Co-occurring *WARS2* and *CHRNA6* mutations in a child with a severe form of infantile parkinsonism

Running title: WARS2 and CHRNA6 variants in infantile parkinsonism

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The authors report no conflicts of interest relevant to the manuscript.

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Abstract

Objective. To investigate the molecular cause(s) underlying a severe form of infantile-onset parkinsonism and characterize functionally the identified variants.

Methods. A trio-based whole exome sequencing (WES) approach was used to identify the candidate variants underlying the disorder. *In silico* modeling, and *in vitro* and *in vivo* studies were performed to explore the impact of these variants on protein function and relevant cellular processes.

Results. WES analysis identified biallelic variants in *WARS2*, encoding the mitochondrial tryptophanyl tRNA synthetase (mtTrpRS), a gene whose mutations have recently been associated with multiple neurological phenotypes, including childhood-onset, levodopa-responsive or unresponsive parkinsonism in a few patients. A substantial reduction of mtTrpRS levels in mitochondria and reduced OXPHOS function was demonstrated, supporting their pathogenicity. Based on the infantile-onset and severity of the phenotype, additional variants were considered as possible genetic modifiers. Functional assessment of a selected panel of candidates pointed to a *de novo* missense mutation in *CHRNA6*, encoding the α6 subunit of neuronal nicotinic receptors, which are involved in the cholinergic modulation of dopamine release in the striatum, as a second event likely contributing to the phenotype. *In silico*, *in vitro* (*Xenopus* oocytes and GH4C1 cells) and *in vivo* (*C. elegans*) analyses demonstrated the disruptive effects of the mutation on acetylcholine receptor structure and function.

Conclusion. Our findings consolidate the association between biallelic *WARS2* mutations and movement disorders, and suggest *CHRNA6* as a genetic modifier of the phenotype.

Glossary: ACh, acetylcholine; DA, dopaminergic; EOP, early-onset parkinsonism; HVA, homovanillic acid; mtARSs, mitochondrial aminoacyl-tRNA synthetases; mtTrpRS, mitochondrial tryptophanyl tRNA synthetase; MTS, mitochondrial targeting sequence;

nAChR, nicotinic acetylcholine receptor; OXPHOS, oxidative phosphorylation; PD, Parkinson’s disease; RCCs, respiratory chain complexes; SEP, sensory evoked potentials; VEP, visual evoked potentials; WB, Western blot; WES, whole-exome sequencing.

Introduction

Early-onset parkinsonism (EOP) is an exceedingly rare condition, usually of monogenic origin, caused by aberrant neurotransmitter synthesis, vesicular trafficking, autophagy, or mitochondrial function [1]. A significant proportion of subjects with a recognized primary genetic cause also shows concomitant variant(s) in Parkinson's disease (PD)-associated genes, acting as phenotypic modifiers which explain, in part, the wide variability of disease onset and progression [2].

Mitochondrial aminoacyl-tRNA synthetases (mtARSs) are essential components of the translation machinery of mitochondria, charging tRNAs with their cognate amino acids during translation of mitochondrial genes [3]. mtARS gene mutations have recently emerged as the molecular cause underlying a wide spectrum of human diseases. Among these, biallelic variants affecting *WARS2*, the gene encoding the tryptophanyl mtARSs (mtTrpRS), have been reported in subjects with a neurological presentation [4]. More recently, the phenotypic spectrum associated with *WARS2* mutations has been expanded by the identification of two individuals with EOP [5,6].

CHRNA6 encodes the α6 subunit of neuronal nicotinic receptors (nAChRs), is highly expressed presinaptically in dopaminergic (DA) neurons of the nigrostriatal-mesolimbic pathway, is activated by acetylcholine/nicotine binding, and is involved in the cholinergic modulation of dopamine release in the striatum [7].

Here, we report co-occurrence of biallelic *WARS2* mutations and a *de novo CHRNA6* variant in a child with a severe form of infantile parkinsonism. Our data confirm the association between *WARS2* loss-of-function and movement disorders, and suggest a role of *CHRNA6* as a modifier gene in EOP.

Methods

Methods are reported as Supplementary Information.

Results

Case report

The proband (AV07) is an 11 year-old male born from non-consanguineous, healthy parents. Between 6-9 months, trunk instability and occasional action jerks of limbs were noticed (video 1). At 10-15 months, loss of postural control, axial hypotonia, dysarthria, and dysphagia became evident. Social interaction and language comprehension remained relatively spared. At 15 months, CSF examination revealed low homovanillic acid (HVA, 185 nmol/L; n.r.295-932), neopterin (7.8 nmol/L; n.r.12-30), and biopterin (5.5 nmol/L; n.r.15-40). Brain MRI, ^{1}H-MRS, EEG, flash and pattern VEP, and SEP were normal. Between 18-36 months, the child developed dystonic posturing of limbs, bradykinesia, rigidity and subcontinuous generalized rest and action rhythmic jerk-like movements (video 2). Levodopa-carbidopa treatment (5/1.25 mg/kg/day) was started at the age of 2 years with relevant motor improvement (video 3), which progressively vanished at the age of 4 with the emergence of on/off phenomena and peak dose dyskinesia (video 4). The child also developed oculogyric crisis, ptosis, supranuclear gaze palsy, exotropia, hypomimia, a severe rigid-akinetic condition (videos 5,6), and versive seizures associated with paroxysmal epileptic alterations in frontal lobe on EEG recording. CSF examination revealed a further decrease of HVA (77 nmol/L; n.r.211-871). Mild cerebral atrophy was evident on brain MRI (Fig. 1A). At 6 years, DaTSCAN imaging revealed a severe derangement of DA striatal pathways (Fig. 1B). Worsening of on-off phenomena required progressive increased levodopa-carbidopa dosage (up to 10/2.5 mg/kg/day). Dopamine receptor agonists, COMT and MAO-B inhibitors were ineffective. At the age of 7, levodopa-carbidopa intestinal gel (240 mg/day) resulted in a relative clinical stabilization for a few years, followed by a subcontinuous levodopa-induced dyskinetic status in waking (video 7).

Molecular findings

A trio-based WES analysis allowed to exclude occurrence of mutations in known EOP-associated genes and hemizygous hits compatible with X-linked inheritance. Among biallelic events, WES identified compound heterozigosity for two missense variants, c.37T>G (p.Trp13Gly) and c.679A>G (p.Met227Val), in *WARS2* (Fig. 2A, Supplementary Table 1). Both variants were predicted to be “deleterious” by CADD. p.Met227Val affected an invariant residue among WARS2 orthologs, and was not reported in public databases (gnomAD/ExAC), while p.Trp13Gly was reported in gnomAD (allele frequency=$3.265e^{-3}$). Furthermore, Sanger sequencing validated three *de novo* variants affecting genes not previously associated with human disease, but encoding proteins with relevant function in neurodevelopment/neurophysiology (*CHRNA6*, *HIBADH*, and *PAK6*). These variants were either private or rare (<1/20,000 in gnomAD), were predicted to be deleterious by CADD (Supplementary Table 1), and were also identified in buccal mucosal epithelial cells and skin fibroblasts, supporting their germline origin. Interrogation of GeneMatcher failed in identifying any relevant match, and mutation scanning performed in two small EOP cohorts was negative, excluding a major role of these genes in EOP.

Functional studies

WARS2 contains an *N*-terminal mitochondrial targeting sequence (MTS) and a *C*-terminal domain with Trp-tRNA ligase activity (Fig. 2A). Trp^{13} is located within the MTS, and its substitution into glycine was previously shown to cause impaired mitochondrial localization [4]. No functional information was available for p.Met227Val (ligase domain). To explore the effect of these lesions, WB analysis was performed in isolated organelles, demonstrating a substantial reduction of WARS2 levels in patient’s fibroblasts compared to controls (Fig. 2B). Indirect evaluation of the oxidative phosphorylation (OXPHOS) status was assessed in mitochondria. Quantification of complex V activity showed a significant reduction of ATP synthesis using different substrates (16%, succinate; 23%, malate; 21%,

pyruvate/malate) (Fig. 2C), indicating impaired function of the respiratory chain complexes (RCCs), while no difference was detected in the expression of RCCs subunits (Fig. 2D). Functional validation of *PAK6* and *HIBADH* variants ruled out their contribution to the phenotype (Supplementary Results, Supplementary Fig. 1). In contrast, the *CHRNA6* c.527T>C (p.Phe176Ser) variant was shown to disrupt protein structure (Supplementary Results, Supplementary Fig. 2A,B) and receptor function. Specifically, different combinations of cDNA clones encoding human α6, β2, β3 and β4 subunits were injected in *Xenopus* oocytes, and ACh-evoked currents were recorded by voltage-clamp technique. Oocytes expressing α6/β4 or α6/β3/β4 displayed functional receptors, while those expressing $\alpha6^{F176S}$/β4 did not (Supplementary Table 2). Of note, oocytes expressing $\alpha6^{F176S}$/β3/β4 showed strongly reduced current amplitudes and number of responsive cells (Supplementary Table 2, Supplementary Fig. 2C-E). Consistently, GH4C1 cells expressing α6/β4 displayed functional receptors, while those expressing $\alpha6^{F176S}$/β4 did not, with expression of the mutant subunit causing a significant reduction in current density and number of responsive cells (Supplementary Fig. 2F-H). Collectively, these data demonstrated a loss-of-function role of p.Phe179Ser in *Xenopus* oocytes and GH4C1 expression systems.

To validate these findings *in vivo*, we used *C*. *elegans* as an experimental model. The assembly of the ACh receptor is well-conserved in nematodes. Among α subunits, *unc-63* more closely resembles the human gene *(https://www.ncbi.nlm.nih.gov/UniGene*). In muscles, UNC-63 is part of the levamisole-sensitive receptor [(L)-AChR], which is susceptible to hypercontraction/paralysis induced by the nicotinic agonist levamisole [8]. UNC-63 is required for locomotion, with null mutants being uncoordinated and resistant to levamisole. Transgenic animals overexpressing *unc-63*^{F169S} (homolog of h*CHRNA6*^{F176S}) under the control of p*myo-3*, driving expression in body-wall muscles, showed normal locomotion in crawling and thrashing assays, excluding a dominant-negative effect of the

mutation (Supplementary Fig. 3A,B). However, while overexpression of *unc-63*^{WT} in an *unc-63*$^{-/-}$ background (*x37*) rescued both uncoordinated phenotype and levamisole resistance, expression of the mutant allele did not (Supplementary Fig. 3 C,D; video 8), demonstrating a loss-of-function role of p.Phe169Ser in muscle receptors. Since muscle and neuronal AChRs exhibit different pharmacological profiles [9,10], we explored the functional impact of the variant in neurons by evaluating the sensitivity to the nicotinic agonist DMPP, whose toxicity is strictly controlled by neuronal UNC-63-containing nAChRs [11]. Exposure of wild-type animals to DMPP caused a lethal phenotype at the L2-to-L3 molt stage, while *unc-63*$^{-/-}$ mutants exhibited partial resistance to the drug (Supplementary Fig. 3E). Neuronal expression of *unc-63*^{WT} (p*unc-63*::*unc-63*) in *unc-63*$^{-/-}$ mutants partially rescued DMPP sensitivity, while expression of *unc-63*^{F169S} did not. More importantly, wild-type worms expressing *unc-63*^{F169S} in neurons acquired partial resistance to the drug, indicating a dominant-negative effect of the mutation in neuronal receptors.

Based on these findings, compassionate use of nicotine was considered as a therapeutic option, with only transient improvement of motor symptoms (Supplementary Results).

Discussion

We report on a severe form of infantile-onset parkinsonism, whose clinical course and response to therapy recapitulated adult PD. WES analysis and functional validation studies identified biallelic mutations in *WARS2* as the molecular cause of the disease and a *CHRNA6* variant potentially acting as a genetic modifier .

WARS2 is a nuclear-encoded protein fundamental to the mitochondrial synthesis of RCCs subunits. Biallelic *WARS2* mutations had previously been reported in sixteen subjects with a phenotype ranging from mitochondrial encephalopathy to variably less severe conditions, including dystonia, epilepsy and ID [4-6]. More recently, *WARS2*

mutations have been identified in two patients with childhood-onset parkinsonism [5,6]. Interestingly, in one of these subjects, mutations affected the same and the adjacent residues found to be mutated in the present case, suggesting possible genotype/phenotype correlation. Previous studies documented that p.Trp13Gly impacts proper subcellular localization [5] and variably affect the OXPHOS system [6], which are in line with the present findings. Compared to the reported EOP subjects [5,6], however, our patient showed a more severe phenotype, with first signs of disease at 6 months, and rapidly progressive neurological deterioration. CSF examination disclosed reduced HVA, a biochemical marker not previously associated with *WARS2* defects. Levodopa/carbidopa treatment was instituted at 24 months with a short “honeymoon” response, followed by increasingly severe motor fluctuations leading to the need for levodopa/carbidopa intestinal gel (7 years). DaTSCAN was confirmatory, revealing a complete derangement of the DA striatal pathways. Collectively, these findings suggest possible occurrence of a second event modulating the phenotype.

Among three *de novo* variants in candidate modifier genes, multiple lines of evidence pointed to the c.527T>C transition affecting *CHRNA6*. Structural modelling and functional profiling demonstrated a disrupting impact of this variant on protein structure and receptor function. Specifically, p.Phe176Ser exhibited a loss-of-function behavior in *Xenopus* oocytes, GH4C1 cells and *C. elegans* neuromuscular junction, while it displayed a dominant-negative effect in neuronal receptors of the nematode. α6*nAChRs have been extensively studied to explore their potential role in PD. Data from preclinical models established that striatal nAChRs influence several biological processes relevant to motor function [12]. However, mice lacking α6, α4, or both subunits, show only minor motor deficits [7], possibly because of functional redundancy. Consistently, *CHRNA6* is not an essential gene given the relatively high number of loss-of-function variants (gnomAD, pLI= 0). Our data do not support a role for *CHRNA6* as a new EOP-associated gene; rather,

they suggest a role of this gene as a modifier able to exacerbate the clinical presentation associated with biallelic *WARS2* mutations (or any other EOP-associated lesion), thus contributing to the unique biochemical (low HVA) and imaging (DaTSCAN) profile of the disease. A transient improvement in motor symptoms and levodopa/carbidopa effectiveness observed after administration of nicotine at an early stage of the disease support this hypothesis, and suggest that nicotine may improve dopamine response in the early stage of PD, in line with data indicating a neuroprotective role of nicotine against nigrostriatal DA neuronal loss [12].

In summary, our findings extend the association between biallelic *WARS2* mutations and EOP, and suggest *CHRNA6* as a genetic modifier of the phenotype. In line with accumulating evidence indicating the relevance of multilocus variation in explaining phenotypic variability, our data emphasize the importance of functional profiling to appreciate the relative contribution of genomic variants to the clinical phenotype.

Figure Legends

Figure 1. Brain imaging. (**A**) Brain MRI of AV07 (5 years) showed mild cerebral atrophy and mega cisterna magna. (**B**) Brain imaging with single-photon emission CT DaTSCAN (6 years) revealed a severe derangement of dopaminergic striatal pathways (left), compared to an age- and sex-matched healthy control (right).

Figure 2. Functional impact of *WARS2* mutations. (A) Location of disease-causing mutations is reported above the WARS2 domain structure scheme (lesions identified in EOP are shown in blue). Mutations found in the present study (red) are reported below the cartoon. The mitochondrial targeting sequence (MTS) and the aminoacyl-tRNA synthetase conserved site are shown in red and yellow, respectively. **(B)** A significant reduction of WARS2 levels was documented in mitochondria from patients' fibroblasts compared to

control cells (*P<0.05; **P<0.0005; Student's t test). WARS2 levels were normalized against VDAC. **(C)** Spectrophotometric determination of complex V activity. Significantly reduced ATP synthesis was documented in mitochondria of patient's fibroblasts, with either substrates used (succinate, malate, pyruvate/malate) (P<0.05). Data are expressed as mean ±SD of three independent experiments. **(D)** Expression of individual subunits of the mitochondrial CI (NDUFS1, NDUFA9), CII (SDHA), CIII (UQCRC2), CIV (COXII), and CV (ATP5A1) complexes.

Declarations of interest

None.

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Final disclosures

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Ethical approval and informed consent

All authors declare that the manuscript is in accordance with the statement of ethical standards for manuscripts submitted to *Parkinsonism and related disorders*. Parents have

consented for video publication and provided a signed release form authorizing the offline and/or online distribution of this video material.

Data Availability

Any anonymized data not published within the article will be shared by request from any qualified investigator.

Authorship

SM: conception and design of the study, analysis and interpretation of data, drafting the article, revising the manuscript critically for important intellectual content

VC: conception and design of the study, analysis and interpretation of data, drafting the article, revising the manuscript critically for important intellectual content

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