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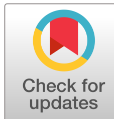

Abnormal function of the UBA5 protein in a case of early developmental and epileptic encephalopathy with suppression-burst.

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/humu.23534.

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ABSTRACT

Early myoclonic epilepsy or Aicardi syndrome (EME) is one of the most severe epileptic syndromes affecting neonates. We performed whole exome sequencing in a sporadic case affected by EME and his parents. In the proband we identified a homozygous missense variant in the ubiquitin-like modifier activating enzyme 5 (*UBA5*) gene, encoding a protein involved in post-translational modifications. Functional analysis of the *UBA5* variant protein reveals that it is almost completely unable to perform its trans-thiolation activity. Although recessive variants in *UBA5* have recently been associated with epileptic encephalopathy, variants in this gene have never been reported to cause EME. Our results further demonstrate the importance of post-translational modifications such as the addition of an ubiquitin-fold modifier 1 (UFM1) to target proteins (ufmylation) for normal neuronal networks activity, and reveal that the dysfunction of the ubiquitous UBA5 protein is a cause of EME.

Keywords

Encephalopathy, UBA5, early myoclonic epilepsy, Ohtahara syndrome.

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Introduction

Early onset epilepsies with suppression-burst (EOEE-SB) are rare devastating epilepsies of infancy imposing an enormous socio-economic burden on family and society. They are characterized by a stormy onset of epileptic activity with frequent seizures and a highly recognizable electroencephalogram (EEG) with periods of silence alternating with bursts of paroxysmal activity (Ohtahara 1976). Two epileptic syndromes associated with a suppression-burst pattern have been described: Ohtahara syndrome (OS), also called early infantile epileptic encephalopathy (EIEE), and Aicardi syndrome or early myoclonic epilepsy (EME) (Ohtahara and Yamatogi 2003). EME is much rarer than OS and it is characterized by a very early onset, with erratic myoclonic jerks and a very poor EEG with periods of silence longer than periods of paroxysmal activity (Aicardi and Goutieres 1978). The distinction between the two syndromes is sometimes difficult to make and the etiologies can overlap, leading most authors to consider these two clinical entities as being part of the same spectrum (Stamberger et al. 2016). Despite considerable progress being made in the last few years for the genetic diagnosis of neonatal onset epilepsies and notably in EOEE-BS (Olson et al. 2017), 40% of patients remain without diagnosis. We performed trio whole exome sequencing (WES) in a French cohort of 29 patients with typical EOEE-SB without molecular diagnosis. Here, we describe the phenotype of a sporadic EME case in which we identified a homozygous variation in the *UBA5* gene (MIM# 610552), encoding the ubiquitin-like modifier activating enzyme 5, and performed functional studies using the variant protein.

Case report

The proband was born from consanguineous parents after normal pregnancy and delivery. Birth parameters were in the normal range (head circumference 25th percentile). He poorly adapted to extrauterine life (APGAR score was 3 at 1 min, 8 at 5 and 10 minutes) and severe global hypotonia was immediately noted. The first epileptic manifestation was noticed 3 hours after birth and consisted in clonic movements of the upper limbs. The patient was

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transfered to NICU for myoclonic *status epilepticus* resistant to phenobarbital. Video/EEG recordings revealed a suppression-burst pattern. The first EEG showed very poor activity with prolonged periods of electrical silence and short bursts of abnormal activity concomitant or not with the myoclonic jerks (**Figure 1**), periods of silence then became shorter than bursts of activity. Brain magnetic resonance imaging and extensive metabolic workup did not reveal any abnormality. The metabolic workup included arterial blood gas; serum glucose, lactate, pyruvate, uric acid ammonnia and pipecolic acid; urinary ketone bodies; chromatography of serum, urine and CSF amino-acids; dosage of very long chain fatty acids; creatine phosphokinase; acylcarnitine profile; study of neurotransmitters in CSF; and chromatography of organic acids in urine. The patient died at day 16 after cardiac arrest due to respiratory failure due to status epilepticus.

Exome sequencing and data analysis

The proband and his parents' DNA samples were sequenced using the TruSeq DNA Exome sequencing kit (Illumina) on a NextSeq 500 sequencing apparatus. Sequencing data were analyzed according to all modes of transmission. We selected the variants affecting the protein sequence and removed the variants present with a minor allele frequency >0.001 in the gnomAD database (http://gnomad.broadinstitute.org/). We also filtered the variants predicted to be pathogenic or likely pathogenic by less than 3 prediction software of the 5 used routinely in our analyses (Supp. Table S1). Finally, we removed the variant located in genes not expressed in the brain after query of the Genotype-Tissue Expression (GTex) portal (https://www.gtexportal.org/home/). Dominant and X-linked modes of inheritance did not lead to the identification of possibly pathogenic variants according to this scheme. Using an autosomal recessive transmission mode (i.e. homozygous or compound heterozygous patient and heterozygous parents), we identified an homozygous variant in the *UBA5* gene (RefSeq NM_024818.4), a gene previously implicated in early infantile epileptic encephalopathy (EIEE44) (Supp. Figure S1). That homozygous variant, c.158A>T in

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NM_024818 (p.Tyr53Phe), has never been described before and is located in the N-terminal region of the UBA5 protein composed of 404 amino acids. The tyrosine at position 53 in the human UBA5 protein is highly conserved throughout evolution, including insects and plants. The variant was submitted as case #152004 to the LOVD variant database (https://databases.lovd.nl/shared/individuals/00152004). Analysis of the UBA5 transcript using cDNAs prepared from the patient's blood cells did not reveal any abnormality, ruling out an effect of the missense variant on the splicing of the UBA5 mRNA.

Functional study of the variant UBA5 protein.

In order to determine the functional consequences of the p.Tyr53Phe variants of the UBA5 protein, functional assays were performed as previously described (Komatsu et al. 2004) (**Figure 2**). Briefly, recombinant ubiquitin-fold modifier 1 (UFM1) (MIM# 610553), UBA5 and the p.Tyr53Phe variation, generated by PCR-based site-directed mutagenesis (Agilent), were N-terminally tagged with glutathione-S-transferase (GST), the His-tag was introduced at the N-terminus of the E2-enzyme, the Ufm1-conjugating enzyme 1 (UFC1) (MIM# 610554). Following expression and purification by chromatography on glutathione sepharose (Sigma-Aldrich) or nickel-charged affinity resin (Bio-Rad), respectively, a time-dependent *in vitro* transthioester formation assay was performed. For this, UFM1 was mixed with UFC1 and UBA5 or the UBA5 variant and incubated in the presence of ATP for 0, 3 and 30 minutes. The transthiolation assay was stopped by the addition of SDS-containing loading buffer followed by SDS-PAGE combined with immunoblotting using anti-UFM1 (BostonBiochem Inc.) anti-UFC1 (Abcam) (**Figure 2A**). For all UBA5 proteins conjugates observed, the p.Tyr53Phe protein variant showed a significantly delayed activity after 3 minutes incubation with 6,8% of remaining transthiolation activity compared to the wild type Uba5 protein (**Figure 2B**). By comparison, the p.Met57Val protein retains 22,6% of wild type activity.

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DISCUSSION

Bi-allelic pathogenic variants in the *UBA5* gene have been described in 16 individuals to date. All but one had severe ID. Despite some degree of heterogeneity, the vast majority of patients (13/16) shows an early-onset developmental encephalopathy with progressive microcephaly, movement disorders, epilepsy and global brain atrophy at the MRI (Muona et al. 2016, Colin et al. 2016, Arnadottir et al. 2017). The epileptic seizures emerged while the development was already abnormal in 13 patients in the form of epileptic spasms (8 patients), myoclonic jerks (2 patients), infantile spasms and myoclonic jerks (2 patients), or other seizure type (1 patient). Thus, the *UBA5*-related phenotype can be defined as a severe early-onset developmental encephalopathy with post-natal microcephaly, with a spectrum of severity ranging from EOEE-BS and early death (the case we describe) to milder phenotypes without epilepsy and moderate ID. Although epilepsy is a prevalent feature, the term "developmental and epileptic encephalopathy" is more accurate than "epileptic encephalopathy" because epilepsy and ID are probably largely independent from each other, as demonstrated by patients without epilepsy (Scheffer et al. 2017). The case we report here illustrates the frequent overlap between OS and EME, with initial EEGs showing periods of silence longer than bursts as described by Aicardi in EME, but this pattern turned out to be the opposite after the first week as described in OS.

The genetic variants identified in all previously published affected individuals with epileptic seizures combine a recurrent p.Ala371Thr hypomorphic allele with a loss of function allele. The p.Ala371Thr allele is not sufficient to cause a genetic disease since it was recently identified in several homozygous individuals demonstrating normal neurological function (Arnadottir et al. 2017). The case reported here is the first homozygous variant case to be affected by an UBA5-related disease. Rather, the patient is homozygous for the p.Tyr53Phe variant inherited from heterozygous

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carrier parents. That missense variant has the most severe effect reported to date on the trans-thioylation activity of UBA5 with >90% of activity loss.

Since drosophila, zebrafish and mice are not viable under a knockout of the UFM1 cascade, the cascade probably possesses conserved essential functions in higher animals. Besides its role in haematopoiesis, the UFM1 cascade was found to influence neurological functions in all animals studied: C. elegans, drosophila, zebrafish and mice (Hertel et al. 2013, Collin et al. 2016, Muona et al. 2016, Duan et al. 2016).

The UBA5 protein is involved in a post-translational mechanism consisting of the addition of an ubiquitin-fold modifier 1 (UFM1) to target proteins. UFM1 is a recently identified novel member of the ubiquitin-like protein (UBL) family (Komatsu et al. 2004). Similar to the process of protein ubiquitination, UFM1 is transferred via an enzymatic cascade to a set of target proteins that largely remain to be identified (Cai et al. 2016). During ufmylation UFM1 is activated by an E1-enzyme, UBA5, conjugated to the E2-enzyme, UFC1, and ligated to protein targets by the E3 enzyme, UFL1 (MIM# 613372). Physiological functions of the UFM1 cascade include cell cycle control, cell differentiation and endoplasmic stress response (Daniel & Liebau 2014; Wei & Xu 2016). The UFM1 cascade is involved in various pathological conditions or diseases like diabetes (Lemaire et al. 2011), tumorogenesis (Yoo et al. 2015; Kim et al. 2013) and ischemic heart disease (Azfer et al. 2006).

While it is currently unknown how UBA5 dysfunction causes a severe epileptic phenotype, it is interesting to note that other E1-activating enzymes in the ubiquitin or ubiquitin-like pathways are also important for neurological development or function, such as the proteins encoded by the *SUMO1* (MIM#601912) (Wilkinson et al. 2010) or *UBE3A* (MIM# 601623) *genes* (LaSalle et al. 2015). Because ufmylation plays such an important role for the development and function of the central nervous system in addition to its previously documented role for erythropoiesis in mouse

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embryos (Tatsumi et al. 2011), it will be most interesting to identify the ufmylated proteins in the central nervous system and the pathogenic mechanisms leading to these severe epileptic encephalopathies. Finally, because of their coordinated action with UBA5 in the ufmylation process, the *UFC1* and *UFL1* genes are also candidates for neurological diseases with or without epilepsy and we propose that they should now be screened in this population of patient in addition to *UBA5*.

ACKNOWLEDGEMENTS

We thank the Centre de Ressources Biologiques of La Timone Children's Hospital (Karine Bertaux, Cécile Mouradian) for assistance with the samples used in this study, Arnaud Lagarde and Christel Castro for their assistance for the production of NGS data. This work was funded by Fondation JED Belgique, Inserm and Aix Marseille Université. The authors declare no conflict of interest.

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FIGURE LEGENDS

Figure 1

Representative EEGs of the patient from birth to the tenth day of life, showing a suppression-burst pattern. EEG (longitudinal montage, right hemisphere : 4 first lines, 20s (left traces), 1 min (right traces) showing initially (D1) very poor traces with prolonged periods of silence ad some bursts of activity either asymptomatic, or concomitant of a myoclonic jerk (deflection of the abdominal movements, ABD line, top panel). Traces then became more active, still very abnormal with shorter periods of silence, visible until the death of the patient. Some myoclonic jerks were recorded (deflection of the ABD line).

ABD : detection of abdominal movements via accelerometer. Position of electrodes : Fp : frontopolar ; T : temporal; O : occipital, C : central.

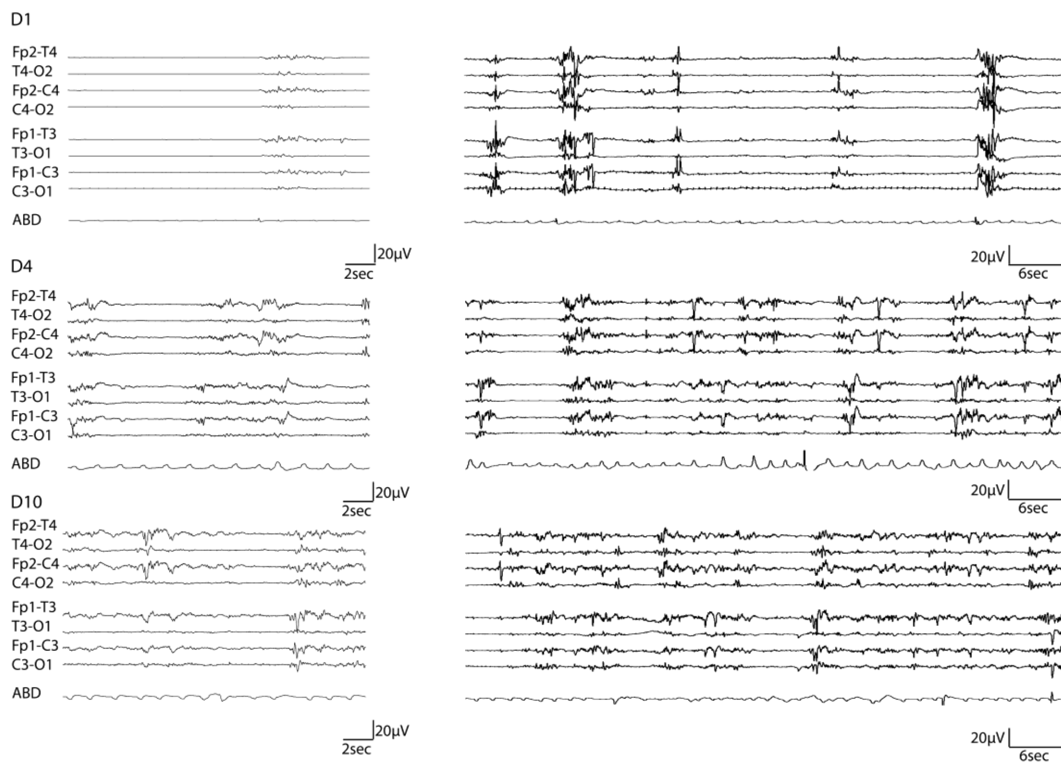

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Figure 2

In vitro activity assay of the UBA5 variant p.Tyr53Phe. UFM1, UBA5 and the UBA5 variants p.Tyr53Phe and p.Met57Val were fused to a GST-tag and UFC1 to a His-tag. Following recombinant expression*,* the purified proteins were used for a transthiolation assay (Komatsu et al, 2004). The thioester activation and conjugation was assessed *in vitro* comparing the activity of wild type UBA5 with the UBA5 variants. For comparison, the UBA5 variant p.Met57Val (Colin et al. 2016) was analyzed in the same assay.

A. Samples were taken after 0, 3 and 30 minutes and analyzed by SDS-PAGE and western blot using anti-UFM1 (1:5000) and anti-UFC1 (1:10 000). **B**. For statistics the formation activity following a 3 minutes incubation was analyzed. Three independent experiments were used for statistical quantification and a one-way ANOVA (Bonferroni‘s multiple comparison test) was performed, ***p<0,001

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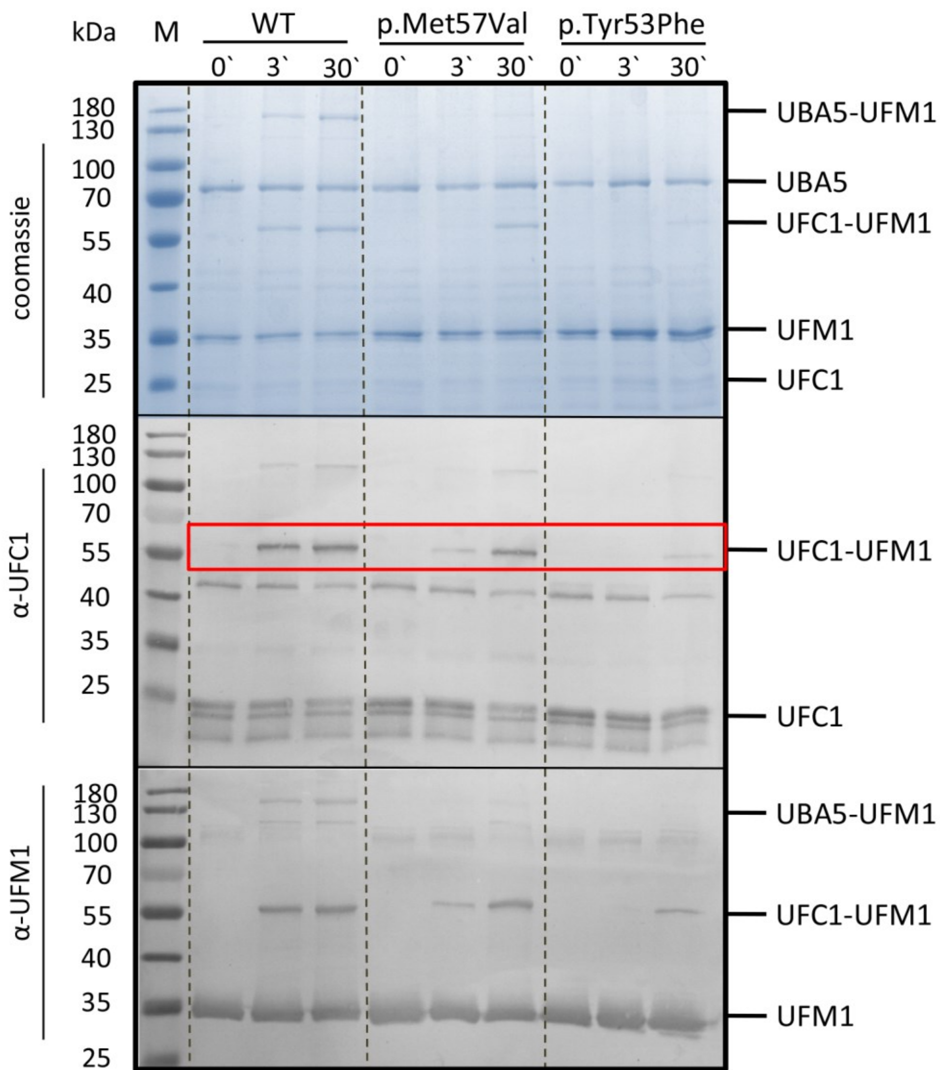

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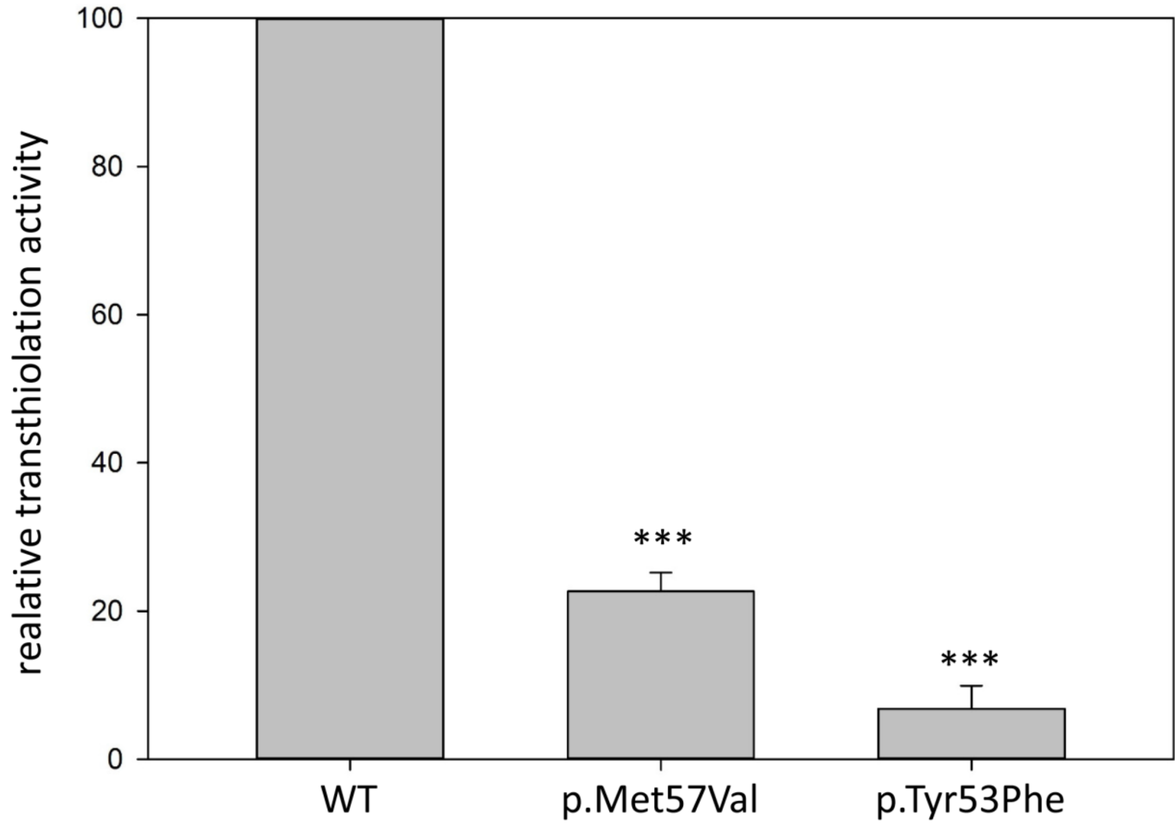

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