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SLC52A2 [p.P141T] and *SLC52A3* [p.N21S] causing Brown-Vialetto-Van Laere Syndrome in an Indian patient: First genetically proven case with mutations in two riboflavin transporters

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ABSTRACT

Background: Brown-Vialetto-Van Laere Syndrome (BVVLS), a rare neurological disorder characterized by bulbar palsies and sensorineural deafness, is mainly associated with defective riboflavin transporters encoded by the *SLC52A2* and *SLC52A3* genes.
Methods: Here we present a 16-year-old BVVLS patient belonging to a five generation consanguineous family from Indian ethnicity with two homozygous missense mutations viz., c.421C>A [p.P141T] in *SLC52A2* and c.62A>G [p.N21S] in *SLC52A3*.
Results: Functional characterization based on ^{3}H-riboflavin uptake assay and live-cell confocal imaging revealed that the effect of mutation c.421C>A [p.P141T] identified in *SLC52A2* had a slight reduction in riboflavin uptake; on the other hand, the c.62A>G [p.N21S] identified in *SLC52A3* showed a drastic reduction in riboflavin uptake, which appeared to be due to impaired trafficking and membrane targeting of the hRFVT-3 protein.
Conclusions: This is the first report presenting mutations in both riboflavin transporters hRFVT-2 and hRFVT-3 in the same BVVLS patient. Also, c.62A>G [p.N21S] in *SLC52A3* appears to contribute more to the disease phenotype in this patient than c.421C>A [p.P141T] in *SLC52A2*.

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1. Introduction

Brown-Vialetto-Van Laere Syndrome is a neurodegenerative disorder characterized by sensorineural deafness, respiratory difficulty, ponto bulbar palsy and muscle weakness due to the involvement of cranial nerves VII, IX and XII [1]. This rare neurological disorder is mostly regarded as autosomal recessive and rarely as autosomal dominant and X-linked inheritance [2]. Recent findings suggest that the severity of this disease is related to riboflavin (RF) deficiency, caused by a defect(s) in either the *SLC52A2* (OMIM: 607882) or the *SLC52A3* (OMIM: 613350) genes that encode RF transporters 2 & 3 (RFVT-2 and RFVT-3), respectively [1,3]. Three RF transporters namely *SLC52A1*/*hRFVT-1* (NM_017986), *SLC52A2*/*hRFVT-2* (NM_033409) and *SLC52A3*/*hRFVT-3* (NM_024531) have been identified in humans and are distributed in a tissue-specific manner [4,5]. The protein sequence of *SLC52A1* is 86.7% identical with that of *SLC52A2* and 44.1% with that of *SLC52A3* [4].

Riboflavin (vitamin B2), a water soluble vitamin is essential for normal cellular functions in its biologically active forms (FMN and FAD), which act as intermediaries in the transfer of electrons in biological oxidation-reduction reactions. Riboflavin transporters (RFVT) play an essential role in the maintenance of RF homeostasis through its absorption in the intestine and reabsorption in the kidney. Several loss-of-function mutations have been identified in *SLC52A2* or *SLC52A3* genes from patients with motor neuron diseases or neuranopathies [1,3,6,7], while mutations in *SLC52A1* have been correlated with glutaric aciduria [8]. The identification of such clinical mutations in *RFVT* genes has led to a better understanding of the pathophysiology of associated neurological disorders and further guided disease management in the affected patients by RF supplementation [9]. In this study, our mutational analysis in a BVVLS patient from Indian ethnicity had identified two homozygous mutations from two different riboflavin transporter encoding genes *SLC52A2* (c.C421A; p.P141T) and *SLC52A3* (c.A62G; p.N21S), which were functionally defective in riboflavin transport activity. Such

Abbreviations: hRFVT, human Riboflavin Transporter; BVVLS, Brown-Vialetto-Van Laere Syndrome; SLC, Solute Carrier; RF, Riboflavin; RFLP, Restriction Fragment Length Polymorphism; DMEM, Dulbecco's Modified Eagle Medium; HuTu, Human duodenum adenocarcinoma cells; U87, Human brain cells; GFP, Green Fluorescent Protein.

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an identification of clinical mutations in both RF transporters *SLC52A2* and *SLC52A3* in a BVVLS patient has been reported for the first time and not been previously reported elsewhere in any ethnicity.

2. Materials and methods

2.1. Clinical testing

All the genetic investigations were performed after getting informed consent as approved by Institutional Ethical Committee of Madurai Kamaraj University, Madurai, India. Blood samples were collected from a 16-year-old BVVLS patient who belongs to a five-generation consanguineous Indian family at Indira Gandhi Institute of Child Health, Bangalore. Detailed clinical records of medical history and scientific examinations were made.

2.2. Genetic testing/mutation screening

DNA was isolated from whole blood using HiPurA blood genomic DNA isolation kit. Polymerase chain reaction was performed to scan for mutations in *SLC52A2* and *SLC52A3* using specific primers designed in intron-exon boundaries (Supplementary Table 1). All 8 exons were sequenced bidirectionally and compared with wild-type *SLC52A2* and *SLC52A3* genes respectively.

To determine the co-segregation of the identified missense mutations that produced either a loss or gain of a restriction site, RFLP analysis was performed with a 219 bp region spanning exon-3 of *SLC52A2* and a 716 bp region spanning exon-2 of *SLC52A3* using specific enzymes *TspRI* and *MnlI*, respectively. The fragments were separated in 12% polyacrylamide gel to differentiate wild-type and mutant alleles. A healthy volunteer donor without a history of BVVLS served as a control.

2.3. In silico analysis

To determine the location of the identified clinical mutations, transmembrane topology of hRFVT-2 and hRFVT-3 was predicted using TMHMM (http://www.cbs.dtu.dk/services/TMHMM/). Pathogenicity of the missense mutations was analyzed using the *in silico* tools Polyphen and Mutation taster. To analyze the conserved nature of the identified clinical mutation, protein sequences of hRFVT-2 and hRFVT-3 from human, cat, dog, and monkey were retrieved from NCBI and multiple sequence alignment was performed.

2.4. Site directed mutagenesis

Site-directed mutagenesis was performed based on *Dpn*I method [10], to generate the mutant constructs using pEGFP-C3-hRFVT-2 and pEGFP-C3-hRFVT-3 as templates with specific mutant primers (Supplementary Table 1).

2.5. Cell culture and transient transfection

Human duodenum adenocarcinoma cells (HuTu-80) and human brain cells (U87) were maintained in DMEM supplemented with 10% FBS, glutamine (0.29 g/L), sodium bicarbonate (2.2 g/L), penicillin (100,000 U/L), and streptomycin (10 mg/L). For RF transport assay, cells were grown in 12-well tissue culture plates and for localization studies, cells were grown in sterile glass-bottomed petri dishes. Cells at 80–90% confluency were transfected with 3 μg of the wild-type and mutant constructs with 3 μL Lipofectamine 2000.

2.6. Riboflavin uptake assay

HuTu-80 cells transfected with wild-type (GFP-hRFVT-2 and GFP-hRFVT-3) and mutant (GFP-hRFVT-2[P141T] and GFP-hRFVT-3[N21S]) constructs of *SLC52A2* and *SLC52A3* were tested for ^{3}H-RF uptake activity by incubating the cells in Krebs-Ringer (KR) buffer containing ^{3}H-RF (14 nM) at 37 °C for 3 min as described previously [11]. Radioactivity was measured using a liquid scintillation counter and normalized with total protein content.

2.7. Live-cell confocal imaging studies

HuTu-80 and U87 cells transfected with wild-type and mutant constructs of hRFVT-2 and hRFVT-3 were imaged using an inverted Nikon C-1 confocal microscopy 24–48 h post transfection as described earlier [11]. Fluorophores were excited using a 488 nm line from an argon ion laser, and emitted fluorescence was monitored with a 530 ± 20 nm band pass (GFP) and the red fluorescent protein (DsRed) was excited with HeNe ion laser at 543 nm.

2.8. Statistical analysis

Data are means ± SE of 3–5 independent experiments. Data were analyzed by one-way ANOVA followed by Tukey's honest significant difference (HSD) test, with statistical significance being set at 0.01 ($^{*}P < 0.01$; $^{**}P < 0.001$).

3. Results

3.1. Clinical observations

The 16-year-old proband of Indian origin was presented with bulbar palsy illness like dysphagia, nasal regurgitation and recurrent aspiration. Respiratory compromise was observed at the age of 9 and deafness was perceived at the age of 11. The patient exhibited typical facial weakness like incomplete closure of eyelids and lower motor neuron limb signs with progressive thinning of muscles. A biochemical profile of the proband showed decreased plasma RF (1.89 μg/L; normal range is 3–15 μg/L) and creatinine levels (0.4 mg/dL; normal range is 0.5–1.5 mg/dL), whereas brain MRI displayed no abnormalities (Fig. 1A). The patient responded well to oral RF supplementation and started to show an improvement in diaphragmatic function by gaining motor function and muscle strength.

3.2. Genetic testing/mutation screening

As a result of mutational screening, two homozygous missense mutations in two different RF transporters were identified in the BVVLS proband. A homozygous novel missense mutation c.C421A; p.P141T was identified in exon-3 of *SLC52A2* and another mutation c.A62G; p.N21S was detected in exon-2 of *SLC52A3*. It is evidenced from the chromatograms that both the mutations were segregated from the parents, as they were heterozygous (Fig. 1B & 1C). The family pedigree illustrates the allelic defect of both the mutations in the affected individuals (Fig. 1D).

RFLP analysis with *Mnl*I and *Tsp*RI for the confirmation of the missense mutation c.C421A in *SLC52A2* and c.A62G in *SLC52A3*, respectively, showed that the proband was homozygous with the elimination of these restriction sites, whereas both the parents were heterozygous due to the presence of both the normal and mutant allele (Fig. 2A & 2B). In addition, the sibling was heterozygous for c.C421A in *SLC52A2*, while there was no variation in *SLC52A3*. The parents and an unaffected sibling of the proband were heterozygous for one or the other mutation, suggesting segregation with disease status within this family and autosomal recessive inheritance.

3.3. In silico analysis

Transmembrane topology predictions using TMHMM for both the hRFVT-2 and hRFVT-3 suggested 11 transmembrane domains (TMD) with the amino terminal facing the cytoplasm while the carboxy

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