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A recurrent single-exon deletion in *TBCK* might be under-recognized in patients with infantile hypotonia and psychomotor delay

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Abstract

Advanced bioinformatics algorithms allow detection of multiple-exon copy-number variations (CNVs) from exome sequencing (ES) data, while detection of single-exon CNVs remains challenging. A retrospective review of Baylor Genetics' clinical ES patient cohort identified four individuals with homozygous single-exon deletions of *TBCK*(exon23, NM_001163435.2), a gene associated with an autosomal recessive neurodevelopmental phenotype. To evaluate the prevalence of this deletion and its contribution to disease, we retrospectively analyzed single nucleotide polymorphism (SNP) array data for 8194 individuals undergoing ES, followed by PCR confirmation and RT-PCR on individuals carrying homozygous or heterozygous exon23 *TBCK* deletions. A fifth individual was diagnosed with the *TBCK*-related disorder due to a heterozygous exon23 deletion *in trans* with a c.1860+1G>A (NM_001163435.2) pathogenic variant, and three additional heterozygous carriers were identified. Affected individuals and carriers were from diverse ethnicities including European Caucasian, South Asian, Middle Eastern, Hispanic American and African American, with only one family reporting consanguinity. RT-PCR revealed two out-of-frame transcripts related to the exon23 deletion. Our results highlight the importance of identifying single-exon deletions in clinical ES, especially for genes carrying recurrent deletions. For patients with early-onset hypotonia and psychomotor delay, this single-exon *TBCK* deletion might be under-recognized due to technical limitations of ES.

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Conflict of interest:

The Department of Molecular & Human Genetics at Baylor College of Medicine receives revenue from clinical genetic testing completed at Baylor Genetics Laboratory.

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Keywords

TBCK ; single-exon deletion; exome sequencing; diagnostic testing; recurrent variant

Introduction

Exome sequencing (ES) has been widely used to identify molecular etiologies contributing to Mendelian disorders. In clinical practice, ES primarily investigates single nucleotide variation or small insertion/deletion (SNV/indel) changes where the sizes of detectable indels varies from one basepair to several hundred basepairs, due to limitations of short-read sequencing, while larger copy number variations (CNVs), sized hundreds of basepairs and larger, have been in the scope of chromosomal microarray(CMA) with ability to detect determined based on array design. New algorithms for CNV detection in ES data based on read depth have been developed, including one in our laboratory (Lalani et al., 2016). However these provide limited resolution, especially for heterozygous, single-exon deletions (Gabrielaite et al., 2021). Consequently, technical challenges are likely preventing identification of such CNVs that may be contributing to diagnoses in patients undergoing clinical ES.

Neurodevelopmental disorders (NDD) have been a major focus for clinical ES due to the large number of potential genes related to the etiology (Evers et al., 2017). ES has contributed to the discovery of many novel causes for NDD, including a severe autosomal recessive condition initially described as caused by biallelic pathogenic SNVs in *TBCK* (TBC1 domain-containing kinase, MIM# 616899, causing infantile hypotonia with psychomotor retardation and characteristic facies-3 (IHPRF3), MIM# 616900) (Alazami et al., 2015; Bhoj et al., 2016). Currently, both disease-causing SNVs and larger CNVs in *TBCK* have been reported in the Human Gene Mutation Database (HGMD) and ClinVar(see Supp. Figure S1 for HGMD SNV/CNV location).

We report a recurrent deletion of exon 23 in *TBCK,* initially identified in a homozygous state in four patients diagnosed with IHPRF3. The recurrent nature of this deletion triggered a thorough reanalysis for this deletion in our ES cohort. Molecular diagnosis with a heterozygous exon 23 deletion *in trans* with a pathogenic splicing variant was reached for a previously unsolved case. Furthermore, functional studies on blood-derived RNA samples from patient 1 revealed abnormal out-of-frame transcripts due to the exon 23 deletion.

Materials and Methods

Editorial policies and ethical considerations

This study was performed in accordance with protocols approved by the institutional review boards at Baylor College of Medicine, Baylor Genetics Laboratory, and National Institutes of Health.

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Patient cohort

The initial patient cohort was composed of 8194 individuals (including probands and their parents where trio ES was performed) submitted to the Baylor Genetics Laboratory for clinical ES with concurrent single nucleotide polymorphism (cSNP) array data available for analysis. ES was conducted according to previously described methods (Yang et al., 2014), and cSNP array was performed following manufacturer's instructions (CytoSNP, HumanExome-24v1.3 array, Illumina, San Diego, CA). In addition to the cSNP array, an *in silico* algorithm to analyze ES read depth/absence of reads was also used to look for homozygous CNVs (Lalani et al., 2016).

Exonic deletion analysis based on SNP array data

Two exonic SNPs, 1KG_4_107092300 (chr4:107,092,300, hg19) and exm417430 (chr4:107,092,341, hg19) on chromosome 4 from Illumina HumanExome-24v1.3 array, located within exon 23 of *TBCK* (NM_001163435.2), were used as screening probes. Samples with logR values for both SNPs less than −0.5 were selected for PCR and Sanger sequencing.

PCR and Sanger sequencing

PCR primers (Supp. Table S1, E23 and E23del) were designed to identify the breakpoints of the exon 23 deletion (Figure 1). Sanger sequencing then was performed using PCR products on the ABL 3730XL DNA Analyzer. Sequencing data then were analyzed by Mutation Surveyor and aligned back to the *TBCK* (NM_001163435.2) reference sequence based on hg19 using Clustal-W.

cDNA study

Total RNA was extracted from whole blood from patient 1 and her parents using RNeasy Mini Kit (QIAGEN), and cDNA was synthesized with oligodT with SuperScript III reverse transcriptase (Invitrogene) according to manufacturer's instructions. The cDNA products were used as templates for PCR (using primers E2125, Supp. Table S1) to amplify the region from the junction of exon 20/21 to exon 25 (Figure 2). PCR products then were run on 2% agarose gel and visualized by ethidium bromide staining. Bands on the gel then were cut out and purified for Sanger sequencing.

Results

Characterization of a 7424bp deletion including the entire exon 23 of *TBCK*

A *TBCK* exon 23 homozygous deletion was identified in four individuals (patients 1–4) with severe central hypotonia and developmental delay (Table 1), by an *in silico* algorithm (Lalani et al., 2016) based on ES read depth/absence of reads. Further investigation as reported here with targeted PCR and Sanger sequencing in patient 1 revealed a ~7.4 kb deletion (chr4:107,091,826-107,099,221, hg19, HGVS recommended as NC_000004.11:g.107091826_107099220delinsCAGAGAATGCCTCTCCATATTTACCAT, referred to as exon 23 deletion hereafter) involving most of intron 22, the entire exon 23 and part of intron 23 as shown in Figure 1 (using *TBCK* transcript NM_001163435.2).

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Sanger sequencing in patient 1 also showed that this deletion was likely the result of a complex rearrangement, as a 28bp noncontiguous segment from intron 22 was retained (chr4:107,096,802-107,096,829, hg19, Figure 1C). Microhomology of 3bp (ATT) was observed between the intron 22 breakpoint and its joined end from the retained intronic segment (chr4:107,099,222-107,099,224, hg19 and chr4:107,096,827-107,096,829, hg19 respectively, see Figure 1C), while no microhomology was observed at the intron 23 breakpoint. These data were suggestive of microhomology-mediated DNA rearrangement, such as microhomology-mediated break-induced replication or microhomology-mediated end joining models, in generating this deletion (Gu et al., 2008; Ottaviani et al., 2014).

The exon 23 deletion yields two different *TBCK* transcripts with early termination codons

To fully investigate the consequence of the exon 23 deletion, we performed RT-PCR on RNA isolated from blood from patient 1 and her parents. As shown in Figure 2, this deletion yields two different transcripts: one with exon 23 absent and the other with inclusion of a new 70bp pseudoexon from intron 23 (chr4:107,085,549-107,085,618, hg19) in addition to absence of exon 23. Both transcripts are out of frame and predicted to cause a premature termination codon leading to nonsense-mediated decay, as the new termination codons are either in exon 24 or the new pseudoexon 23 out of 26 total exons in the gene (NM_001163435.2:c.2060_2235del:p.Glu687Valfs*9 and NM_001163435.2:c.2060_2235delins70:p.Ile688Asnfs*17). In comparison, the two abnormal transcripts and wild type transcripts were detected in both heterozygous parents of patient 1, while only the wild type transcript was detected in the control (Figure 2B). Looking for supporting evidence on whether our observed RNA splicing pattern is individual/family specific, we find a patient from the Undiagnosed Diseases Network (UDN, https://undiagnosed.hms.harvard.edu/) cohort with the identical homozygous *TBCK* exon 23 deletion with fibroblast RNA sequencing data, which showed the same pattern of two abnormal splicing events, including the inclusion of the 70bp pseudoexon (Supp. Figure S2) (Murdock et al., 2021).

Recurrence of the exon 23 deletion in the general population and a clinical ES patient cohort

Within a CNV database from the general population (Database of Genomic Variants, DGV), a similar deletion (minimally sized as 7414bp, deleting exon 23) in *TBCK* is reported as esv3601677 in a heterozygous state once from 2504 samples (http://dgv.tcag.ca/dgv/app/variant?id=essv11555782&ref=GRCh37/hg19, see Supp. Figure S3). Given the presence of this deletion in the nonconsanguineous parents of patient 1 and in the DGV database, we sought to identify additional occurrences of this event (homozygous or heterozygous). We screened all cases in the clinical ES cohort based on concurrent SNP array data using two SNPs located within exon 23. Samples that screened positive were further tested by PCR and Sanger sequencing confirmation. It should be noted that our screening method is not fully validated, and there are technical limitations, as only 2 SNPs on our array fall within the deletion; therefore it is possible that the number of carriers in our ES cohort could be underestimated.

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Out of a total of 8194 individuals from 5305 families, eight families (including the families of patients 1–4) were confirmed to carry exon 23 deletions. In addition to the four homozygotes, a fifth individual was diagnosed with a *TBCK*-related disorder due to the presence of a heterozygous exon 23 deletion in trans with a c.1897+1G>A pathogenic variant (Table 1, patient 5). The remaining 3 families had heterozygous carriers without a second allele identified. While consanguinity was reported in the family of patient 2, in the remaining three families with children who carry the homozygous deletion, parental ES data excluded consanguinity, suggesting a total of 11 unrelated carriers out of 7110 unrelated individuals undergoing ES, as shown in Supp. Table S2.

Sanger sequencing showed that all exon 23 deletions have identical breakpoints with retained intronic sequence as originally characterized in patient 1. These eight families came from different ethnic groups (3 European Caucasian, 1 Middle Eastern, 2 Hispanic, 1 African American and 1 consanguineous South Asian). While this observation might suggest this is a recurrent event, a distant founder mutation is also possible. To evaluate for the possibility of a founder mutation, we analyzed SNP data in the region surrounding the deletion in the eight families and observed a conserved SNP haplotype (consisting of 29 SNPs surrounding this deletion) (Supp. Table S3). An additional three carriers of this SNP haplotype were identified who did not carry the exon 23 deletion. While these observations could support a founder mutation, the incomplete association of exon 23 deletion and the 29 SNP haplotype cannot rule out a recurrent rearrangement occurring on a predisposing haplotype.

Discussion

To date, a majority of ascertained pathogenic variants in *TBCK* have been SNV/indels, while structural variants involving deletions larger than 1kb have only been reported a few times in HGMD and ClinVar. Our work suggests that the role of structural variants in *TBCK*-related neurodevelopmental syndrome may be underappreciated. Supporting this assertion of under-recognition of *TBCK* CNVs, three of the five diagnoses in this cohort were in patients enrolled in the Undiagnosed Diseases Network (UDN), as were two other reported individuals with pathogenic *TBCK* CNVs (Lee et al., 2020; Murdock et al., 2021). The UDN enrolls patients who have completed thorough diagnostic evaluations, frequently including ES, that were unrevealing, showing that traditional testing methodologies are not detecting and reporting these variants (Splinter et al., 2018).

Interestingly, two of the three *TBCK* deletions reported in HGMD occurred around exon 23 (Supp. Figure S1). Lee et al.(2020) reported a *de novo* splicing variant *in trans* with a heterozygous inherited 35kb deletion including the entire exon 23 and parts of introns 22 and 23 in a 3-year-old female with significant global developmental delay, hypotonia, short stature, macrocephaly, nystagmus, periventricular leukoencephalopathy and seizures. Sumathipala et al. (2019) reported a Norwegian patient with fatal encephaloneuropathy, hypotonia, global deterioration, and seizures. This patient had two homozygous deletions: one is similar to the 7.4kb exon 23 deletion reported here, and one is a homozygous 6.8kb deletion entirely within intron 22. Studies of cDNA extracted from the patient's fibroblasts showed that exon 23 skipping occurred in the *TBCK* transcripts without other complexity.

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In addition to these exon 23-containing deletions in HGMD, in our laboratory we identified a heterozygous 49kb deletion from intron19 to intron 23, including exons20–23, *in trans* with a c.456-2A>G splicing variant in a patient with intellectual disability, generalized hypotonia, abnormality of the cerebral white matter, electroencephalogram abnormality, and cerebral visual impairment, who underwent whole genome sequencing as part of the UDN (representing a sixth *TBCK* CNV-related diagnosis within the UDN).

Combined with our cohort of this recurrent 7.4kb deletion around exon23, these data strongly suggest that there might be a “hotspot” region for structural variants arising around exon 23 of *TBCK*. Further investigation to reveal the molecular mechanism for the hotspot and to more accurately estimate the prevalence of these CNVs is warranted to help improve diagnosis for this severe neurodevelopmental disorder. This finding also highlights the importance of CNV evaluation during ES when *TBCK*-related disorder is suspected.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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Figure 1:

A 7.4kb complex exon 23 deletion was identified in *TBCK*. A) Primers were designed to detect the deletion and to pinpoint the breakpoints. Red line shows the deletion region. B) Homozygous exon 23 deletion in patient 1 was confirmed through PCR. WT: control. PT1, patient 1 with a homozygous deletion C) Sequencing (hg19 coordinates) of the exon 23 deletion allele in patient 1 defines a likely complex rearrangement scenario with a 28bp fragment from intron 22 retained and 3bp (ATT) of microhomology at the intron 22 breakpoint's junction with the 28bp fragment.

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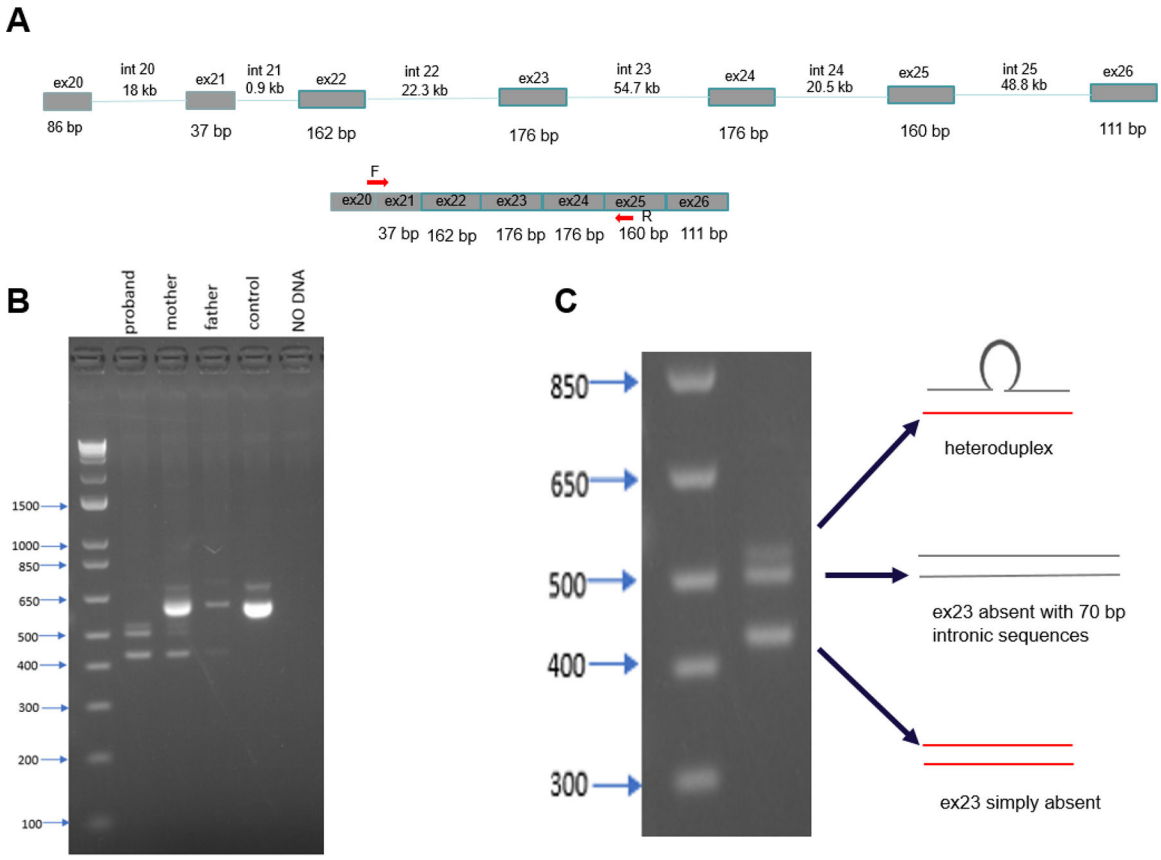

Figure 2:

cDNA studies show that exon 23 deletion causes two different frameshift transcripts. A) Genetic structure around *TBCK* exon 23 (NM_001163435.2) and the locations of RT-PCR primers. Note the forward primer is located across the junction of exon 20 and exon 21. B) RT-PCR products from proband (patient 1) with homozygous exon 23 deletion, her parents with heterozygous deletions, and a control. Three abnormal bands of products were observed in patient 1 and both parents compared to a control. C) The composition of three bands of mutant transcripts revealed by PCR/Sanger sequencing: the lower two bands differ by 70bp, which sequencing showed to be a 70bp pseudoexon from intron 23 included in the larger transcript (chr4:107,085,549-107,085,618, hg19; data not shown). The top band is a heteroduplex of the two different products formed during PCR.

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Table 1.

Homozygous *TBCK* exon 23 deletion and compound heterozygous deletion with a pathogenic splicing variant provided molecular diagnoses for hypotonia, infantile, with psychomotor retardation and characteristic facies 3 [MIM:616900].

	Patient 1	**Patient 2**	**Patient 3**	**Patient 4**	**Patient 5**
Sex, age at diagnosis	F, 1.2 years	F, 3 years	M, 8.9 years	F, 0.8 years	M, 2.6 years
Ethnicity	Caucasian	South Asian (India)	Caucasian	Middle Eastern	Caucasian
TBCK exon 23 deletion	Homozygous	Homozygous	Homozygous	Homozygous	Compound heterozygous with c.1860+1G>A
Parental *TBCK* exon 23 deletion	Heterozygous	Heterozygous	Heterozygous	Heterozygous	Heterozygous father
Consanguinity	-	+	-	NA	-
Growth (percentiles)					
Height	2nd	43rd	38th	95th	30th
Weight	48th	60th	75th	79th	13th
Head size	79th	31st	97th	37th	18th
Neurologic					
Developmental delay	Profound, global	Severe, global	Severe, global	Global	Severe, global
Hypotonia	Severe	Severe	Profound, with increased leg tone	+	Severe
Reflexes	Absent	Absent	Absent	Present	Absent
Seizures	-	+	Myoclonic, GTC, Infantile spasms	-	+
EEG	Generalized slow activity	Abnormal		NA	NA
Regression	-	-	+	-	-
Speech	Absent	Absent	Absent	Absent	Absent
MRI	Normal at 3 months	Tortuous cerebral arteries, Thin CC, Ventriculomegaly, Mild cerebral white matter atrophy	Severe bilateral mesial temporal sclerosis, Cortical and white matter atrophy, Periventricular white matter gliosis, Encephalomalacia, Atrophy of deep gray matter	Normal prenatally	Periventricular leukomalacia
Ophthalmology	Cortical visual impairment, Nystagmus, Esotropia, Delayed responses on ERG	Ptosis, Esotropia	Nystagmus, Ptosis	NA	Nystagmus, Esotropia, Mild myopia
Dysmorphic features		Coarse			Plagiocephaly
Bitemporal narrowing	-	+	NA	NA	-
Eyes	Normal	Deep-set	NA	NA	Deep-set, Arched eyebrows
Nose	Normal	Prominent nasal bridge	NA	NA	Prominent nasal bridge

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	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5
Mouth/lips	Bifid uvula, Long philtrum, Tented upper lip	High palate, Absent uvula, Tented upper lip	NA	NA	High palate, Tented upper lip, Mild macroglossia
Chin	Small	Normal	NA	NA	Dimple
Hirsutism	-	Low anterior hairline	NA	NA	-
Musculoskeletal	Weakness, Decreased muscle bulk, Clubfoot, Camptodactyly	Torticollis, 5th finger clinodactyly, Mild 2–3 toe syndactyly	Upper arm fasciculations, Contractures, Mild scoliosis, Myotonic activity on EMG	Weakness	Pes planus, Proximal weakness
Respiratory	Laryngomalacia	Stridor	Ventilator-dependent	NA	Laryngomalacia, Sleep apnea
Gastrointestinal	-	Moderate GERD	Dysphagia, GERD, Uses G-tube	Elevated liver enzymes, Normal ultrasound	Dysphagia, Constipation
Other	Hypogammaglobulinemia	Sacral dimple	Kidney stones, Asymmetric kidneys, Nephrocalcinosis, Hypertension	Similarly affected sister (not tested), deceased at 3 years	

+ = feature present; - = feature absent; CC = corpus callosum; ERG = electroretinography; F = female; GERD = gastroesophageal reflux; GTC = generalized tonic-clonic; M = male; NA = not available