Received: 11 February 2022 | Revised: 29 March 2022 | Accepted: 14 April 2022

DOI: 10.1111/cge.14142

CLINICAL GENETICS WILEY

LETTER TO THE EDITOR

Decipher non-canonical *SPAST* splicing mutations with the help of functional assays in patients affected by spastic paraplegia 4 (SPG4)

Dear Editor,

This study aims to improve our knowledge about the splicing mutations in the *SPAST* gene (chr2:32288625-32382706-NM_014946.3) responsible for Spastic paraplegia type 4 (SPG4). Splicing events in *SPAST* account for about 10% of patients with a pathogenic variant.[1] Such a prevalence is underestimated since only a few splicing mutations are placed within the essential dinucleotide considered in diagnostic pipelines.[1]

In the last 10 years, we performed genetic analysis on 100 patients with a clinical phenotype compatible with an upper motor neuron syndrome (UMNS) selectively affecting lower limbs. Sixteen of them are carriers of mutations in SPG4 (7 missense, 3 frameshift, and 5 splicing variants), identified respectively in five familial and 11 sporadic patients.

Among five *SPAST* splicing mutations identified in six patients, four involve variants falling within the essential dinucleotides site and they are considered "conventional splice-site mutations" (classified as 4 or 5 according to ACMG), while c.1537-8T>G identified in two families is considered as "non-canonical splice-site mutation" since it falls at +8 from the exon 14, and according to ACMG was classified as VoUS (PM2-BP) and reported in ClinVar as a benign variant. To decipher the pathogenicity and molecular mechanisms of such non-canonical splicing variants, we combined in silico, in vitro (Minigene-assay) and in vivo (RNA-analysis) approaches along with segregation analysis in two different families (Families 1373 and 120, Figure 1).

These families showed the presence of Hereditary Spastic Paraplegia with an autosomal dominant pattern of inheritance. Genetic analysis identified variant c.1537-8T>G in *SPAST* in the probands of III:6 of family 1373 and III:2, III:3 and II:2 of family 120. This variant was not found in healthy subjects 1373 (III:5, IV:1, IV:5, IV:7, IV:8). In silico analysis suggested no influence on splicing (varSEAK SSP, NNSPLICE, EX SKIP, CRYP-SKIP). Both Minigene and in-vivo RNA analysis on lymphocytes identified abnormal splicing causing retention of intron 13 on probands and III:6 of family 1373, and II:3 and III:2 of family 120. These data, along with segregation analysis, reclassified the variant as Pathogen (ACMG:5,PM1-PM2-PM4-PP3-PS3-PVS1;ClinVar: SCV001745881).

The present study remarks on the need to improve the identification and interpretation of non-canonical splice-site mutation in SPG4 patients, demonstrating that the proposed approach is able to correctly interpret the involvement of this variant in the disease onset.[2]

Although in silico analysis is useful to assess potential pathogenic mechanisms, and ACMG guidelines are essential for establishing variant pathogenicity, sometimes these tools are not sufficient for this kind of variant. Molecular insights, such as the Minigene-assay and RNA analysis, have proved useful in correctly deciphering splicing variants first interpreted as VoUS, and then reclassified as pathogenic, also when in in silico analysis by several different tools evidenced the lack of any abnormal splicing events.

Moreover, these data suggest that *SPAST* splicing events, now reported in about 10% of patients, are more frequent in SPG4. Here we characterized six *SPAST* splice variants out of 16 mutations representing 37% of SPG4 patients, thus confirming that the frequency of *SPAST* splice variants is underestimated.

FUNDING INFORMATION

Italian-Ministry of Health (CR2019-2023: Identification of New Variants/Genes Responsible for Ataxia and Spastic-Paraplegia)

CONFLICT OF INTEREST

The authors declare no conflict of interest.

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1111/cge.14142.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

Rosangela Ferese,[1] Simona Scala,[1] Antonio Suppa,[1,2] Rosa Campopiano,[1] Francesco Asci,[1,2] Maria Antonietta Chiaravalloti,[1] Alessandro Zampogna,[2] Carmelo D'Alessio,[1] Filomena Fittipaldi,[1] Fabio Buttari,[1] Alba Di Pardo,[1] Emiliano Giardina,[3] Stefania Zampatti,[3] Francesco Fornai,[1,4] Giuseppe Novelli,[1,5] Mirco Fanelli,[6] Chiara Zecca,[7]

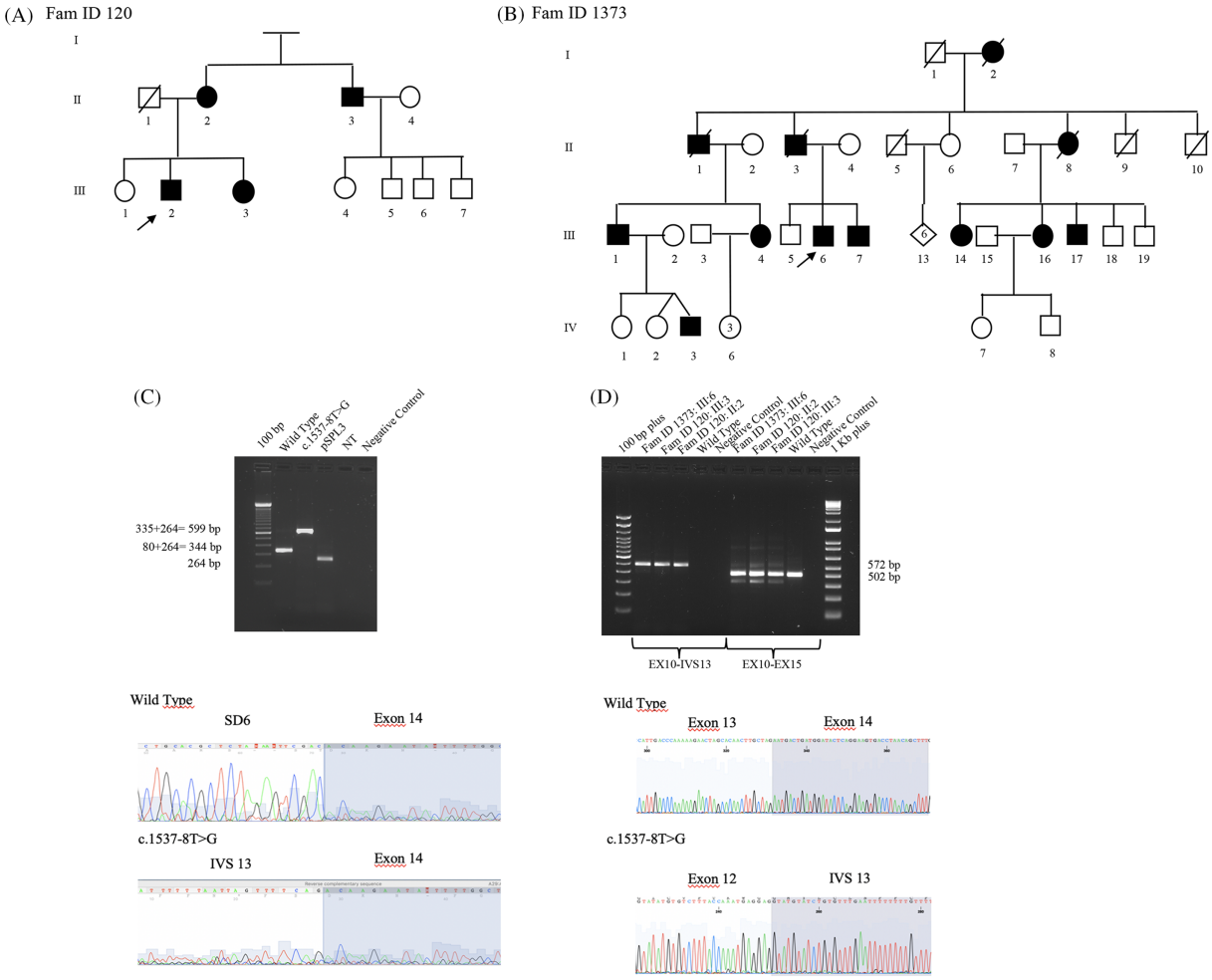

FIGURE 1 Functional analysis of c.1537-8T>G. (A) Pedigree of Family 120; (B) Pedigree of Family 1373. C.Minigene-assay for variant c.1537-8T>G. Lane 1: wild-type genotype (344 bp, 80 bp, normal splicing of Exon 14 + 264 bp of pSPL3 Exon); Lane 2: abnormal splicing, [599 bp, 80 bp normal splicing (Exons 14) + 255 bp (Intron 13)] + 264 bp of pSPL3 Exon); Lane 3: pSPL3 empty vector; Line 4: HEK293T cDNA without transfection (NT) of pSPL3; Lane 5: PCR negative control. Sanger sequence shows intron13 retention. (D) RNA analysis from peripheral blood of Fam ID 1373: III:6 and Fam ID 120: II:2. Lane 1 to 4: First set of Primers showing amplification of mutated allele (572 bp, Exon 10Fw/Intron13Rw) in mutated samples (Line 1, 2 3) and no amplification in the control sample. Sanger sequence shows wt and the retention of intron 13. Lane 6/10: Second set of Primers (Exon10Fw/Exon15Rw), showing preferential amplification of weight allele. [Colour figure can be viewed at wileyonlinelibrary.com]

Giancarlo Logroscino,[7] Diego Centonze,[1,8] and Stefano Gambardella,[1,6]

[1]*IRCCS Neuromed, Pozzilli, Italy*
[2]*Department of Human Neurosciences, Sapienza University of Rome, Rome, Italy*
[3]*Genomic Medicine Laboratory, IRCCS Fondazione Santa Lucia, Rome, Italy*
[4]*Department of Translational Research and New Technologies in Medicine and Surgery, University of Pisa, Pisa, Italy*
[5]*Department of Biomedicine and Prevention, University of Rome "Tor Vergata", Rome, Italy*
[6]*Department of Biomolecular Sciences, University of Urbino "Carlo Bo", Urbino, Italy*
[7]*Center for Neurodegenerative Diseases and the Aging Brain, Department of Clinical Research in Neurology, Bari University, "Pia Fondazione Card G.Panico" Lecce, Bari, Italy*
[8]*Department of Systems Medicine, Tor Vergata University, Rome, Italy*

Correspondence
Stefano Gambardella, IRCCS Neuromed, Pozzilli, Italy.
Email: stefano.gambardella@uniurb.it

Rosangela Ferese and Simona Scala equally contributed to the paper.

REFERENCES

1. Wang GS, Cooper TA. Splicing in disease: disruption of the splicing code and the decoding machinery. *Nat Rev Genet*. 2007;8(10):749-761. doi:10.1038/nrg2164
2. He WB, Xiao WJ, Dai CL, et al. RNA splicing analysis contributes to reclassifying variants of uncertain significance and improves the diagnosis of monogenic disorders. *J Med Genet*. 2022;108013. doi:10.1136/jmedgenet-2021-108013