Research Article

Identification of Missense Mutation (I12T) in the BSND Gene and Bioinformatics Analysis

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Nonsyndromic hearing loss is a paradigm of genetic heterogeneity with 85 loci and 39 nuclear disease genes reported so far. Mutations of BSND have been shown to cause Bartter syndrome type IV, characterized by significant renal abnormalities and deafness and nonsyndromic hearing loss. We studied a Pakistani consanguineous family. Clinical examinations of affected individuals did not reveal the presence of any associated signs, which are hallmarks of the Bartter syndrome type IV. Linkage analysis identified an area of 18.36 Mb shared by all affected individuals between markers D1S2706 and D1S1596. A maximum two-point LOD score of 2.55 with markers D1S2700 and multipoint LOD score of 3.42 with marker D1S1661 were obtained. BSND mutation, that is, p.I12T, cosegregated in all extant members of our pedigree. BSND mutations can cause nonsyndromic hearing loss, and it is a second report for this mutation. The respected protein, that is, BSND, was first modeled, and then, the identified mutation was further analyzed by using different bioinformatics tools; finally, this protein and its mutant was docked with CLCNKB and REN, interactions of BSND, respectively.

1. Introduction

Deafness or hearing loss can be due to genetic or environmental causes or a combination of both. The genetic hearing loss is classified as syndromic or nonsyndromic. Among the many disorders classified as syndromic hearing loss, the pathology varies widely, but in nonsyndromic hearing loss, the defect is generally sensorineural. Seventy percent of deafness has genetic causes and is classified as nonsyndromic. Autosomal recessive nonsyndromic deafness is genetically heterogeneous and is the most common form of inherited hearing loss. Autosomal recessive genes are responsible for about 77% of the cases of hereditary nonsyndromic deafness, with over 85 loci and 21 different genes identified to date (<http://hereditaryhearingloss.org/>). The high degree of genetic heterogeneity of deafness reflects the great diversity of specialized proteins that are required to make sense of sound, and continuing discovery of common and rare mutations associated with deafness in humans has provided

many serendipitous points of entry into the biology of hearing.

Bartter's syndrome (BS) is characterized by hypokalemic, hypochloremic metabolic alkalosis with normal or low blood pressure despite high plasma renin activity and serum aldosterone. The inheritance pattern is autosomal recessive. Antenatal BS with bilateral sensorineural deafness (BSND) was first described in children born to a consanguineous couple from a Bedouin family of Southern Israel [1]. BS type IV, BSND variant, occurs because of a mutation in the BSND gene on chromosome 1p31 coding for protein "barttin" which forms the β subunit of CICKb and CICKa channels located on the basolateral membrane of TAL and inner ear epithelium [2]. The clinical features of Bartter syndrome type IV include sensorineural deafness and peculiar facies, distinguished by the triangular face, large eyes, and protruding ears (1).

Mutation in BSND gene is analyzed by model prediction using SAM.T08 server. Comparison of the protein sequence

of BSND with closely related species takes place and find out conserved regions. Other bioinformatics analysis such as protein modeling and proteins docking are also carried out for more bioinformation.

2. Materials and Methods

Before the onset of the study, approval from the Institutional Review Board, Islamabad, and informed consent was obtained from all individuals. Clinical examination was performed at hospitals of the respective area. Audiometry was performed on selected individuals to detect the level of hearing loss. The families were visited at their places of residence to generate pedigrees [3] and collect other relevant information. Blood samples from available affected and normal individuals of each family were collected for DNA extraction.

2.1. Genotyping and Linkage Studies. High molecular weight DNA was extracted from leukocytes following the standard method as described by Sambrook et al. [4]. Genomic DNA was quantified by spectrophotometer readings at OD₂₆₀ and diluted to 40 ng/μL for amplification by polymerase chain reaction (PCR).

To elucidate the gene defect in the family presented here, an initial search for linkage was carried out by using polymorphic microsatellite markers mapped within autosomal recessive nonsyndromic deafness loci listed on the hereditary hearing loss homepage [5]. Genome-wide screening was conducted with microsatellite markers (Linkage Mapping Set 10, Invitrogen, USA). Two-point linkage analysis was carried out using MLINK of the FASTLINK computer package [6]. Multipoint linkage analysis was performed using ALLEGRO [7]. For the analysis, an autosomal recessive mode of inheritance with complete penetrance and a disease allele frequency of 0.001 were assumed. Equal allele frequencies were used in the analysis. However, since it is well known that using allele frequencies, which are too low, can lead to false positive results, a sensitivity analysis was performed. Haplotypes were constructed using SIMWALK2 [8, 9].

2.2. Mutation Screening. To screen for mutation in the BSND gene, exons and splice junction sites were PCR amplified from genomic DNA using the four primer sets. The purified PCR products were subjected to cycle sequencing using big dye terminator V 3.1 ready reaction mix and sequencing buffer (PE Applied Biosystems, Foster City, Calif, USA). The sequencing products were purified to remove unincorporated nucleotides and primers with Centriflex™ Gel Filtration Cartridge (Edge Biosystems, Gaithersburg, Md, USA). These purified products were resuspended in 10 μL of TSR (Template Suppression Reagent) and were placed in 0.5 mL septa tubes to be directly sequenced in an ABI Prism 310 Automated Sequencer (PE Applied Biosystems, Foster City, Calif, USA). Chromatograms from normal and affected individuals were compared with the corresponding control gene sequences from NCBI (National Center for Biotechnology Information) database to identify the aberrant nucleotide base-pair change. (<http://www.ncbi.nlm.nih.gov/>).

2.3. Protein Modeling, Docking, and Phylogenetics Analysis. SAM.T08 [10–14] was used to model the BSND protein and its interacting protein with highest confidence score (i.e., 0.990) CLCNKB. For their further evaluation, RAMPAGE server [15] was considered. ClustalW program was subjected to analyze the phylogenetics relationship of different animals having BSND protein. Conserved amino acids were predicted by using UniportKB tool available on ExPASy server.

Identified BSND mutation was generated by using Swisspdb server. Protein interactor of BSND was found out by STRING server, an online database of known and predicted protein interactions which includes direct (physical) and indirect (functional) associations [16]. Protein docking of both the normal and the mutant BSND was carried out using HEX tool with its interactive proteins CLCNKB and REN.

3. Results

3.1. Clinical Assessments. Ages of affected individuals varied between 12 and 65 years at the time of study. Clinical examination of the hearing impaired individuals did not reveal the presence of signs or symptoms, which are hallmarks of the Bartter syndrome type IV. Urinary and blood biochemistry was tested at the Islamabad Diagnostic Centre, including testing for liver function, renal function, electrolytes, and hematology. Blood hematology and biochemistry markers were within the normal range, except that renin level was at borderline in one of the affected subject (VI: 5, 2.53 mL/h). Detailed clinical and biochemical features of normal and affected members are mentioned in Table 1. The height and weight were within normal range for the local population. An audiometric evaluation of selected affected members by measuring the threshold of hearing at 250–8000 Hz for pure-tone air conduction and bone conduction showed severe hearing loss across all frequencies.

Renal sonography ruled out the presence of nephrocalcinosis or metabolic alkalosis in these affected individuals. Nor have the affected individuals displayed problems with polydipsia, polyuria, nocturnal enuresis, and hypocalciuria.

3.2. Genotyping Results. Analysis of the results obtained from genome search performed on the ten individuals of the family (III-1, III-3, IV-1, V-1, V-2, V-4, V-5, VI-1, VI-3, and VI-5) (Figure 1) identified an area of interest on chromosome 1. Two-point analysis generated LOD score of 2.027 at marker D1S193 (73.21 cm) and 2.05 at marker D1S3462 (247.23 cm) on chromosome 1 (Table 2). In order to test linkage to these two regions, additional markers located in the vicinity of D1S3462 and D1S193 were chosen from Marshfield map [17] and genotyped in all the ten family members. The maximum two-point LOD score was increased to 2.55 with marker D1S2700. Multipoint linkage analysis for the family derived a maximum LOD score of 3.42 at marker D1S1661 (Table 3). The three-unit multipoint support interval contained a 10.48 Mb region, which span from markers D1S3721 to D1S2690. Haplotypes analysis delimited the centromeric boundary defined by a recombination between markers D1S1596 and D1S2770

TABLE 1: Clinical and biochemical features of normal and affected members of studied family.

Features	Reference ranges	Normal members		Affected members from the family with hearing loss		
		V:3	V:5	V:1	VI:3	VI:5
Age (y)		30	35	45	12	10
Other symptoms of BSIV		no	no	No	No	No
S ^d ·Na (mmol/L)	136–148	141	140	135	137	134
S·K (mmol/L)	3.6–5.0	3.9	4.6	3.4	3.5	3.5
S·Cl (mmol/L)	104–114	105	107	97	102	101
S·Mg (mg/dl)	1.9–2.5	2.3	2.2	2.0	1.9	2.1
S·HCO ₃ (mmol/L)	17.5–27.5	30.0	27.5	30.0	30.2	31.0
S·Ca (mg/dl)	8.6–10.5	8.7	9.5	9.2	9.8	10.0
S·Creatinine (mg/dL)	0.85–1.35	0.7	0.8	0.6	0.5	0.4
P ^e Renin (ng/ml/hr)	0.15–2.33	0.31	0.6	2.13	2.03	2.53
S·Aldosterone (ng/dL)	1–6; ^f 4–31	>1.7 ^f	13.0	10.3	11.9	6.3
S·Osmolality (mosm/kg)	275–295	293	289	281	290	287
U ^g Na (mmol/L)	30–150	17.5	154.6	39.1	16.9	13.3
U·K (mmol/L)	20–67	3.4	91.31	12.13	8.43	4.12
U·Ca (mg/dL)		6.0	16.0	15.3	11.3	13.0
U·Mg (mg/dL)		3.6	14.4	3.1	2.4	1.12
U·Cl (mmol/L)	46–168	16.3	174.3	46.56	19.2	10.5
Uosmolality (mosm/kg)	50–1400	74	903	179	105	56
NephrocacinosiS		Absent	Absent	Absent	Absent	Absent

in individual V-1. Recombination event between D1S2706 and D1S3721, defined the telomeric boundary in the same individual (V-1). Therefore, the minimum critical region of 18.36 Mb identified for disease locus and shared by all the affected individuals between the markers D1S2706 and D1S1596. The critical interval (17.27 cm) identified in this Pakistani family overlaps with the critical region to which DFNA2 was mapped on 1p34 in a large Indonesian family with autosomal dominant, progressive and sensorineural hearing loss [18]. The DFNA2 locus maps between markers D1S255 (65.47 cm) and D1S211 (73.81 cm), and thus shares a region of 2.68 cm with DFNB interval identified in our family. Riazuddin et al. [19] identified 1.5 Mb in four kindred segregating nonsyndromic deafness at the chromosome 1.

3.3. Mutation Analysis of Candidate Genes. Through a database search, we identified several genes mapping between the linkage interval in the family (Human Genome Project-Santa Cruz; <http://genome.ucsc.edu/>, May 2004). Among these, KCNQ4 (MIM 603537), CLDN19, FOXE3 (MIM 601094), FOXD2 (MIM 602211), TSPAN1, and BSND (MIM 606412) were plausible candidate candidates in the interval of our family. We started sequencing of these and completely sequenced two genes (KCNQ4 and CLDN19), but we found no mutation in both. Recent studies of Riazuddin et al. [19] showed that mutation in BSND can cause nonsyndromic deafness and is the molecular basis of

DFNB73 locus in four Pakistani families segregating non-syndromic deafness [19]. As the critical interval identified in the family contains potential candidate gene barttin (BSND, MIM 606412) as well; therefore, it was sequenced to search for the mutation. Sequence analysis of exon 1 of the BSND gene in our family also revealed missense mutation involving T to C transition at nucleotide number 35 (35T > C), which resulted in substitution of isoleucine to threonine at amino acid position 12 (I12T) (Figure 2). BSND mutation; that is, p.I12T cosegregated in all extant members of the pedigree. This mutation was present in the heterozygous state in obligate carriers within the family.

3.4. Bioinformatics Analysis. Models of BSND CLCNKB and REN proteins were generated by SAM.T08 server and visualized by Rasmol (Figure 3(a)–3(c)). Rampage values for BSND were number of residues in favored region 95.6%, the number of residues in allowed region 3.1%, and the number of residues in outlier region 1.4% (Figure 4(a)); however, the CLCNKB and REN values were little varying. Its values were the number of residues in favored region: 94.8%, 97%, the number of residues in allowed region: 4.2%, 3%, and the number of residues in outlier region is 0.9% for CLCNKB and 0% for REN (Figure 4(c)).

Comparison of conserved amino acid of human BSND with closely related species like mouse, rat, and rabbit shows that isoleucine at position 12 is highly conserved, so

FIGURE 1: Pedigree of family with nonsyndromic autosomal recessive hearing loss associated haplotypes. Haplotypes for the most closely linked STRPs are shown below each symbol. The genetic map distances according to the Marshfield genetic map in centimorgans (cM) are shown in parenthesis next to the marker name. Arrows adjacent to the haplotypes indicate key recombination events. The alleles are denoted 1–3 according to their allele size.

TABLE 2: Two-point LOD score results between the locus identified in family and chromosome 1p34.2–p32.1 markers.

Marker	deCode map postion ¹	Marshfield map postion ²	Physical map postion ³	LOD score AT $\theta =$					
				0.00	0.01	0.05	0.1	0.2	0.3
D1S472	57.95	65.47	37,102,828	−999.99	−0.68	−0.05	0.14	0.20	0.13
D1S186	60.14	67.22	37,900,316	−999.99	−0.069	−0.10	0.05	0.10	0.06
D1S432	—	69.86	39,046,164	−1.334	−0.69	−0.18	−0.02	0.03	0.01
D1S1598	63.51	70.41	40,011,183	−1.544	−0.61	−0.09	0.03	0.04	0.00
D1S2706	65.31	71.13	40,741,361	−999.99	−1.34	−0.18	0.15	0.24	0.14
D1S3721	65.87	72.59	41,395,560	2.027	1.97	1.75	1.49	1.49	0.56
D1S193	66.64	73.21	42,688,369	2.027	1.97	1.75	1.49	0.99	0.56
D1S2713	67.87	73.81	44,182,569	1.726	1.67	1.47	1.24	0.80	0.43
D1S2134	71.29	75.66	47,993,241	1.690	1.66	1.52	1.32	0.91	0.52
D1S1661	72.42	78.25	51,142,551	1.228	1.19	1.06	0.90	0.60	0.33
D1S2652	77.15	80.77	55,178,852	1.319	1.28	1.13	0.96	0.62	0.34
D1S2690	79.63	83.07	56,867,560	2.027	1.97	1.75	1.49	0.99	0.56
D1S2700	83.07	87.31	58,828,955	2.556	2.48	2.21	1.88	1.23	0.66
D1S2770	85.02	88.40	59,442,255	−999.99	−0.26	0.26	0.35	0.29	0.17
D1S1596	—	89.49	59,670,589	−1.55	−0.52	−0.00	0.10	0.08	0.01
D1S2737	85.47	90.58	61,038,037	−999.99	−1.10	0.07	0.40	0.46	0.29
D1S2788	87.25	93.86	61,568,857	−999.99	−2.16	−0.90	−0.46	−0.14	−0.03
D1S230	88.12	95.31	62,314,306	−999.99	−2.16	−0.90	−0.46	−0.14	−0.03
D1S1648	99.62	101.48	73,125,563	−999.99	−2.27	−1.00	−0.55	−0.20	−0.06
D1S1665	99.62	102.02	73,941,402	−999.99	−2.34	−1.04	−0.57	−0.20	−0.06

¹Sex-average Kosambi cM map distances from the deCode genetic map [7].²Sex-average Kosambi cM map distances from the Marshfield genetic map [17].³Sequence-based physical map distance in bases according to the Human Genome Project-Santa Cruz (<http://genome.ucsc.edu/>, Assembly: May 2004).

TABLE 3: Multipoint LOD score results between linked locus identified in family and chromosome 1p34.2–p32.1 markers.

Marker	Physical position	Location	LOD
ATA79C10	—	0.0000	−2.0630
D1S472	37,102,828	3.0940	nan
D1S186	37,900,316	5.1350	nan
D1S432	39,046,164	8.2290	−2.4708
D1S1598	40,011,183	9.2390	−0.0755
D1S2706	40,741,361	10.2490	−8.2103
D1S3721	41,395,560	11.2590	3.0063
D1S193	42,688,369	12.2690	3.2113
D1S2713	44,182,569	14.3100	3.3672
D1S2134	47,993,241	16.3510	3.4190
D1S1661	51,142,551	18.3920	3.4201
D1S2652	55,178,852	21.4860	3.3868
D1S2690	56,867,560	24.5800	3.2867
D1S2700	58,828,955	28.7490	2.8398
D1S2770	59,442,255	29.7590	1.3404
D1S1596	59,670,589	30.7690	nan
D1S2737	61,038,037	31.7800	−inf
D1S2788	61,568,857	34.8730	nan
D1S230	62,314,306	36.9140	−inf
D1S1648	73,125,563	43.3060	−inf
D1S1665	73,941,402	44.3160	nan
GATA152F05L	85,238,770	45.3260	nan

mutation at this point can be significant (Figure 5(a)). The evolutionary relationship between human, mouse, rat, and rabbit were evaluated by phylogenetic analysis. The analysis represents that the BSND of mouse and rat is closely related to each other and shows homology with human and rabbit (Figure 5(b)).

Our mutation (I12T) of BSND was generated by Swis-spdb server and was analyzed by Viewerlite. This substitution of isoleucine with threonine altered the bonding capacity with other side chains as well as other interactors (Figure 6(a)-(b)).

According to STRING database, the physical and functional interaction of BSND was very much significant with CLCNKB protein. Clinical assessment showed the reduced chlorine level in all the affected individuals. According to further clinical assessments, renin level was at border range in one affected individual, so REN protein was also selected to check the effect of the respected mutation on this protein. However, other interactors are COL7A1, ARL2, CLCN5, KCNJ1, CASR, SLC12A1, SLC12A3, and CLCNKA (Figure 7). Both the proteins were docked with HEX v 6.1, taking BSND as receptor and CLCNKB and REN as a ligand. The total energy value calculated for BSND-CLCNKB molecules was −229094.6 and BSND-REN was −1145.7. However, the total energy value for the mutant (I12T) on docking with CLCNKB was maximized to −218782.3 and with REN was −1149.8. This values fluctuation ultimately results in reduced binding affinity with CLCNKB and

FIGURE 2: Representative chromatogram generated by Big Dye Terminator, sequencing of translated exon1 of BSND gene indicating a T to C transition at nucleotide 35 (35T > C) from an affected female individual (VI-1) (a), a heterozygous carrier (VI-1) (b) and a normal male individual (V-2) (c) of the family. The T to C transition at nucleotide position 35 results in isoleucine to threonine change at amino acid 12 (I12T). Arrows indicate the nucleotide change in the sequence.

FIGURE 3: Protein models of BSND (a) CLCNKB (b), and REN (c) generated by SAM_T08 and visualized by Rasmol (display = ribbon; colors = group).

(a)

(b)

FIGURE 4: Continued.

FIGURE 4: Ramapage results of residues for protein model of human BSND protein (a) CLCNKB (b) and REN (c).

increased binding affinity with REN and so may be the case with other interacting proteins.

4. Discussion

Pathophysiological pathway leading from a specific mutation to a specific phenotype has remained elusive in syndromic as well as nonsyndromic hearing loss families. Individuals with the same mutation can fall along a clinical spectrum ranging from asymptomatic to severely affected and can even have completely different diseases with different mutation in the same gene. Our results also support the findings of Riazuddin et al. [19] that pathogenic mutation of BSND gene can cause the nonsyndromic hearing loss. But there are no subclinical renal metabolic changes in our family as reported by Riazuddin et al. [19]. Renin level is at boarder range only in one affected individual, while renal sonography ruled out the presence of nephrocalcinosis in these affected individuals. Nor have the affected individuals displayed problems with polydipsia, polyuria, nocturnal enuresis, metabolic alkalosis, and hypocalciuria. It might be possible that partial loss of function of barttin induced by T12I allele cause only selectively hearing loss and appear to

have less pronounced effects. However, the genotype does not always predict the clinical phenotype, which varies both within and between families carrying the mutation in the same gene, implying the existence of other genetic and/or environmental factors that influence phenotype. Several disease-causing BSND mutations have been identified and functionally analyzed. In all cases, there is a genotype-phenotype relationship in that the level of function of mutant barttin predicts the renal phenotype. Our results support the relationship between missense BSND mutation (I12T) and nonsyndromic hearing loss. It is a secondary report that the BSND as causal gene in nonsyndromic deafness.

The protein sequence of BSND is highly conserved in closely related species of human, mouse, rat, and rabbit, and it contains 2 putative transmembrane domain starting from 7 to 26 amino acid and 31 to 53 amino acid [20].

Bioinformatics analysis of mutation in BSND protein suggest that isoleucine, a hydrophobic amino acid, is being converted into threonine, a polar amino acid, which is more susceptible to posttranslation modifications, affecting the quaternary structure of protein resulting in mutant protein.

Potassium (K^+) secretion by strial marginal cells and vestibular dark cells require Cl^- to recycle in the basolateral membrane via a major Cl^- conductance. This Cl^- conductance is composed of the Cl^- channels CLCNKA/BSND and CLCNKB/BSND. Cl^- channels CLCNKA/BSND and CLCNKB/BSND consist of the pore-forming CLCNKA

FIGURE 7: The interactions of BSND with other functionally similar proteins retrieved by STRING online database. CLCNKB showed highest confidence score, that is, 0.990 with BSND.

α -subunits and CLCNKB and the BSND β -subunit. Mutations of BSND reduce channel conductivity and surface expression [22].

Estévez et al. [2] proved that BSND protein is an essential β subunit for CLCNKA and CLCNKB chloride channels, with which it colocalizes in basolateral membranes of renal tubules and of potassium-secreting epithelia of the inner ear. Disease-causing mutations in either CLCNKB or BSND compromise currents through heteromeric channels [2].

The CLCNKA and CLCNKB channels are members of the ClC family, which comprises at least 9 mammalian chloride channels. Each is believed to have 12 transmembrane domains and intracellular N and C termini. The prototype of the family in torpedo is gated by both voltage and chloride [23].

Docking results of both normal and mutant interaction with CLCNKB suggested that there is a reduced binding affinity on mutation which may results the reduced down regulating or up regulating interactions; however, the mutant does not effect the interaction with renin (REN). So, we can conclude that the boarder range of renin level in one affected individual is not dependent upon this mutation.

Identification of mutation in BSND gene in more families will give valuable insight into the genetic mechanisms underlying nonsyndromic hearing disorder. This work is a fundamental step in the bioinformatics analysis of BSND and provides basics for further analysis.

Electronic-Database Information

- (i) Marshfield Medical Research Foundation database website: <http://research.marshfieldclinic.org/genetics/>.
- (ii) National Center for Biotechnology Information: <http://www.ncbi.nlm.nih.gov/>.
- (iii) UCSC Genome Browser: <http://genome.ucsc.edu/>.

Abbreviations

BSND:	Bilateral sensorineural deafness
CLCNKB:	Chloride Channel, Kidney, B
REN:	Renin
ARF:	ADP-ribosylation factor
I:	Isoleucine
T:	Threonine.

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