luciferase activity was normalized to Renilla luciferase activity, which was measured by adding 100 μl Stop & Glo® reagent and measuring light output for 5 seconds. ANOVA using Dunnett's test (GraphPad Prism®, version 5) was used to test for significance of differences in the means, expressed as false discovery rate q values.

RESULTS

HNF1β Gene Sequencing

DNA samples from 32 probands affected with PBS were identified in the repository, including 30 males (23 white non-Hispanics, 3 black non-Hispanics, 4 white Hispanics) and 2 females (both white non-Hispanics). One heterozygous mutation was discovered in exon 1 of the *HNF1β* gene, yielding a missense mutation encoding glycine instead of valine at amino acid position 61 (V61G, fig. 1). Parental samples of this individual with PBS were unavailable to assess whether the mutation was inherited or *de novo*. This nsSNP was not reported in the NCBI dbSNP and is not contained in the 1000 Genome Project. However, the V61G mutation was previously reported in 2 patients without PBS, 1 with VACTERL association,[14] and 1 with müllerian anomaly and a solitary kidney.[15] Neither of these publications described functional testing of the V61G missense mutation to prove causality.

The V61G mutation is found in exon 1 of the *HNF1β* gene between the dimerization domain and the DNA binding domain,[16] and is conserved across multiple species (see Appendix). However, both in silico analyses predict that the V61G substitution is not functionally significant. PolyPhen-2 analysis predicts V61G to be benign (PolyPhen-2 score = 0.000

[scale of 0 to 1.0]) and SIFT analysis predicts V61G is tolerated (SIFT score = 0.08 [scale of 0 to 1.0, with score less than 0.05 considered damaging]).

Functional Assays

HNF1β consists of an N-terminal dimerization domain, a Pit-1/Oct-1/Unc-86 domain and homeodomain that mediate DNA binding, and a C-terminal transcriptional activation domain. *HNF1β* is known to regulate the expression of *Pkhd1* by directly binding to the *Pkhd1* promoter.[13] To test whether V61G *HNF1β* can activate the *Pkhd1* promoter, reporter gene assays were performed in HeLa cells that do not endogenously express *HNF1β*. Plasmids encoding either the WT *HNF1β*, V61G *HNF1β* or empty parent vector were cotransfected with the luciferase reporter plasmid containing the *Pkhd1* promoter into HeLa cells, and luciferase activity was measured 48 hours later (fig. 2, *A*). To correct for differences in transfection efficiency, cells were transfected with a constant amount of pRL encoding Renilla luciferase, and transcription activity was calculated from the ratio of firefly and Renilla luciferase activities. Each transfection was completed in triplicate and each experiment was repeated 3 times. Calculated average ± SE relative luciferase activity for WT *HNF1β* and V61G *HNF1β* was 2.99 ± 0.06 and 2.79 ± 0.47, respectively, suggesting that the V61G mutation does not affect the ability of *HNF1β* to bind to the *Pkhd1* promoter and drive expression of luciferase.

Activation of the *Pkhd1* promoter by *HNF1β* is stimulated by cyclic adenosine monophosphate responsive element binding protein and p300/CBP associated factor, which directly interact with the transcriptional activation domain at the C-terminal of *HNF1β*. These findings suggest that *HNF1β* activates *Pkhd1* transcription by recruiting coactivators that promote histone acetylation and chromatin remodeling at the promoter. Deletion mutants lacking the C-terminal domain (ΔC *HNF1β*) function as dominant negative mutants, possibly by preventing the recruitment of coactivators.[17] To determine if the V61G mutation encodes for a dominant negative mutant form of *HNF1β*, reporter assays were performed in mouse inner medullary collecting duct cells (mIMCD3), which endogenously express *HNF1β*. Plasmids encoding either the WT *HNF1β*, V61G *HNF1β*, ΔC *HNF1β* or an empty vector were cotransfected with the luciferase reporter plasmid containing the *Pkhd1* promoter into mIMCD3 cells, and luciferase activity was measured 48 hours later (fig. 2, *B*).

To correct for differences in transfection efficiency, cells were transfected with a constant amount of pRL encoding Renilla luciferase, and transcription activity was calculated from the ratio of firefly and Renilla luciferase activities. Each transfection was completed in triplicate and each experiment was repeated 3 times. Calculated average ± SE relative luciferase activity for WT *HNF1β* and V61G *HNF1β* was 17.0 ± 1.3 and 18.1 ± 1.5, respectively, and for the dominant negative mutant ΔC *HNF1β* was 5.661 ± 0.601. Statistical analysis of these averages suggests that the V61G mutation does not encode for a dominant negative form of *HNF1β* (q = 2.14).

DISCUSSION

PBS, also known as Eagle-Barrett syndrome, is lethal in approximately 50% of affected individuals and is associated with high morbidity in the survivors. In addition to anomalies of the abdominal wall and urinary tract and cryptorchidism, accompanying birth defects can include cardiovascular, respiratory, orthopedic and gastrointestinal anomalies. Although the majority of surviving patients with PBS have normal cognitive functioning, many surviving children have lifelong disabilities, including renal insufficiency and renal failure requiring renal replacement therapy. Multiple surgical interventions are necessary in surviving children, and despite surgical advances, the disorder remains challenging. PBS is associated

with long-term diminished self-esteem from poor cosmesis, constipation, pulmonary and urinary dysfunction, impaired physical mobility and sexual health issues. For PBS survivors and their families the lifelong disabilities can be devastating psychologically, financially, socially and medically.

Two mechanistic theories have been proposed to explain embryonic maldevelopment, which begins at approximately 6 to 10 weeks of gestation. In the first theory, known as the theory of mesodermal arrest, an unknown primary defect in the development of the lateral plate mesoderm between 6 and 10 weeks of gestation produces primary maldevelopment of the abdominal wall and urinary tract musculature. In the second theory, involving in utero bladder obstruction, a hypoplastic/dysplastic prostate or abnormal urethra prevents urine passage. This obstruction of urine flow causes bladder, ureteral and renal distention with secondary abdominal wall and urinary muscle maldevelopment. Despite these anatomical and mechanistic theories, the molecular basis is unknown.

Originally a possible genetic basis for PBS was largely discounted due to reports of monozygotic twins discordant for PBS. Nevertheless, 2 publications have described concordant PBS occurring in monozygotic twins.[3,4] Additionally 12 published case reports of familial PBS primarily affecting brothers have suggested a possible autosomal or X-linked recessive mode of inheritance (see table).[3–6,18–25] Thus, a genetic basis for PBS is highly suggested.

Currently *HNF1β* is the only candidate PBS gene based on 2 published PBS cases with chromosome 17q12 microdeletions encompassing the *HNF1β* gene.[8,9] In 2008 an adult with PBS, type 2 diabetes, early onset gout and pancreatic atrophy was tested for mutations in *HNF1β*, which identified no variants.[8] Further testing identified a *de novo* heterozygous chromosome 17q12 microdeletion of 3.2Mb deleting the entire *HNF1β* gene. In 2010 a severely affected infant with PBS, who died at birth, was described to have a *de novo* 1.3Mb chromosome 17q12 interstitial microdeletion including the *HNF1β* gene.[9]

HNF1β is a transcription factor that regulates gene expression necessary for normal mesodermal and endodermal development, and is expressed in numerous tissues, including kidney, prostate, mesonephric duct derivatives, pancreas, gut and liver. The renal cysts and diabetes syndrome (Online Mendelian Inheritance in Man code 137920, also known as maturity onset diabetes of the young, type 5) represents the most common *HNF1β* phenotype.[11,26–28] Other clinical features described in association with the *HNF1β* phenotype include müllerian duct anomalies, increased liver enzymes and hyperuricemia.[15,29,30] Given the temporal and spatial expression pattern of *HNF1β*, it is conceivable that *HNF1β* haploinsufficiency or *HNF1β* loss of function mutations could disturb normal intermediate mesoderm differentiation, yielding the urinary tract maldevelopment seen in PBS.[10,11]

Given the rarity of PBS, nearly all publications on PBS are single case studies describing large genomic rearrangements. Currently there are no publications investigating the genetic basis of PBS in a large cohort. Thus, our cohort of 32 patients with PBS may represent the largest series published to date. Despite our large-scale screening, only 1 mutation of *HNF1β*, V61G, was detected in 1 patient with PBS. This missense mutation has previously been reported in 2 individuals without PBS, 1 with VACTERL association,[14] and 1 with müllerian duct anomaly and solitary kidney.[15] However, no testing was performed by either group to confirm or refute whether the V61G substitution disturbed *HNF1β* function. This valine is highly conserved between species but its location between important protein motifs decreases the impact of amino acid substitution in this position.

Since the V61G substitution had not been detected in normal controls but had been detected in 2 patients with congenital genitourinary anomalies, we performed functional characterization of the V61G variant. The V61G mutant *HNF1β* protein revealed normal transcription factor binding without dominant negative effect via luciferase assay, suggesting that V61G is not a functionally significant mutation.

Nevertheless, this study does not completely discount the role of *HNF1β* in PBS. Given that all of our patients are living survivors with PBS, we could have enrollment bias in our study. *HNF1β* may have a strong genetic role in PBS nonsurvivors, a cohort that is not included in our series.

Also our patients may have *HNF1β* mutations positioned outside the regions analyzed in our study, such as within regulatory regions or introns. In addition, mutations in upstream or downstream signaling events in the *HNF1β* pathway may affect *HNF1β* expression and function. However, these pathways are only currently being elucidated and may comprise hundreds of genes. Our functional assay does not comprehensively assess these unknown pathways.

Finally, polymerase chain reaction sequencing does not test for copy number variations or gene rearrangements. Since polymerase chain reaction does not test for the previously reported *HNF1β* heterozygous deletions found in the 2 PBS cases, an *HNF1β* copy number variation assay is necessary to discount the role of *HNF1β* in PBS. Having screened the only known candidate gene for PBS, mutations of genes other than *HNF1β* might cause PBS. Until additional candidate genes are identified, the genetic basis of PBS remains unknown.

CONCLUSIONS

Despite PBS case reports of multi-exonic *HNF1β* deletions, functionally significant mutations detectable by coding and splice site sequencing of *HNF1β* are uncommon in PBS. The V61G *HNF1β* mutation was detected in 1 of 34 patients (3%) with PBS in this large-scale screening but was observed to be functionally normal. Further genetic study is warranted in PBS to lead ultimately to prevention and improved treatments for this rare but severe disease.

Acknowledgments

Supported by the Prune Belly Syndrome Network, Inc. (http://www.prunebelly.org/), NIH Grant R01DK042921 and the UT Southwestern O'Brien Kidney Research Core Center (P30DK079328).

Dr. Moshe Yaniv, CNRS Institut Pasteur, Paris, supplied the plasmids. Emma Sanchez, research coordinator, provided integral help with recruitment and consenting PBS probands and families. Wen-Xiu Zhang and Patricia Cobo-Stark assisted with research.

Abbreviations and Acronyms

HNF1β	hepatocyte nuclear factor-1beta
mIMCD3	mouse inner medullary collecting duct cells
NCBI	National Center for Biotechnology Information
PBS	prune belly syndrome
Pkhd1	autosomal recessive polycystic kidney and hepatic disease 1
SIFT	sorting intolerant from tolerant

SNP	single nucleotide polymorphism
WT	wild-type

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APPENDIX

Conserved region of V61G mutation (red) within *HNF1β* across species

Species	Sequence
HNF1β human	DTKPVFHTL
HNF1β mouse	DTKPVFHTL
HNF1β rat	DTKPVFHTL
HNF1β frog	DNKPVFHTL
HNF1β zebrafish	DSKPVFHTL
HNF1β cattle	DTKPVFHTL

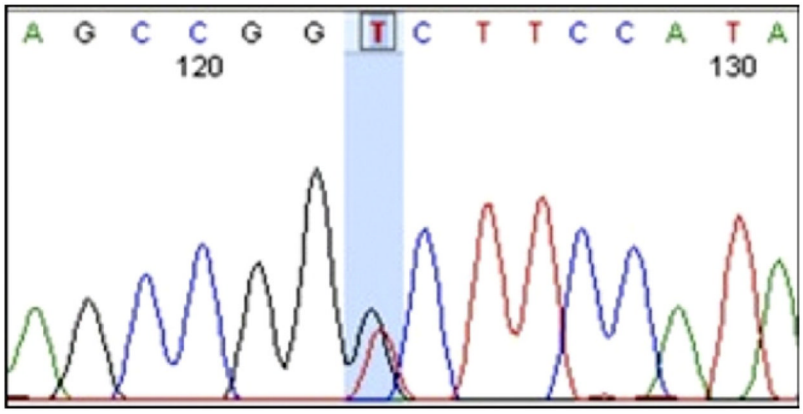

Figure 1.
Electropherogram of V61G mutation (blue shaded area) in patient with PBS, occurring at position 36, 104, 694 (hg19).

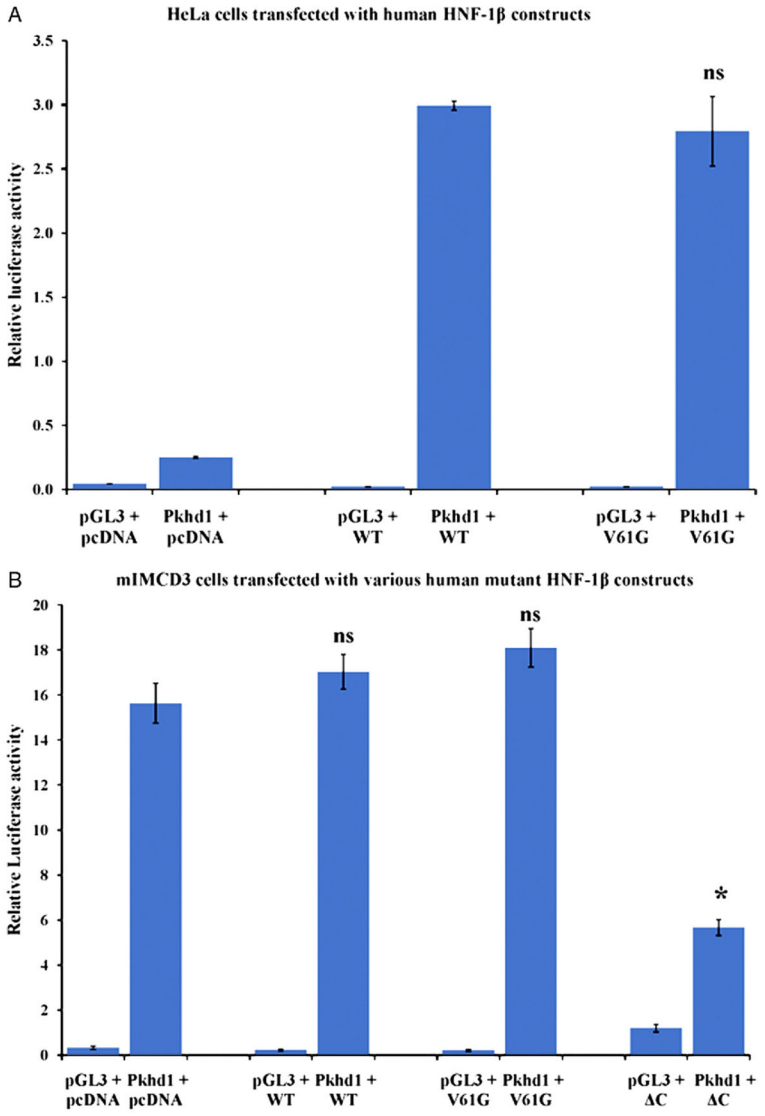

Figure 2.

Transactivation of Pkhd1 promoter by WT and mutant V61G *HNF1β* proteins. *A*, HeLa cells were cotransfected with 0.6 μg pGL3-Pkhd1 and 0.3 μg pcDNA3.1 or pcDNA3.1-WT *HNF1β* or pcDNA3.1-V61G *HNF1β*. Cells were cotransfected with 2 ng pRL, and luciferase activity was normalized to Renilla luciferase. Data are means ± SE for 3 independent transfections. *B*, mIMCD3 cells were cotransfected with 0.6 μg pGL3-Pkhd1 and 0.3 μg pcDNA3.1, pcDNA3.1-WT *HNF1β*, pcDNA3.1-V61G *HNF1β* or pcDNA3-*HNF1β*ΔC. Cells were cotransfected with 2 ng pRL, and luciferase activity was normalized to Renilla luciferase. Data are means ± SE for 3 independent transfections. Asterisk indicates $p < 0.001$ compared to cells transfected with pcDNA3.1. *ns*, nonsignificant.

Table 1

Reported cases of familial prune belly syndrome

References	PBS Cases	Suggested Mode of Inheritance
Grenet et al[18]	2 Brothers	Autosomal/X-linked recessive
Harley et al[19]	2 Brothers	Autosomal/X-linked recessive
Afifi et al[20]	2 Brothers of first cousin parents	Autosomal/X-linked recessive
Garlinger and Ott[4]	2 Brothers	Autosomal/X-linked recessive
Riccardi and Grum[21]	2 Brothers	Autosomal/X-linked recessive
Lockhart et al[5]	2 Brothers + 1 sister	Autosomal recessive
Gaboardi et al[22]	2 Brothers + 1 sister	Autosomal recessive
Adeyokunnu and Familusi[23]	1 Brother + 1 sister + 1 cousin	Autosomal recessive
Feige et al[24]	1 Brother + 1 sister	Autosomal recessive
Balaji et al[3]	2 Monozygotic male twins	Autosomal recessive
Chan and Bird[25]	Family (2 brothers + mother + maternal grandmother)	Autosomal dominant/mitochondrial
Ramasamy et al[6]	2 Brothers	Autosomal/X-linked recessive