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LETTER TO THE EDITOR

Aberrant splicing induced by the most common *EPG5* mutation in an individual with Vici syndrome

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Sir,

Recently, a study describing the clinical and genetic data of 50 patients (30 families) with Vici syndrome (OMIM 242840) was published in *Brain* (Byrne *et al.*, 2016*b*). Vici syndrome is a severe autosomal recessive condition recently found to be caused by mutations in the *EPG5* gene (NG_042838.1) (Cullup *et al.*, 2013). Principal features of Vici syndrome include neurological sequelae (agenesis of the corpus callosum, profound developmental delay, and progressive microcephaly), neonatal cataracts, hypopigmentation involving both skin and hair, progressive dilated or hypertrophic cardiomyopathy, and both B and T cell-related immunodeficiency (Byrne *et al.*, 2016*a*, *b*). The most common mutation identified in Vici syndrome patients is *EPG5* NM_020964.2; c.1007A>G p.Gln336Arg. Based on the two large cohorts of patients recently studied (Cullup *et al.*, 2013; Byrne *et al.*, 2016*b*), this mutation accounts for >10% (9/86) of mutations. Ancestry data from previous studies show that this mutation may be associated with Ashkenazi descent (Byrne *et al.*, 2016*a*, *b*). This variant was not detected in whole genome sequencing data from our cohort of 4456 unrelated adults seen at the Inova Translational Medicine Institute (Bodian *et al.*, 2014, 2016). This variant is seen with a frequency of 3.129×10^{-5} in the European (non-Finnish) population of Exome Aggregation Consortium (ExAC) and has thus far never been reported in other ExAC populations (ExAC, 2016; http://exac.broadinstitute.org).

The *EGP5* c.1007A>G mutation has been suggested to affect splicing through *in silico* prediction models, though multiple predictions are contradictory [Human Splicing Finder (Desmet *et al.*, 2009; http://www.umd.be/HSF3/HSF.html: −6%) and NNSPLICE (Reese *et al.*, 1997; http://www.fruitfly.org/seq_tools/splice.html: −100%)]. Likewise, *in silico* predictions of the missense mutation are in disagreement with regards to its pathogenicity [PolyPhen-2 (Adzhubei *et al.*, 2010; http://genetics.bwh.harvard.edu/pph2/: probably damaging, 0.998/1.00); SIFT (Kumar *et al.*, 2009; http://sift.jcvi.org/: tolerated, 0.36 > 0.05); and MutationTaster (Schwarz *et al.*, 2014; http://www.mutationtaster.org/: disease causing)]. Unlike other mutations causing Vici syndrome, full functional characterization of this

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common mutation is lacking (Cullup *et al.*, 2013; Byrne *et al.*, 2016*b*). Therefore, it is imperative that definitive proof of whether this mutation induces aberrant splicing or not is assessed. Furthermore, understanding if the patient phenotype is due to a null allele (splice mutation) or a missense mutation (p.Gln336Arg) is important in order to comprehend the molecular mechanism of Vici syndrome.

To investigate the functional effect of this mutation, including whether splicing is affected, we studied samples from a patient with prototypical features of Vici syndrome including static encephalopathy, absent corpus callosum, and neonatal cataracts. This individual had been found via commercial exome sequencing with Sanger validation (GeneDx) to be homozygous for the *EPG5* c.1007A>G mutation (Patient 29 in Byrne *et al.*, 2016*b*). Samples were obtained with full informed consent through a National Human Genome Research Institute IRB-approved protocol (76-HG-0238). Cultured primary dermal fibroblasts from this individual were harvested and after Sanger validation of the variant, RNA was extracted; fibroblasts from an unaffected individual (PCS-201-010, ATCC) were cultured and processed simultaneously as a control. Extracted mRNA was reverse transcribed using the SuperScript® VILO™ cDNA synthesis kit (Invitrogen).

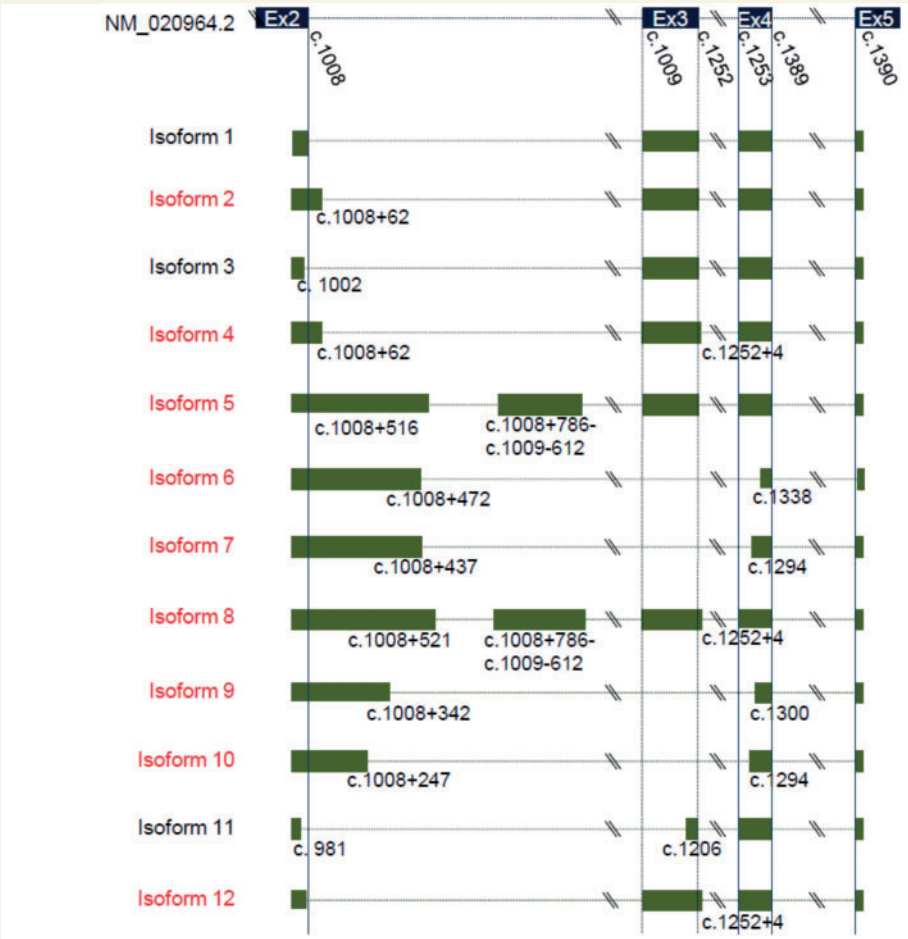
We first analyzed the *EPG5* mRNA level in the affected patient's cells. For this, we performed quantitative polymerase chain reaction (PCR) (TaqMan® assay Hs00826959_m1, Life Technologies), which showed a decrease of ~50% in the affected fibroblasts compared to control (normalized to *POLR2A* expression, Hs00172187_m1), suggesting that the c.1007A>G, p.Gln336Arg variant induces a decrease of *EPG5* mRNA (data not shown). This reduction of mRNA is most likely due to nonsense-mediated decay (NMD) of misspliced isoforms (Hentze and Kulozik, 1999). The next question we addressed was whether the observed, stable *EPG5* mRNA is the product of the correctly spliced isoform or not.

To analyze the residual mRNA, we PCR amplified patient and control cDNA using primers in exon 2 (5'-atgctaaaagtcggctgtgg-3') and exon 5 (5'-gccaactctttgtagcacgg-3') with AmpliTaq Gold®360 (Applied Biosystems). In the control sample, one prominent PCR product was observed with an apparent length of 450 nucleotides. In the patient, however, bands at ~470, 540 and >1000 nucleotides were observed by electrophoresis (data not shown). These different PCR products observed in the patient sample represent several different splice isoforms that are generated in the context of the missense mutation but not degraded by NMD.

To better characterize these different isoforms observed in the patient cDNA, we sequenced the amplified cDNA. For this, the PCR products were cloned into a pCR4-TOPO bacterial vector (Life Technologies) and over 100 individual clones were isolated and sequenced with both M13 forward and reverse primers. Surprisingly, 25% of clones sequenced from the patient sample showed normal mRNA splicing (isoform 1, Fig. 1). The other products observed in the patient sample represented several different splice isoforms (isoforms 2–11, Fig. 1; variant splice sites are indicated relative to the normal splice isoform cDNA positions). The most frequently occurring isoform, isoform 2, uses an alternate splice donor site 62 nucleotides into intron 2 (58% of clones, Fig. 1). Other splice isoforms include both an in-frame deletion of six nucleotides from the end of Exon 2, removing the c.1007A>G variant, as well as complex isoforms that include nucleotides from intron 2 with partial or complete skipping of exon 3. We occasionally (<6%) identified isoforms with missplicing in exon 4 (Fig. 1), incorporating 4 bp of intron 4. Although this missplicing was also seen in the control sample, of the 55 control clones sequenced only one harbored this 4 bp insertion at the end of Exon 4 (isoform 12, Fig. 1), with 98% of the clones mapping to the wild-type splice product, isoform 1 (Fig. 1).

Most of the isoforms (70%) identified would harbour a premature termination codon (PTC) (Fig. 1). For the splice products that encode part of intron 2, a PTC is inserted three residues downstream of the p.Gln336Arg missense mutation, such that the predicted protein sequence would be p.Gln336ArgfsX4. These isoforms encoding a PTC have, at least partially, evaded NMD. NMD is not a fully efficient process (Isken and Maquat, 2007) and different mechanisms can explain this escape, such as exon junction proximity (Nagy and Maquat, 1998), presence of upstream open reading frames (Stockklausner *et al.*, 2006) or downstream translation reinitiation (Hamid *et al.*, 2010). In our patient, the remaining 30% of transcripts that do not harbor a PTC are either normal splice products with the p.Gln336Arg missense mutation or carry an in-frame deletion (isoforms 3 and 11, Fig. 1).

In summary, we show that the most common pathogenic mutation in the Vici syndrome-associated gene *EPG5*, the missense NM_020964.2; c.1007A>G, p.Gln336Arg, induces a more complex effect than expected. It not only produces the normal isoform with the missense mutation but also multiple misspliced mRNA isoforms. Some of these isoforms are targeted for NMD, as shown by the decreased level of *EPG5* mRNA. All of the variants in EPG5 associated with Vici syndrome are null alleles (splice site, stop gain or frameshift) with the exception of few missense alleles (Byrne *et al.*, 2016*a*). With the proof that the missense p.Gln336Arg induces a splicing defect, it is possible that the other missense variants may affect the splicing, either directly by deleting or creating a splice site or affecting splicing regulatory elements (Ward and Cooper, 2010). Therefore, performing mRNA analysis in patients with missense variants might reveal that Vici syndrome is only associated with loss of function due to null alleles, where phenotype variability could be associated with the amount of residual protein produced (Byrne *et al.*, 2016*a*). With the increased generation of sequencing data, laboratory-based evidence of pathogenicity is important, especially for mutations that appear relatively common within a specific population. We hope that this functional evidence will be useful for genetic counseling, as well as contribute to the understanding of the biological underpinnings of this disease.



Isoform	% of Ct (*n*=55)	% of Pt (*n*=108)
1	98.2%	24.6%
2	0%	59.0%
3	0%	8.4%
4	0%	4.7%
5	0%	1.86%
6	0%	0.93%
7	0%	0.93%
8	0%	0.93%
9	0%	0.93%
10	0%	0.93%
11	0%	0.93%
12	1.8%	0%

Figure 1 Aberrant splicing. Different splicing events identified after cDNA amplification of *EPG5* (between exon 2 and 5) in control (Ct) and patient (Pt) fibroblasts compared to the normal isoform (NM_020964.2). The per cent of sequenced clones from each sample that map to the indicated isoforms are provided in the table along with the total number (*n*) of clones sequenced. Isoforms that are predicted to encode a premature termination codon are labelled in red. Coordinates for the aberrant splice sites are provided below each specific isoform and reference the normal isoform cDNA sequence.

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References

Adzhubei IA, Schmidt S, Peshkin L, Ramensky VE, Gerasimova A, Bork P, et al. A method and server for predicting damaging missense mutations. Nat Methods 2010; 7: 248–9.

Bodian DL, Klein E, Iyer RK, Wong WS, Kothiyal P, Stauffer D, et al. Utility of whole-genome sequencing for detection of newborn screening disorders in a population cohort of 1,696 neonates. Genet Med 2016; 18: 221–30.

Bodian DL, McCutcheon JN, Kothiyal P, Huddleston KC, Iyer RK, Vockley JG, et al. Germline variation in cancer-susceptibility genes in a healthy, ancestrally diverse cohort: implications for individual genome sequencing. PLoS One 2014; 9: e94554.

Byrne S, Dionisi-Vici C, Smith L, Gautel M, Jungbluth H. Vici syndrome: a review. Orphanet J Rare Dis 2016a; 11: 21.

Byrne S, Jansen L, U-King-Im JM, Siddiqui A, Lidov HG, Bodi I, et al. EPG5-related Vici syndrome: a paradigm of neurodevelopmental disorders with defective autophagy. Brain 2016b; 139 (Pt 3): 765–81.

Cullup T, Kho AL, Dionisi-Vici C, Brandmeier B, Smith F, Urry Z, et al. Recessive mutations in EPG5 cause Vici syndrome, a multisystem disorder with defective autophagy. Nat Genet 2013; 45: 83–7.

Desmet FO, Hamroun D, Lalande M, Collod-Beroud G, Claustres M, Beroud C. Human splicing finder: an online bioinformatics tool to predict splicing signals. Nucleic Acids Res 2009; 37: e67.

Hamid R, Hedges LK, Austin E, Phillips JA III, Loyd JE, Cogan JD. Transcripts from a novel BMPR2 termination mutation escape nonsense mediated decay by downstream translation re-initiation: implications for treating pulmonary hypertension. Clin Genet 2010; 77: 280–6.

Hentze MW, Kulozik AE. A perfect message: RNA surveillance and nonsense-mediated decay. Cell 1999; 96: 307–10.

Isken O, Maquat LE. Quality control of eukaryotic mRNA: safeguarding cells from abnormal mRNA function. Genes Dev 2007; 21: 1833–56.

Kumar P, Henikoff S, Ng PC. Predicting the effects of coding non-synonymous variants on protein function using the SIFT algorithm. Nat Protoc 2009; 4: 1073–81.

Nagy E, Maquat LE. A rule for termination-codon position within intron-containing genes: when nonsense affects RNA abundance. Trends Biochem Sci 1998; 23: 198–9.

Reese MG, Eeckman FH, Kulp D, Haussler D. Improved splice site detection in Genie. J Comput Biol 1997; 4: 311–23.

Schwarz JM, Cooper DN, Schuelke M, Seelow D. MutationTaster2: mutation prediction for the deep-sequencing age. Nat Methods 2014; 11: 361–2.

Stockklausner C, Breit S, Neu-Yilik G, Echner N, Hentze MW, Kulozik AE, et al. The uORF-containing thrombopoietin mRNA escapes nonsense-mediated decay (NMD). Nucleic Acids Res 2006; 34: 2355–63.

Ward AJ, Cooper TA. The pathobiology of splicing. J Pathol 2010; 220: 152–63.