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Genetic Characterisation of Pantothenate Kinase Associated Neurodegeneration (PKAN) in a Consanguineous Family from Jammu and Kashmir, India

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ABSTRACT Pantothenate kinase-associated neurodegeneration (PKAN), a rare neurological disorder occurs by variation(s) in the PANK2 (Pantothenate kinase 2) gene and is linked to iron accumulation in the basal ganglia. The researchers have carried out targeted gene sequencing of all exons of *PANK2* in a patient with suspected phenotype of PKAN. A missense variant in exon 6 of PANK2 gene (NM_153638.3:c.1583C>T,NP_705902.2:p.Thr528Met) has been identified in the patient. Further, sequencing of the exon in extended consanguineous family showed autosomal recessive mode of inheritance in the family. It is emphasised that inclusion of molecular diagnostics in clinical evaluation procedures of potential genetic or uncharacterised abnormalities is critical especially if the family has known history of high consanguinity. It is anticipated to provide effectively, such families with access to a variety of genetic counselling programmes, thus reducing illness burden in the affected family.

INTRODUCTION

Pantothenate Kinase-associated Neurodegeneration (PKAN), also referred as Hallervorden-Spatz disease, is a autosomal recessive rare condition characterised by progressive extrapyramidal dysfunction and accumulation of non-heme iron in the brain, particularly in the basal ganglia region of the bilateral globus pallidus (Zhou et al. 2001; Hayflick et al. 2003). It is an inborn error in biosynthesis of mitochondrial coenzyme A (CoA), primarily associated with variations in PANK2 gene encoding a 50.5 kDa mitochondrial enzyme, Pantothenate kinase 2 (Angural et al. 2017). Pantothenate kinase plays a vital role in other mechanisms too, such as neurotransmitter and glutathione metabolism, fatty acid production, degradation and energy metabolism (Karim et al. 2000). PKAN is the most prevalent cause of neurodegeneration caused by brain iron accumulation (Levi and Tiranti 2019), accounting for about thirty to fifty percent of worldwide Neurodegeneration with Brain Iron Accumulation (NBIA) cases (Rump et al. 2005; Brezavar and Bonnen2019).The estimated prevalence of PKAN is 1-3 per million, with a carrier frequency of one in 275-500 individual (Svetel et al. 2019). Based upon the age of onset, progression and symptoms, PKAN is divided into two categories, that is, classic and atypical (Hayflick et al. 2003). Classic PKAN usually appears around the age of three and before the age of six in ninety percent of cases (Nassif et al. 2016). The most prevalent symptom is gait issues, but other indicators include pyramidal and extrapyramidal abnormalities, as well as significant dystonia (Hayflick 2006; Schneider et al. 2012). The "eye-of-the-tiger" pattern is a common magnetic resonance imaging (MRI) sign in PKAN. On a T2-weighted MRI, eye-of-the-tiger pattern is developed by accumulation of iron in the globus pallidus region with hyperin-

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tensity in the anteromedial area. Neurodegeneration due to neuronal loss, gliosis, and edema are thought to be the causes of hyperintensity in the anteromedial area (Akcakaya et al. 2017). Atypical PKAN is more diverse than classic PKAN, presenting in the later decades of life and progressing more slowly. Atypical PKAN is characterised by psychiatric symptoms and speech abnormalities, which include sadness, aggressiveness, emotional ability and impulsivity as well as physical and vocal tics. Dysarthria, hypophonia, spasmodic dysphonia and palilalia are other examples of speech problems in the disease (Hayflick et al. 2003; Pellecchia et al. 2005; Gregory et al. 2009; Hogarth 2015).

Objectives

The objective of the study was targeted gene sequencing of all exons of *PANK2* in a patient with suspected phenotype of PKAN for confirmation and identification of the casual variation. The researchers explained a familial case of atypical Pantothenate kinase- associated neurodegeneration (PKAN) from the region of Jammu and Kashmir and identified NM_153638.3(PANK2):c.1583C>T (p.Thr528Met) as the casual variant, segregating in autosomal recessive manner with disease, in the family (Fig.1).

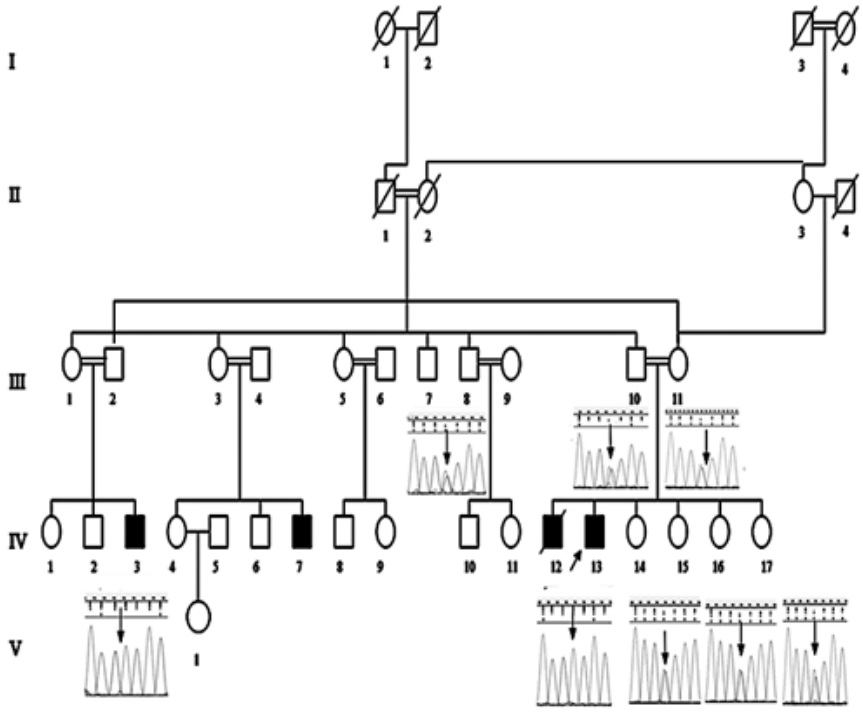


Fig. 1. PKAN family Pedigree. Squares represents males and circles are females (affected with solid representations). The proband IV(13) has been marked with a black arrow. The screenshots represents electropherogram data of Sanger Sequencing (reverse strand) indicating the genotypes of the individuals under study for the variation. An arrow on the electropherogram indicates variation (missense) NM_153638.3(PANK2):c.1583C>T, in exon6 of *PANK2* gene in the reverse strand of the samples. The proband IV(13) along with his affected cousin IV (3) from maternal side, is found to be homozygous (A>A) for the found variant. His parents III(10) and III(11) along with his siblings IV(15), IV(16), IV(17) and paternal uncle III(8) were heterozygous carriers (G>A). Thus, the variant was observed segregating in autosomal recessive mode of inheritance in the family

Clinical Presentation

The proband, 21-year-old IV(13), born from consanguineous parents [III(10) 60 years and III(11) 45 years], presented with an extra-pyramidal symptoms such as progressive generalised dystonia. Medical history revealed that at age of 13 years, proband had difficulty in eating (difficulty in jaw closing) and writing with his right hand. However, other routine activities were normal. After some years, that is, at the age of 16 years, the patient suffered from tremors and dysarthric speech. His magnetic resonance imaging (MRI) investigations depicted a typical "eye of tiger" sign on T2-weighted images, demonstrating diffused bilateral and symmetric hypointensity of both the globus pallidus and hyperintensity at central part in T2-weighted images (Supplementary Fig.1). His biochemical assay profile was otherwise normal with an exception of serum alkaline phosphatase that showed elevated level of 258.0 IU/L (normal range: 37-147 IU/L). Ophthalmological evaluation of the proband IV(13) did not reveal a Kayser-Fleischer (KF) ring around eye iris and there was no evidence of retinitis pigmentosa. Parents reported that one of the affected brothers IV(12) of the proband died whereas rest of the offsprings IV(15) 12 years, IV(16) 15 years, and IV(17) 18 years of age are unaffected. Also, his paternal cousin IV(3) (25 years), born from consanguineous parents [III(1) 50 years and III(2) 47 years], also showed extra pyramidal dystonic features.

METHODOLOGY

Sample Collection and Extraction of DNA

Whole blood samples from extended family of the proband were collected (both affected as well as unaffected), with their informed consent, in the study verified by the Institutional Ethical Review Board SMVDU (IERB Serial No: SMVDU/IERB/18/67). DNA from collected samples was extracted and the quality of the extracted DNA samples was checked on a 0.8 percent agarose by gel electrophoresis and DNA quantification was performed using an Eppendorf BioSpectrometer®. The genomic DNA concentration of isolated genomic DNA was measured using NanoDrop (ND1000, USA), dilutions of 50ng/ul were made and then stored at -20 °C till further use.

Genetic Screening

Sanger Sequencing and In-silico Analysis of PANK2 Gene

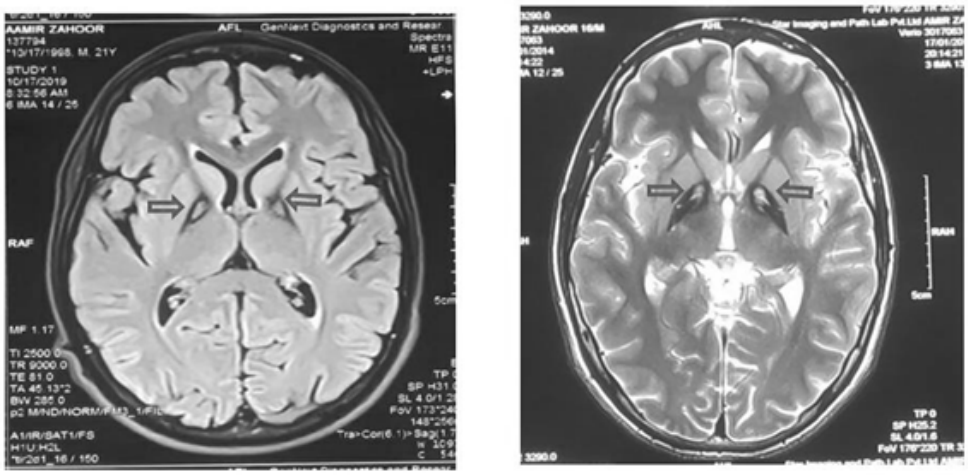
All exons of *PANK2* (exon 1 to 7) including their boundaries of exons and introns, amplified using polymerase chain reaction (PCR). The mRNA RefSeq NM_153638.3 reference sequence was used to build primers. The designed primer sequences are available upon request. The amplified DNA was subjected to Sanger sequencing utilising primers according to ABI BigDye Terminator Cycle Sequencing Kit. The data obtained from Sanger sequencing was visualised and analysed using Seq scanner 2 software (Applied Biosystems®) and NCBI BLAST (Johnson et al. 2008). Mutation Taster 2 (Schwarz et al. 2014) tool was used to characterise the pathogenicity of the found variant.

Additional Information

All data and other associated information regarding this study have been provided as supplementary information in the form of Supplementary Figure 1 (MRI of proband depicting "eye-of-tiger" sign as characteristic feature of PKAN) and Supplementary Figure 2 (UCSC genome screenshot depicting conservation of identified variant among mammals). Raw sequence reads as .ABI files are also provided.

RESULTS

Data obtained through targeted sequencing of all exons of PANK2 gene were further analysed through Sequence Scanner Software v2.0 (Applied Biosystems®) using genomic DNA reference sequence NM_153638.3GRCh37/hg19 assembly. The researchers have identified missense variation [NM_153638.3:c.1583C>T(G>A in reverse strand),NP_705902.2:p.Thr528Met]in exon 6 of PANK2 gene. As in electropherogram (Fig. 1), the proband IV(13) and his affected maternal cousin IV (3) are found to be homozygous (A>A) for the found variant, whereas his parents III(10) and III(11) along with his siblings IV(15), IV(16) and IV(17) and paternal uncle III(8) were heterozygous carriers (G>A). These results indicated that affected individuals were homozygous and unaffected were heterozygous carriers for PKAN in the extended



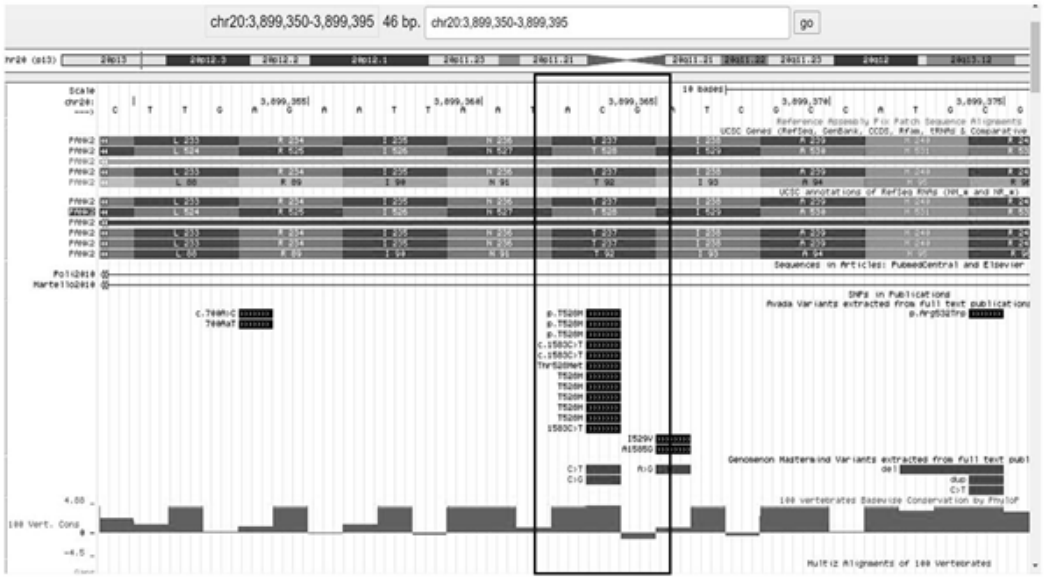
Supplementary Fig.1. Brain Magnetic resonance-sequences of the affected proband IV(13). Blue arrows on the these sequences reveal (a) areas of hypo-intensities in globus pallidus bilateral region on brain T1W MR-sequence, and (b) areas of hyper-intensities in globus pallidus bilateral region on brain T2W MR-sequence

family. Also, the variant segregates in autosomal recessive mode of inheritance in the family, hence confirms the inheritance pattern of PKAN.

This missense variation is known to cause PKAN (Svetel et al. 2019; Svetel et al. 2020), as it results in sequence change, which replaces threonine with methionine at codon 528 (NP_705902.2:p. Thr528Met) of the PANK2 protein that potentially leads to the disruption of gene function and outcome of it is cell damage neurotoxicity, and neurodegeneration (Suwanpakdee et al. 2018**).** This characteristic feature of neurodegeneration due to iron accumulation has been noticed in the form of "eye-of-the-tiger" sign on MRI findings of the proband (Supplementary Fig.1). Mutation Taster (Schwarz et al. 2014), an online tool, also predicted identified variant pathogenicity with a high score (Mutation Taster score=1.00) and variant was observed to be conserved in most of the mammalian species (Supplementary Fig. 2). The detected variant has also been identified as pathogenic in the ClinVar database, and reported as disease causing in earlier published genetic investigations of NBIA (Hartig et al. 2006; Landrum et al. 2018).

DISCUSSION

The clinical presentations in the study indicated a typical PKAN based on the onset of disease, neurological symptoms, and disease development. It was finally confirmed by the presence of missense variation NM_153638.3:c.1583C>T(NP_705902.2: p.Thr528Met). Classical PKAN has been related more often with nonsense variations whereas, missense variations are associated with atypical PKAN (Pan and Zhu 2020). The found Variant p. Thr528 Met has been associated with atypical PKAN in literature (Hartig et al. 2006; Svetel et al. 2019; Svetel et al. 2020; Werning et al. 2020) with late age onset (Hashemi et al. 2008). It is also reported as the second most common pathogenic PANK2 mutation (Zhang et al. 2006) and in the European population (p.T528M) is one of the most prevalent variation associated in with NBIA (Svetel et al. 2019). Neurodegeneration with brain iron accumulation (NBIA) are basically types of neurodegenerative heterogeneous disorders in which deposition of iron in the area of basal ganglia of brain is a common feature (Pan and Zhu 2020). PANK2 is a critical enzyme in the manufacture of coenzyme A in the mitochondria, impacting important metabolic activities (Leonardi et al. 2007). Coenzyme A is an important molecule that is involved in over 100 metabolic pathways (Wang et al. 2019). Co-A is involved in several metabolic pathways, including the heme biosynthesis, citric acid cycle, fatty acid and amino acid metabolism, steroid and



Supplementary Fig.2. Screenshot of UCSC genome browser indicating conservation at NM_153638.3 (PANK2):c.1583C>T (p.Thr528Met) substitution in PANK2 gene across 100 vertebrates

sterol biosynthesis, and hence their deficiency caused by PANK2 variations results in variety of metabolic defects as well as possible mitochondrial dysfunction. Also, Pantothenate kinase catalyses the phosphorylation of N-pantothenoyl-cysteine, pantothenate (vitamin B5), and pantetheine, which is essential for coenzyme A (CoA) production (Suwanpakdee et al. 2018).The detrimental variations in the PANK2 gene results in mitochondrial cysteine and pantetheine buildup (which binds iron effectively), results in rapid auto-oxidation and generation of cytotoxic free radicals, which cause cell damage neurotoxicity, and neurodegeneration (Suwanpakdee et al. 2018)**.** Non-functional PANK2 proteins have a significant impact on cell energy operations (Zhang et al. 2006). This variant impairs the enzyme's catalytic domain, and it is commonly associated with an atypical form of PKAN, which supports biochemical evidence of residual enzyme function (Svetel et al. 2020). Although the specific patho-physiology of PKAN is unknown, it is possible that this mutation alters PANK2 function by a mechanism that is only relevant in the context of environmental stressors or advancing age (Kotzbauer et al. 2005). The substitution of methionine for threonine at position 528 due to c.1583C>T variant in exon six of the PANK2 gene affects the catalytic domain of the enzyme (Hayflick et al. 2003; Svetel et al. 2019). Consequently, it limits the biosynthesis of coenzyme A (CoA), which causes production of high cysteine levels, resulting in secondary iron build-up and oxidative stress-induced neuronal damage, cysteine being an effective iron chelator (Kurian and Hayflick 2013).The main characteristic imaging feature of PKAN (classic as well as atypical PKAN) is the "eye-of-the tiger" sign related to iron deposition in the area of globus pallidus due to degeneration of neurons (Pan and Zhu 2020), also observed in the present study. On T2-weighted imaging, the eye-of-the-tiger sign, which is defined by bilateral hypointensity in the globus pallidus region with a area of central hyperintensity, is a characteristic feature of PKAN as seen in the proband (Supplementary Fig. 2). Some patients may have hypo-intensity in the substantia nigra. On T2-weighted examples, areas of hypointensity at sites of iron deposition can be seen (Amaral et al. 2015; Angural et al. 2017).

Pathologically, the area of hypointensity corresponds to improper iron accumulation, whereas the centre hyperintense signal is caused by gliosis-induced neuronal death (Guillerman 2000). Despite its rare prevalence, patients with progressive extrapyramidal sickness should be evaluated for neurodegeneration with brain iron build-up syndromes, especially if there is presence of brain iron deposition on magnetic resonance imaging. PKAN is a relatively rare condition with only about 180 variants identified worldwide (Chang et al. 2020) with no effective treatment (Sharma et al. 2018). As majority of PANK2 mutations diminish the enzyme's function, new therapy strategies have focused on pantothenate, the PANK2 enzyme substrate (Aoun et al. 2017), certain reported in-vitro investigations show that this medication can stabilise *PANK2* expression levels only in specific mutations, emphasising the need of accurate PANK2 genotyping in each PKAN case (Álvarez-Córdoba et al. 2019). The treatment of PKAN, like that of other NBIAs, is symptomatic, and multidisciplinary rehabilitation programmes can assist these patients in achieving their goals and improving their quality of life (Pellecchia et al. 2005) required that the disorder is diagnosed in a timely manner.

CONCLUSION

Targeted sequencing of all exons of PANK2 gene identified variation (missense) NM_153638.3: c.1583C>T, causing amino acid change NP_705902.2:p.Thr528Met in PANK2 protein and found variant segregated in the autosomal recessive manner with the disease in the family. The study concludes that missense variation p.Thr528Met results in PKAN in the family from Jammu and Kashmir.

RECOMMENDATIONS

The prevalence of the rare disorders is relatively high in Jammu and Kashmir's population due to the practice of consanguinity and endogamy. Most of the rare disorders like PKAN, are being misdiagnosed or remained undiagnosed due to lack of availability of resources, scientific literature, diagnostic tools and clinical management facilities, thus their number keeps on increasing every year. As the region has remained relatively unexplored in terms of genetics-based studies due to lack of resources, it adds to the burden a huge number of genetic disorders that are most likely limited to specific extended families or entire communities. Thus, this study recommends to focus on the screening and management of such disorders. Further, it is anticipated that community-based screening approaches of such variants identified in populations of the region, may act as biomarkers to find carriers in these population, where presence of such rare variants is high due to social practices of consanguinity, thus substantially may reduce the disease burden of such rare diseases in the region.

LIMITATIONS

Due to lack of availability of scientific literature, the researchers are unable to report the incidence of PKAN in Jammu and Kashmir's population. Also, gene expression and other functional aspects of the effect of identified variant on the protein are lacking in the present study and warranted for better understanding of the disease.

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DECLARATION

All authors declared that this article has not been published or sent to any other publication elsewhere.

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The authors have nothing to declare.

AUTHORS CONTRIBUTION

All the authors have critically reviewed the manuscript and contributed in its finalisation.

ETHICAL DECLARATIONS

The study was approved under IERB Serial No: SMVDU/IERB/18/67) by the Institutional Ethical Review Committee (IERB), SMVDU, J&K, India

meeting relevant ethical guidelines. Also, all necessary patient/participant consents have been obtained and the appropriate institutional forms have been archived. For minors, consent has been taken from the parents and this study does not involve any kind of financial support from the recruited family.

ABBREVIATIONS

IERB: Institutional Ethical Review Committee
KF:Kayser-Fleischer
NBIA: Neurodegeneration with Brain Iron Accumulation
Met: Methionine
MRI:magnetic resonance imaging
PKAN:Pantothenate Kinase-Associated Neurodegeneration
PANK2: Pantothenase Kinase 2
PCR: Polymerase Chain Reaction
Thr: Threonine

NOTE

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