ORIGINAL ARTICLE

Mutation profile of *BBS* genes in Iranian patients with Bardet–Biedl syndrome: genetic characterization and report of nine novel mutations in five *BBS* genes

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Bardet–Biedl syndrome (BBS) is a rare ciliopathy disorder that is clinically and genetically heterogeneous with 18 known genes. This study was performed to characterize responsible genes and mutation spectrum in a cohort of 14 Iranian families with BBS. Sanger sequencing of the most commonly mutated genes (*BBS1*, *BBS2* and *BBS10*) accounting for ~50% of BBS patients determined mutations only in *BBS2*, including three novel mutations. Next, three of the remaining patients were subjected to whole exome sequencing with 96% at 20× depth of coverage that revealed novel *BBS4* mutation. Observation of no mutation in the other patients represents the possible presence of novel genes. Screening of the remaining patients for six other genes (*BBS3*, *BBS4*, *BBS6*, *BBS7*, *BBS9* and *BBS12*) revealed five novel mutations. This result represents another indication for the genetic heterogeneity of BBS and extends the mutational spectrum of the disease by introducing nine novel mutations in five *BB*S genes. In conclusion, although *BBS1* and *BBS10* are among the most commonly mutated genes in other populations like Caucasian, these two seem not to have an important role in Iranian patients. This suggests that a different strategy in molecular genetics diagnostic approaches in Middle Eastern countries such as Iran should be considered.
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INTRODUCTION

Bardet–Biedl syndrome (BBS, MIM no. 209900) is a rare genetic ciliopathy disorder in which different body systems are affected with a wide range of clinical symptoms. The clinical signs are divided into two categories: primary and secondary features. Primary features, hallmarks of the disorder, are the most prevalent signs, including rod–cone dystrophy leading to night blindness and eventually considerable blindness, polydactyly, obesity, hypogonadism, renal anomalies and mental retardation.[1,2] However, considering mental retardation as one of the cardinal features has been challenged.[1,3,4] Secondary or minor features include speech disorder, diabetes mellitus, hearing loss, liver disease, Hirschprung disease, congenital heart defects, craniofacial abnormalities and many others. Owing to the observation of pleiotropy in BBS, clinical diagnosis is based on the presence of at least four primary features or three primary plus at least two secondary features.[5,6]

The prevalence of this disorder is variable from approximately 1 in 125 000–160 000 in North America and Europe[7–9] and 1 in 156 000 in Tunisia of North Africa[10] to higher prevalence such as 1 in 17 000 among the Bedouins in Kuwait,[11] 1 in 13 000 in Newfoundland[12] and 1 patient in every 3700 individual in The Faroe Islands,[7,13] suggesting the role of founder effect in these specific populations.

Classically, autosomal recessive pattern of inheritance has been considered for this disorder. However, regarding the variable penetrance and expressivity of the clinical symptoms observed in the patients, a more complex and also controversial pattern named 'triallelic inheritance' or 'digenic trait' has been proposed in <10% of the BBS families.[14,15]

BBS is a heterogeneous ciliopathy disorder, with 18 known genes that account for about 80% of the cases. These include *BBS1*, *BBS2*, *BBS3* (*ARL6*), *BBS4*, *BBS5*, *BBS6* (*MKKS*), *BBS7*, *BBS8* (*TTC8*), *BBS9* (*PTHB1*), *BBS10*, *BBS11* (*TRIM32*), *BBS12*, *BBS13* (*MKS1*), *BBS14* (*CEP290*), *BBS15* (*C2orf86*), *BBS16* (*SDCCAG8*), *BBS17* (*LZTFL1*) and *BBS18* (*BBIP1*).[7,14,16,17]

Variations in the contribution of each gene in different reports, in addition to the presence of some clinically diagnosed BBS patients with no responsible mutations in the known genes, all together offers the possibility of other novel genes (the remaining 20%), not being identified yet. Now, it is clear that all these genes have a specific role in the ciliogenesis and maintenance of the cilium, mostly by forming BBSome complex (*BBS1*, *BBS2*, *BBS4*, *BBS5*, *BBS7*, *BBS8* and *BBS9*)

or being a part of chaperonin complex (*BBS6*, *BBS10* and *BBS12*). *BBS7* protein interacts with *BBS2* and *BBS9* proteins to form the BBSome core complex, which is an important intermediate complex during the sequential assembly of BBSome. These assembly reactions are stabilized and regulated by BBS chaperonic complex (*BBS6*, *BBS12*, *BBS10* and *CCT/TRiC*). *BBS3* (*ARL6*) is one of the GTP-binding proteins with an essential role in proper localization of BBSome. However, other genes located in or at the distal end of basal bodies interacting with different signaling pathways; such as, sonic hedgehog and Wnt.[1,2,6,14,18,19] The recently identified genes *BBS17* (*LZTFL1*) and *BBS18* (*BBIP1*) have been presented with roles in BBSome entry into cilia for *BBS17* and being the eighth subunit of the BBSome for *BBS18*, respectively.[16,17]

All the 18 known genes in BBS account for about 80% of the clinically diagnosed cases. *BBS1* and *BBS10* are the most frequently mutated genes that account for about 23% and 20% of BBS patients in European and North American populations, respectively. However, some regional variations have been reported as patients originating from Middle East and North Africa are mostly mutated in *BBS4*, *BBS5* and *BBS8*,[14] and *BBS4* is reported as the most prevalent gene among Turks and Pakistanis. There are several reports in Pakistani families with mutations in *BBS1*, *BBS3*, *BBS10* and *BBS12*.[7,20–22] Owing to the high heterogeneity in BBS, a practical approach is needed for molecular diagnosis, which cannot be achieved unless prioritization based on the prevalence of pathogenic mutations.

The spectrum of BBS gene mutations in Middle Eastern countries, especially Iran, is not entirely clear, which indicates that more study is needed to determine a strategy for molecular diagnosis of this syndrome in populations. This study was performed to characterize responsible genes and mutation spectrum in a cohort of 14 Iranian families with BBS.

MATERIALS AND METHODS

Recruitment of the subjects and clinical diagnosis
Clinical examination was performed considering the presence of at least four cardinal signs or three primary features with expressing at least two minor symptoms as the inclusion criteria for BBS diagnosis.

Fourteen unrelated patients with BBS, 13 of which were of various ethnic origins within Iran and 1 immigrant from Afghanistan were recruited in this study. In 7 out of 14 (50%) referred families, there were only one affected child, but the remaining families had more children (2 and 3) with BBS phenotype. Consanguinity was absent in two families (Table 1). Informed consent from all patients was obtained, according to the ethics committee recommendations. The genomic DNA of the patients was extracted from their blood's white blood cells as described elsewhere.[23] RNA extraction and establishment of Epstein–Barr virus-transformed lymphoblastoid cell lines were performed whenever it was required.[24]

Mutation analysis
Initial screening for the three most commonly mutated genes. Initially, mutation analysis of all the 14 patients was performed by screening the coding regions of the three most commonly mutated genes, including *BBS1*, *BBS2* and *BBS10*. Primer3 (http://frodo.wi.mit.edu/primer3/) and Oligo-analyzer 3.1 (http://eu.idtdna.com/analyzer/applications/oligoanalyzer/) were used for designing 41 primer sets to cover all 36 coding exons of the three genes plus 50 bp from introns in the both sides of each exon.[25] Conventional Sanger sequencing using Big Dye Terminators (Applied Biosystems 3130 Genetic Analyzer; Applied Biosystems, Foster City, CA, USA) was used for sequencing the amplified DNA amplicons. Mutation nomenclature was made according to the guidelines of Human Genome Variation Society (HGVS v.2.0) and checked with the use of Mutalyzer (https://mutalyzer.nl/). Functional effect of the novel missense mutations were evaluated using bioinformatics tools such as PolyPhen (http://genetics.bwh.harvard.edu/pph2/), SIFT (http://sift.jcvi.org/) and Conseq server (http://conseq.tau.ac.il/) if possible and also cosegregation analysis in the families.[26–28]

Exome sequencing. Next, three of the remaining patients were chosen and whole exome sequencing was performed. The Agilent SureSelect Human Exome Kit (v.4), (Agilent Technologies, Inc., Santa Clara, CA, USA) was used for target enrichment and the three sample DNAs were pooled together and used for paired-end sequencing of 100 cycles (generating 100 bp reads) on a single lane of Illumina HiSeq2000's flow-cell (Illumina Inc., San Diego, CA, USA).

The sequence reads were aligned to the reference sequence of the human genome using Burrows–Wheeler transform algorithm. The Unified Genotyper module of the GATK package was used for calling both single-nucleotide polymorphisms and indels.[29] Regions with at least eightfold depth of coverage were used for calling variants, and a different nucleotide from the reference sequence seen in at least 25% of the reads aligned to a given position were called as a variant.

Analysis was performed with more focus on all 18 known *BBS* genes for which the coverage was 96%, 97% and 97% at eightfold depth of coverage for the three samples. First, all the synonymous variants were removed. Then all the common variants (minor allele frequency >1%) reported in public databases were removed keeping our focus on rare variants only (minor allele frequency ⩽1). The dbNSFP database for predicting the functional effect of the missense variants was used.[30] All missense variants predicted to be benign based on the dbNSFP database were removed from the list of variants. At last, confirmation of variants detected by exome sequencing was performed with Sanger sequencing of the specified region.

Final screening for mutation detection
Finally, six other *BBS* genes including *BBS3*, *BBS4*, *BBS6*, *BBS7*, *BBS9* and *BBS12*, which account for about 20% of BBS patients, were screened in the remaining patients. Methods used for designing primers, sequencing and variant calling were the same as those used in the initial screening of three commonly mutated *BBS* genes. Eighty amplicons were sequenced for covering all 72 coding exons of the six *BBS* genes in this step.

To study the cosegregation of the detected variants, all the family members were analyzed for the specific variant, using Sanger sequencing. In cases of novel variants, additional 100 normal Iranian chromosomes were screened.

RT–PCR analysis
Total RNA was extracted from fresh blood using QIAamp RNA Blood Mini Kit (Qiagen, Hilden, Germany) or established Epstein–Barr virus-transformed lymphoblastoid cell lines with the help of RNeasy Mini Kit (Qiagen) and the following cDNA synthesis was carried out by QuantiTect Reverse Transcription Kit (Qiagen).

To investigate the effect of potentially splicing mutations on the expressed, novel variants located in or near splice sites were assessed by reverse transcription-polymerase chain reaction (RT–PCR) using primers designed to amplify the specified regions on cDNA to confirm the variant effect on the expression level. Amplified RT–PCR products were analyzed on 2% agarose gel and confirmed by Sanger sequencing of the purified bands with the help of QIAquick Gel Extraction Kit (Qiagen), according to the manufacturer's protocol.

RESULTS
Screening of the three most commonly mutated genes (*BBS1*, *BBS2* and *BBS10*) results in the identification of causal mutations in *BBS2* gene for four patients. That includes a nonsense mutation (p.Gln335*), two splicing variants (c.471G>C and c.1910+1G>T) and one frameshift insertion (c.256_278dup23). No mutation was found in *BBS1* and *BBS10* genes (Table 2).

RT–PCR analysis for the identified novel splicing mutation (c.1910+1G>T) in *BBS2* gene revealed an unexpectedly 200 bp band, resulting in two alternatively spliced mRNA for *BBS2* that is deletion of exon 14 and/or 15 for the mutated allele.[31] Gel purification and Sanger sequencing of the cDNA products revealed

Table 1 Clinical evaluation of all the patients in this study

Patient code	Consanguinity	Ethnicity	Family history	Obesity[a]	Visual symptoms						Learning[a] disability	Polydactyly[a]		Renal[a] anomaly	Hypogonadism[a] (male)	Speech disorder	Other symptoms
					Rod–cone dystrophy[a]	RP	Cataract	Night blindness	Strabismus	Myopia		Hand[a]	Feet[a]				
34754	Y	Fars	Y	Y	N/A	Y	N	Y	N	N	Mild	N	Y	Y	N/A (female)	Y	
34885	Y	Zaboli	Y	Y	Y	N/A	Y	N/A	Y	N	Moderate	Y	Y	N	N/A	N/A	
38498	Y	Afghan	N	Y	N	N	N	N	N	Y	Mild	Y	Y	Y	N/A (female)	N	IUGR Hyperactivity/astigmatism
38931	Y	Fars	N	Y	N/A	Y	N	N	N	N/A	Mild	Y	Y	N/A	Y	N	Cryptorchidism/brachydactyly
34792	Y	Mazani	N/A	Y	N	Y	N	N	N	Y	N	Y	Y	N	Y	N/A	Nystagmus/congenital cardiac anomaly/Hirschprung syndrome/brachydactyly
41931	Y	Turk	Y	Y	N/A	N/A	Y	Y	Y	N/A	Moderate	Y	Y	N/A	N/A	Y	Behavioral problem /developmental delay/dental anomaly/Hirschprung syndrome
37709	N	Southern	N	Y	N/A	N/A	N	N	N	Y	N	Y	Y	N	N/A(female)	N	Short stature/brachydactyly/syndactyly/increased creatinine/glycosuria/mild renal cortical atrophy and parenchymal disease
35201	Y	Southern	Y	Y	N	Y	N	N	N	Y	N	N	Y	Y Multiple renal cyst	N/A (female)	N	Diabetes insipidus/brachydactyly/urethra, general abnormality/renal dysplasia/hydronephrosis/vaginal atresia/malformed uterus
36000	Y	Southern	Y	Y	N/A	Y	N	N	N	Y	N	N	Y	N	Y	N	Behavioral problem/hypothyroidism/growth delay
40568	Y	Fars	Y	Y	Y	N/A	N	Y	N	N	Y	Y	Y	Y Multiple renal cyst	Y	N	Club foot/behavioral problem
34881	Y	Fars	Y	Y	Y	N/A	N	N	Y	N/A	Severe to profound	Y	Y	N	N/A (female)	Y	Hirschprung syndrome/poor coordination
40302	N	Fars	N	Y	N/A	Y	N	N	N	Y	N	Y	Y	Y	Y	Y	Nystagmus/congenital cardiac anomaly/behavioral problem/Hirschprung syndrome/brachydactyly/IUGR
34842	Y	Fars	Y	Y	N	N	N	N	N	N	Mild	Y	Y	N/A	N/A (female)	N	Congenital cardiac anomaly(ASD/TR)/behavioral problem/hyperactivity/poor concentration/brachydactyly
34677	Y	Fars	Y	Y	N/A	N/A	N	N	N	Y	Mild to moderate	Y	Y	N	Y	N	Brachydactyly/clinodactyly

Abbreviations: ASD, atrial septum defect; IUGR, intrauterine growth retardation; N/A, not available; TR, tricuspid regurgitation.
[a]The cardinal feature in classic Bardet–Biedl syndrome.

Table 2 Mutations identified in this study

Patient no.	Gene	Accession no.	Gene locus	Variation	Type of variation	Genotype	Recurrence in Iranian patients	Reference
34754	BBS2	NM_031885.3	16q21	c.1003C>T p.(Gln335*)	Nonsense	Homozygote	1	This study
34885	BBS2	NM_031885.3	16q21	c.471G>C p.(T157T) (IVS3 ds G-C −1)	Splicing	Homozygote	1	This study/IVS3 ds G-T −1: Deveault et al.[34]
38498	BBS2	NM_031885.3	16q21	c.256_278dup23 p.(Val94Serfs*8)	Duplication	Homozygote	1	Hjortshøj et al.[36]
38931	BBS2	NM_031885.3	16q21	c.1910+1G>T	Splicing	Homozygote	1	This study
34792	BBS9 (PTHB1)	NM_198428.2.	7p14	c.1789+1G>A	Splicing	Homozygote	1	Nishimura et al.[52]
41931	BBS9 (PTHB1)	NM_198428.2.	7p14	c.263+1G>A r.776_777 ins ATAA	Splicing	Homozygote	1	This study
37709 35201	BBS12	NM_001178007.1	4q27	c.1156_1157 CG>TA p.Arg386*	Nonsense	Homozygote	2	This study
36000	BBS12	NM_001178007.1	4q27	c.2019del p.(Trp673Cysfs*7)	Deletion	Homozygote	1	This study
40568	BBS4	NM_033028.4	15q22.3-q23	c.77_220del p.(Pro27_Ala74del)	Deletion	Homozygote	2	Mykytyn et al.[33]
34881	BBS4	NM_033028.4	15q22.3-q23	c.829G>A p.(Gly277Arg)	Missense	Homozygote	1	This study
40302	BBS7	NM_176824.2	4q27	c.1342_1346 del CAGGC p.(Gln448Argfs*13) c.1443T>A p.(Cys481*)	Deletion/ nonsense	Compound heterozygote	1	This study

that parents are heterozygous and the affected child is homozygous. Both alternatively spliced transcripts were observed in all members of the family and not the control samples. These clearly confirm the deleterious effect of this novel splicing mutation on BBS2 gene product (Figure 1).

Regarding the high conservation of the last guanine nucleotide and its possible role in splicing, the homozygous substitution in BBS2, c.471G>C (IVS3 ds G-C −1/p.T157T), is considered to have drastic impact on splicing machinery. As there was no further access to the family members, variant verification on RNA level was not feasible.

Whole exome sequencing for three of the patients revealed a novel BBS4 homozygous mutation (p.Gly277Arg) in one patient (Table 2). No pathogenic variation was identified in the coding regions of all the 18 known BBS genes in two other patients, suggesting possible presence of noncoding pathogenic variations in known genes or the possibility of novel genes for BBS phenotype in these patients.

In the final screening, direct sequencing of other six BBS genes resulted in the identification of causal mutations for seven other patients in BBS4, BBS7, BBS9 and BBS12. Those mutations include one deletion (c.77_220 del144) in BBS4, a compound heterozygous mutations (c.1342_1346 del CAGGC and p.Cys481*) in BBS7 in a non-consanguineous family, two splicing mutations in BBS9 (c.263+1G>A, c.1789+1G>A), one nonsense mutation (p.Arg386*) and one deletion (c.2019del) in BBS12 (Table 2).

RT–PCR analysis of the novel splicing mutation (c.263+1G>A) in BBS9 revealed neither exon skipping nor intron retention. This splice donor variant is reported as rs137962929 in dbSNP137 with a minor allele frequency of 0.0077 (Exome Variant Server, evs.gs.washington.edu/) and there is no report regarding its pathogenicity in HGMD mutation database causing BBS.[32] RT–PCR products for all the family members, including parents, two affected children and one normal child, were not differentiable on agarose gel. Sanger sequencing of the PCR products revealed the addition of four

nucleotides (ATAA) in mature mRNA in affected individuals leading to a premature stop codon (r.776_777 ins ATAA/ p.Gly89*), which cosegregates in the family. It should be noted that previous sequencing of the genomic regions did not reveal any insertion, proposing the disturbance in splicing machinery. The inserted ATAA could be originated from the third intron between these two exons, which has at least 14 ATAA repeats in its sequence, as well as at the beginning of the third exon's sequence (Figure 2).

Deletion of exons 3 and 4 (c.77_220del144) of BBS4 gene in one family was confirmed by RT–PCR. This known deletion was previously observed in another Iranian patient (unpublished data), suggesting that it is a recurrent BBS4 mutation in Iranian BBS patients.[33]

It is noteworthy to mention another mutation: c.1156–1157 CG>TA (p.Arg386*) in BBS12 gene that is identified in two out of three BBS12 disease-associated patients of this study, which is a small-scale evidence for its predominance in BBS12-related Iranian patients.

DISCUSSION

This study was performed to characterize responsible genes and mutation spectrum in a cohort of 14 Iranian families with BBS. In total, nine BBS genes (BBS1, BBS2, BBS3, BBS4, BB6, BBS7, BBS9, BBS10 and BBS12) were completely screened in these patients and the causal mutations in 12 of them were identified in five BBS genes.

Generally, the highest proportion of contributing genes in BBS belongs to BBS1 and BBS10, accounting for 23% and 20% of the cases, respectively. It should be noted that these contributions varies in different populations as the proportion of BBS1 and BBS10 extends to 40–50% in Northern European patients. In addition, the most common reported mutations are M390R in BBS1 and C91fs*95 in BBS10.[2,14] Screening the three most prevalent genes: BBS1, BBS2 and BBS10 in this study showed no mutation in BBS1 and BBS10, which is quite different from other populations, whereas 28.6% of patients of
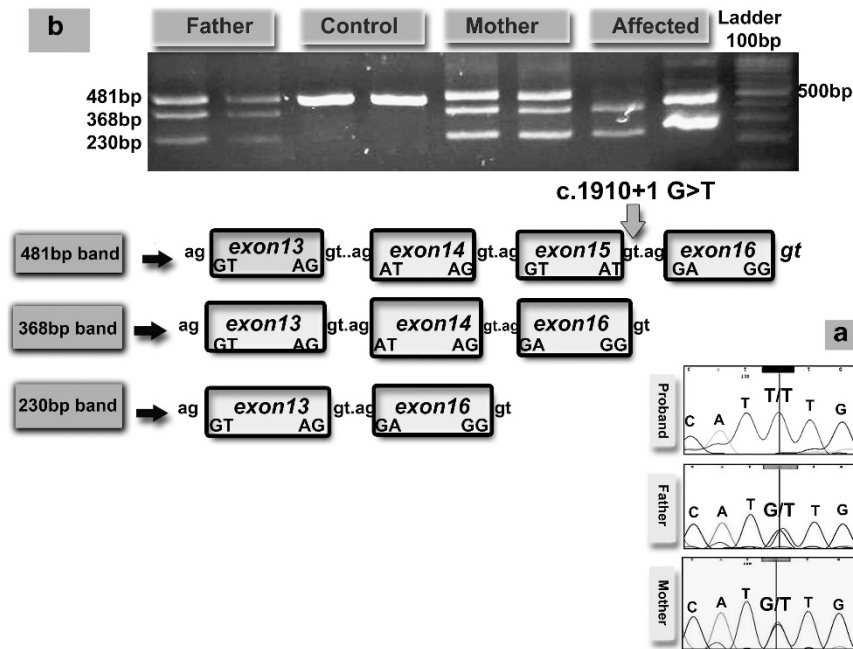
Figure 1 (**a**) Sanger sequencing result on genomic level confirmed cosegregation of the splicing variant in the family. (**b**) Reverse transcription-polymerase chain reaction (RT–PCR) analysis for the novel identified splicing variant: c.1910 + 1 G>T in *BBS2* gene. These entire bands were gel purified followed by sequencing. As schematically shown, the 481 bp band on gel electrophoresis indicated the presence of normal allele in parents as well as in the control sample, whereas there is no sign of amplification for the affected child. The second band, 368 bp, is the product of exon 15 skipping due to the abnormal splicing. The smallest band represents skipping of both exons 14 and 15 in all members of the family comparing to control samples. A full color version of this figure is available at the *Journal of Human Genetics* journal online.
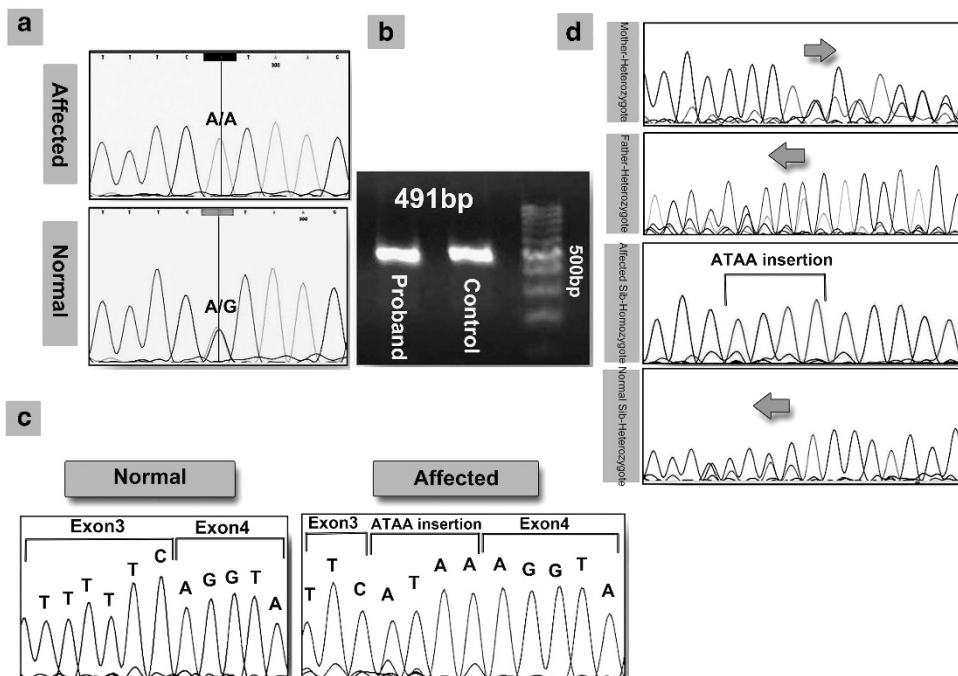

Figure 2 (**a**) Sanger sequencing on genomic level revealed c.263 + 1G>A in *BBS9*. (**b**) Reverse transcription-polymerase chain reaction (RT-PCR) analysis for the novel identified splicing variant: c.263 + 1G>A in *BBS9* gene. The amplified products in the affected individual showed the same size as the control sample. (**c**) Sanger sequencing of the amplified region of cDNA revealed splicing inaccuracy or inefficiency that leads to insertion of ATAA on RNA level. (**d**) Sanger sequencing result on cDNA confirmed cosegregation of the splicing variant in the family. A full color version of this figure is available at *Journal of Human Genetics* online.

this study carried *BBS2* mutations compared with the reported average contribution of 8–12% for *BBS2* in most populations.[34] It is noteworthy to mention that mutation hotspots are not common among *BBS* genes, except M390R in *BBS1* and also C91fs*95 mutations, which are commonly (48%) found in patients with *BBS10* disease-associated alleles in many populations such as European, as well as Middle Eastern and Arab populations.[2,35] The absence of *BBS1* and *BBS10* disease-associated mutations and also higher frequency of *BBS2* mutations in a cohort of BBS patients with Iranian origin is completely different from Caucasian populations.

Although recognizable genotype–phenotype correlation is absent in BBS, there are some reports of milder phenotypes in *BBS1*- and *BBS12*-related disease compared with *BBS10*.[34] Hjortshøj *et al.*[36] observed that milder phenotype was mostly observed in patients who carried recurrent p.M390R mutation in the *BBS1* gene. They concluded that the observed milder phenotype in *BBS1* disease-associated patients could be explained by the type of mutation (missense in *BBS1*) compared with mostly truncating mutations in *BBS10*. The majority of our patients carry truncating mutations in *BBS* genes. This is consistence with the observation of mostly severe symptoms in our patients.

The only missense variant in this study was c.829G>A (p.Gly277Arg) identified by whole exome sequencing in the *BBS4* gene. Bioinformatic predictions and multiple sequence alignment revealed high conservation for the Gly277, with deleterious effects on the protein level if this substitution happens (Figure 3). *BBS4* is one of the conserved BBS proteins constituting BBSome, a protein complex with a role in biogenesis of primary cilium.[37] It is proposed that centriolar satellites transport BBSome to the basal body of the cilium and the *PCM1* helps the aggregation of BBSome by chaperonic functions. There are published data on interactions between BBS4-PCM1 and BBS8-PCM1, which indicates association of BBSome via these two proteins.[36] The Gly277 in *BBS4* is located in the region

necessary for interaction with PCM1 protein. In the other word, this missense variant seems to disrupt the association between BBSome and PCM1 that may lead to its incorrect or disrupted transport. All these data together suggest the pathogenicity of c.829G>A (p.Gly277Arg) in this patient (Figure 3).

Four out of 12 variants identified in this study were splicing variants. This was not beyond our expectation, as almost 10% of all the mutations reported in diseases can be caused by variants located in splice site sequences.[38] This percentage could be extended to even 50% of disease-causing variants in genes with large number of exons.[39] As it has been proposed such mutations could display different effects on pre-mRNA splicing process, such as, exon skipping, intron retention, introducing novel splice sites within exonic or intronic regions or activating cryptic splice sites located in the vicinity.[40] These splicing effects are explained by the examples identified in the present work.

Regarding the RT–PCR results of c.1910+1 G>T in the *BBS2* gene, the reason may lie in the essence of splicing reactions. In this case, there might be a cryptic splice site, which is activated under the impact of this substitution leading to deletion of both exons in the mature product. However, this activation seems to be not fully penetrant owing to the presence of PCR product including deletion of only one exon. Overall, regardless of whether one or two exons are deleted in the mature mRNA, this splice site variant removes at least a chain of 37 amino acids that might disrupt BBS2 protein interaction mostly with BBS7 and BBS9 proteins in BBSome complex.[37] As stated, impairment of pre-mRNA splicing could be due to mutations in every sequence of the gene, but mostly located in highly conserved 5′ and 3′ locations. It is clear that there are other nucleotides in the vicinity that are highly conserved and their alterations might affect the normal pre-mRNA splicing reactions such as c.471G>C (IVS3 ds G-C −1/p.T157T) in *BBS2* in the present work.[39] It is noteworthy to mention that c.471G>T (p.T157T) has been reported in two patients
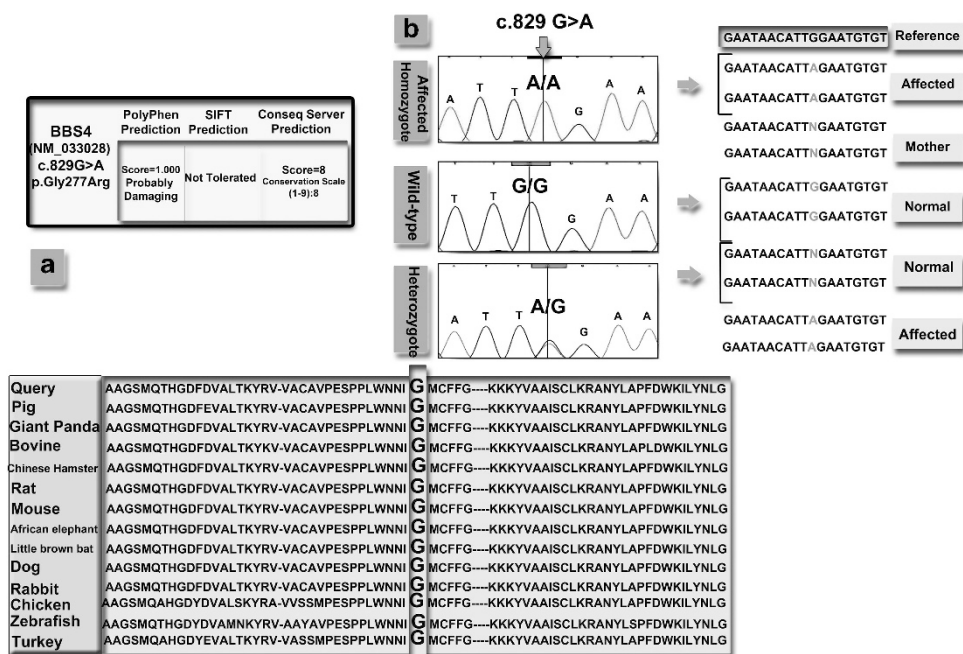

Figure 3 (a) Bioinformatic predictions and multiple sequence alignment (obtained from PolyPhen2 software) revealed high conservation for the Gly277 with destructive effects on the protein level if c.829G>A happens in *BBS4*. (b) Cosegregation of c.829G>A in family members is shown. A full color version of this figure is available at *Journal of Human Genetics* online.

originating from Lebanon. Although the nucleotide change on genomic level is different (G>T in the study by Deveault et al.[34] and G>C in our study), the repeated report of c.471G substitution in BBS patients, who interestingly all come from Middle East, make another strong reason for involvement of this variant in the disease.[34]

In addition, based on the RT–PCR analysis, the impact of c.263+1G>A in BBS9 leads to creation of premature stop codon (PTC). This result could be explained by the fact that such errors in splicing regions could cause inefficient or inaccurate splicing reactions leading to the generation of PTC and inducing probable downstream reactions of nonsense-mediated decay followed by mRNA degradation, which is in fact the surveillance mechanism for the cell.[41–44] Regarding the location of PTC, which confines upstream of the 3′-most exon–exon junction with more than 50–55 nucleotides, nonsense-mediated decay might be activated to destroy the mature mRNA yielding no protein product.[45] However, the presence of the specific mRNA in our study could be explained by the fact that translation is considered as the prerequisite of nonsense-mediated decay, meaning that mature mRNA will not be degraded unless the translation begins in the cytoplasm.[44]

According to the results achieved by this study, no mutation was identified in the following genes: BBS1, BBS3, BBS6 and BBS10.

BBS3 (ARL6) is one of the GTP-binding proteins with essential role in proper localization of BBSome.[19] Regarding its unusual predominance in Saudi patients with BBS compared to the rare frequency of 0.4% in Caucasian populations plus an additional previous report of BBS3 in an Iranian family, a decision was made to screen the gene in the cohort of this study.[46,47] However, no mutation was identified in our cohort that reaffirms the BBS3 low frequency same as the Caucasian population.

Overall, nine BBS genes (BBS1, BBS2, BBS3, BBS4, BBS6, BBS7, BBS9, BBS10 and BBS12) each that accounted for >5% of BBS patients in previous reports were completely screened. However, the mutation frequency for BBS4 and BBS7 was just 2%. These two genes were selected based on the published higher frequency for BBS4 in Middle Eastern populations and previous observation of BBS7 in a cohort of 136 Iranian families with intellectual disability.[48]

This study represents the distribution of causal mutations among Iranian BBS patients, which is significantly different from the other reports, especially from Caucasian populations. The commonly mutated BBS1 and BBS10 genes were not mutated in our patients and may not have an important role in Iranian patients while other genes such as BBS2 (28.6% in this study), BBS4 (~14% of this study), BBS12 (21.4% in this study) and BBS9 (~14% of this study) seem to have a greater role in Iranian patients with BBS compared with other populations.

Although a small sample size is presented in this study, the obtained results indicated high and nearly the same prevalence for both BBS2 and BBS12 in our cohort of Iranian patients. There is not a common statement about the choice of BBS2 as the third most mutated gene. As proposed by Muller et al.,[49] regarding the small coding region of BBS12, this gene could be considered in diagnostic approaches before BBS2, for efficient and rapid genotyping. However, this should not cause the underestimation about the large proportion of BBS2 among the patients in diagnostic approaches.[49] These results suggest a different strategy in molecular genetics diagnostic approaches for BBS in Iran and possibly other Middle Eastern countries. However, the advent of high-throughput sequencing in recent years has changed the molecular diagnostic protocols for such disorders with clinical and genetic heterogeneity.[50] The importance of

studies such as the present one is represented by identifying the most common causes of this disorder in each population, which could elucidate an algorithm for clinical genetic practice that would clarify the screening protocols before applying more advance technologies such as next-generation sequencing.[51] In conclusion, this study represents another strong indication for heterogeneity of the disorder and extends the mutational spectrum of the known BBS genes by introducing nine novel mutations in five BBS genes.

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