De novo mutations in *PURA* are associated with hypotonia and developmental delay

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Abstract *PURA* is the leading candidate gene responsible for the developmental phenotype in the 5q31.3 microdeletion syndrome. De novo mutations in *PURA* were recently reported in 15 individuals with developmental features similar to the 5q31.3 microdeletion syndrome. Here we describe six unrelated children who were identified by clinical whole-exome sequencing (WES) to have novel de novo variants in *PURA* with a similar phenotype of hypotonia and developmental delay and frequently associated with seizures. The protein Purα (encoded by *PURA*) is involved in neuronal proliferation, dendrite maturation, and the transport of mRNA to translation sites during neuronal development. Mutations in *PURA* may alter normal brain development and impair neuronal function, leading to developmental delay and the seizures observed in patients with mutations in *PURA*.

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INTRODUCTION

Whole-exome sequencing (WES) provides a comprehensive strategy to identify pathogenic genetic variants in patients with developmental abnormalities (Veltman and Brunner 2012; Yang et al. 2013, 2014). The 5q31.3 microdeletion syndrome is associated with hypotonia, feeding difficulties, severe developmental delay, and epilepsy. Genomic 5q31.3 deletions range in size from 101 kb to 5 Mb and include the purine-rich element binding protein A (*PURA*), a candidate gene for the developmental manifestations (Shimojima et al. 2011; Hosoki et al. 2012; Brown et al. 2013). *PURA* is essential for normal brain development, synapse formation and proliferation of neurons, oligodendrocytes, and astrocytes in the central nervous system (Khalili et al. 2003; Johnson et al. 2006; Hokkanen et al. 2012). Mutations in *PURA* have previously been associated with moderate to severe developmental delay, learning disability, hypotonia, neonatal respiratory issues, feeding difficulties, and seizures or "seizure-like" movements in 15 patients (Hunt et al. 2014; Lalani et al. 2014). Here we describe six unrelated patients with de novo mutations in *PURA* identified through WES associated with the consistent phenotype of hypotonia and developmental delay and frequently associated with seizures.

RESULTS

Clinical Presentation

Six unrelated patients all share similar clinical features of hypotonia and developmental delay (Table 1). The children range in age from 6 mo to 15 yr, and none of the children are functionally verbal. There is no history of regression in any of the children. Patient 1 had poor suck, bradycardia, and apnea during the neonatal period. "Seizure-like" activity was observed in two patients. One had "seizure-like" activity (twitching, stiffening, staring spells, collapsing) with a normal EEG, and a second developed myoclonic movements and possible gelastic seizures at 3 yr of age. One child has a history of infantile spasms. Vision has been variably and minimally impaired in five of the children. All have normal hearing. One child also has mild osteopenia and a history of fractures. There is no evidence of other significant medical problems or birth defects. Four of the children are dysmorphic with varying features (Fig. 1). Two of the patients have only minor features such as epicanthal folds or highly arched palate, while two other patients have more significant findings such as dolichocephaly, hypertelorism, broad forehead, and persistent fetal pads. Height and weight are within the normal range. Head circumferences ranged from less than the 10th to the 97th percentile. Brain MRI demonstrates delayed myelination and nonspecific enlargement of the subarachnoid spaces, cortical sulci, and ventricular system in one patient, periventricular white matter changes reflective of a stroke in the second patient, periventricular leukomalacia in the third, and mild corpus callosum volume loss in the fourth. Three of the patients have constipation and one also has gastroesophageal reflux disease. All children had clinical WES as part of a trio analysis wherein the proband and both parents were sequenced. There was no known history of consanguinity in any of the families.

Exome sequencing produced an average of ~13 Gb of sequence per sample (Table 2). The mean coverage of captured regions was ~150× per sample with >98% covered with at least 10× coverage, an average of 92% of base call quality of Q30 or greater, and an overall average mean quality score of >Q36. Filtering of common SNPs (>10% frequency present in 1000 Genomes database) resulted in ~4500 variants per proband sample. In total, 232 genes (257 unique sequence changes) of interest were identified across the six families when considering all possible modes of inheritance. Evaluation of these 232 genes eliminated 231 genes lacking clinical overlap with the patients' phenotype, leaving one gene.

Novel de novo heterozygous variants in *PURA* were detected by WES and confirmed by Sanger sequencing in six affected children (Table 1 and Fig. 2). The *PURA* p.Ile188Thr (c.563T>C) mutation in Patient 1 represents a nonconservative amino acid substitution of a hydrophobic residue replaced by a polar residue. Amino acid isoleucine 188 is highly conserved throughout evolution (Fig. 2B). The c.768dupC (p.Ile257Hisfs*37) mutation in Patient 2 produces a frameshift starting with codon isoleucine 257 that changes this amino acid to a histidine residue and creates a premature termination codon that deletes the last 66 amino acids after the insertion of 36 novel amino acids. This mutation is predicted to cause a loss of normal protein function through protein truncation. In Patient 3, the de novo c.1A>T (p.Met1?) mutation alters the initiator methionine codon (Met1). It is not known whether the loss of Met1 disrupts all protein translation from that allele or whether an abnormal protein is produced using an alternate Met start codon. The c.697_699delTTC (p.Phe233del) mutation in Patient 4 is an in-frame deletion, previously reported in a patient by Hunt et al. (2014). The c.4_8delGCGGA (p.Ala2Profs*197) mutation identified in Patient 5 causes a frameshift starting with codon alanine 2, changes this amino acid to a proline residue and creates a premature termination codon and replaces the last 321 residues with 196 different ones. This mutation is predicted to cause a loss of normal protein function through protein truncation. Patient 6 has a c.302_310delCTCTCTCCA (p.Thr101_Ser103del) in-frame deletion of three

Table 1. Clinical features of patients with mutations in *PURA*

Patient	Age	Sex	Mutation	Hypotonia	Head circumference	Age at sitting	Age at walking	Verbal skills	Vision	Brian MRI	EEG	Seizure
1	8 yr	M	c.563T>C p.Ile188Thr	Y	97th %ile	1 yr	3 yr	One word approximations	Normal	Slightly delayed myelination	Normal	"Seizure-like" activity
2	4 yr	M	c.768dupC p.Ile257Hisfs*37	Y	Unknown	1 yr	4 yr with a walker	Nonverbal	Optic nerve pallor, esotropia	White matter changes	Normal	None
3	10 yr	F	c.1A>T p.Met1?	Y	50th %ile	1 yr	4 yr	Nonverbal	Strabismus, esotropia	Normal	Unknown	Myoclonic, possible gelatic seizures
4	6 mo	F	c.697_699delTTC p.Phe233del	Y	5–10th %ile	N/A	N/A	N/A	Cortical visual impairment	Periventricular leukomalacia	Normal	None
5	15 yr	F	c.4_8delGCGGA p.A1a2Profs*197	Y	25–50th %ile	Unknown	4 yr	Nonverbal	Exotropia	Mild corpus callosum volume loss	Abnormal	Infantile spasms
6	5 yr	F	302_310delCTCTCTCCA p.Thre101_Ser103del	Y	33rd %ile	5 yr	Not walking	Nonverbal	Myopia and strabismus	Normal	Normal	Spasticity in foot and lower extremities
Hunt et al. #1	4 yr	F	c.726_727delGT p.Phe243Tyrfs*50	Y	9–25th %ile	2 yr	Nonambulatory	Nonverbal	Nystagmus with preserved optokinetic reflex, dysconjugate gaze	Delayed myelination	Normal	"Seizure-like" episodes
Hunt et al. #2	14 yr	F	c.847delG p.Glu283Argfs*45	N	<0.4th %ile	1 yr	2 yr	Sentences; limited vocabulary	Normal	Normal	N/A	None
Hunt et al. #3	12 yr	F	c.616A>T p.Ile206Phe	Y	75–91st %ile	1 yr	1 yr	Short phrases, repetitive	Normal	Normal	Abnormal	"Seizure-like" episodes
Hunt et al. #4	6 yr	F	c.697_699delTTC p.Phe233del	Y	75th %ile	Cannot sit unassisted	Nonambulatory	Nonverbal	Early cortical visual impairment, eye movements dysconjugate	Delayed myelination	Abnormal	Infantile spasms, progressed to tonic, focal dyscognitive seizures
Lalani et al. #1	6 mo	M	c.812_814delTCT p.Phe271del	Y	86th %ile	Cannot sit unassisted	N/A	N/A	Normal	Normal	Abnormal	Infantile myoclonus, partial onset with secondary clonic generalization
Lalani et al. #2	7 mo	M	c.307_308delTC p.Ser103Hisfs*97	Y	20th %ile	Cannot sit unassisted	N/A	N/A	Intermittent exotrophia	Dysmorphic corpus callosum	N/A	None
Lalani et al. #3	10 mo	M	c.556C>T p.Gln186*	Y	73rd %ile	Cannot sit unassisted	N/A	N/A	Normal	Normal	Normal	"Seizure-like" episodes in the neonatal period, myoclonic jerks

Continued

Table 1. *Continued*

Patient	Age	Sex	Mutation	Hypotonia	Head circumference	Age at sitting	Age at walking	Verbal skills	Vision	Brian MRI	EEG	Seizure
Lalani et al. #4	1 yr	F	c.289A>G p.Lys97Glu	Y	65th %ile	10 mo	Nonambulatory	Nonverbal	Strabismus	Normal	N/A	Myoclonic jerks, exaggerated startle
Lalani et al. #5	4 yr	F	c.299T>C p.Leu100Pro	Y	66th %ile	1 yr	Nonambulatory	Nonverbal	Nystagmus, Brown syndrome	Mild myelin maturation delay	N/A	Single generalized tonic clonic seizure at 5 mo
Lalani et al. #6	2 yr	F	c.363C>G p.Tyr121*	Y	91st %ile	1 yr	Non-ambulatory	Nonverbal	Normal	Absent septum pellucidum	Abnormal	Myoclonic jerks
Lalani et al. #7	2 yr	F	c.783C>G p.Tyr261*	Y	76th %ile	1 yr	Non-ambulatory	Nonverbal	Left eye estropia	Normal	Abnormal	Seizure-like episode in the neonatal period, myoclonic jerks
Lalani et al. #8	5 yr	F	c.470T>A p.Mct157Lys	Y	85th %ile	Cannot sit unassisted	Nonambulatory	Nonverbal	Nystagmus, strabismus	Normal	N/A	None
Lalani et al. #9	12 yr	M	c.265G>C p.Ala89Pro	Y	>97th %ile	Cannot sit unassisted	Nonambulatory	Nonverbal	Nystagmus	Hypomyelination	Abnormal	Myoclonic, generalized tonic and atonic seizures
Lalani et al. #10	12 yr	F	c.263_265delTCG p.Ile88_Ala89delins Thr	Y	50th %ile	2 yr	3 yr	Nonverbal	Strabismus, myopia	Normal	Abnormal	Lennox-Gastaut syndrome, intractable generalized epilepsy
Lalani et al. #11	15 yr	F	c.596G>C p.Arg199Pro	Y	N/A	Unknown	Nonambulatory	Nonverbal	Nystagmus, extremely farsighted	N/A	Abnormal	Lennox-Gastaut syndrome, myoclonic drops, tonic clonic, startle seizures

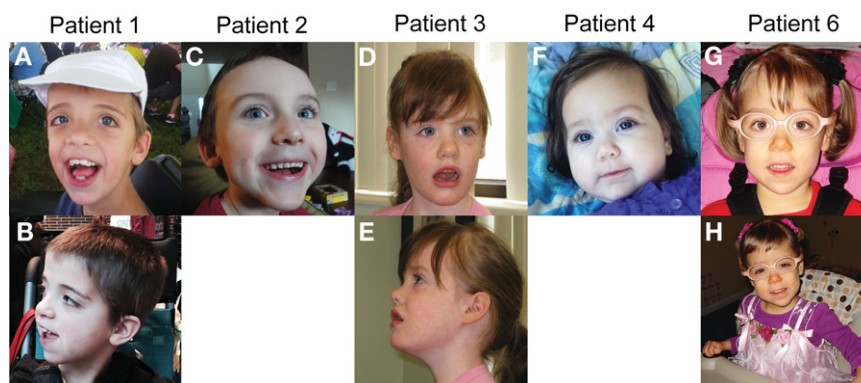MRI, magnetic resonance imaging; EEG, electroencephalogram.

Figure 1. Photographs of patients. (*A,B*) Patient 1. (*C*) Patient 2. (*D,E*) Patient 3. (*F*) Patient 4. (*G,H*) Patient 6. Patients 1 and 2 exhibit dolichocephaly and Patients 1–5 all have a broad forehead. Note hypertelorism and highly arched palate in Patient 1 and epicanthal folds in Patient 4.

amino acids in the highly conserved Pur I domain. None of the de novo variants identified in these six patients were detected in the Database of Single Nucleotide Polymorphisms (dbSNP; http://www.ncbi.nlm.nih.gov/SNP/), 1000 Genomes (1000G; http://www.1000genomes.org/), Exome Aggregation Consortium (ExAc; http://exac.broadinstitute.org), or Exome Variant Server (ESP; http://evs.gs.washington.edu/EVS/). All six mutations are located in highly conserved regions and all except for the mutation of Patient 3 are predicted to be deleterious by SIFT (http://sift.jcvi.org/), CADD (http://cadd.gs.washington.edu/), and MutationTaster (http://www.mutationtaster.org/). The de novo c.1A>T (p.Met1?) mutation in Patient 3 is presumed deleterious by CADD and Mutation Taster.

DISCUSSION

Six unrelated patients ranging in age from 6 mo to 15 yr with common clinical features of hypotonia and developmental delay were all found to have heterozygous de novo predicted deleterious rare or novel variants in *PURA*, identified through WES (Table 1). *PURA* is the leading candidate gene responsible for the developmental phenotype in the 5q31.3 microdeletion syndrome (Brown et al. 2013; Hunt et al. 2014; Lalani et al. 2014). Two papers

Table 2. Sequencing results

Patient	10× cov. (%)	Mean cov.	Yield (Gb)	Q30	MeanQ	Filtered vars	*PURA* mean CDS cov.	Var. total fam. cov.	Samples	Mean per-sample var. cov.
1	98.57	122	14.6	88	34	4973	105	491	3	164
2	98.79	166	12.5	94	36	4057	128	721	3	240
3	98.61	165	11.6	95	37	4475	143	63	3	21
4	98.71	201	14.6	88	35	4613	149	811	3	270
5	98.45	155	11.1	93	36	4504	125	41	3	14
6	97.22	122	12.3	91	36	5445	70	244	3	81
Mean	98.39	155	13	92	36	4678	120	395	3	132

cov., coverage; CDS, coding sequence; var., variance; fam., family.

A

B

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Human       STQGQTIALPAQGLIEFRDAL
Chimpanzee  STQGQTIALPAQGLIEFRDAL
Mouse       STQGQTIALPAQGLIEFRDAL
Sheep       STQGQTIALPAQGLIEFRDAL
Xenopus     LQSGQTIALPAQGLIEFRDAL
Zebrafish   STQGQTIALPAQGLIEFRDAL
Drosophila  ---RSQIALPAQGMIEFRDAL
```
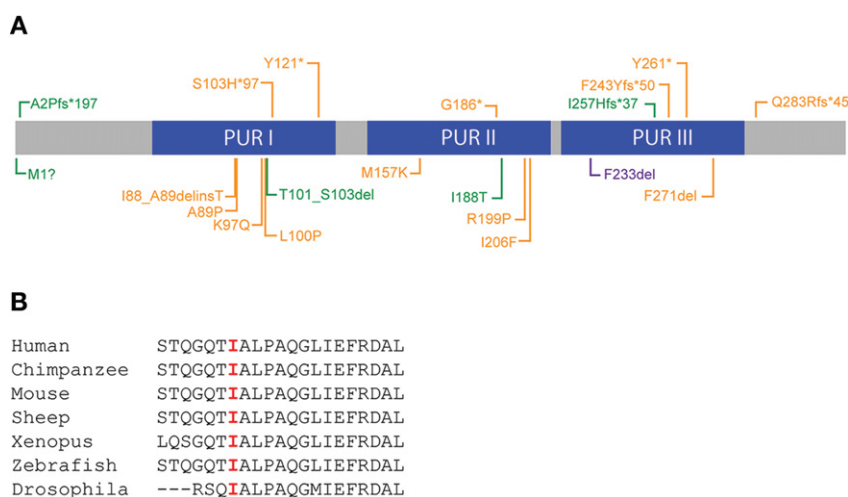
Figure 2. Mutations in *PURA*. (*A*) Diagram of Purα with mutations identified in our patients in green. Previously identified mutations are in orange (Hunt et al. 2014; Lalani et al. 2014). Missense and in-frame deletion mutations are shown *below* the protein diagram and gene disrupting truncating mutations are shown *above*. (*B*) Sequence alignment of Ile188 in PUR II region illustrating conservation of amino acids at amino acid 188.

recently reported de novo mutations in *PURA* in 15 individuals with developmental features equivalent to the 5q31.3 microdeletion syndrome (Hunt et al. 2014; Lalani et al. 2014). A separate large-scale WES study implicated 12 novel genes enriched for damaging de novo mutations with evidence for a role in developmental disorders (The Deciphering Developmental Disorders Study 2015). The analysis of the exomes of 1133 children with severe, undiagnosed developmental disorders and their parents identified *PURA* in three of the patients. We report six additional unrelated patients with de novo mutations in *PURA* to provide further evidence that *PURA* is largely, if not solely, responsible for the developmental delay, hypotonia, and seizures observed in the 5q31.3 microdeletion syndrome. Our additional patients expand the number and location of mutations in *PURA* and the associated phenotypes. Four of our patients had variants in the PUR domains, which are highly conserved throughout evolution (Fig. 2).

PURA is located on 5q31.2 and is encoded by a single exon that encodes a highly conserved multifunctional protein, Purα. *PURA* is expressed ubiquitously, including the brain, muscle, heart, and blood. Purα is a member of the Pur family of nucleic acid binding proteins which consist of a glycine-rich flexible amino terminus, a central core region and a potential carboxy-terminal protein binding region. All human Pur proteins have three highly conserved sequence-specific repeats, Pur repeats I–III, of 64–80 amino acids that are the hallmark of the Pur proteins. Purα has helix-unwinding capability (Khalili et al. 2003) and has been shown to bind specific sequences of ssDNA, dsRNA, and ssRNA with preference for GGN-repeats (Gallia et al. 2000; Graebsch et al. 2009; Johnson et al. 2013) to regulate a variety of cellular processes including DNA replication, gene transcription, RNA transport, and mRNA translation (White et al. 2009; Johnson et al. 2013).

Studies on mice have shown that Purα is involved in neuronal proliferation, dendrite maturation, and the transport of mRNA to translation sites in hippocampal neurons (Khalili et al. 2003; Kanai et al. 2004; Johnson et al. 2006; Hokkanen et al. 2012). Two independently generated knockout mouse models demonstrate that mice appear normal at birth but develop neurologic features, including ataxic gait, hind limb weakness, and continuous and increasingly severe tremors (Khalili et al. 2003; Hokkanen et al. 2012). The *PURA* knockout mice eat and sleep normally but do not gain weight. Throughout postnatal

development, these mice exhibit mislamination of the cerebellum and cerebrum and low numbers of Purkinje cells in the hippocampus and cerebellum associated with uncoordinated movements, tremors, and lethargy with death by 21–25 d. Heterozygous $PURA^{+/-}$ mice appeared normal but exhibited neurologic and myeloid defects that were intermediate in severity compared with $PURA^{-/-}$ mice. Both $PURA^{-/-}$ and $PURA^{+/-}$ mice had myeloid defects with reduced splenic monocyte development; however, the effect on $PURA^{+/-}$ mice was not as severe as the knockout mice due to *PURA* haploinsufficiency. Heterozygous $PURA^{+/-}$ mice also had occasional spontaneous seizures upon handling (Khalili et al. 2003; Hokkanen et al. 2012).

In the central nervous system, Purα has been detected in large neuronal mRNA-containing complexes with the fragile X mental retardation protein (FMRP), encoded by *FMR1*, an RNA-binding protein that is required for normal neural development. Johnson et al. (2006, 2013) showed that Purα is specifically expressed in the dendrites of hippocampal neurons in rats, and colocalizes with FMRP at dendritic junctional translation sites. Purα also binds to CGG repeats in *FMR1*, which is silenced in individuals with fragile X syndrome (FXS) and is overexpressed in patients with fragile X–associated tremor/ataxia syndrome (FXTAS). It is possible that impaired binding of Purα to CGG repeats in FMR1 may play a role in disease progression, resulting in defective neural and brain development in both settings.

Purα is also known to regulate expression of the myelin proteolipid protein Plp1, which is the predominant structural component of myelin sheaths in the central nervous system (Dobretsova et al. 2008). This interaction may be related to the delayed and decreased myelination observed in the brain MRIs. Mutations in *PLP1* are associated with Pelizaeus–Merzbacher disease (PMD; MIM#312080) (Torii et al. 2014). We hypothesize that abnormal or decreased binding of Purα to its targets may be responsible for defective brain development and function and related to the seizures seen in patients with mutations in *PURA*. Additional studies on patients with *PURA* mutations are necessary to better understand the correlation between genotype and phenotype and further investigation of the molecular mechanism of *PURA* during brain development and function.

METHODS

Whole-Exome Sequencing

Genomic DNA was extracted from whole blood from 1098 affected children with developmental delay and their parents. Exome sequencing was performed on exon targets isolated by capture using the Agilent SureSelect Human All Exon V4 (50-Mb) kit (Agilent Technologies). One microgram of DNA from blood specimen was sheared into 350–400-bp fragments, which were then repaired, ligated to adaptors, and purified for subsequent PCR amplification. Amplified products were then captured by biotinylated RNA library baits in solution following the manufacturer's instructions. Bound DNA was isolated with streptavidin-coated beads and reamplified. The final isolated products were sequenced using the Illumina HiSeq 2000 or 2500 sequencing system with 100-bp paired-end reads (Illumina). DNA sequence was mapped to the published human genome build UCSC hg19/GRCh37 reference sequence using BWA with the latest internally validated version at the time of sequencing, progressing from BWA v0.5.8 through BWA-Mem v0.7.8 (Li and Durbin 2009; Li 2012). Targeted coding exons and splice junctions of known protein-coding RefSeq genes were assessed for average depth of coverage with a minimum depth of 10× required for inclusion in downstream analysis. Local realignment around insertion-deletion sites was performed using the Genome Analysis Toolkit v1.6 (DePristo et al. 2011). Variant calls were generated simultaneously on all sequenced family members using SAMtools v0.1.18 (Li

Table 3. Variants identified from whole-exome sequencing of six families

	Filtering results	Manual review	Resulting genes of interest
Homozygous (# seq changes)	80 (83)	0 (0)	0 (0)
Compound heterozygous (# seq changes)	15 (32)	5 (10)	0 (0)
De novo (# seq changes)	127 (132)	1 (1)	1 (1)
X-linked genes (# seq changes)	10 (10)	1 (1)	0 (0)
Total genes (# seq changes)	232 (257)	7 (12)	1 (1)

et al. 2009). All coding exons and surrounding intron/exon boundaries were analyzed. Automated filtering removed common sequence changes (defined as >10% frequency present in 1000 Genomes database). The targeted coding exons and splice junctions of the known protein-coding RefSeq genes were assessed for the average depth of coverage and data quality threshold values. Whole-exome sequence data for all sequenced family members were analyzed using GeneDx's XomeAnalyzer (a variant annotation, filtering, and viewing interface for WES data), which includes nucleotide and amino acid annotations, population frequencies (NHLBI Exome Variant Server and 1000 Genomes databases), in silico prediction tools, amino acid conservation scores, and mutation references. Variants were filtered based on inheritance patterns, gene lists of interest, phenotype and population frequencies, as appropriate. Resources including the Human Gene Mutation Database (HGMD), 1000 Genomes database, NHLBI Exome Variant Server, OMIM, PubMed, and ClinVar were used to evaluate genes and detected sequence changes of interest (Table 3). Additional searches were performed using specific gene lists related to ID. Identified sequence changes of interest were confirmed in all members of the trio by conventional dideoxy DNA sequence analysis using an ABI3730 (Life Technologies) and standard protocols with a new DNA preparation.

ADDITIONAL INFORMATION

Ethics Statement

The study was approved by the Institutional Review Board of Columbia University and written consent was obtained for collecting blood samples and sequencing from all study participants.

Data Deposition and Access

Whole-exome sequencing data is not publicly available because patient consent could not be obtained. The *PURA* variants found in this study have been deposited in ClinVar under accession numbers SCV000223979, SCV000223981, SCV000223986, SCV000223987, SCV000223989, and SCV000223991.

Author Contributions

A.J.T. analyzed the data, drafted, and critically reviewed the manuscript. R.B. analyzed the data and critically reviewed the manuscript. M.T.C. analyzed the data and critically reviewed the manuscript. K.A.-Y. provided the clinical data and critically reviewed the manuscript. P.A.

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