CLINICAL COMMENTARY

Age-dependent epileptic encephalopathy associated with an unusual co-occurrence of *ZEB2* and *SCN1A* variants

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ABSTRACT

Mowat-Wilson syndrome is a genetic disorder associated with a variable phenotype including peculiar facial features associated with intellectual disability, epilepsy, language impairment, and multiple congenital anomalies caused by heterozygous mutation of the *ZEB2* gene. The ZEB2 protein is a complex transcription factor that encompasses multiple functional domains that interact with the regulatory regions of target genes including those involved in brain development. Recently, it has been documented that *ZEB2* regulates the differentiation of interneuron progenitors migrating from the medial ganglionic eminence to cortical layers by repression of the *Nkx2-1* homeobox transcription factor. It has therefore been suggested that the deficit in *ZEB2* may induce an imbalance of neuronal inhibition/excitation leading to epileptic seizures. Given the phenotypic variability of Mowat-Wilson syndrome, to date, a distinctive genotype-phenotype correlation has not been delineated. Here, we report a patient with a severe phenotype of Mowat-Wilson syndrome, associated with a novel heterozygous *de novo* frame-shift variant in the *ZEB2* gene, as well as an additional novel heterozygous missense variant in the *SCN1A* gene, the mutation of which is known to affect NaV1.1-mediated sodium current in GABAergic interneurons. We hypothesize that the severe neurological phenotype of our patient may be influenced by the coexistence of both genetic mutations.

Key words: Mowat-Wilson syndrome, epilepsy, GABAergic interneurons, genotype-phenotype correlation, EEG, *ZEB2, SCN1A*

Mowat-Wilson syndrome (MWS) (OMIM #235730) is characterized by peculiar facial features associated with intellectual disability/global developmental delay (ID/GDD), and variable structural anomalies including agenesis of the corpus callosum, microcephaly, hypospadias, cardiac defects, Hirschsprung disease (HSCR) and eye defects (Garavelli *et al*., 2009; Yamada *et al.*, 2014; Ivanoski *et al*., 2018). In addition, epilepsy and language impairment are the main features of the syndrome. MWS is caused by *de novo* heterozygous variants in the *ZEB2* gene on chromosome 2 and its haploinsufficiency appears to be the primary pathogenetic mechanism (Wakamatsu *et al.,* 2001). Although more than 300 individuals have been reported so far, the variability in phenotype, which may be categorised into subgroups with only small numbers of patients, makes it challenging to establish genotype-phenotype correlations (Ivanoski *et al*., 2018).

Here, we report a patient with a severe neurological phenotype of MWS associated with a newly described intestinal defect and a novel heterozygous frame-shift *de novo* variant in the *ZEB2* gene, as well as an additional novel heterozygous missense variant in the *SCN1A* gene inherited from his healthy father. This report aims to contribute to better diagnosis of the disorder and help establish possible genotype-phenotype correlations.

Case study

This study was approved by the ethics committee of Palermo 1 University Hospital.

The detailed clinical evaluation of the patient was performed after obtaining written informed consent for publication from the patient's parents. The patient, a 10-year-old girl, was the third offspring born to healthy Italian unrelated parents. Her family history was remarkable for intellectual disability, psychiatric disorders, and epilepsy in her paternal mother and two great aunts. She was born at term by elective Caesarean section following an uneventful pregnancy. APGAR scores were 8-10, head circumference 33 cm (10^{th} centile), birth weight 3,240 g (50^{th} centile), and height 51 cm (75^{th} centile). At birth, she was diagnosed with anal atresia and rectoperineal fistula corrected by anoplasty and, later at six months of age, by posterior anal relocation. At three months of age, cardiac assessment revealed patent ductus arteriosus and apical ventricular septal defect with small left-to-right shunting.

Her developmental milestones were delayed: head control, sitting without support, and walking were achieved at 3, 18, and 36 months, respectively. At 14 months, she was referred to our department due to developmental delay. On admission, neurological and psychomotor examination revealed microcephaly (42 cm [$<3^{th}$ centile]), mild hypotonia, normal deep tendon reflexes, difficulty in unsupported sitting, poor spontaneous motility, inability to grasp objects, poor eye

contact, absence of social smiling, repetitive sticking-out of the tongue, under-reaction to pain, and a high level of sensory seeking. In addition, the patient showed craniofacial dysmorphic features including a broad nasal bridge, hypotelorism, a thin upper lip, a high arched palate, a narrow forehead, and four spots (variable in size) on the left groin and thigh (*see video sequence*).

The EEG displayed a symmetric irregular posterior 5-6-Hz activity associated with isolated spike-wave discharges mainly in both frontal regions (*figure 1*).

At three years of age, the patient first experienced atypical absence seizures with head oscillation and drop attacks with a frequency of 2-3/die, associated, during drowsiness, with abnormal interictal EEG displaying isolated or short series of recurring and generalized 3-Hz spike-wave complex discharges (*figure 1*). In addition, the child showed unsteady posture, worsening of uncertain walking, drooling, restlessness, irritability, self-injury, and poor responsiveness to environmental stimuli. She could speak only a few meaningful sounds, used in association with a restricted conventional repertoire of gestures, to communicate with other individuals, but she understood simple sentences. The clinical course, to date, is characterized by alternating remission and relapse of an electroclinical pattern, partially refractory to common antiepileptic drugs. Indeed, an increase in seizure frequency was constantly associated with increased EEG abnormalities (leading to continuous spike-wave activity during slow sleep [CSWS]) and a worsening of the above-mentioned behaviour and motor abnormalities (worsening of ataxic-like gait and speech language, drooling, irritability, and self-injury). In addition, the relapse of seizures was adversely influenced by febrile illness, suggesting a phenotype with fever-triggered seizures. However, a combination of levetiracetam and ethosuximide was the most effective treatment leading to a long period of seizure freedom. Brain MRI performed at two and four years of age showed a mild unmodified thinning of the corpus callosum isthmus (*figure 2*).

Comprehensive genetic testing was performed during the clinical course. A next-generation sequencing panel showed a new heterozygous missense variant (c.25C>G) corresponding to within the N-terminal domain of the *SCN1A* gene (NM_001165963.1), resulting in substitution of a highly conserved amino acid (p.Pro9Ala). This variant, not reported in the ExAC database, was inherited from her healthy father. Bioinformatics assessment of the significance of the *SCN1A* missense mutation using two different algorithms, PolyPhen2 (http://genetics.bwh.harvard.edu/pph2/) and Mutation Taster (http://www.mutationtaster.org/), predicted a possible deleterious effect on protein function (probably damaging with a score of 0.989 based on PolyPhen2, and disease-causing with a score of 27 based on Mutation Taster).

In addition, whole-exome sequencing showed a novel heterozygous *de novo* frame-shift variant, c.2264dupA p.Asp755fs, in the *ZEB2* gene (NM_014795.3), not reported in the ExAC database (*for more details see supplementary data online*).

Discussion

The ZEB2 protein is a complex transcription factor that encompasses multiple functional domains that interact with the regulatory regions of target genes. In particular, the *ZEB2* plays a key role in several processes associated with nervous system development. It was found to be highly expressed in all brain regions of the human foetal telencephalon, involving several neuronal populations including pyramidal neurons of the hippocampus, cortico-spinal neurons, dopaminergic neurons in the brainstem (Nishizaki *et al*., 2014), and cerebellum Bergmann glia precursors (He *et al*., 2018). Recently, *ZEB2* has been shown to regulate the differentiation of interneuron progenitors, migrating from the medial ganglionic eminence to cortical layers by repression of the *Nkx2-1* homeobox transcription factor (McKinsey *et al.*, 2013). It has therefore been suggested that the deficit in *ZEB2*, resulting in decrease in cortical interneurons associated with increased striatal GABAergic interneurons, may induce an imbalance of inhibition/excitation, leading to epileptic seizures, the main component of Mowat-Wilson syndrome (Cordelli *et al.*, 2013). In addition, our patient has a novel *SCN1A* variant (c.25C>G; p.Pro9Ala) inherited from her unaffected father.

Whereas we are only just starting to identify genotype-phenotype correlations for Kv7.2 channelopathies (Piro *et al.*, 2019), *SCN1A* mutations, resulting in reduction of NaV1.1-mediated sodium currents in GABAergic interneurons, are associated with a spectrum of epilepsy syndromes, ranging from relatively mild phenotypes with febrile seizures plus to severe myoclonic epilepsy of infancy, also known as Dravet syndrome (DS) (Claes *et al.,* 2001). Although the father of the presented patient carries a *SCN1A* variant (c.25C>G), and has never had any epileptic seizure, we have been cautious in classifying the variant as benign. Indeed, previous studies have highlighted that *SCN1A* variants may have a low penetrance and a familial recurrence that affects the descendants of non-symptomatic parents (Gataullina and Dulac, 2017). Furthermore, the neurodevelopmental profiles of *ZEB2* and *SCN1A* mutations show some overlap, making it difficult to establish the role of the *SCN1A* variant in our patient's phenotype. In addition, this unusual co-occurrence underlines similarities with some features of Dravet syndrome including ataxic gait, language, cognitive, and behaviour deficit, hyperthermia sensitivity and a distinct profile of language deficit with significant impairment of expressive language development but relatively good receptive language (Battaglia *et al.*, 2013; Gataullina and Dulac, 2017).

To date, there is no precise genotype-phenotype relationship for Mowat-Wilson syndrome or Dravet syndrome as their respective genotypes are associated with significant phenotypic variability. In recent years, however, intrafamilial and clinical heterogeneity have been widely discussed in the literature and the results seem to point to a relationship between loss of function of $Na_v1.1$ channels in GABAergic interneurons and the spectrum of severity of $Na_v1.1$-associated epilepsy syndromes (Catterall *et al.*, 2010; Zuberi *et al.*, 2011; Nissenkorn *et al.*, 2019). On the other hand, a recent collaborative international study carried out to further characterize the phenotype, natural history, and genotype-phenotype correlation of 87 patients with a genetic diagnosis of MWS revealed that some retention of ZEB2 protein function may lead to a milder clinical phenotype. Moreover, a mild phenotype may be associated with mutations resulting in a loss of function of ZEB2 protein (Ivanovski *et al.*, 2018). Remarkably, the two genes affected by mutation in our patient regulate the differentiation and functioning of GABAergic interneurons, of which the action potential firings (of at least 75%) are supplied by sodium current of $Na_v1.1$ channels. In addition, based on studies to investigate the phenotype of patients with *SCN1A* and *ZEB2* mutation, authors hypothesize that other genetic and environmental factors may be responsible for the phenotypic variability.

Our findings therefore suggest that the *SCN1A* variant, c.25C>G, together with the *ZEB2* mutation, may have influenced the severity of our patient's neurological phenotype, playing a role in the dysfunction of interneurons. Furthermore, the lack of structural brain abnormalities on MRI in our patient supports the hypothesis that the electroclinical pattern is caused by cortical interneuron dysfunction (Cordelli *et al.*, 2013). Clearly, further studies will be needed to confirm this hypothesis. Furthermore, the clinical features of our patient included anal atresia (not previously reported in literature) which may reflect the role of *ZEB2* in the regulation of target genes leading to different congenital defects, further expanding the spectrum of phenotypes associated with *ZEB2*-related disorders.

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None of the authors have any conflicts of interest to declare.

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Legends

Figure 1. Progression of EEG abnormalities in the patient showing isolated spike-wave discharges mainly in both frontal regions (**A**), a brief duration of generalized 2.5-Hz spike-wave discharge (**B**), a moderate duration of generalized 3-Hz spike-wave discharge (**C**), and sub-continuous spike-wave activity during slow sleep (**D**).

Figure 2. Brain T1-weighted sagittal MRI performed at four years of age showing mild thinning of the corpus callosum isthmus.

Video
Video recording during a seizure-free period with relatively healthy status, demonstrating typical facial features, homophonic vowel sounds, and ataxic-like walking.

Short questions with answers:

1) Which of the following statements about families with Mowat-Wilson syndrome carrying *ZEB2* pathogenetic variants is correct?

   A. A mild phenotype is associated with mutations resulting in loss of function of *ZEB2* protein;
   B. A mild phenotype is always associated with mutations resulting in some retention of ZEB2 protein function;
   C. There is not a precise genotype-phenotype relationship for the disorder.

Right answer C

2) Which of the following statements about Mowat-Wilson syndrome is correct?

   A. The association of language impairment, ataxic gait and electroclinical pattern may be a significant biomarker of the disorder;
   B. Language impairment is a transient symptom;
   C. Na-blockers are the first line drugs.

Right answer A

3) In patients carrying *ZEB2* and *SCN1A* pathogenic variants the language and cognitive impairment, and ataxic gait

   A. Result from electroclinical pattern;
   B. Are influenced by epileptic activity;
   C. Are independent of each other.

Right answer B

Supplementary data

Analysis of *SCN1A* (NM_001165963. 1).
Genomic DNA of the patient was extracted from peripheral leukocytes, using QIAsymphony S (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Genomic DNA was enriched using HaloPlex Target Enrichment System (Agilent Technologies Inc., 2013). Genomic DNA samples were digested with eight pairs of restriction enzymes to create a library of gDNA restriction fragments. Restricted fragments were pooled and hybridized with customized probes hybridizing to exons and 10-bp flanking regions of the selected genes*. Circularized target DNA-HaloPlex probe hybrids, containing biotin, were captured with streptavidin-coated magnetic beads and DNA ligase was added to the capture reaction to close nicks in the circularized probe-target DNA hybrids. Subsequently, we amplified the samples by PCR and purified using AMPure XPbeads (Beckman Coulter, Inc.) and a magnetic plate. Prior to sample pooling, the quality of the library was inspected using Tapestation (Agilent Technologies Inc.). A multiplexed 150-bp paired-end-read exome sequencing was carried out on Illumina MiSeq (Illumina Inc.) Sequencer running on MiSeq Control Software (HCS). Raw data from MiSeq sequencing runs were processed using two software pipelines, SureCall 4.0 (Agilent Technologies Inc) and the Biomedical Genomics Workbench software version 3.5.2 (Qiagen). Sequencing reads were filtered for low-quality reads, trimmed for adapter sequences and tagged as belonging to the specific patient according to the barcode.
Using the spectrum of the expected mutations in the training set, the parameters for variant calling were established to minimize the number of false-positive results and guarantee the characterization of all the true-positive calls; the following filter thresholds were considered: minimum allele frequency for single-nucleotide polymorphism (SNP) and indel (SNP% ≥20), phred-like quality score of the called variant (Qcall ≥20) and depth of coverage (Depth ≥20).
Using Sanger sequencing, we analysed the exons classified as uncovered in order to reach the percentage of target region correctly covered; moreover, the new non-synonymous nucleotide variants identified were also confirmed by Sanger sequencing. Classification of identified variants was made according to guidelines (Matthijs *et al*.; Guidelines for the diagnosis of next-generation sequencing EJHV v.24 2016): (1) benign, (2) probably benign, (3) of uncertain meaning (VUS), (4) probably pathogenic, and (5) pathogenic. Interpretation of variants was carried out according to the guidelines (Richards *et al*.; Standards and guidelines for the interpretation of sequence variants.) ACMG - Genetics in Medicine 2015).
*list of genes *ALDH7A1* (NM_001182. 2), *PNPO* (NM_018129. 3), *ARHGEF9* (NM_015185. 2), *SLC25A22* (NM1191060. 1), *PLCB1* (NM_015192. 3), *TBC1D24* (NM_001199107. 1), *PNKP* (NM_007254. 3), *KCNT1* (NM_020822. 2), *KCNQ2* (NM_172107. 2), *SCN2A* (NM_021007. 2), *SCN8A* (NM_014191. 3), *STXBP1* (NM_003165. 3), *SCN1A* (NM_001165963. 1), *PCDH19* (NM_001184880. 1), *CDKL5* (NM_003159. 2), *SPTAN1* (NM_001130438. 2), *SLC2A1* (NM_006516. 1), *ST3GAL3* (NM_174963. 3), *GRIN2A* (NM_001134407. 2), *CHD2* (NM_001271. 3), *HCN1* (NM_021072. 3), *SYNGAP1* (NM_006772.2), *SLC35A3* (NM_012243. 2), *KCNQ3* (NM_004519.3).

Analysis of *ZEB2* (NM_014795.3)
Clinical whole-exome sequencing (WES) was performed for the proband and her unaffected parents. The Nextera Rapid Capture Enrichment kit (Illumina) was used according to the manufacturer's instructions. Libraries were sequenced with an Illumina HiSeq3000 using a 100-bp paired-end protocol. Sequence alignment with the human reference genome (UCSC hg19) and variants call and annotation were performed using an in-house pipeline, as described elsewhere (Mencacci *et al*., 2016). The raw data of single nucleotide variants (SNVs) and indels was then

filtered. Only exonic and donor/acceptor splicing variants were considered. In accordance with the pedigree and phenotype, priority was given to rare variants (<1% in public databases, including the1000 Genomes project, NHLBI Exome Variant Server, Complete Genomics 69, and Exome Aggregation Consortium [ExAC v0.2]) that fit a recessive or a *de novo* model and are located within genes previously associated with EOEE. The *de novo ZEB2* variant identified by WES in the proband (c.2264dupA; p.Asp755fs) was confirmed by traditional Sanger sequencing. The detailed conditions for sequencing analysis are available upon request.