Rare novel *CYP2U1* and *ZFYVE26* variants identified in two Pakistani families with spastic paraplegia.

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Keywords: spastic paraplegia, SPG15, SPG56, next generation sequencing, consanguinity

Word count: 1894

Abstract

Bakground: Hereditary Spastic paraplegias (HSPs) are a clinically and genetically heterogeneous group of degenerative disorders characterized by progressive spasticity and weakness of the lower limbs. This study aimed to identify causative gene variants in two unrelated consanguineous Pakistani families presented with 2 different forms of HSP.

Methods: Whole exome sequencing (WES) was performed in the two families and variants were validated by Sanger sequencing and segregation analysis.

Analysis: In family A, a homozygous pathogenic variant in *ZFYVE26* was identified in one family. While in family B, a frameshift variant in *CYP2U1* was identified in 4 affected individuals presented with clinical features of SPG56. Our study is the first report of *ZFYVE26* mutations causing HSP in the Pakistani population and the second report of *CYP2U1* in a Pakistani family.

Conclusions: Our findings enhance the clinical and genetic variability associated with two rare autosomal recessive HSP genes, highlighting the complexity of HSPs. These findings further emphasize the usefulness of WES as a powerful diagnostic tool.

Hereditary spastic paraplegias (HSPs) are a group of clinically and genetically heterogeneous inherited neurological disorders characterized by progressive lower extremities spasticity and weakness. HSPs are caused by defects that primarily affect the upper motor neurons in the brain and spinal cord, disrupting the normal transmission of neuronal signals to muscles and often associate degeneration of the pyramidal tracts, mainly resulting mainly in progressive spasticity of the lower limbs and walking difficulties. Depending upon the presence of additional symptoms, HSP is divided into two main categories, complicated and uncomplicated.[1] In uncomplicated or pure HSP, only the lower body is affected with bilateral leg spasticity and weakness being prominent feature while complicated forms are accompanied by variable additional neurological features including impaired vision, ataxia, epilepsy, cognitive impairment and peripheral neuropathy.[2] There are more than 80 genetic types of HSPs [3] and mode of inheritance include autosomal dominant and X-linked and autosomal recessive. To date, over than 100 causative genes have been identified underlying different HSPs, despite some affected individuals and families still remain genetically undetermined.[4]

Spastic paraplegia type 15 (SPG15) is a complex autosomal recessive (AR) HSP typically characterized by slowly progressive spastic paralysis of the lower limbs in association with thin corpus callosum on brain magnetic resonance imaging (MRI). Additional clinical features include intellectual disability, hypotonia, sensory and motor neuropathy, cerebellar ataxia and visual impairment.[5] SPG15 is caused by mutations in the *ZFYVE26* gene (OMIM: 612012) that encodes for spastizin, a protein part of the AP5 complex (adaptor related protein complex 5) which is critical for autophagic lysosomal reformation (ALR), a pathway that generates new lysosomes. Similarly, its binding partner spatacsin (the SPG11 protein) is also important in autophagy, where lysosomal targeting of spastizin requires an intact FYVE domain to bind the phosphatidylinositol 3-phosphate. A mutation in either of these proteins can result in dysfunction of the autophagy/lysosomal biogenesis machinery leading to neurodegeneration. Both spastizin and spatacsin are proteins widely expressed in the nervous system where they are also involved in axonal development and maintenance.[6] SPG15 is considered one of the commonest causes of AR-HSP associated with thin corpus callosum, representing together with SPG11 approximately 70% of such cases.[7] Another AR-HSP type, SPG56, is caused by mutations in the *CYP2U1* gene (OMIM: 610670), which encode for a member of the cytochrome P450 family 2.[4] Disease mechanism associated with biallelic *CYP2U1* mutations implicate the inhibition of P450 hydroxylase enzyme activity, resulting in either modification of protein structure or loss of protein's ability to bind heme.[8] The SPG56-associated clinical features include mental deterioration with cerebellar ataxia, neuropathy and retinal impairment. [4]

Herein, we aimed to study and identify causative gene variants in two nonrelated consanguineous Pakistani families segregating HSP, as part of the SYNaPS Study Group. This large patient cohort, sponsored by the Wellcome Trust, aims to recruit consanguineous families from Central Asia. Next generation sequencing was conducted as part of the Queen Square Genomics group at University College London. We report a novel variant of *ZFYVE26* (NM_015346: c.2084_2085delinsATG; p.Glu694_Gln695delinsMet), in a consanguineous

Pakistani family presented with developmental regression, spasticity, weakness of the lower limbs and behavioral problems in a single individual (family A). In a second HSP family (family B), presenting with (mild to severe) intellectual disability, and visual impairment, we identified a novel homozygous variant in *CYP2U1* (NM_183075: c.725_726del; p. Asp242ValfsTer3) in four affected individuals. Notably, our study is the first report of *ZFYVE26* mutations causing HSP in the Pakistani population. Recently Zulfiqar *et al.* (2019) previously reported *SPG56* variants in three patients born to consanguineous Pakistani parents.

Next generation sequencing

After informed consent, we collected blood samples from the probands, their parents and unaffected siblings, and extracted DNA using standard procedures. To investigate the genetic cause of the disease, WES was performed in the affected proband. Nextera Rapid Capture Enrichment kit (Illumina) was used according to the manufacturer instructions. Libraries were sequenced in an Illumina HiSeq3000 using a 100-bp paired-end reads protocol. Sequence alignment to the human reference genome (UCSC hg19), and variants calling, and annotation were performed as described elsewhere.[9] After removing all synonymous changes and variants not shared by the patient and the two parents, we filtered single nucleotide variants (SNVs) and indels, only considering exonic and donor/acceptor splicing variants. In accordance with the pedigree and phenotype, priority was given to rare variants [<1% in public databases, including 1000 Genomes project, NHLBI Exome Variant Server, Complete Genomics 69, and Exome Aggregation Consortium (ExAC v0.2)] that were fitting a recessive or a *de novo* model.

Results

Whole-exome sequencing in family A revealed a homozygous frameshift variant (NM_015346: c.2084_2085delinsATG; p.Glu694_Gln695delinsMet) in the *ZFYVE26* gene. This variant was present within the most significant homozygous block (chr14: 67,347,517-70,030,202) identified in the family WES-based homozygosity mapping analysis.

In family B, we identified through WES a frameshift variant (NM_183075: c.725_726del; p. Asp242ValfsTer3) in the *CYP2U1* gene. This variant was present within homozygosity block (chr4: 108,014,614-113,333,298). Upon segregation using traditional Sanger sequencing both parents were found to be heterozygous carriers of the variants. In-silico predictors (including SIFT and Polyphen) confirmed the severe impact of the mutations in *ZFYVE26* and *CYP2U1*.

Clinical Features

The proband of family A (II-2) is an 11-year old male born to consanguineous parents from Pakistan with no family history of neurological diseases. His infantile motor and developmental

milestones were reported as normal but, since the age of 8 years, he presented with moderate regression of his motor, social and speech skills. The proband is still able to stand and walk unsupported but not able to do more than 40-50 steps independently. There is no remarkable cognitive impairment and he is performing adequately at school, although he is having some speech problems. Manual ability is also quite normal and the proband can perform most of his daily activities unaided. Finally some behavioural disturbances are present consisting of aggressiveness and impaired social interaction. Neurological examination revealed lower limb spasticity with brisk deep tendon reflexes and extensor plantar responses. Brain MRI imaging studies showed bilateral T2-weighted and FLAIR signal hyperintensities affecting the white matter mostly in posterior brain regions and a slight thinning of the anterior part of the body of corpus callosum was also noticed. (Figure 1)

The proband of family B (V-1) is a 9-year-old male born to consanguineous Pakistani parents. Since the first months of life he presented with generalised hypotonia, delayed developmental milestones and febrile seizures, which first appeared at the age of 3 months. Lower limbs slowly progressed to spasticity, while febrile seizures disappeared after infancy and were not followed by epileptic seizures. The other three affected family members (IV-3, IV-6 and IV-7) also presented similar features with variable severity. Patient IV-7 was affected with marked hypotonia since birth and severely delayed motor and developmental milestones and presented a more aggressive disease course. Affected individuals from the family never attained the ability to stand or walk. They all had lower limb spasticity, intellectual disability, dysarthria, visual impairment, and cerebellar signs on neurological examination (Video 1). Brain imaging revealed T2-weighted and FLAIR hyperintense white matter signal abnormalities in periventricular areas. (Figure 1)

Discussion

In this study, two unrelated consanguineous Pakistani families were genetically investigated by WES. They presented with two different types of HSPs. In family A, the affected individual showed a disease onset with onset in late childhood, as is commonly observed in *ZFYVE26*-related SPG15.I In family B, all four affected individuals from the family presented a typical early infantile onset of the SPG56 disease. Affected individuals from both families presented a neurodevelopmental impairment with delay or regression of motor milestones, variable intellectual disability and behavioral abnormalities. Patients from family B also had neonatal-onset hypotonia and infantile-onset febrile seizures as part of their clinical phenotype. In family A, a novel frameshift variant in the *ZFYVE26* gene (c.2084_2085delinsATG) was identified in the proband, while the WES-based genetic analysis in family B identified a novel segregating frameshift variant (c.725_726del) in the *CYP2U1* gene.

ZFYVE26 encodes a protein called spastizin, a protein implicated in autophagy and in the formation of autophagosomes which participate to the degradation and recycling of intracellular materials. Previously, it has been showed that AR SPG15-related *ZFYVE26* mutations impair

autophagy by causing an accumulation of immature autophagosomes, leading to cell dysfunction and often cell death.[7]

Biallelic *CYP2U1* mutations causing AR SPG56 caused by have previously been reported in association with altered phospholipid metabolism, ultimately leading to cellular dysfunction and neurodegeneration.[8] The *CYP2U1* gene is critical for the synthesis and the function of the P450 enzyme, involved in hydroxylation of fatty acids and phospholipid degradation.

Both novel variants herein reported were predicted to be damaging by different in-silico prediction tools including SIFT, Polyphen, and Mutation Taster. The disease mechanism for SPG15 in Family A is most likely due to the destabilization of spastizin protein with consequent impaired autophagy. In Family B the *CYP2U1* mutation could have impacted cytochrome P450 protein structure probably by disrupting interaction between the functional domains. However, further functional studies will be necessary to address the effect of these mutations and the exact disease mechanisms underlying HSPs in these families.

Both families reported here have in common the presence of white matter abnormalities as revealed in brain MRI scans. The presence and significance of white matter changes has already been demonstrated in various types of HSP[10], and our findings in these two families with SPG15 and SPG56 respectively, further contribute to the overlap of HSP with white matter disorders.

Conclusion

In two Pakistani families, we identified novel homozygous variants in *ZFYVE26* and *CYP2U1* implicated in two rare AR HSPs. To the best of our knowledge, this report represent the first case of SPG15 identified in Pakistan and in general only the second in South Asian population, with another individual previously reported in India.[11] Most SPG15 cases have been so far described in countries part of the Mediterranean area (Table 1), including North African and South European countries [12-14] . Our finding indicates that founder *ZFYVE2* mutations could also be present in the South Asian-Punjab area, resulting in a more worldwide distribution of SPG15. This highlight the importance of considering SPG15 as a possible diagnosis of AR HSP, irrespectively of the individuals' ethnic background.

This is also the second report of SPG56 in a Pakistani family with SPG56 while additional kindreds have been previously described in India [15] and Iran [1], suggesting that SPG56 may represent not so uncommon cause of complicated AR HSP in the South Asia area. The phenotype of SPG56 can be broad, including both pure and complicated forms (Supplementary Table 1), with a variable presence of additional signs and symptoms.[1 2 16] The family reported in this study presented with features such as early hypotonia and febrile seizures, that can represent an expansion of the phenotypic spectrum of this rare disease. The clinical presentation along with MRI findings suggestive of white matter involvement, further implicate delayed/abnormal myelination in the pathogenesis of this disease, as indicated in previous studies.[1 2]

Our report highlight a possible founder effect for SPG15 in South Asia and broaden the phenotypic spectrum of SPG56. Next generation sequencing based studies involving populations from different ethnic backgrounds will help in the future to understand the exact worldwide distribution of the rarest AR HSPs, also contributing to the further delineation of the associated clinical phenotypes. In the long-term, this information will consequently help affected families in terms of clinical management and prognosis, family planning and prenatal diagnosis.

Acknowledgments

The authors thank all the participating patients and their families for their cooperation. Families was collected as part of the SYNaPS Study Group collaboration funded by The Wellcome Trust. Research for was conducted as part of the Queen Square Genomics group at University College London, supported by the National Institute for Health Research University College London Hospitals Biomedical Research Centre.

Funding

We are supported by the Rosetree Trust, Ataxia UK, MSA Trust, Brain Research UK, Muscular Dystrophy UK, Muscular Dystrophy Association (MDA USA), Higher Education Commission of Pakistan, The MRC (MR/S01165X/1, MR/S005021/1, G0601943), Wellcome Trust (WT093205MA, WT104033AIA and the Synaptopathies Strategic Award, 165908) and the National Institute for Health Research University College London Hospitals Biomedical Research Centre.

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Researcher	Year	Cases	Ethnicity	Mutation
ZFYVE26				
Hanein *et al.*	2008	6 cases	Tunisian, Italy, Ireland, Moroccan, Algeria, Israeli Arab	p.Gln493X, p.Phe683LeufsX685, p.Arg1209fsX1220, p.Arg1438X, c.5485-1G>A, p.Trp2234CysfsX2238
Goizet *et al.*	2009	13 cases	Turkish, Syrian, French, Tunisian, Belgian, Austrian, Portuguese	p.Gln1808X, p.Leu1679RfsX8, p.Ser2004T, p.Leu2099LfsX12, p.Glu103X, p.Glu143X, p.Glu414X, p.Arg728X, p.Asp778RfsX15, p.Cys1356X, p.Ala1931PfxX1957X, p.Arg2329RfsX2337, p.Gln15X
Denora *et al.*	2009	1 case	Italian	p.Asp599fsX613, p.Lys2314X
Schüle *et al.*	2009	7 cases	German, Turkish	p.Arg198X/ c.7128+1G>C, p.Ser615F, c.2332+7delT, p.Pro1467P, p.Cys1871Y
Yoon *et al.*	2013	1 case	Filipino	c.273+8G>A (r.spl?)
Pensato *et al.*	2014	8 cases	Italian	p.Gln491*, p.Ile508Asn, p.Asn577Ilefs*36, p.Gln752*, p.Ser1312*, p.Arg1378*, p.Gln1735*, p.2248delLys
Chakrabarty *et al.*	2016	1 case	Indian	p.Arg1602Ter
Morais *et al.*	2017	1 case	Portuguese	p.Asn2100Glufs*12
Özdemir *et al.*	2019	1 case	Turkish	p.Arg2133Asnfs*15
CYP2U1				
Tesson *et al.*	2012	5 cases	Saudi Arabian, Italian, Egyptian, Spanish/Vietnamese	p.Asp316Val, p.Cys262Arg, p.Glu380Gly, p.Leu21Trpfs*19, p.Arg488Trp
Citterio *et al.*	2014	1 case	Egyptian	p.Met1?
Leonardi *et al.*	2016	1 case	Italian	p.Arg390*
Masciullo *et al.*	2016	1 case	Italian	p.Ser2*, p.Val430Glyfs*18
Kumar *et al.*	2016	1 case	Indian	p.Cys262*
Kariminejad *et al.*	2016	2 cases	Iranian	p.Gly353Arg, p.Gln211*
Iodice *et al.*	2017	1 case	Caucasian	p.Val430Glyfs*18, p.Pro516*
Durand *et al.*	2017	2cases	Italian, Turkish	p.Gly115Ser, p.Arg384Ile, p.Cys490Tyr
Zulfiqar*et al.*	2019	1 case	Pakistani	p.Glu202Lys

Table 1. Previous studies reporting families with HSP harboring pathogenic variants In *ZFYVE26* and *CYP2U1* genes.

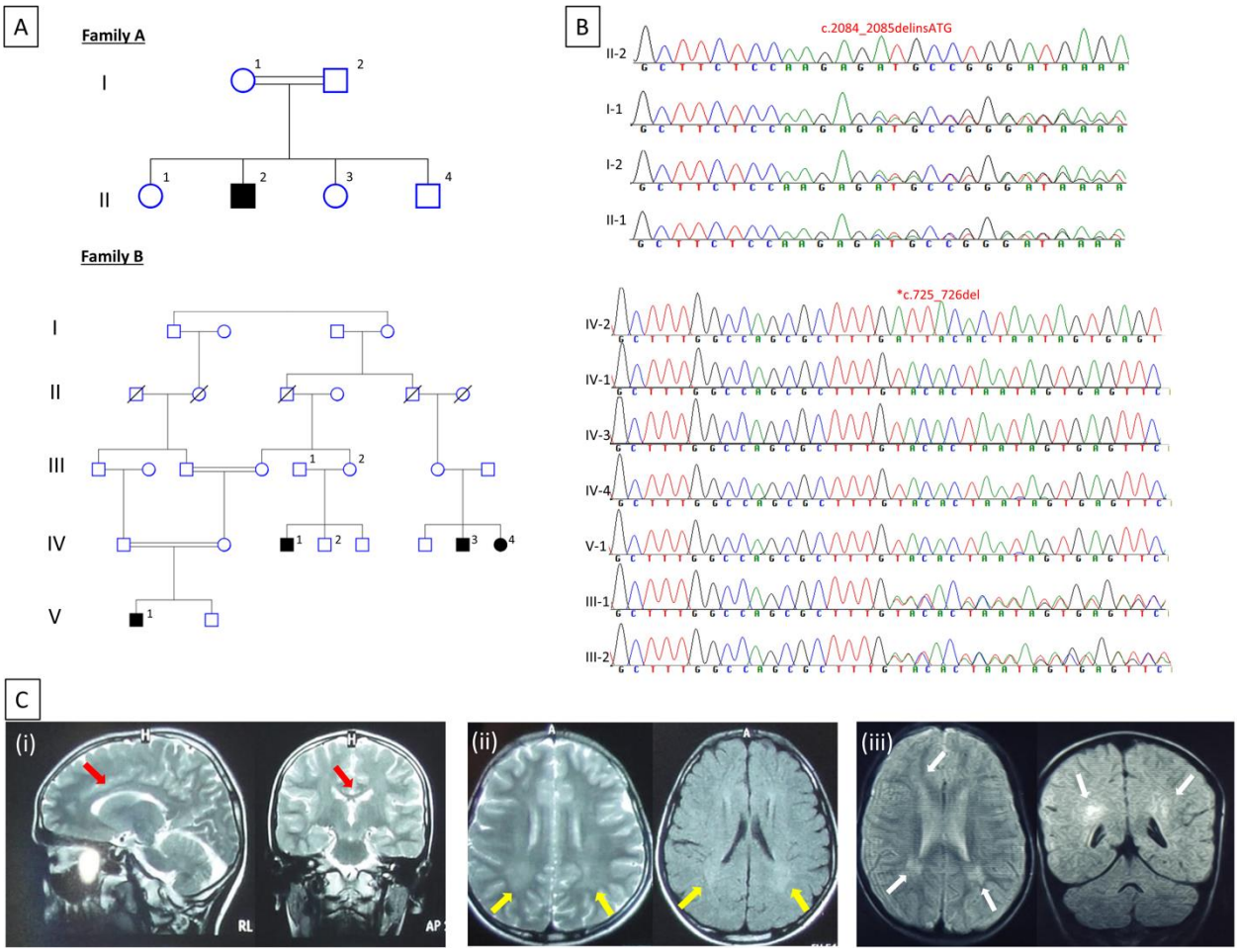

Figure 1. Pedigrees, Sanger sequencing and clinico-radiological natural history of patients. (A) Pedigrees of the 2 families carrying the homozygous frameshift variants in *ZFYVE26* (in affected individual II-2) and *CYP2U1* (in 4 affected individuals IV-1, IV-3, IV-4 and V-1). (B) Mutations and Sanger sequencing electropherograms confirming the mutations in the families. (C) MRIs of patients. (i) Sagittal and coronal brain T2-weighted MRI images of proband of family A showing slight thinning of the anterior part of the body of corpus callosum (red arrows), (ii) T2-weighted and FLAIR brain MRI images of proband of family A showing signal hyperintensities in posterior white matter (yellow arrows), (iii) T2-weighted and FLAIR brain MRI images of proband of family B showing signal hyperintensities in periventricular regions (white arrows)