Exome Sequencing in Brown-Vialetto-Van Laere Syndrome

To the Editor: Brown-Vialetto-van Laere syndrome (BVVL [MIM 211530]) is a rare, progressive, childhood neurodegenerative disease that is characterized by pontobulbar palsy, sensorineural hearing loss, and respiratory problems. BVVL is clinically heterogeneous, presenting as early as the neonatal period and as late as the third decade of life.[1] BVVL has a prominent familial component, consistent with an autosomal-recessive mode of inheritance, in all but one family reported. The genetic cause of BVVL was until recently unknown; therefore, we read with interest the recent article by Green and colleagues that described mutations of *C20orf54* (MIM 613350) as the underlying cause of BVVL.[2] We congratulate the authors on this exciting new finding.

At the time of publication by Green and colleagues, we were working on the identification of the genetic cause of BVVL, using predominantly whole-exome sequencing but also whole-genome sequencing. We had performed sequence enrichment for the exome using the SureSelect Human Exome Kit (Agilent Technologies), followed by second-generation sequencing on a GAIIx (Illumina) in two siblings and two unrelated patients with BVVL. In addition, we had performed exome enrichment and sequencing on one sample from family 2008, using a service from Roche which used NimbleGen array capture and 454 sequencing. Sequence alignment, quality control, and variant calling was performed with BWA,[3] SAMTools,[4] the Genomic Analysis Toolkit (GATK),[5] and Picard. When necessary, sequences were viewed with the use of IGV.

DNA from human subjects was collected after written, informed consent was given, and this study was approved by the institutional review board of the National Institute on Aging. Variants were identified with GATK's SingleSampleGenotyper and IndelGenotyper tools. The single-nucleotide variants were then filtered for the removal of low-quality variant calls with GATK's VariantFiltrationWalker tool, with filtering based on the LodThreshold, FisherStrand, ClusteredSnps, AlleleBalance, and IndelArtifact attributes. Prior to variant calling, each lane of exome and genome sequence data was aligned with the use of BWA. Samtools and Picard were used to convert, sort, and index the aligned data files. The sequence quality scores were recalibrated with GATK. Picard was then used to identify and remove duplicate reads from each lane and then merge the multiple lanes of sequencing data per patient.

Because we believed that underlying genetic mutation(s) causing BVVL was likely to be extremely rare, all identified variants were then filtered against dbSNP and 1000 Genomes, removing all previously reported variants. Restricting the variants to 937 nonsynonymous alterations detected in at least one of the four samples, we selected only those changes that were homozygous, or when a variant was heterozygous, an additional heterozygous variant was present in the same gene, in the same sample. This resulted in a sample-specific list of 37 genes with 39 homozygous nonsynonymous mutations and 48 genes with 140 compound-heterozygous nonsynonymous changes. We then compared these gene lists across samples and identified genes present in the affected siblings and at least one of the two other BVVL patients. This resulted in a list of eight genes, three of which contained a large number of variants, and we see a large number of previously unreported changes consistently in these transcripts across exome studies, suggesting that these variants are likely an artifact of the methodology (*CTBP2* [MIM 602619], *OR4C3* and *CDC27* [MIM 116946]; unpublished data), most likely because of sequencing error, a systematic artifact of the selection process, or an error in the underlying alignment. The remaining five genes were our primary candidates for BVVL: *LOC100130581*, *KIR2DL3* (MIM 604938), *ANKRD20B*, *C20orf54*, and *USP17L2*.

At this point we read Green and colleagues' work. We established that patient 2008-410 (II:2) in our study, corresponds to case 4 in the study by Green et al. Notably, although we also found the c.639C>G (p.Y213X; nucleotide sequence numbering is based on sequence NM_033409) mutation in *C20orf54* (NM_033409.3) reported by Green et al., in our exome-sequencing data, we observed this as a heterozygous mutation, rather than as a homozygous mutation as reported. Further, in this sample and in the DNA sample from the affected sibling, exome sequencing identified an additional rare heterozygous variant not reported by Green and colleagues, c.211G>A (p.E71K). To establish whether our observations were an artifact of the enrichment or of the second-generation sequencing process, we performed additional Sanger-based sequencing. This confirmed that both variants were present in both affected siblings as heterozygous changes (Figure 1). Analysis of DNA from the parents of 2008-410 (II:2) and 2008-411 (II:1) demonstrated that these two variants were indeed compound heterozygotes, the c.639C>G (p.Y213X) variant being inherited from the mother (I:1) and the c.211G>A (p.E71K) variant being inherited from the father (I:2) (Figure 1).

In sample 48111 (IV:1) from family DZ, exome sequencing revealed a rare homozygous c.82C>A (p.P28T) variant in *C20orf54*, and this was confirmed by Sanger sequencing (Figure 1). It was subsequently confirmed that this alteration segregates with disease in an affected niece (V:1) and an affected sibling (IV:2) (Figure 1). We failed to identify a rare nonsynonymous

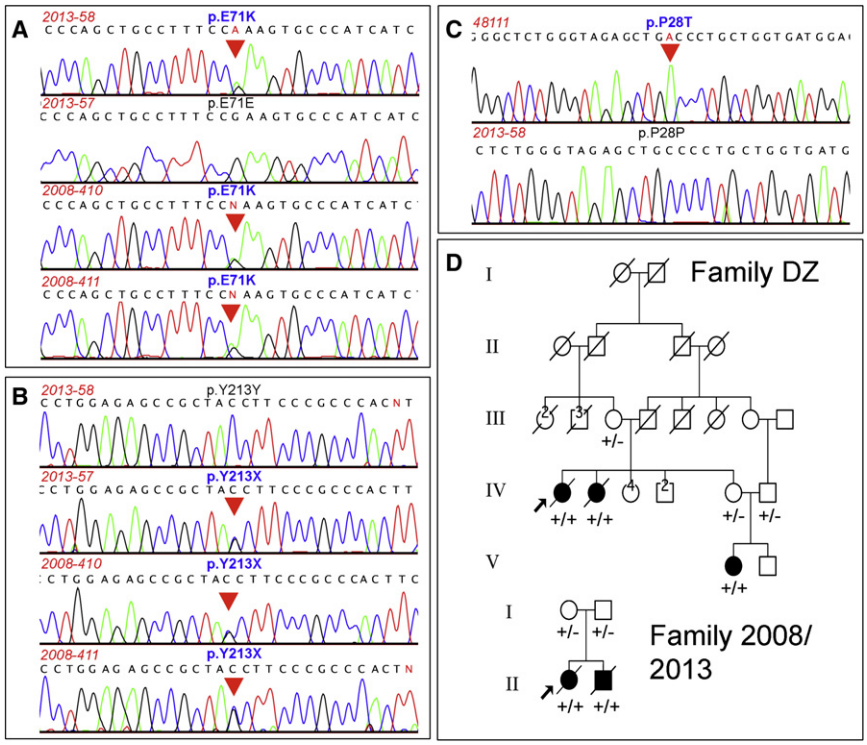

Figure 1. *C20orf54* **Mutations in Patients with BVVL**
(A) Heterozygous c.211G>A (p.E71K) mutation carried by both affected children (2008-410, II:2 and 2008-411, II:1) and the patients' father (I:2) but not by the patients' mother (I:1).
(B) Heterozygous c.639C>G (p.Y213X) mutation carried by both affected siblings and their mother (2008-410, II:2; 2008-411, II:1; and I:1) but not by the father (I:2).
(C) Homozygous c.82C>A (p.P28T) mutation carried by patient 48111 (IV:1) from family DZ.
(D) Pedigrees of families DZ and 2008/13, probands indicated by arrows.

variant in *C20orf54* in sample HH, the proband from a recessive family with typical childhood-onset BVVL, suggesting genetic heterogeneity in this disorder. However, the family was not informative enough to be useful for linkage analysis, and therefore we cannot exclude the possibility that we have missed a mutation.

Family DZ, which originated from Eastern Turkey, shows multiple consanguinity and has a total of three affected children with BVVL. Clinical features were identical, but there was over 20 yrs difference in the age at onset, with one case available for postmortem examination. The proband (IV:1) developed normally until she presented with acute respiratory distress, stridor, and paralysis of vocal cord abduction, requiring ventilation. She later developed cranial nerve palsies with ophthalmoplegia, dysarthria, dysphagia, tongue fasciculation, facial weakness, and weakness and wasting of the limbs. She died at the age of 8 yrs from respiratory failure. A postmortem examination was carried out, which revealed replacement gliosis of the cranial nuclei, particularly at the bulbo-pontine level, but only mild anterior horn cell involvement. The proband's affected sister (IV:2) developed hearing loss at the age of 10 yrs over a matter of months. In the following years she developed features identical to those of her deceased older sister, with progressive bulbar palsy, dysarthria, dysphagia, tongue fasciculation, facial weakness, weakness and wasting of the limbs, and breathing problems. Investigations revealed a motor neuronopathy and axonal degeneration on a nerve biopsy. She died at the age of 29 yrs from respiratory failure. A niece of the proband (V:1) is also clinically affected but is in the early stages of the disease. She has hearing problems and cranial nerve palsies. In family DZ the p.P28T mutation segregated with the disease as a homozygous trait and was not present in controls.

Family 2008 is from the United States, with European and Asian ancestry. The proband (II:1) developed normally until 16 mo of age, when she was diagnosed with progressive bulbar palsy. Over the next 17 mo she developed multiple cranial nerve palsies, beginning with bilateral vocal fold paralysis necessitating tracheostomy, facial and pharyngeal muscle weakness necessitating percutaneous endoscopic gastrostomy, hearing loss, and weakness and wasting of her limbs distally and of her neck muscles, requiring a brace. She was a bright child and was able to use sign language to communicate with her family. She became ventilator dependent in the last few months of her life and passed away from pneumonia, a complication of her tracheostomy. The brother (II:2) of the proband was born with transposition of the great vessels, requiring surgery at birth. He was well until the age of 9 mo, when he developed a unilateral ptosis that had been intermittent since birth but became permanent at this time. At this time he had abnormal bilateral auditory evoked potentials, and at the age of 12 mo he developed stridor due to bilateral adductor vocal fold paralysis. He was stable for 5 mo, babbling, playing, starting to stand and walk, and eating without difficulty or aspiration, but he developed an overactive gag reflex and over the next few months developed problems very similar to those of his sister and died of respiratory failure.

In summary we report accurate mutation detection of disease-causing mutations for BVVL by exome sequencing. We describe a compound heterozygous c.639C>G/c.211G>A (p.Y213X/p.E71K) mutation in a family previously reported to have disease caused by a homozygous p.Y213X mutation, and we report a p.P28T mutation,

absent from dbSNP and 1000 Genomes data, that we propose to be a cause of BVVL. We also describe the clinical and genetic heterogeneity that is present in BVVL.

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Web Resources

The URLs for data presented herein are as follows:

1000 Genomes, http://www.1000genomes.org/page.php
Burrows-Wheeler Aligner (BWA), http://bio-bwa.sourceforge.net/
dbSNP, http://www.ncbi.nlm.nih.gov/projects/SNP/
Genetic Alliance, http://www.geneticalliance.org
Genome Analysis Toolkit, https://www.broadinstitute.org/gsa/wiki/index.php/The_Genome_Analysis_Toolkit
Integrative Genomics Viewer (IGV), http://www.broadinstitute.org/igv/
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/
Picard, http://picard.sourceforge.net/index.shtml
SAMTools, http://samtools.sourceforge.net/

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Response to Johnson et al.

To the Editor: Johnson et al. report that a second mutation in *C20orf54* (MIM *613350) is seen in their BVVLS family "2008," which appears to be the same individual as our "case 4,"[1] implying that this patient is a compound heterozygote (p.E71K and p.Y213X) rather than homozygous for Y213X. Upon reexamination of the sequence traces, even with the benefit of hindsight, we still see a homozygous change at Y213X, although we do see a heterozygous change at E71K. We concur that this patient is most likely a compound heterozygote on the basis of the results of Johnson et al. This clearly demonstrates the advantage of testing additional family members, including parents whom we did not have access to at the time of paper submission.

We can confirm that we have also identified the homozygous mutation p.P28T in a sample provided to us by H. Houlden, which appears to be the second BVVLS patient reported by Johnson et al. (sample 48111). This result was not published in our report.[1]

We read with interest the exome-sequencing data the authors provide in their Letter. Before we submitted our paper, Dr. Singleton, upon learning that we had identified mutations in our BVVLS patients, provided us with a short list of 364 variants in 223 candidate genes from his study. Neither *c20orf54*, nor the other four genes mentioned in their letter appeared in this short list. The approach of whole-exome sequencing for determining causative mutations in rare monogenic disorders is one of the many exciting developments of next-generation sequencing.[2] It is currently still a very expensive, sledgehammer approach, which will undoubtedly become more cost effective in the near future. Some of the pitfalls demonstrated by Johnson et al. may also be ameliorated by technical improvements. Notably, sequencing several patients, filtering by dbSNP, and shortlisting mutations that occur in all patients may yield confusing results if, as in the