Hereditary spastic paraplegia associated with a novel intronic non canonical splice site variant in AP4B1 gene.

Clara Gómez-González[1]*, Cristina Pizarro[1]*, Carlos Rodríguez-Antolín [2, 3], Samuel I. Pascual [4], Mar García[4], Carmen Rodríguez[5], Rubén de Sancho[1], Ángela del Pozo [6], Mario Solís[6], Carmen Prior [1,] Rosa J. Torres [7,8],

*These authors contributed equally to the work.

1. Department of Genetics, INGEMM, La Paz University Hospital, Madrid, Spain.

2. Cancer Epigenetics Laboratory, INGEMM, La Paz University Hospital, Madrid, Spain.

3. Biomarkers and Experimental Therapeutics in Cancer, IdiPaz, Madrid, Spain.

4. Department of Pediatric Neurology. La Paz University Hospital, Madrid, Spain.

5. Department of Next Generation Sequencing, INGEMM, La Paz University Hospital, Madrid, Spain.

6. Department of Bioinformatics, INGEMM, La Paz University Hospital, Madrid, Spain.

7. La Paz University Hospital Health Research Institute (FIBHULP), IdiPaz, Madrid, Spain.

8. Center for Biomedical Network Research on Rare Diseases (CIBERER), ISCIII, Spain.

Correspondence to: Rosa Torres, MD, PhD,
Edificio de Laboratorios, Planta 3
Hospital Universitario "La Paz"
Paseo de la Castellana 261
28046 Madrid. Spain.
Phone: +34917277343
e-mail: rtorres.hulp@salud.madrid.org

Abstract.

Pathogenic variants in the *AP4B1* gene conduct to a rare form of hereditary spastic paraplegias (HSP) known as SPG47 (spastic paraplegia 47), characterized by progressive spastic paraplegia with progression to tetraplegia, developmental delay, neonatal/early infant hypotonia, postnatal microcephaly and seizures. We report a patient, with clinical suspicion of complicated HSP of lower limbs with intellectual disability, and a novel homozygous non canonical splice site variant in *AP4B1* gene, in which the effect on splicing of the detected variant was validated by blood RNA analysis.

A total of 152 genes associated with SPG were sequenced in the patient by Next Generation Sequencing (NGS) using a custom gene panel. Total RNA was isolated from peripheral blood of the patient, his parents, and a control subject, and cDNA generated by RT-PCR. A fragment of *AP4B1* mRNA was amplified by PCR employing primers forward and backward located in exon 8 and exon 11 of the *AP4B1* gene, respectively. Fragment obtained from the PCR reaction were purified from agarose gel and sequenced.

A homozygous non canonical splice site variant in the *AP4B1* gene c.1511-6C>G (NM_006594.4) was found in the proband by NGS. The variant was also found in heterozygosity in both parents and classified as variant of uncertain significance according to the standards and guidelines provided by the ACMG. Two different *AP4B1* mRNA fragments were obtained in both the patient and his parents. The shorter fragment was the predominant in the patient and revealed a deletion c.1511_1792del p. (Glu504_Ser597del) with skipping of the *AP4B1* exon 10. The patient longer

fragment, correspond to an insertion c.1510_1510+1insCTCAG p.(Glu504Alafs*22) of the last 5 nucleotides of *AP4B1* intron 9. We confirmed that this non-canonical variant affects the normal splicing of RNA and, thus, sustains the molecular diagnosis of SPG47 in the patient and provides genetic counseling in the family.

Keywords:

Introduction.

Hereditary spastic paraplegias (HSP) are a heterogeneous group of neurodegenerative diseases, with variable age of onset (1). They are characterized by the progressive decline of the corticospinal tract that leads to spasticity and associated disability. Adaptor protein complex 4 (AP-4) belongs to a family of adaptor proteins involved in vesicle formation and protein trafficking, and it is composed of four subunits encoding by four different genes: *AP4B1* (β1), *AP4E1* (ε1), *AP4M1* (μ1) and *AP4S1* (σ1). AP-4 is ubiquitously expressed in human tissues, including the central nervous system, and mediates the transmembrane transport of cargo proteins from the trans-Golgi network to endosomes by recruiting the machinery necessary to build the vesicles in which proteins are comprised (2,3).

The presence of bi-allelic loss-of-function variants in any of the subunits encoding AP-4 genes cause the entire complex dysfunction which is associated to a similar phenotype, consisting of spastic paraplegia and developmental delay (1,4). So, AP-4-associated HSP include four different conditions: SPG47 (AP4B1, OMIM #614066), SPG50 (AP4M1, OMIM #612936), SPG51 (AP4E1, OMIM #613744), and SPG52 (AP4S1, OMIM #614067), according to the subunit encoding gene mutated (4).

Pathogenic variants in the *AP4B1* gene conduct to a rare form of HSP known as SPG47. There are about 30 pathogenic variants described in this gene related to SPG47, according to previous reports and HGMD database (4). The most common types are frameshift or nonsense variants in the coding region, but only a small proportion are splice site variants. Clinical symptoms associated with *AP4B1* variants include progressive spastic paraplegia with progression to tetraplegia, developmental delay, neonatal/early infant hypotonia, postnatal microcephaly or seizures (2-6).

Here we report a patient with a novel homozygous non canonical splice site variant in *AP4B1* gene identified by next generation sequencing (NGS). In this patient, the effect on splicing of the detected variant has been validated by RNA analysis allowing confirmation of the molecular diagnosis of SPG47 in the patient.

Patients and Methods.

Clinical characterization

The patient is a 16-year-old boy born to non-consanguineous and healthy parents but coming from two little villages only 30 km away. He was born by natural delivery at 42 weeks of gestation, with weight and height in normal ranges (3570 g; 50 cm) and head circumference (HC) of 36 cm (p75). Apgar scores were 9-10 and there were no perinatal complications.

The boy started to hold his head at the age of 3 months, independent sitting at 12 months, crawling at 19-20 months and walking at the age of 2 years. Clinical presentation at the age of 2 and half years was microcephaly (HC 46 cm; <p3), psychomotor developmental delay and increased tonus of lower limbs. By that time, he could only speak a few words and named objects. Brain magnetic resonance imaging (MRI) showed thinned splenium of corpus callosum and ventriculomegaly with no other signs of white matter lesions or cerebral atrophy. The initial clinical suspicion was cerebral palsy.

At 7 years of age, magnetic transcranial stimulation revealed a disorder of the pyramidal pathway more pronounced when stimulating the lower limb than the upper one. Moreover, an alteration of myelination of visual pathways is noticed; visual evoked potential test showed delayed latencies and abnormal conduction. Physical

examination showed external rotation of the hips and knees, flat foot and equinovarus deformity as well as trunk hypotonia. When he was 13 years old, he experienced an episode of non-convulsive seizure with environment disconnection, fixed gazed without loss of consciousness lasting about 60 minutes. He started antiepileptic therapy and has never present with another episode since then.

Nowadays, he can walk without assistance; fine motor activity in upper limbs is normal; he is able to control sphincters and eats without assistance; he can talk, knows lots of words, understands complex orders and he is learning to read. It is a social boy with outgoing character and shows interest for meeting other children and interact with people. He went to special-needs education school.

Electroencephalography (EEG) evaluations have always been normal. Electromyography (EMG) evaluations showed no alterations in motor and sensory nerve conduction. Computerized tomography (CT) at the time of onset was also normal.

The clinical suspicion was complicated HSP of lower limbs with intellectual disability. Informed consent was obtained from the patient and his parents to perform DNA and RNA analysis in peripheral blood, and for publication.

DNA analysis.

DNA was extracted from peripheral blood from the patient and his parents using standard techniques (Chemagic DNA blood kit, PerkinElmer, Waltham, MA, USA). 152 genes associated with spastic paraplegia were sequenced in the patient by NGS using a custom gene panel with SeqCap EZ Roche technologies. Library preparation and exome enrichment steps were performed according to manufacturer´s workflow and sSequencing was performed on a HiSeq4000 (Illumina, San Diego, California, USA)

platform and bioinformatics analysis was carried out by the Clinical Bioinformatics Unit of La Paz University Hospital (Human reference genome: hg19), including in silico bioinformatics tools for splicing defect prediction Alamut Visual (v2.14.), Adaboost , RandomForest [ref_ada_rf] and SpliceAI [ref_spAI]. To classify the variants identified by NGS, we followed the recommendations of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology (ACMG) (7). NGS results were confirmed in the patient and studied in his parents´ DNA by Sanger sequencing.

RNA analysis.

Total RNA was isolated from peripheral blood of the patient, his parents, and a control subject using the QIAamp RNA Blood Mini Kit (QIAGEN GmbH, D-40724, Hilden, Germany). A first-strand cDNA template was generated using the ImProm-II™ Reverse Transcriptase system (Promega, Promega Corporation, WI, USA) with oligo (dT) as a random primer for RT-PCR. Primers forward and backward were designed in exon 8 and exon 11 of the *AP4B1* gene, respectively to amplify, by PCR, a 593 pb fragment from the single strand cDNA including the entire 9 and 10 exons of the *AP4B1* gene. DNA fragments obtained from the PCR reaction were purified from agarose gel employing the MinElute Gel Extraction kit (QIAGEN GmbH, D-40724, Hilden, Germany). The purified DNA fragments were sequenced by an automated sequencer using the BigDye® Terminator Cycle Sequencing kit (Applied Biosystems) in an ABI PRISM® 377 DNA Sequencer (Applied Biosystems).

Results.

The analysis of the data obtained by NGS of the proband revealed a homozygous non canonical splice site variant in the *AP4B1* gene in the proband NM_006594.4: c.1511-6C>G (chr1: 114438666). The variant was confirmed by Sanger sequencing and also found in heterozygosity in both parents. This variant had not been previously described and had no minor allele frequency data according to the 1000 genomes, Gnomad and ESP6500 databases. The variant was expected to modify the splicing site according to *in silico* bioinformatics tools for splicing defect prediction (Table 1, Figure 1). Hence, according to the standards and guidelines provided by the ACMG, the variant was considered as variant of uncertain significance (VUS) until their effect on splicing has been validated.

To confirm the splicing defect hypothesis, analysis of *AP4B1* mRNA was performed in the proband, his parents and a control subject. As the NM_006594.4: c.1511 -6 C>G (change is located in the 3' intronic sequence between exon 9 and 10 of the *AP4B1* gene, primers forward and backward were designed in exon 8 and exon 11 of the *AP4B1* gene, respectively. By RT-PCR and subsequent PCR amplification, a single DNA fragment of 593 bp was obtained from the control RNA. However, two different size fragments, of about 593 bp and 311 bp respectively, were observed in both the patient and his parents in the agarose gel (Figure 2A). The shorter band of about 311 bp was predominant in the patient. In contrast, the longer band of about 593 bp was more intense in both parents. Sequencing of purified shorted fragments from patient and his parents revealed a normal 311 pb *AP4B1* mRNA sequence except for the skipping of exon 10. The variant with the mentioned deletion c.1511_1792del p. (Glu504_Ser597del) does not change the reading frame (Figure 2B). The purified longer

fragment obtained from the patient RNA showed an insertion of 5 bp CTCAG corresponding to the last 5 nucleotides of intron 9 following the C>G change. The variant c.1510_1510+1insCTCAG p.(Glu504Alafs*22) alters the reading frame and results in a premature stop codon (Figure 2B). The purified longer fragments obtained from the parents and the control RNA showed a 593 bp normal sequence. However, with Sanger sequencing, the presence of a minimum amount of the longer fragment with the 5 nt insertion in the parents could not be definitively discarded.

Discussion.

We report the clinical and molecular characterization of a patient with a classical SPG47 phenotype and a non-canonical intronic bi-allelic variant, c.1511-6C>G, in the *AP4B1 gene*, not previously reported, and detected by NGS. Non-canonical splice site variants are difficult to interpret and, according to the standards and guidelines provided by the ACMG, these variants have to be considered as VUS unless their effect on splicing has been validated. We have confirmed that this non-canonical variant affects the normal splicing of RNA and, thus, sustains the molecular diagnosis in the patient and provides genetic counseling in the family.

HSP presents a wide clinical and genetic heterogeneity. SPG47 is a rare form of HSP due to autosomic recessive *AP4B1* gene mutation. To date, the vast majority of pathogenic variants described in this gene involve nonsense or frameshift mutations but a small number of missense mutations and canonical splice site variants have also been described (4). The variant identified in this report is the first one described, to our knowledge, in a non-canonical site affecting the splicing in the *AP4B1* gene.

NGS approaches, used for genetic diagnostics of HSP in routine clinical settings, report a diagnostic yield between 20-50% according to different authors (8-12), so

probably an important percentage of diagnosis are missing. Estimates of the relative contribution of canonical and non-canonical mutations within the Human Gene Mutation Database gives an estimated non canonical contribution of about 30-43% (13-14), and the ClinVar proportion of non-canonical mutations is about 17% (15). Lord et al. (16), using mutational burden analysis in a large cohort of proband–parent trios, estimate that 35%–40% of pathogenic variants in non-canonical splice site positions are missing from public databases. The introduction of NGS technologies in routine clinical diagnostics has come accompanied by the uncertainty in ascribing pathogenicity to novel variants, especially in deep intronic or non-canonical splice regions. These variants are classified as VUS as it was, in the first moment, the variant described in our patient. Although a combination of *in silico* tools, focused on the alteration of the conserved 3' or 5' splice sites, may be used to distinguish pathogenic variants, the correct prediction of a potential effect on splicing of variants located in non-canonical splicing regulatory elements is elusive and is still mandatory to confirm the splicing defect, when transcript analysis in patient samples is possible. Wai HA et al. (17) examined effects on splicing of a large cohort of clinically identified variants and compared performance of bioinformatic splicing prediction tools commonly used in diagnostic laboratories as Alamut Visual (v2.11, including MES, NNSplice, and SSF), HSF and SpliceAI. They found that the analysis of splicing alteration, using patient blood RNA, identifies diagnostically important splicing abnormalities in 33% of the cases and clarifies functional effects of a significant proportion of VUS. Their work demonstrates that bioinformatic predictions are improving but still make significant errors and support that RNA analysis should therefore be routinely considered in genetic disease diagnostics.

In this work, we have examined the effect on splicing of the *AP4B1* variant: NM_006594.3: c.1511-6C>G (Chr 1:114438666: G: C with Alamut Visual (v2.14.) and Ada, RandomForest and SpliceAI bioinformatics tools (Table 1). In silico predictions indicated a decreased probability of the normal acceptor splicing site position 1:114438660 (=114438666-6) and the creation of a new acceptor splicing site in position 1:114438665 (=114438666-1) (Figure 1). The decrease in the score of the predictors of the normal acceptor splicing site foresee the skipping of the exon 10. According to predictions, we found two different mRNA products in the patient. Sequencing of the purified preponderant shorted fragments from the patient revealed a 311 pb *AP4B1* mRNA sequence with the skipping of exon 10. The exon 10 deletion c.1511_1792del p. (Glu504_Ser597del) does not change the reading frame (Figure 2B), and it is present in a minor amount in the heterozygous parents. But predictors of in silico analysis also recognize a new alternative splicing site with high scores in a site other than the canonical one. Accordingly, the purified longer fragment obtained from the patient RNA showed a CTCAG insertion, corresponding to the last 5 nucleotides of intron 9 following the C>G change. This fact confirms the generation of a new AG alternative splicing site, as it was predicted, with the addition of the 5 intronic bp to the exon 10 sequence (Figure 1). The variant c.1510_1510+1insCTCAG p.(Glu504Alafs*22) alters the reading frame and results in a premature stop codon. Either way, is probable that none of the RNA products results in a useful protein, leading to the loss of function of the whole complex.

One limitation of this study is the use of blood RNA to detect aberrant splicing. Although AP-4 is ubiquitously expressed in human tissues, including the central nervous system, we cannot discard a tissue specificity of splicing of *AP4B1* gene. We

have assumed that similar splicing events are taking place in central nervous system and in blood, which is not necessarily the case.

In summary, AP4B1-associated HSP and other AP-4-deficiency syndromes should be suspected in infants and children with early developmental delay, intellectual disability, thin corpus callosum, microcephaly and neonatal or infant hypotonia that progresses to symmetric spastic paraplegia. The presence of an intronic mutation when NGS analysis is performed has to be taken into account, as it could modify the regular splicing leading to an abnormal protein complex. These variants are relevant for counseling of affected families and for further investigation, in order to establish a more accurate phenotype-genotype correlation. In this study we have performed splicing analysis of *AP4B1* gene in blood RNA of a patient with a homozygous non canonical intronic variant and have identified two splicing abnormalities. These findings reclassified the variant as probably pathogenic and permits a diagnosis of the patient and a genetic counseling of the family. Bioinformatics' predictions are important tools that can be used for prioritizing candidate genetic variants but RNA analysis in blood, or in other available tissues, should be routinely employed to help in genetic disease diagnostics.

Acknowledgments

This work was supported in part by Grants from Instituto de Salud Carlos III (ISCIII) PI15/01000, from Mutua Madrileña Foundation, and by Center for Biomedical Network Research on Rare Diseases (CIBERER), ISCIII; Spain.

Conflict of Interest Statement

The authors declare that they have no conflict of interest.

References.

1. Hirst J. AP-4-Associated Hereditary Spastic Paraplegia Summary Genetic counseling GeneReview Scope. 2019;1–18.
2. Ebrahimi-Fakhari D, Cheng C, Dies K, Diplock A, Pier DB, Ryan CS, et al. Clinical and genetic characterization of AP4B1-associated SPG47. Am J Med Genet Part A. 2018;176(2):311–8.
3. Behne R, Teinert J, Wimmer M, D'Amore A, Davies AK, Scarrott JM, et al. Adaptor protein complex 4 deficiency: A paradigm of childhood-onset hereditary spastic paraplegia caused by defective protein trafficking. Hum Mol Genet. 2020;29(2):320–34.
4. Ebrahimi-Fakhari D, Teinert J, Behne R, Wimmer M, D'Amore A, Eberhardt K, et al. Defining the clinical, molecular and imaging spectrum of adaptor protein complex 4-associated hereditary spastic paraplegia. Brain. 2020;143(10):2929–44.
5. Szczałuba K, Mierzewska H, Śmigiel R, Kosińska J, Koppolu A, Biernacka A, et al. AP4B1-associated hereditary spastic paraplegia: expansion of phenotypic spectrum related to homozygous p.Thr387fs variant. J Appl Genet. 2020;61(2):213–8.
6. Abou Jamra R, Philippe O, Raas-Rothschild A, Eck SH, Graf E, Buchert R, et al. Adaptor protein complex 4 deficiency causes severe autosomal-recessive intellectual disability, progressive spastic paraplegia, shy character, and short stature. Am J Hum Genet. 2011;88(6):788–95.
7. Laboratories KD, Genetics M, Health O, Road P, Molecular C, Children N, et

al. Standards and guidelines for the interpretation of sequence variants. Acta Ophthalmol. 2018;96(S261):134–134.

8. Kumar KR, Blair NF, Vandebona H, Liang C, Ng K, Sharpe DM, Grünewald A, Gölnitz U, Saviouk V, Rolfs A, Klein C, Sue CM. Targeted next generation sequencing in SPAST-negative hereditary spastic paraplegia. J Neurol. 2013 Oct; 260(10):2516-22. doi: 10.1007/s00415-013-7008-x. Epub 2013 Jun 28. PMID: 23812641.

9. Iqbal Z, Rydning SL, Wedding IM, Koht J, Pihlstrøm L, Rengmark AH, Henriksen SP, Tallaksen CM, Toft M. Targeted high throughput sequencing in hereditary ataxia and spastic paraplegia. PLoS One. 2017 Mar 31; 12(3):e0174667. doi: 10.1371/journal.pone.0174667. Erratum in: PLoS One. 2017 Oct 12;12 (10):e0186571. PMID: 28362824; PMCID: PMC5375131.

10. Burguez D, Polese-Bonatto M, Scudeiro LAJ, Björkhem I, Schöls L, Jardim LB, Matte U, Saraiva-Pereira ML, Siebert M, Saute JAM. Clinical and molecular characterization of hereditary spastic paraplegias: A next-generation sequencing panel approach. J Neurol Sci. 2017 Dec 15; 383:18-25. doi: 10.1016/j.jns.2017.10.010. Epub 2017 Oct 10. PMID: 29246610.

11. Kara E, Tucci A, Manzoni C, Lynch DS, Elpidorou M, Bettencourt C, Chelban V, Manole A, Hamed SA, Haridy NA, Federoff M, Preza E, Hughes D, Pittman A, Jaunmuktane Z, Brandner S, Xiromerisiou G, Wiethoff S, Schottlaender L, Proukakis C, Morris H, Warner T, Bhatia KP, Korlipara LV, Singleton AB, Hardy J, Wood NW, Lewis PA, Houlden H. Genetic and phenotypic characterization of complex hereditary spastic paraplegia. Brain. 2016 Jul;

139(Pt 7):1904-18. doi: 10.1093/brain/aww111. Epub 2016 May 23. PMID: 27217339; PMCID: PMC4939695.

12. D'Amore A, Tessa A, Casali C, Dotti MT, Filla A, Silvestri G, Antenora A, Astrea G, Barghigiani M, Battini R, Battisti C, Bruno I, Cereda C, Dato C, Di Iorio G, Donadio V, Felicori M, Fini N, Fiorillo C, Gallone S, Gemignani F, Gigli GL, Graziano C, Guerrini R, Gurrieri F, Kariminejad A, Lieto M, Marques LourenǴo C, Malandrini A, Mandich P, Marcotulli C, Mari F, Massacesi L, Melone MAB, Mignarri A, Milone R, Musumeci O, Pegoraro E, Perna A, Petrucci A, Pini A, Pochiero F, Pons MR, Ricca I, Rossi S, Seri M, Stanzial F, Tinelli F, Toscano A, Valente M, Federico A, Rubegni A, Santorelli FM. Next Generation Molecular Diagnosis of Hereditary Spastic Paraplegias: An Italian Cross-Sectional Study. Front Neurol. 2018 Dec 4; 9:981. doi: 10.3389/fneur.2018.00981. PMID: 30564185; PMCID: PMC6289125.

13. Krawczak M, Thomas NS, Hundrieser B, Mort M, Wittig M, Hampe J, Cooper DN. 2007. Single base-pair substitutions in exon–intron junctions of human genes: nature, distribution, and consequences for mRNA splicing. Hum Mutat 28: 150–158. doi:10.1002/humu.20400.

14. Caminsky N, Mucaki EJ, Rogan PK. 2014. Interpretation of mRNA splicing mutations in genetic disease: review of the literature and guidelines for information-theoretical analysis. F1000Res 3: 282. doi:10.12688/

15. Landrum MJ, Lee JM, Benson M, Brown G, Chao C, Chitipiralla S, Gu B, Hart J, Hoffman D, Hoover J, et al. 2016. ClinVar: public archive of interpretations of clinically relevant variants. Nucleic Acids Res 44: D862–D868. doi:10.1093/nar/gkv1222 f1000research.5654.1.

16. Lord J, Gallone G, Short PJ, McRae JF, Ironfield H, Wynn EH, Gerety SS, He L, Kerr B, Johnson DS, McCann E, Kinning E, Flinter F, Temple IK, Clayton-Smith J, McEntagart M, Lynch SA, Joss S, Douzgou S, Dabir T, Clowes V, McConnell VPM, Lam W, Wright CF, FitzPatrick DR, Firth HV, Barrett JC, Hurles ME; Deciphering Developmental Disorders study. Pathogenicity and selective constraint on variation near splice sites. Genome Res. 2019 Feb; 29(2):159-170. doi: 10.1101/gr.238444.118. Epub 2018 Dec 26. PMID: 30587507; PMCID: PMC6360807

17. Wai HA, Lord J, Lyon M, Gunning A, Kelly H, Cibin P, Seaby EG, Spiers-Fitzgerald K, Lye J, Ellard S, Thomas NS, Bunyan DJ, Douglas AGL, Baralle D; Splicing and disease working group. Blood RNA analysis can increase clinical diagnostic rate and resolve variants of uncertain significance. Genet Med. 2020 Jun;22(6):1005-1014. doi: 10.1038/s41436-020-0766-9. Epub 2020 Mar 3. Erratum in: Genet Med. 2020 Apr 1;: PMID: 32123317; PMCID: PMC7272326.

[ref_ada_rf] In silico prediction of splice-altering single nucleotide variants in the human genome
Nucleic Acids Res. 2014 Dec 16; 42(22): 13534–13544.
Xueqiu Jian, Eric Boerwinkle and Xiaoming Liu

[ref_spAI] Predicting Splicing from Primary Sequence with Deep Learning
Jaganathan et al.
Cell. 2019 DOI:https://doi.org/10.1016/j.cell.2018.12.015

Table 1: Splicing effect of Ada, Rf and SpliceAI bioinformatic tools for the variant c.1511-6C>G in *AP4B1* gene.

Bioinformatic tools	Splicing effect predictions
Ada (cutoff 0.612)	Score: 0,999879 Prediction: Possibly damaging splicing site
Rf (cutoff 0.598)	Score: 0,9419999 Prediction: Possibly damaging splicing site
SpliceAI (0-1)	Chr1:114438665 (=114438666-1) DS_AG Delta score (acceptor gain) = 0,89 Chr1:114438660 (=114438666-6) DS_AL Delta score (acceptor loss) = 0,41

Figure 1: Splicing effect of the variant c.1511-6C>G in AP4B1 gene as predicted by the Alamut Visual v2.14

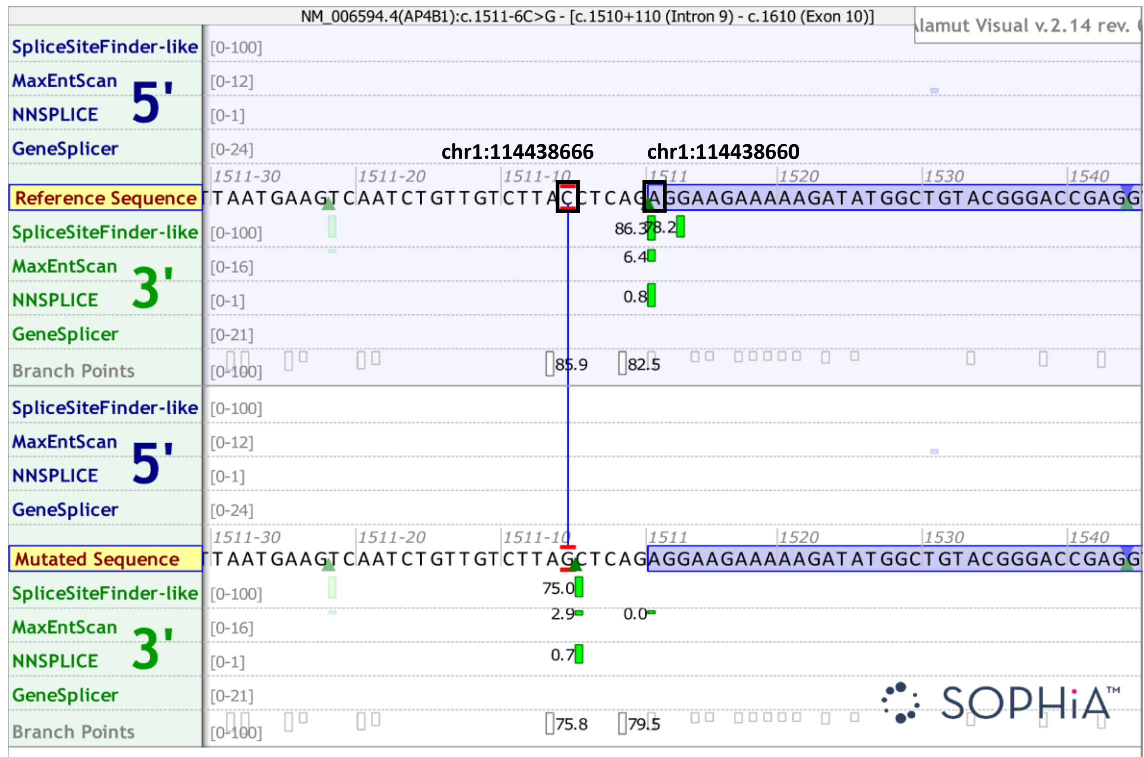
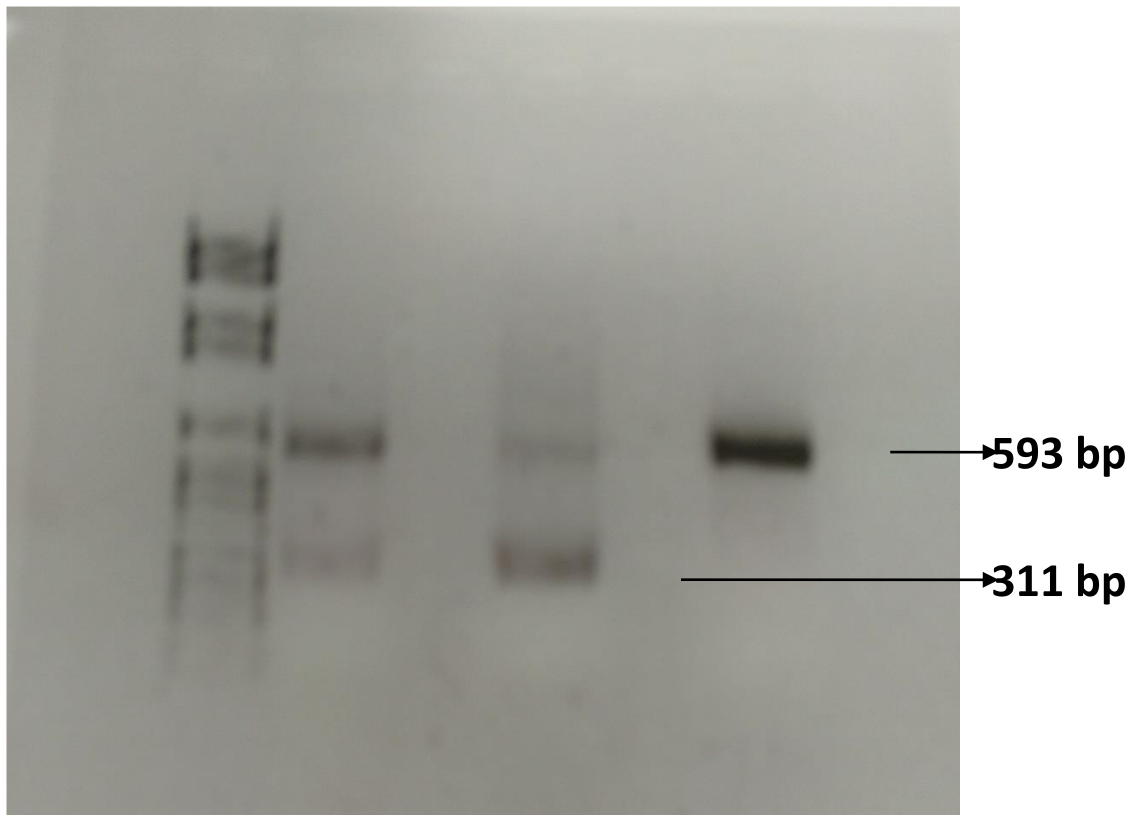


Figure 2:

Figure 2 A: Agarose gel DNA fragments obtained from the PCR reaction with primers forward and backward in exon 8 and exon 11 of the AP4B1 gene, respectively



VI Father Patient Control

Figure 2 B: Purified sequences of 311 bp and 593 bp DNA fragments from the patient