Novel mutations identified in EIF2B5 gene in Kashmiri patients as susceptibility factor for multiple sclerosis

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White matter disease refers to a set of diseases that affect the white matter of the brain and all of which have different consequences on brain function. Most of the studies have shown that it results from the defects during protein synthesis, with the gene defects in EIF2B1–5, encoding the five subunits of eukaryotic translation initiation factor 2B (eIF2B) α, β, γ, δ and ε, respectively. eIF2B plays a crucial role in protein translation and its regulation under different conditions. The previous studies have shown that mutations in five subunits of eIF2B cause white matter disease of the brain and thus EIF2B is the main culprit in development of white matter disease. In this study, the mutational screening of EIF2B5 gene encoding eIF2Be was performed for the first time in 12 Kashmiri patients, each having a unique white matter disease condition. We found two novel missense mutations in EIF2B5: c.580A>G, p.Thr194Ala and c.611C>T, p.Ala204Val among the patients with demyelinating disease (multiple sclerosis), but no mutation was found in other patients. In conclusion our study suggests involvement of the EIF2B5 gene in MS development, thus suggesting p.Thr194Ala to be a susceptibility factor for the development of multiple sclerosis.

Keywords: White matter disease, EIF2B (1-5), Mutational screening, Protein translation, Multiple sclerosis

White matter disease covers a large group of disorders in which white matter of the brain is affected. Almost all categories of pathology may involve white matter of the brain. White matter defects have been observed in congenital, inflammatory, neoplastic, post-traumatic, metabolic, toxic, vascular, degenerative and demyelinating disease. The primary white matter disorders are mainly divided into three groups: dysmyelinating disease, demyelinating disease and the hypomyelinating disease1. A wide number of conditions fall under the broad category of white matter disease, each with a wide range of symptoms and severity.

White matter disease can begin at birth or in adulthood and the clinical spectrum varies with disease type and its onset. The diagnosis is usually made on the basis of magnetic resonance imaging (MRI) findings and clinical history. In the last decade, the molecular genetics in white matter disease has begun to be explored, and these investigations have increased our understanding regarding its genetics. White matter disease seems to have an obvious genetic background with a number of candidate genes like glial fibrillary acidic protein (GFAP), β-galactosyl ceramidase (GALC) and aryl sulfatase A (ARSA) have been identified2-4. Despite the impediments in mapping genes for such a complex disorder, some progress has been made and recent studies on families with this disorder have reported significant linkage to regions on chromosomes 12q24.3, 14q24, 1p34.20, 2p23.3 and 3q27, encoding the five subunits of eukaryotic translation initiation factor 2B (eIF2B): α, β, γ, δ and ε, respectively5,6. Mutations in each of the five eIF2B subunits are known to affect white matter of brain in different white matter diseases, such as vanishing white matter disease (VWM) (OMIM 603896)6. These mutations cause different white matter abnormalities, leading to the concept of eIF2B-related disorders.
EIF2B disorders are related to a defect in translation initiation factor, leading to a defective regulation of translation initiation. The initiation factor eIF2B plays a vital role in regulation of protein synthesis initiation by catalyzing nucleotide exchange on eIF2. It changes an inactive eIF2-guanosine diphosphate (GDP) into the active complex eIF2-guanosine triphosphate (GTP), thus promoting regeneration of active eIF2 by exchange of GDP for GTP. This is a crucial point in regulation of protein synthesis initiation under wide range of cellular conditions, as it is involved in the regulatory pathway for the prevention of synthesis of denatured proteins during stress conditions in the cell.

In diverse stress conditions, Ser\(^{51}\) of the \(\alpha\)-subunit of eIF2 is phosphorylated by several protein kinases like general control non-depressible 2 (GCN2) or protein kinase R (PKR)-like endoplasmic reticulum (ER) protein kinase [PERK]\(^{9,10}\). The phosphorylated eIF2 has a higher affinity for eIF2B, but instead of promoting nucleotide exchange, it diminishes the nucleotide exchange activity\(^{7,8}\). Various other mechanisms known to regulate the activity of eIF2B involve the phosphorylation of its \(\varepsilon\)-subunit on several Ser/Thr residues\(^{11,12}\). eIF2B5 which encodes the catalytic subunit of multi-subunit complex contains most of the mutations and such mutations may be fatal\(^{13,14}\). Therefore, mutations in this gene apparently affect the activity of the complex.

Multiple sclerosis (MS) is an inflammatory condition in which neurodegeneration occurs, governed by demyelination. MS appears to be a multigenic condition in which a genetic predisposition along with an infection plays a central role in disease development\(^{15}\). Different lines of evidence have reported that genetic factors play an important role to disease susceptibility\(^{16}\). Then probably the genetic heterogeneity exists. In our study, we have chosen EIF2B5 gene, as most of the mutations reported in this gene not only cause VWM disease, but also VWM with milder phenotype and MS\(^{17,18}\), although previous studies have not shown any relationship between EIF2B5 variations and MS\(^{19}\).

Despite some major differences between MS and VWM, there are certain similarities between the two, suggesting the association between EIF2B5 gene and MS susceptibility\(^{20-22}\). Since both MS and VWM are sensitive to heat, therefore, variations in the EIF2B5 gene might be a susceptibility factor, increasing the risk for developing MS. Moreover, previous studies have reported cases with similar genetic, biochemical and MRI data of MS patient to that observed in VWM\(^{21}\). Thus, mutations in EIF2B5 gene have drawn strong attention as a possible cause of MS. However, contribution of mutations in EIF2B5 gene to MS development in the Indian population remains to be determined.

The present study has been aimed to investigate the mutational status of EIF2B5 gene and to elucidate its role in white matter disease occurrence and development in Kashmiri patients. To the best of our knowledge, the present study is first to examine mutational status of EIF2B5 gene in a group of Kashmiri patients, each presenting a particular white matter disease. We have screened 42 (12 affected and 30 controls) subjects for mutations in EIF2B5 gene. We report two novel mutations in two patients with demyelinating type of white matter disease i.e., MS.

**Materials and Methods**

**Patients and samples**

Patients presenting for treatment of white matter disease at the Department of Neurology, Sher-I-Kashmir Institute of Medical Sciences (SKIMS), Srinagar, Jammu and Kashmir, India, were recruited for this study with prior informed consent, following the ICMR (GOI) guidelines. Twelve venous blood samples were collected from patients diagnosed with a unique white matter disease condition, following the ethical procedures of the Institute. Samples were collected after the complete clinical investigation. Subjects were encouraged to narrate all the details relevant to this study. This included age of the subject, gender, personal habits, family history, dietary history, provisional diagnosis, marital status, socioeconomic factors, history of disease onset and any associated complications. As a control group, the blood samples were also collected from 30 age- and sex-matched healthy subjects from the same geographic area. Blood samples from both affected as well as controls were collected in 0.5 M EDTA (50-100 µl) for DNA extraction and stored at -80°C until analysis.

**DNA extraction and PCR amplification**

Total genomic DNA was isolated from blood by modified salting out method\(^{23}\). Proteinase K digestion and ZymoBead\(^{TM}\) genomic DNA kit (Cat. No. D3004, Zymo, USA). DNA concentration was measured by spectrophotometric method.
PCR amplification was carried out by using two designed sets of primer pairs (Table 1) to amplify exon 4 and exon 8 of EIF2B5 (OMIM 603945). PCR was carried out in Applied Biosystems thermal cycler at respective annealing temperature (Table 1). PCR was carried out in a reaction volume of 50 µl containing 100–200 ng of genomic DNA, 0.2 mM dNTPs (BRIT, India), 1X PCR buffer (BRIT, India), 0.5 U of Taq DNA Polymerase (BRIT, India) and 10 pmol of each primer (Sigma-Aldrich®, USA). Amplification was carried for 25-30 cycles, with each cycle consisting of initial denaturation at 95°C for 5 min, followed by denaturation at 95°C for 30 s, annealing at 58°C and 60°C (for primer sets ‘4’ and ‘8’, respectively) for 45 s, extension at 72°C for 1 min and a final extension at 72°C for 10 min, following the last cycle.

**Sequencing and prediction of protein tertiary structure**

Screening for sequence variants was performed using the services of Macrogen Inc. Seoul, Korea. Nucleotide sequences were analyzed and compared with NCBI reference sequence of EIF2B5 (accession number NG_015826.1/NM_003907.2). All identified mutations were confirmed by reverse sequencing. Simultaneously, 30 control individuals were also analyzed for the presence of same mutations. The amino acid sequence for both wild type and mutant proteins in fasta format obtained from NCBI (http://www.ncbi.nlm.nih.gov) was submitted to an automated server I-TASSER (http://www.zhang.bioinformatics.ku.edu/I-TASSER) for 3-dimensional (3D) structure prediction24,25. The server furnished the predicted 3D structure in a Pdb format. The Pdb files were analyzed using Swiss-Pdb Viewer 4.0.4 and the total energy of the predicted 3D structures was computed subsequently.

**Results**

In the present study, we did mutational screening of those exons in which most of the mutations have been reported. To investigate the role of EIF2B5 gene in the genesis of white matter disease, we screened its exon 4 and exon 8 for mutations in different patients from Kashmir. Agarose gel electrophoresis revealed successful isolation of genomic DNA from blood samples of both diseased as well as controls (data not shown). The DNA isolated was used as a template for amplification and PCR showed specific product as per expected size (178 bp) (Fig. 1).

Sequencing revealed the presence of two mutations in exon 4 of two patients having MS. Both were novel missense mutations: A to G change at base 580 of cDNA, resulting in an amino acid exchange of Thr to Ala (ACT to GCT) at codon 194 and other C to T change at base 611 of cDNA, resulting in substitution of Ala with Val (GCT to GTT) at codon 204 (Fig. 2 & Table 2). Both were seen to be present in heterozygous state. Additionally, the predicted 3D structure and energy status of mutant proteins showed decreased stability as the energy of mutant proteins was higher than wild type (Wild type, -29787.375; Codon 194, -14181.535 and Codon 204, -29773.381) (Fig. 3). Our results suggest that these missense mutations are probably the risk factors for MS development in Kashmiri population and the contribution of different variations in MS development varies from population to population and therefore depends on both genetic as well as environmental factors, however, the role of mutations in other genes cannot be ruled out by this study.

Among 12 patients, we identified mutations in 2 patients with MS. The two novel missense variations in exon 4 (EIF2B5, c.580A>G, p.Thr194Ala; EIF2B5, c.611C>T, p.Ala204Val) were
probably the primary cause of disease, since they were absent in control group of healthy individuals. So far, none of these mutations are reported in the single nucleotide polymorphism (SNP) databases. The mutation observed at codon 194 could be considered disease-causing mutation, as this resulted in replacement of polar residue (Thr) with non-polar residue (Ala) and would probably be expected to cause conformational change in eIF2B5 subunit and consequently perturb eIF2B function in these individuals. The functional consequences of these mutations can be evaluated by conducting functional analysis using mutant eIF2B5 protein. Moreover, no change was found in exon 8 of EIF2B5.

**Discussion**

Investigating the genetics of common and complex disorders, such as white matter disorder remains one of the great challenges in human genetics. White matter disease is considered to be a complex and multigenic condition involving several overlapping pathways, each one mediated by a group of distinct genetic profiles. Therefore, studying the genetic variations of white matter disease-related genes can further clarify the relationship between molecular genetics of white matter disease and its development. Previous studies suggest that white matter disease susceptibility can be related to EIF2B gene. Impaired eIF2B function in humans may cause
abnormal glial cell development. This abnormal development of white matter could increase sensitivity of cells containing mutated eIF2B to stress.

The mutations in EIF2B gene hamper the regulatory pathway, thus resulting in the synthesis of denatured proteins during cellular stress. Of the five eIF2B subunits, eIF2Bε is the largest and the most important, as it exhibits guanine nucleotide exchange factor activity (GEF), when expressed alone. EIF2B5 mutations impair the function of the eIF2B complex in diverse ways, leading to the formation of shortened proteins, hampering holocomplex assembly and causing partial loss of GEF activity. The previous studies have shown that the activity of mutant eIF2B is diminished.

Moreover, a recent study in the Chinese population has reported the effect of mutations identified in EIF2B5 gene on the GEF activity of eIF2B protein, phosphorylation level of eIF2Bε, interaction between different subunits to form the eIF2B complex and interaction with the eIF2 substrate. Also, it is known that leakage of blood in the brain acts as an early trigger for switching on the brain’s inflammatory response, creating a neurotoxic environment that damages nerve cells in some white matter disease conditions. A similar study in mice model has shown that a biodegradable nanoparticle could be used as a vehicle to deliver antigen that modulates the immune system to stop its attack on myelin and halting MS. These findings provide the groundwork for much needed therapies to treat white matter diseases. Henceforth, EIF2B5 screening was done for mutations in a group of Kashmiri patients with white matter disease. Mutational analysis of exon 4 and exon 8 of EIF2B5 gene in 12 samples of white matter disease patients from Kashmir revealed two novel missense mutations, among which mutation A to G resulted in exchange of Thr to Ala (ACT to GCT) at codon 194 and other mutation C to T produced the substitution of Ala with Val (GCT to GTT) at codon 204 (Table 2).

Furthermore, their respective chromatograms on comparison with the reference sequence obtained from NCBI database showed overlapping peaks representing A to G and C to T changes (Fig. 2). Moreover, the 3D structure and energy status of mutant proteins predicted by I-TASSER showed decrease in stability (Fig. 3). The decrease was significant in codon 194 variant, suggestive of effect of this mutation on the functioning of eIF2B5 protein. This amino acid change from Thr (a polar amino acid) to Ala (a non-polar amino acid) can be very significant and can play a crucial role in altering the functional properties of eIF2B5 protein. These alterations cannot be nullified and may probably be contributing to the MS susceptibility in Kashmiri population. Although the findings from this study were unique, they needed further confirmation as the sample size in our study was small.

In conclusion, our data strongly suggest a possible role of the EIF2B5 mutations in the development of MS in Kashmiri population. The findings from this study suggest that mutations in EIF2B5 gene may be a causative factor in MS development and the eIF2B5 protein could possibly be used as a novel diagnostic, prognostic or therapeutic marker in white matter disease. However, further studies are needed to search for more number of white matter disease patient’s candidate genes to identify association of these genes with the development of the disease.

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**References**

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