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Haploinsufficiency of *ATP6V0C* possibly underlies 16p13.3 deletions that cause microcephaly, seizures and neurodevelopmental disorder

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Data available on request from the authors

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DISCLOSURE STATEMENT

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

S.B and R.J.T conceived and designed the study. S.B , S.G and M.S were involved in patient care, diagnosis, and treatment. All authors acquired, analyzed, and interpreted the data.

R.J.T and S.B drafted the manuscript, which was critically revised by all the authors.

ABSTRACT

Introduction: We recently contributed to the description of eight individuals with a novel condition caused by 16p13.3 microdeletions encompassing *TBC1D24*, *ATP6V0C*, and *PDPK1* and resulting in epilepsy, microcephaly and neurodevelopmental problems. The phenotypic spectrum, the minimum overlapping region and the underlying disease mechanism for this disorder remain to be clarified.

Results: Here we report a 3.5-year-old male, with microcephaly, autism spectrum disorder and a *de novo* 16p13.3 microdeletion. We performed detailed *in silico* analysis to show that the minimum overlapping region for the condition is ~80Kb encompassing five protein coding genes. Analysis of loss of function constraint metrics, transcript-aware evaluation of the population variants, GeVIR scores, analysis of reported pathogenic point variants, detailed review of the known functions of gene products and their animal models showed that the haploinsufficiency of *ATP6V0C* likely underlies the phenotype of this condition. Protein-protein interaction network, gene phenology and analysis of topologically associating domain showed that it was unlikely that the disorder has an epistatic or regulatory basis.

Conclusions: 16p13.3 deletions encompassing *ATP6V0C* cause a neurodevelopmental disorder. Our results broaden the phenotypic spectrum of this disorder and clarify the likely underlying disease mechanism for the condition.

MAIN TEXT

Introduction

Copy number variants (CNVs) are an important cause for neurodevelopmental disorders (Cooper et al., 2013; Coe et al., 2013; Mefford et al., 2014). Disease causing CNVs result in diverse phenotypes and have significant medical and socioeconomic impact (Coppola et al., 2019; Burghel et al., 2020). Variability of CNVs can make their clinical correlation challenging. Individuals with overlapping CNVs can help in determining the minimum overlapping regions for specific disorders (Kasher et al., 2016). A significant proportion of copy number losses are driven by haploinsufficiency of dosage sensitive genes (Rice et al., 2017). Studying CNVs can, therefore, also provide insights into genetic basis of single gene (Yagi et al., 2003; Cuvertino et al., 2017) or complex disorders (Banka et al., 2015). Other mechanisms by which chromosome deletions can convey phenotypes include contiguous gene deletions, gene interruption, generation of fusion genes, unmasking of deleterious variants resulting in recessive phenotypes, epistasis, position effects such as disruption of the regulatory regions of the genome and disruption of topologically associating domains (TADs) (Stankiewicz et al., 2002; Lupski et al., 2005; Spielmann et al., 2018).

We recently contributed to the description of eight individuals with epilepsy, microcephaly, neurodevelopmental problems and overlapping 205 kb to 504 kb 16p13.3 microdeletions and proposed that it is a novel genetic condition (Table 1) (Mucha et al., 2019). The defined minimum critical region of ~112 kb (GRCh37:Chr16:2,530,000–2,642,000) that included seven known genes - *TBC1D24*, *ATP6V0C*, *AMDHD2*, *CEMP1*, *MIR3178*, *PDPK1* and *DQ577714*. Since the original report, there has been no other peer-reviewed publication on this condition. The phenotypic spectrum, the minimum overlapping region and the underlying disease mechanism for this disorder remain to be clarified. Here we report an additional individual with an overlapping 16p13.3 deletion and a similar phenotype, which further establishes this microdeletion syndrome as a distinct entity. We also present results of *in silico* analysis that clarifies the possible underlying disease mechanism.

Methods and Results

Case Report

The proband is a 3.5-year-old male, born to non-consanguineous parents with no relevant family history. His mother's pregnancy was complicated by antenatal bleeding and placenta praevia. He was born via Caesarean section at 38 weeks gestation with a birth weight of 2.41kg (-2SD). The immediate postnatal period was uneventful apart from a brief period of hypothermia.

At the age of 10 months, the child presented with a tonic-clonic seizure during a febrile illness. Subsequently he had multiple febrile tonic-clonic seizures. 24-hour electroencephalograms did not show any epileptic activity. A brain magnetic resonance imaging scan at 1 year did not identify any abnormality. He has not been formally diagnosed with epilepsy and no anti-epileptic treatment has been required to be initiated so far. He

first rolled over at the age of 10 months but then did not roll over again until several months later. He attained independent walking at 18 months of age. An Early Social cognitive Battery assessment performed at the age of 38 months showed his early social interaction and play skills to be impaired. An Autism Diagnostic Observation Schedule (ADOS Module 2) performed at 38 months of age showed him to be on moderate autism spectrum. Social affect score was 8 and restricted repetitive behaviours score of 1 with calibrated severity score of 5, meeting the criteria for autism spectrum disorder. Weschsler Preschool and Primary Scale of Intelligence – Fourth UK edition (WPPSI-IV) performed at 36 months of age gave a Full Scale Intelligence Quotient of 90 within the average range (25th centile). His verbal comprehension index was 91 on the 27th centile; (scaled scores: Receptive vocabulary = 10; Information = 7) and Visual spatial index was 97 on the 42nd centile (scaled scores: Block Design = 10; Object Assembly = 9). Working memory and vocabulary index could not be computed due to lack of compliance but he scored within low average range for Picture memory (scaled score of 7) and Picture Naming (scaled score 7).

The child also has a history of frequent infections including recurrent upper respiratory tract infections, several episodes of glue ear and one episode of enteroviral meningitis. He also required tonsillectomy.

At age 2 years, the child's occipital frontal head circumference was 45 cm (-3SD), weight was 10.2 kg (-1.8SD) and height was 84.6 cm (-0.25SD). A neurological examination at this age was unremarkable. Facially the child is not dysmorphic.

Array comparative genomic hybridisation

Array comparative genomic hybridisation (a-CGH) analysis using OGT CytoSure Constitutional v3 Array (6x60K) revealed a 220kb loss of chromosome 16p13.3 ([GRCh37] 16p13.3(2,415,389_2,635,921)x1) (Figure 1A). The deletion encompassed eight known protein coding genes (*CCNF*, *C16orf59*, *NTN3*, *TBC1D24*, *ATP6V0C*, *AMDHD2*, *CEMP1* and *PDPK1*) and two non-protein coding genes (*MIR3178* and *DQ577714*). Targeted a-CGH performed on DNA samples of both parents revealed normal results indicating that the proband's deletion had occurred *de novo*. Comparison of the clinical features of our individual, with the phenotypes of previously published individuals (Table 1) showed remarkable overlap (Mucha et al., 2019). We, therefore, classed this deletion as pathogenic based on the joint consensus recommendation of the American College of Medical Genetics and Genomics (ACMG) and the Clinical Genome Resource (ClinGen) (Riggs et al., 2020). The variant details were uploaded on DECIPHER (#363843).

In silico analysis and literature review

Combining our data with the previously published individuals revealed the minimum overlapping region for the condition to be ~80Kb (GRCh37:chr16:2,555,682-2,635,921) encompassing five protein coding (*TBC1D24*, *ATP6V0C*, *AMDHD2*, *CEMP1* and *PDPK1*) and two non-protein coding genes (*MIR3178* and *DQ577714*) (Figure 1A).

To investigate the possible disease mechanism, we first compiled the pLI and LOEUF scores of the genes in the deleted region (Table 2) (Karczewski et al.,2020). *PDPK1* and *ATP6V0C* were found to have the highest pLI (0.98 and 0.74 respectively) and the lowest LOEUF (0.29 and 0.69 respectively) scores. Closer inspection of the gnomAD data revealed that the relatively low pLI and higher LOEUF scores of *ATP6V0C* are, at least in part, likely attributable to the relatively small size of this gene (only 3 exons encoding a protein of 155 amino acids). Furthermore, the only observed loss of function population variant is located in part of its exon 2 that is not included in two out of four known transcripts of the gene (Supplement Figure S1). Next, we obtained Gene Variation Intolerance Ranking (GeVIR) scores for the genes in this region (Table 2) (Abramovs et al.,2020). GeVIR is able to prioritize short genes, for which loss of function constraint cannot be estimated with confidence. Amongst the five protein coding genes *ATP6V0C* was found to be the highest ranked autosomal dominant disorder gene in GeVIR, followed by *PDPK1*. Next, we quantified the levels of gene expression in selected brain regions using GTEX portal (Figure 1B) (Supplement Table S1) (Gtex Consortium.2015). Expression of *ATP6V0C* was found to be highest across multiple brain tissues. Our analyses thus far indicated that the haploinsufficiency of *PDPK1* or *ATP6V0C* or both possibly underlies 16p13.3 deletions that cause microcephaly, seizures and neurodevelopmental problems.

Next, we investigated the known functions, animal models and pathogenic point variants in these two genes in more detail. *PDPK1* (also known as PDK1) encodes a protein kinase that is involved in intracellular signalling pathway facilitating cell migration (Bergfeld et al.,2012). *PDPK1* murine knockout model is incompatible with life but mice with reduced residual PDK1 activity are smaller but fertile and without any obvious neurological phenotype (Alvarez-Pérez et al.,2006). No *PDPK1* point variants were detected in ClinVar (Landrum et al.,2016). Three possible disease causing missense *PDPK1* variants were listed in Human Gene Mutation Database (HGMD) (Stenson et al.,2003). However, the associated phenotypes were congenital heart defect (Russell et al.,2019), premature ovarian insufficiency (Oral et al.,2019) and modification of phenotype in Williams syndrome (Masson et al.,2019). None of these phenotypes overlap significantly with what has been observed in individuals with 16p13.3 deletions. *ATP6V0C* (ATPase, H⁺ transporting, lysosomal 16 kDa, V0 subunit C) encodes for a component of vacuolar ATPase (V-ATPase), a subunit enzyme that acidifies the intracellular organelles of eukaryotes (Bayascas et al.,2008). This process maintains endocytic and exocytic pathways (Bayascas et al.,2008). The subunit is present in synaptic vesicles, endosomes, lysosomes, clathrin-coated vesicles, and the Golgi complex (Abbas et al.,2020). *ATP6V0C* has a neuron-specific expression at presynaptic vesicles in zebrafish embryos and facilitates neurotransmitter storage (Wullschlegel et al.,2011). An individual with Dravet syndrome and a *de novo* frameshift *ATP6V0C* variant has been previously reported (Carvill et al.,2014). De novo DB also lists a frameshift *ATP6V0C* identified in a proband in the Deciphering Developmental Disorders study (Deciphering Developmental Disorders Study., 2017 ;Tychele et al.,2017).

ClinVar lists a single missense *ATP6V0C* variant of uncertain significance without any associated clinical data (Landrum et al.,2016).These analyses indicated that

haploinsufficiency of *ATP6VOC* is more likely to explain the phenotype caused by 16p13.3 deletions.

Next we considered the possibility of an epistatic model to explain the phenotype of 16p13.3 deletions (Veltman et al.,2010). We first looked for known interactions between the protein products of these genes. Protein-protein interaction network analysis was conducted using String (Szklarczyk et al.,2019) and the gene phenology was studied using Panther (Mi et al.,2019). These analyses did not reveal any known interactions or obvious functional overlaps (Supplement Tables S2 and S3). Finally we looked at the possibility of this deletion disrupting any TAD boundaries. Analysis of the deleted region by ClinTAD (<https://www.clintad.com/>, accessed April 2020) (Lupiáñez et al.,2016) showed that the breakpoints did not overlap with known TAD boundaries.

Discussion

We present a boy with microcephaly and autistic spectrum disorder (ASD) with a *de novo* chromosome 16p13.3 deletion, which spans between 2,415,389 and 2,635,921 [GRCh37]. The similarity of the phenotype of our individual with our eight recently described individuals and overlap of this deletion with the previously defined minimum critical region are strongly indicative that this deletion is causal (Mucha et al.,2019). Our observations further establish chromosome 16p13.3 deletion syndrome microcephaly, seizures and neurodevelopmental problems as a distinct genetic disorder. Of note, this condition should not be confused with other non-overlapping 16p13.3 deletions in this region resulting in alpha-thalassemia-intellectual disability syndrome (Babbs et al.,2020) or Rubinstein-Taybi syndrome (Breuning et al.,1993;Hennekam et al.,1993).

Epilepsy (including tonic clonic and myoclonic seizures) and microcephaly are the most frequent features in individuals with this syndrome (Mucha et al.,2019). Notably, although the individual reported here had history of febrile seizures, he has not been diagnosed as having epilepsy. The individual described in this study was diagnosed with ASD. Reported neuropsychiatric developmental disorders in the disorder include developmental delay (DD), Attention deficit hyperactivity disorder (ADHD), Intellectual disability (ID) and bipolar disorder (Mucha et al.,2019). Of note, the assessments in our patient suggest social communication impairments, but with average range cognitive ability. Our observations suggest that the phenotype of this condition is variable.

Our patient had history of repeated infections. None of the previous individuals have been reported to with this phenotype. At present it is not possible to be certain if this observation is linked to the 16p13.3 deletion or if it is coincidental. Of interest, PDPK1 has been reported to regulate macrophages metabolism, T cells differentiation and B cells haemostasis (Yang et al.,2016).

Previously the phenotype of this condition was attributed to haploinsufficiencies of *TBC1D24*, *ATP6VOC* and *PDPK1*. Our *in silico* analyses indicated that haploinsufficiency of either *ATP6VOC* or *PDPK1* or both the genes may underlie the microcephaly, seizures and neurodevelopmental disorders in individuals with these 16p13.3 deletions. Of the two genes

haploinsufficiency of *ATP6V0C* provides a more compelling explanation for the phenotype of this condition. It is because of its known function in acidification of synaptic vesicles (Bayascas et al.,2008), its high level of expression in brain tissues (Fig 1B), high GeVIR ranking (Table 2) and previous descriptions of individuals with frameshift variants and epilepsy or developmental disorders (Carvill et al.,2014; Deciphering Developmental Disorders Study, 2017)

Of note, the pLI score of *ATP6V0C* is only 0.74, which is lower than expected for a gene, loss of function variants in which may cause a severe early-onset developmental disorder. However, our transcript-aware evaluation of population variants demonstrates that the deletions or loss of function variants that affect all the transcripts of this gene may be more deleterious than those variants which affect only some transcripts. *TBC1D24* encodes a member of the Tre2-Bub2-Cdc16 (TBC) domain-containing RAB-specific GTPase-activating protein family (Falace et al.,2010). Bi-allelic *TBC1D24* loss-of-function variants cause Deafness (OMIM 614617) (Rehman et al.,2014), DOORS syndrome (OMIM 220500) (Campeau et al.,2014) , Rolandic epilepsy with paroxysmal exercise-induced dystonia and writer's cramp (OMIM 608105) (Luthy et al, 2019), Early Infantile Epileptic encephalopathy (OMIM 615338) (Duru et al.,2010) and infantile myoclonic epilepsy (OMIM 605021) (Falace et al.,2010). Heterozygous carriers of *TBC1D24* loss-of-function variants do not show clinical features such as epilepsy, microcephaly or developmental delay. Of note, a heterozygous missense p.(S178L) *TBC1D24* variant has been proposed to cause autosomal dominant deafness-65 (OMIM 616044) (Azaiez et al.,2014 ;Zhang et al.,2014). However, the proposed mechanism in this variant was gain of function or a dominant-negative. Heterozygous loss of *TBC1D24* is unlikely to be responsible for the phenotype seen with these deletions. Our analyses also indicate that epistatic or regulatory dysregulation are unlikely to explain the phenotypes of this condition. However, it is impossible to rule out the contribution to the phenotype from loss of other genes within the deletions without further studies.

In summary, we describe an additional individual with a rare chromosome 16p13.3 deletion that establishes the condition as a distinct entity and clarifies its phenotypic spectrum. Our analysis suggests that the haploinsufficiency of *ATP6V0C* is likely to underlie the pathology of this condition. Further data and experiments will be needed to prove these assertions conclusively.

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