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Two *De Novo* Novel Mutations in One *SHANK3* Allele in a Patient with Autism and Moderate Intellectual Disability

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Abstract

SHANK3 encodes for a scaffolding protein that links neurotransmitter receptors to the cytoskeleton and is enriched in postsynaptic densities of excitatory synapses. Deletions or mutations in one copy of the *SHANK3* gene cause Phelan-McDermid syndrome, also called 22q13.3 deletion syndrome, a neurodevelopmental disorder with common features including global developmental delay, absent to severely impaired language, autistic behavior, and minor dysmorphic features. By whole exome sequencing, we identified two *de novo* novel variants including one frameshift mutation and one missense change in a 14-year-old boy with delayed motor milestones, delayed language acquisition, autism, intellectual disability, ataxia, progressively worsening spasticity of the lower extremities, dysmorphic features, short stature, microcephaly, failure to thrive, chronic constipation, intrauterine growth restriction and bilateral inguinal hernias. Both changes are within the CpG island in exon 21, separated by a 287 bp sequence. Next generation sequencing of PCR products revealed that the two variants are most frequently associated with each other. Sanger sequencing of the cloned PCR products further confirmed that both changes were on a single allele. The clinical presentation in this individual is consistent with other patients with a truncating mutation in exon 21, suggesting that the missense change contributes none or minimally to the phenotypes. This is the first report of two *de novo* mutations in one *SHANK3* allele.

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Keywords

Phelan-McDermid syndrome; SHANK3; whole exome sequencing; de novo; double mutations on one allele; variant phasing

Introduction

The *SHANK3* (SH3 and multiple ankyrin repeat domains 3) gene encodes a scaffold protein of the postsynaptic density (PSD) of excitatory synapses, functioning by interacting directly or indirectly with various proteins, including major types of glutamate receptors such as NMDARs, AMPARs, and mGluRs [Ehlers 1999; Sheng and Kim 2000]. It is preferentially expressed in the striatum, cerebral cortex and cerebellum. SHANK3 belongs to the SHANK family of proteins which contain five conserved protein domains – an ankyrin repeat (ANK), a Src homology 3 (SH3), a PSD-95/Discs large/ZO-1 (PDZ), a proline-rich region containing homer- and cortactin-binding sites (Pro), and a sterile alpha motif (SAM). The homer- and cortactin-binding sites and sterile alpha motif are at the C-terminus of the SHANK3 protein. Functional studies have demonstrated that these domains are crucial for synaptic targeting and postsynaptic assembly of SHANK3 multimers [Boeckers et al., 2005].

Haploin sufficiency of *SHANK3* is responsible for the major neurological symptoms in Phelan-McDermid syndrome (PHMDS, MIM 606232), also known as 22q13.3 deletion syndrome, which is a condition characterized by neonatal hypotonia, global developmental delay, absent to severely delayed language acquisition, normal to accelerated growth, autistic behavior, and minor dysmorphic features [Wilson et al., 2003]. Patients with *SHANK3* deletions and frameshift mutations have speech or developmental delay with variable severity among different types of variants. Mutations in the *SHANK3* gene were identified in ~1-2% of patients with autism spectrum disorder (ASD) and up to 2.12% of the cases with moderate to profound intellectual disability [Boccuto et al., 2013; Leblond et al., 2014; Mongan et al., 2002]. *SHANK3* mutations were also reported in patients with childhood-onset schizophrenia [Gauthier et al., 2010].

Herein, we report the first double mutant *SHANK3* allele identified by whole exome sequencing (WES), in a patient with autism, intellectual disability, ataxia, dysmorphic features, short stature, progressive spasticity and microcephaly.

Methods

Whole Exome Sequencing (WES)

WES was performed and the data were analyzed as described previously [Yang et al., 2013; Yang et al., 2014]. The Exome was captured using in-solution exome capture reagent VCRome, version 2.1 (Roche NimbleGen), and 100-bp paired-end sequencing was performed on Illumina HiSeq 2000 platform. Uniquely aligned sequence was 11.6 Gb with an average coverage of 145 times for the targeted region, and 98.26% of targeted coding bases of the exome were covered by at least 20 reads.

PCR amplification of the *SHANK3* variants, cloning and sequencing of PCR product

Amplification of genomic DNA from the patient and parents was performed on DNA from peripheral blood to amplify a 473 bp segment covering both variants identified in the patient using primers: forward 5'- CTC TTC GCT CCG TCC AAG - 3' and reverse 5'- GTG AGT GGG TGG ATG AAG GT -3'. PCR was performed using TaKaRa LA Taq DNA polymerase (Takara Bio, Otsu, Japan) with 250 ng of genomic DNA in a reaction with final volume of 50 µL. Betaine was added at final concentration of 1M. After first round of denaturing at 95°C for 5 minutes, 36 cycles amplification was performed using the conditions of denaturing 98°C for 10 seconds, annealing 62°C for 30 seconds, and extension 68°C for 3 minutes. A final extension was performed at 68°C for 10 minutes. The PCR product from the proband was TA cloned (pGEM-T easy, Promega) and individual clones were analyzed by Sanger sequencing to determine the phase of the two mutations.

Next generation sequencing (NGS) of PCR products

PCR products from the proband and both parents were fragmented by sonication and indexed for the subsequent NGS analysis by high throughput sequencing on MiSeq (Illumina Inc., San Diego, CA) with 2×100 paired-end reads. The raw data in base call files (.bcl format) were converted and demultiplexed by MiSeq (Illumina). Demultiplexed sequence reads were aligned to the human genome 19 (hg19) reference sequence. NGS data were visualized using integrative genomics viewer (IGV) with the setting of paired-end (PE) alignments. NGS was also performed directly on PCR product from the proband with adaptors added at both ends by two rounds of PCR. 15 cycles were performed for the 1st round PCR using primers P1-F 5'- ACA CGA CGC TCT TCC GAT C CTC TTC GCT CCG TCC AAG-3' and P1-R 5'- ACG TGT GCT CTT CCG ATC TGT GAG TGG GTG GAT GAA GGT - 3' and 30 cycles for the 2nd round PCR to add adaptors using primers DAN-I5-D501 5'- AAT GAT ACG GCG ACC ACC GAG ATC TAC ACT ATA GCC TAC ACT CTT TCC CTA CAC GAC GCT CTT CCG ATC - 3' and DAN-I7-D712 5'- CAA GCA GAA GAC GGC ATA CGA GAT CTA TCG CTG TGA CTG GAG TTC AGA CGT GTG CTC TTC CGA TCT - 3'.

Clinical Report

The proband was a 14-year-old boy of European descent with microcephaly, ataxia, moderate intellectual disability and dysmorphic features. The mother has a history of infertility and the proband was conceived by *in vitro* fertilization. He was delivered prematurely at 29 6/7 weeks gestation by C-section to his 35-year-old mother and 32-year-old father. He was born a fraternal triplet to his co-triplets who are identical to each other. The pregnancy was complicated by preeclampsia. Birth weight was 0.845 kg, length 34 cm, head circumference 24 cm. His Apgar scores were 4 and 7. He was intubated twice for respiratory distress and also had a PDA that required Indocin for closure. Surgery was required for bilateral inguinal hernias. He was hospitalized for about 3 months before going home. His development was delayed; he did not walk until he was 2 ½ years old. He had stereotypical behaviors such as spinning toys and repeatedly opening and closing the cabinets. Developmental progression was slow but there was no regression.

Upon physical exam at 13 years of age, his height was 150.1 cm (22nd percentile), weight 38.1 kg (14th percentile), head circumference 50.5 cm (1st percentile, -2.5 SD). He had long nasal columella and slightly thick lips with wide smile but noted family resemblance. Extremity exam revealed mild bilateral 5th figure clinodactyly, bilateral high arched feet with mild inward curving, bilateral mild overlapping of 2nd toe over 3rd and hunched over posture. He had moderate intellectual disability and autism. His gait examination was notable for ability to walk for short distances but requiring a wheelchair for longer distances due to spasticity. He could feed himself with his fingers and was able to use a spoon. He had hypertonia of his lower extremities, facial hypotonia and difficulty with coordination of eating. For language, he was only able to babble and communicated with simple augmentative communication software, hand guiding, and directing caregivers to what he wanted. He exhibited aggressive and self-stimulatory behaviors such as hitting himself. There was concern for low extremity neuropathy due to a severe foot deformity and gait disturbance; however, follow-up electromyogram and nerve conductive velocity were normal. A gastrostomy-tube was present that was primarily used for medications and fluids supplementation. His parents had never observed seizures. He had chronic constipation and poor gut motility.

Family history indicted a female maternal cousin with attention deficit hyperactivity disorder, math learning disability, and a concern for autism spectrum disorder. Both parents and two triplet brothers were generally healthy.

The proband had a normal karyotype, microarray (Agilent 108k), UBE3A sequencing and testing for congenital disorders of glycosylation (affinity chromatography/MS), plasma amino acids and plasma organic acids. Brain MRI at 6.5 years of age was normal. An MRI of the spinal cord was performed due to his progressive lower extremity spasticity and revealed a tethered cord which was surgically released.

Results

Whole Exome Sequencing (Baylor Genetics Laboratories) was ultimately pursued and revealed a heterozygous c.2860dupC (p.Leu954Profs342) pathogenic variant and a heterozygous c.3236C>T (p.P1079L) variant of unknown significance (VUS) in the *SHANK3* gene (RefSeq NM_033517.1). Both variants have not been previously reported in the literature and are absent from the 1000 Genomes database (healthy individuals) and the Exome Aggregation Consortium database (60,706 unrelated individuals). Both variants are in the penultimate exon (exon 21), the largest exon of *SHANK3*. The truncating mutation is located in a string of five cytosines. The duplication of a single base pair at nucleotide position 2860 is predicted to result in a frameshift at amino acid 954 and premature translation termination. The missense change is 286 bp downstream of the truncating mutation.

The two *SHANK3* variants were confirmed by targeted Sanger sequencing. Sanger sequencing analysis of this region in the parental samples did not detect these variants, suggesting that both changes arose *de novo* in the patient. Examination of 15 highly

polymorphic markers confirmed that both are biological parents. No other rare variants related to the phenotypes were identified by WES.

To determine the phase of the two *SHANK3* mutations, a genomic fragment of 473 bp encompassing both variant sites, one close to each end, were amplified by PCR. A sequencing library was constructed of the fragmented PCR products and 2×100 bp paired-end NGS was performed. Of the 256k total reads, 11k matched paired-end reads with expected gap distance were obtained. 77 randomly selected PE reads with sequences covering both *SHANK3* variant sites were analyzed. The duplication of C at position c.2860 was most frequently associated with a mutant T at position c.3236 (N=28). *In cis* configuration was observed in 62% (28/45) of PE reads with at least one mutation, suggesting that the two mutants were on the same allele.

To avoid any potential artifacts introduced by fragmentation of PCR products in library construction, NGS was also performed directly on PCR products without fragmentation. 893k matched paired-end reads with expected gap distance, out of total 1.17 million reads, were obtained. Randomly selected PE reads with sequences covering both *SHANK3* variant sites were analyzed. 84% (47/56) PE reads with at least one mutation showed *in cis* configuration (Figure 2), which is much higher than that observed in NGS data on the fragmented PCR product.

To further confirm the *in cis* configuration, the PCR product from the proband was cloned and 10 individual clones were subsequently Sanger sequenced. Sequence analysis of all the clones consistently showed that the c.2860dupC and c.3236C>T were on the same allele of *SHANK3* (Figure 3).

Discussion

***Since the *SHANK3* protein acts as a scaffolding protein, such a prematurely truncated peptide could act in a dominant negative fashion in cells where it is expressed. Alternately, the aberrantly spliced transcript may be degraded, leading to reduced levels of *SHANK3* protein. This would be in agreement with Durand et al. [2007] who showed that abnormal *SHANK3* gene dosage or premature truncation of the peptide are associated with ASD [Durand et al., 2007].

We identified two *de novo* *SHANK3* variants, including a truncating mutation and a missense change, in a boy with intellectual disability and autism. Both variants were within exon 21 on a single allele with the truncating mutation 286 bp upstream of the missense change.

Double *de novo* single nucleotide germline mutations in the same gene have been reported in only a few genes. Two *de novo* mutations in fibrillin (FBN1), c.T3212G and c.A3219T, were reported in a patient with neonatal Marfan syndrome [Wang et al., 1996]. A *de novo* double missense mutant allele in *RET* in a patient with MEN2A, c.634T>C and c.640C>G, was reported [Tessitore et al., 1999]. Two *de novo* missense changes, c.2930C>G and c.2955T>C, in the androgen receptor gene located on the X chromosome were reported in monozygotic twins diagnosed with complete androgen insensitivity syndrome [Mongan et

al., 2002]. All these changes are in a single allele within fourteen bases of each other. The distance of the two *de novo* *SHANK3* mutations in this patient is farther away than these previously reported double single allele mutations. Since these changes are *de novo* in close proximity on the same allele, these mutations were generated most probably from a single event. *De novo* nucleotide substitutions and indels have been observed in combination with *de novo* structural variations such as genomic deletion/duplications as a single event [Brandler et al., 2016]. Although previous CMA study is normal, we cannot rule out the presence of additional small structural variations in a close proximity with the two *SHANK3* mutations.

SHANK3 is one of the most GC-rich genes in the genome with 66.7% of GC for the 7096 bp coding region, while the average of the human genome is 41%. The GC content in the exon 21 of 2254 bp region is as high as 72.4%. The c.2860dupC truncating mutation is in a string of 5 C while the c.3236C>T missense change is in a CpG dinucleotide sequence. Both changes are within one of the multiple CpG islands in *SHANK3*.

Individuals with *SHANK3* truncating mutations in exon 21 displayed autism and moderate to severe/profound intellectual disability [Leblond et al., 2014]. Our patient had moderate intellectual disability and autism, global developmental delay and hypotonia, which have been consistently observed in patients with *SHANK3* truncating mutations. In addition, our patient also showed features that have been previously reported in a subset of patients, including microcephaly, feeding difficulty, ataxia and constipation. Epilepsy and regression, which have been observed in patients with *SHANK3* haploin sufficiency, were not present. In general the clinical presentation of our patient was consistent with the clinical features in patients with *SHANK3* truncating mutations.

The c.2860dupC truncating mutation is expected to cause a frameshift and a premature stop codon at position 954, leading to the loss of critical protein interaction domains (Homer, Abpl, cortactin) in the C-terminal region of *SHANK3*. The mutation is in the penultimate exon, more than 1.7 kb upstream of the last splice donor site. The mutant mRNA is expected to escape non-sense mediated decay according to “55-bp rule”. Truncating mutations are enriched in the exon 21 [Leblond et al., 2014]. Multiple functional studies have been performed on mutations in exon 21 resulting in truncating proteins without C-terminal critical domains. A similar frameshift mutation, c.2497dupG, resulted in the truncated protein accumulating in the nucleus of transfected cells and the cells had reduced complexity of the dendritic tree, less spines, and less excitatory [Cochoy et al., 2015]. Another similar frameshift mutation, causing a premature stop codon at position 1227, strongly affected spine development and morphology, as well as growth cone motility [Durand et al., 2012].

The missense change, c.3236C>T (p.P1079L), is in the same exon and only 125 amino acids downstream of the truncating mutation. The proline at position 1079 is a moderately conserved amino acid (considering 12 species) and this variant was predicted to be tolerable by SIFT (<http://sift.jcvi.org/>) and benign by Polyphen-2 (<http://genetics.bwh.harvard.edu/pph2/>). Since the two changes are on the same allele, the functional effect of the frameshift mutation is unlikely to be affected by this missense change. However, *SHANK3* contains multiple intragenic promoters and alternative splicing [Maunakea et al., 2010]. An extensive

array of mRNA and protein isoforms has been identified, but the complex transcriptional regulation of *SHANK3* is incompletely understood [Waga et al., 2014]. Therefore, the contribution to our patient's phenotype from this missense change cannot be completely ruled out.

Previously described methods for determining the phase of two changes in one gene include testing parents or relatives, restriction enzyme digestion, allele specific PCR, sequencing of cloned PCR products and digital PCR followed by DNA sequencing. Massively parallel sequencing has also been used for phase determination through mate-pair sequencing of a segment enriched by long range amplification [Cradic et al., 2014]. The relationship between two alleles was established by comparing the base calls at each heterozygous position in fragments covering both heterozygous positions with the pairs of sequence reads. A small percentage of incorrectly linked bases have also been observed, with recombination events during PCR amplification were the most common source of error, accounting for 7% of total reads. Other factors, such as random error in NGS data and ligation of fragments from opposite alleles during mate pair library preparation, may also be attributed to error. Our NGS sequencing revealed that majority of PE reads have both *SHANK3* mutations indicating a cis configuration. A small percentage of PE reads with a mutation in one position and a reference allele in the other position were observed in this study, mainly due to *in vitro* recombination during PCR, possibly through incomplete PCR products as primers. In comparison with the NGS data generated after fragmentation of PCR products, the NGS data generated without fragmentation have much higher rate of PE reads within expected gap distance over total reads (75% vs 4%). The percentage of incorrectly linked bases is much lower in NGS data generated directly from PCR amplification without fragmentation than those with fragmented PCR products. Our study demonstrated that direct NGS sequencing of a PCR product with adaptors added during amplification represents a simple tool for gene phasing.

In summary, for the first time, we identified two *de novo* mutations in one *SHANK3* allele in a patient. Considering the phase and characteristics of the two variants, the clinical features in this individual are most likely the consequence of the truncating mutation with no or minimum contribution from the missense change.

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Figure 1.

Two mutations were detected in a single patient by WES and confirmed by Sanger sequencing. (A) Chromatograms displaying the truncating and missense mutations present in the proband and absent in the parents. (B) Both mutations located in exon 21 (GenBank RefSeq:NM_033517). (C) The truncating mutation located after the PDZ domain and before the homer binding region. The putative truncating protein deletes critical protein interaction domains in the SHANK3C-terminal region. Domains were drawn based on GenBank NP_277052.1.

Figure 2.

Representatives PE reads from Next-generation sequencing (NGS) demonstrate the two *SHANK3* variants are on the same allele. 84% of PE reads with at least one mutation *in cis* configuration. Each horizontal bar stands for one paired-end read with the gray thick bars representing sequences same as reference sequences. The purple bar indicates the c.2860dupC variant and the red bar indicates the c.3236C>T variant. The few PE reads with only one mutation detected, which were most likely generated from recombination during PCR, are indicated by blue arrows.

Figure 3. Chromatograph of sequencing of one clone of PCR products from the proband demonstrates that the *SHANK3* variants, c.2860dupC and c.3236C>T, are on the same allele. Top is the mutation trace and bottom are the enlarged regions around the mutations. The mutations are indicated by red arrows.