Characterization of novel CACNA1A splice variants by RNA -sequencing in patients with episodic or congenital ataxia

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SHORT REPORT

Characterization of novel *CACNA1A* splice variants by RNA-sequencing in patients with episodic or congenital ataxia

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Abstract

Loss of function variants in *CACNA1A* cause a broad spectrum of neurological disorders, including episodic ataxia, congenital or progressive ataxias, epileptic manifestations or developmental delay. Variants located on the AG/GT consensus splice sites are usually considered as responsible of splicing defects, but exonic or intronic variants located outside of the consensus splice site can also lead to abnormal splicing. We investigated the putative consequences on splicing of 11 *CACNA1A* variants of unknown significance (VUS) identified in patients with episodic ataxia or congenital ataxia. In silico splice predictions were performed and RNA obtained from fibroblasts was analyzed by Sanger sequencing. The presence of abnormal transcripts was confirmed in 10/11 patients, nine of them were considered as deleterious and one remained of unknown significance. Targeted next-generation RNA sequencing was done in a second step to compare the two methods. This method was successful to obtain the full cDNA sequence of *CACNA1A*. Despite the presence of several isoforms in the fibroblastic cells, it detected most of the abnormally spliced transcripts. In conclusion, RNA sequencing was efficient to confirm the pathogenicity of nine novel *CACNA1A* variants. Sanger or Next generation methods can be used depending on the facilities and organization of the laboratories.

KEYWORDS

CACNA1A, congenital ataxia, episodic ataxia, genetic diagnosis, RNA analysis, splicing defect

1 | INTRODUCTION

CACNA1A variants have been associated with hemiplegic migraine and episodic ataxia in 1996.[1,2] In recent years, the increase of NGS screening has allowed to widen the phenotypic spectrum of the diseases linked to *CACNA1A* to congenital or progressive ataxia, epilepsy, developmental delay, or a combination of several.[3–9] *CACNA1A* is therefore widely tested in NGS panels in patients with various phenotypes. While the variants responsible for hemiplegic migraine are missense variants leading to a gain of function, the variants responsible for episodic ataxia are loss of function variants and variants located on the AG/GT consensus splice sites are considered deleterious. However, other intronic or exonic variants may prevent normal splicing of the mRNA. Many splicing predictive tools have been developed but the predicted consequences of variants must be confirmed by functional tests. Here, we report the analysis of the cDNA extracted from fibroblasts of 11 patients with permanent or episodic ataxia in whom a variant of unknown significance with a putative effect on the splicing was detected, and we compare specific RT-PCR analyze with NGS RNA sequencing.

2 | PATIENTS AND METHODS

Eight patients presenting typical manifestation of episodic ataxia (P1 to P8) and three patients with congenital cerebellar ataxia (P9 to P11) are reported. Congenital ataxia was defined by the presence of clinical cerebellar symptoms before the age of 2 years, without any regression. All patients harbored variants of unknown significance that were tested for splicing on RNA extracted from fibroblasts.

Patients, or their parents if they were minors, signed an informed consent prior to blood sampling, skin biopsy and genetic analysis, in accordance with French law for diagnostic genetic testing. Testing was done in a hospital laboratory approved for genetic molecular diagnosis. The analyses were performed in accordance with French regulations and the principles of the Declaration of Helsinki.

3 | MOLECULAR SCREENING

The 47 exons of *CACNA1A* and their nearby intronic regions were sequenced. The prediction of the splicing effect of the variants was assessed using SpliceSiteFinder- like, MaxEntScan, NNSPLICE, GeneSplicer, SPiP[10] and SpliceAI.[11]

Total RNAs were extracted from fibroblasts obtained from patient's skin biopsy.

Specific primers were designed in exons spanning each variant and RT-PCR products had a Sanger sequencing.

Targeted RNA sequencing was done with the SureSelect XT HS2 RNA system (Agilent Technologies) and sequencing was performed in an Illumina MiSeq instrument. A reference RNA composed of total RNA from 10 human cell lines (Agilent Universal Human Reference RNA) and a normal RNA obtained from fibroblastic cell lines were used as controls. The reads were mapped to the reference human genome GRCh37 (Ensembl annotation, release 87) with the STAR aligner (v.2.5.2b), using default parameters. The resulting BAM alignment files were indexed using samtools (v.1.9) and implementation into the Integrated Genome Viewer (IGV) browser was performed to obtain Sashimi plots.

4 | RESULTS

4.1 | Detection of the variants in DNA

Eight patients with episodic ataxia and three patients with congenital ataxia had a *CACNA1A* variant of unknown significance with in silico predictions in favor of abnormal splicing. A short description of the patients is available in Table 1.

Nine variants were intronic and two variants were exonic. The variant c.631+5G>A in IVS4 had previously been reported in a family with episodic ataxia but cDNA analysis was not conducted[12]; the other variants were novel. Nine variants were absent in gnomAD v2, the two others were <1/10 000 (Table 1). The results of the prediction softwares are reported in Table 2.

4.2 | Sanger RNA sequencing

RT-PCR sequencing showed that 10/11 variants led to abnormal splicing: seven led to the deletion of the adjacent exon, and three led to the use of a cryptic intronic splice site and the insertion of intronic nucleotides in the cDNA (Table 2 and Supplemental Figure 1). No splicing defect was detected for the last one (P10).

Nine variants, eight detected in patients with episodic ataxia (P1–P8), and one detected in a patient with congenital ataxia (P9), were considered damaging by creating a frameshift leading to a premature stop codon, or leading to the deletion of an exon coding for a transmembrane domain essential for the conformation of the protein. Patient 9 has two variants in *CACNA1A*, c.1082G>A/p.Gly361Glu inherited from her symptomatic father that co-segregates in six members in the paternal branch with episodic ataxia manifestations, and c.783A>T inherited from her asymptomatic mother (penultimate nucleotide of exon 5). The second variant is responsible of a decrease in the strength of the splice site, which results in a reduction in exon 5′ splice efficiency (Supplemental Figure 2). The RNA degradation of either allele was excluded by the normal rate of the second variant c.1082G>A on the cDNA Sanger electropherogram. The variant c.5404-3C>T (P10) was considered as benign since no effect on RNA was detected (heterozygous polymorphisms in exons 16 and 42 excluded a degradation of the transcript). The interpretation of the variant c.6193-3C>A (P11) was doubtful since it led to an in-frame insertion of 60 intronic nucleotides in the cDNA supposed to result in the insertion of 20 amino acids in the intracytoplasmic C-terminal tail of the protein; this variant was inherited from an asymptomatic parent and is present 20 times in gnomAD v2.

TABLE 1 Patients and *CACNA1A* variants.

Patient	Pathology	Spo /Fam	Age at onset (years)	Age at referral (years)	Intercritic ataxia or nystagmus	Cerebellar atrophy	Other	Affected relatives	Genomic DNA	Frequency in gnomAD
P1	EA	Spo	35	71	Cerebellar syndrome	Yes (vermis)		–	IVS4: c.631+5G>A	Absent
P2	EA	Spo	30	41	Cerebellar syndrome	Yes (vermis)	Low IQ	–	IVS5: c.5403+3_5403+6del	Absent
P3	EA	?	12	22	Ataxia and nystagmus	No (22 yo)		2 Sisters: episodes of vertigo	IVS33: c.5137-9A>G	Absent
P4	EA	Fam	12	21	Nystagmus	No (21 yo)		Mother and brother[a]: EA	Exon 37: c.5628G>C	Absent
P5	EA	Fam	27	48	Ataxia and nystagmus	No		Father, brother and daughter[a]: EA	IVS13: c.1785-3C>G	Absent
P6	EA	Spo	2	3	No	No	Walking delay		IVS35: c.5403+4A>C	Absent
P7	EA	Fam	2	8	Nystagmus	Unknown	Learning delay	Father[a]: migraine and vertigo	IVS14: c.1917-3C>A	Absent
P8	EA	Spo	20	55	Nystagmus	No		–	IVS19: c.3092+3_3092+6del	Absent
P9[b]	CA	Fam	0	5	Ataxia and nystagmus	Yes		Father[b], grandfather[b], 2 uncles[b], 2 cousins[b]: EA Aunt[b]: gait disturbance	Exon 5: c.783A>T Exon 7: c.1082G>A	Absents
P10	CA	Spo	0	11	Ataxia and nystagmus	Yes		–	IVS35: c.5404-3C>T	6 / 249 082
P11	CA	Spo	0	5	Ataxia and nystagmus	No		–	IVS42: c.6193-3C>A	20/279 696

Abbreviations: CA: congenital ataxia; EA: episodic ataxia; Fam: familial; Spo: sporadic.

[a]Affected relatives tested for the variant detected in the proband and mutated.

[b]Affected relatives carrying the variant c.1082G>A/p.(Gly361Glu) that co-segregates with episodic ataxia manifestations in the paternal family.

TABLE 2 Splice predictions (acceptor splice sites and donor splice sites) and consequences on RNA.

Variant	Acceptor Splice Site location	SSF [0–100]	MaxEnt [0–12]	NNSPLICE [0–1]	GeneSplicer [0–24]	SPiP (Risk to alter splicing)	SPLICE AI acceptor site	RT PCR (exons)	Consequence on RNA (cDNA sequencing)	Predicted protein
c.1785-3C>G	Exon 14—c.1785 ℕ	−13.3%	8.87 ⇒ −	0.75 ⇒ −	4.86 ⇒ −	98.41%	AL: 0.92	11–16	Del exon 14 c.1785_1916del	p.(Tyr596_Gln639del*)
c.1917-3C>A	Exon 15—c.1917 ℕ	−11.2%	−25.4%	−17.2%	−48.3%	98.41%	AL: 0.57	11–16	Del exon 15 c.1917_1989del	p.(Phe640Tyrfs*118)
c.5137-9A>G	**Intron 33—c.5137–8**	**− ⇒ 80.56**	**− ⇒ 6.46**	**− ⇒ 0.49**	**− ⇒ 2.34**	98.41% Alteration of the consensus splice site and creation of a new splice site	**AG: 0.97** AL: 0.27	32–38	Creation of a cryptic splice site (ins 8 nt) c.513_514insTTCCACAG	p.(Met172Phefs*10)
	Exon 34—c.5137 ℕ	82.79 ⇒ −	−58.8%	−0.3%	9.17 ⇒ −					
c.5404-3C>T	Exon 36—c.5404 ℕ	70.59 ⇒ −	−17.0%	-	−22.3%	69.33%	AL: 0,01	30–37	No abnormal splice detected	p.=
c.6193-3C>A	**Intron 42—c.6193–60**	**= 81.88**	**= 9.37**	**= 0.97**	**= 14.09**	98.41%	**AG: 0.44** AL: 0.52	41–45	Use of a cryptic splice site (ins 60 nt) c.6192_6193ins60	p.(Gln2064_Ser2065ins TPGIWRSGPRAHLALPSAPQ)
	Exon 43—c.6193 ℕ	78.30 ⇒ −	−47.4%	0.67 ⇒ −	−81.2%					

Variant	Donor Splice Site location	SSF [0–100]	MaxEnt [0–12]	NNSPLICE [0–1]	GeneSplicer [0–24]	SPiP (Risk to alter splicing)	SPLICE AIDonor site	RT PCR (exons)	Consequence on RNA (cDNA sequencing)	Predicted protein
c.631+5G>A	Exon 4—c.631 ℕ	72.72 ⇒ −	6.68 ⇒ −	0.53 ⇒ −	3.88 ⇒ −	98.41%	DL: 0.87	2–6	Del exon 4+/−5 c.540_631del	p.(Ile181Phefs*47)
c.783A>T	Exon 5—c.784 ℕ	−9.5%	−18.6%	−5.7%	−50.1%)	85.91%	DL: 0.21	2–8	Del exon 5 c.632_784del	p.(Ser211_Asp262delinsAsn)
c.3092+3_3092+6del	Exon 19—c.3092 ℕ	84.81 ⇒ −	9.82 ⇒ −	0.99 ⇒ −	5.80 ⇒ −	98.41%	DL: 0.94 **DG: 0.28**	19–20	Use of a cryptic splice site (ins 31 nt) c.3092_3093ins31	p.(Glu1032Trp*2)
	Intron 19—c.3092+35	**= 72.75**	**= 6.72**	**= 0.78**	**+308.4%**					
c.5403+3_5403+6del	Exon 35—c.5403 ℕ	−18.0%	−69.8%	0.99 ⇒ −	5.48 ⇒ −	98.41%	DL: 0.95	30–37	Del exon 35 c.5253_5403del	p.(Ser1752Cysfs*2)
c.5403+4A>C	Exon 35—c.5403 ℕ	−12.4%	−17.9%	−15.4%	−79.7%	98.41%	DL: 0.63	30–37	Del exon 35 c.5253_5403del	p.(Ser1752Cysfs*2)
c.5628G>C	Exon 37—c.5628 ℕ	74.32 ⇒ −	5.13 ⇒ −	0.51 ⇒ −	-	98.41%	DL: 0.03	35–40	Del exon 37 c.5532_5628del	p.(Arg1846Phefs*23)

Note: Bold indicates the values for potential of cryptic splice sites creation.
ℕ indicates the value at the normal splice site.

4.3 | Targeted next-generation RNA sequencing

Sequencing of RNA extracted from fibroblasts showed a good coverage of all exons of *CACNA1A* except exon 44 that was poorly covered. Exons 37a and 37b, respectively, included in transcripts NM_001127221 and NM_001127222 are both present in fibroblasts. The alternative use of two different splice acceptor sites at the junction depending on the transcript leads to a reading difficulty at this place (Supplemental Figure 3).

RNAseq analysis showed good quality for all patients except one for whom the coverage was low (P6). The effect on RNA was clearly observed on Sashimi plots for seven variants (Supplemental Figures 4a, b). Variants leading to abnormal splicing on exon 35 or 37 were poorly visible.

5 | DISCUSSION

Nine *CACNA1A* deleterious variants including eight novel were identified that were absent in polymorphism databases and resulted in aberrant splicing in fibroblastic cells. The variant c.5404-3C>T was considered as probably benign and the variant c.6193-3C>A leading to a predicted insertion in-frame of 20 amino acids in the C-terminal segment remained of unknown significance.

In silico software gave globally good predictions. MaxEntScan and SSF software delivered the clearest and exact predictions for all variants. SpliceAI and SPIP that are more recent tools gave also a good rate of accurate predictions for our patients. The combination of in silico prediction softwares was an effective way to predict modifications of splice.

RNASeq combined with STAR analysis and Sashimi plot visualization was efficient except for variants with an impact on splicing of exons 35 and 37, notably due to the presence of several isoforms.

Interestingly, one patient carried two pathogenic variants, each inherited from a parent. The presence of homozygous or compound heterozygous *CACNA1A* variants has been reported in severe patients with epileptic encephalopathy.[13,14] In our patient, the presentation was more severe than in the paternal family members carrying one of the variants but less severe than previously published cases with two *CACNA1A* variants. The variant inherited from the asymptomatic mother has a partial effect on splicing; it may not cause clinical signs on its own but enhances severity when combined with a second deleterious variant in trans. This is the first case described with a decrease in splice site strength in *CACNA1A*.

At last, the search for anomalies by Sanger sequencing on RNA obtained from fibroblasts was successful. This technique needs a specific design of primers depending on the anomaly suspected but does not require any special equipment and can be easily realized. Next-generation RNA sequencing has the advantage of screening the entire sequence, allowing detection of potential heterozygous SNPs and exclusion of allele degradation in cases where no abnormal transcript is detected, but is impacted by the presence of multiple isoforms in fibroblast RNA (several different CACNA1A transcripts are present in fibroblastic cells). It is more expensive and demands a specific computer analysis but it could also detect putative splicing defect due to deep intronic variants not detected by exonic genomic sequencing. The choice between the two methods depends on the facilities and organization of the laboratories and on the position of the variant to be tested. RNA targeted sequencing has allowed to conclude on the pathogenicity of 9/11 variants in our patients. RNAseq analysis provides access to the whole transcript and identifies splicing defects for the majority of the variants but is impacted by the presence of multiple isoforms of the gene.

6 | CONCLUSION

Aberrant RNA splicing of the *CACNA1A* gene is a pathogenic mechanism in patients with episodic or congenital ataxia. Predictive splicing software give good performance for *CACNA1A* intronic variants located outside the consensus sites and synonymous exonic but consequences need to be confirmed by cDNA studies. The RNA obtained from a culture of fibroblastic cells provides a good quality sequence of the CACNA1A cDNA over almost the entire gene (exon 44 is not well covered). This technique allows the validation of most splicing anomalies suspected by predictive software.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

PEER REVIEW

The peer review history for this article is available at https://www.webofscience.com/api/gateway/wos/peer-review/10.1111/cge.14358.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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