Functional analysis of a novel mutation in the *TIMM8A* gene that causes Deafness-Dystonia-Optic Neuronopathy Syndrome

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Abstract

Background*:* The rare, X-linked neurodegenerative disorder, Mohr-Tranebjaerg syndrome (also called Deafness-Dystonia-Optic Neuronopathy (DDON) syndrome), is caused by mutations in the*TIMM8A* gene. DDON syndrome is characterized by dystonia, early-onset deafness, and various other neurological manifestations. The *TIMM8A* gene product localizes to the intermembrane space in mitochondria where it functions in the import of nuclear-encoded proteins into the mitochondrial inner membrane. Frameshifts or premature stops represent the majority of mutations in *TIMM8A* that cause DDON syndrome. However, missense mutations have also been reported that result in loss of the *TIMM8A* gene product.

Methods: We report a novel *TIMM8A* variant in a patient with DDON syndrome that alters the initiation codon and employed functional analyses to determine the significance of the variant and its impact on mitochondrial morphology.

Results*:* The novel base change in the *TIMM8A* gene (c. 1A>T, p.Met1Leu) results in no detectable protein and a reduction in *TIMM8A* transcript abundance. We observed a commensurate decrease in the steady-state level of the Tim13 protein (the binding partner of Tim8a) but no decrease in *TIMM13* transcripts. Patient fibroblasts exhibited elongation and/or increased fusion of mitochondria, consistent with prior reports.

Conclusion*:* This case expands the spectrum of mutations that cause DDON syndrome and demonstrates effects on mitochondrial morphology that are consistent with prior reports.

Introduction

- Referred herein as DDON syndrome, the synonymous condition Mohr-Tranebjaerg syndrome (MTS) was first described nearly sixty years ago as an X-linked condition affecting Scandinavian populations (1-3).
- Characterized primarily by progressive deafness in early childhood, this condition also manifests with dystonia, spasticity, and dysphagia (4-6).
- Located on Xq22, the gene associated with DDON syndrome, *TIMM8A* (originally called DDP for deafness dystonia peptide; OMIM#300356) encodes a small protein that localizes to the intermembrane space in mitochondria (2,3). Tim8 forms a complex with other small TIM proteins, to facilitate the import of nuclear-encoded proteins into the mitochondrial inner membrane (8-10).
- The pathogenetic mechanism of DDON is not fully defined. However, several studies have implicated impaired transport through the intermembrane space, and subsequent mitochondrial dysfunction when Tim8 is unable to associate with its binding partner, Tim13, as the primary driver of pathogenesis.
- In this report, we describe a male patient harboring a novel base change in the *TIMM8A* gene (c. 1 A>T, p. Met1Leu) with features of DDON syndrome and provide functional studies to confirm the pathogenic status of this variant.

Clinical Summary

Our male patient was the product of a non-consanguineous normal pregnancy. Term delivery was via C-section due to placental hemorrhage and fortunately there were no perinatal problems from delivery. Prior to age 3 years, he met all developmental milestones on time without stagnation or regression. At 3 years, his parents began to notice some regression in his expressive language. He was eventually diagnosed with an auditory neuropathy and at age 6 years received cochlear implants. His most recent ophthalmological examination, at age 6, did not show optic neuropathy or retinal involvement. His neurological examination was otherwise without abnormality, in particular, findings of ataxia or dystonia were absent. Due to early auditory neuropathy, a massively parallel gene sequencing panel was sent for commercial testing for hearing loss gene abnormalities (Prevention Genetics, Marshfield, WI). A likely pathogenic variant in the *TIMM8A* gene, c. 1A>T, p. Met1Leu was found. On parental testing mother was found to be a carrier, with low copy numbers of the variant compared to wild type, suggesting that mother may be a mosaic for this genetic change.

Methods

Patient information and ethical compliance: Informed consent was obtained from the parents of the proband prior to participation in the research study. All procedures employed were reviewed and approved by the appropriate institutional review committee in the GGC IRB protocol.

Isolation of nuclear/mitochondria by subcellular fractionation: A subcellular fractionation protocol was utilized in order to enhance the signal of the TIMM proteins trapped within the mitochondrial intermembrane. Patient cells lines were cultured, fractionated into two lysates, with the first containing both nuclei and mitochondria and a second lysate including cytoplasmic and cellular membrane contents.

SDS-PAGE and Western blotting: Fractionated samples from both patient and control cell lines were resolved on a 15% SDS-PAGE gel followed by transfer to nitrocellulose membranes and blocked using 5% non-fat powdered milk in TBS-T solution for 1 hr at room temperature. Ponceau S staining was performed to visualize total protein load.

Mitochondrial staining: Fibroblasts were seeded onto coverslips in a twelve well cell culture plate. A rabbit polyclonal anti-P5CS (ALDH18A1) antibody was used to label mitochondria in staining buffer for one hour at room temperature followed by incubation with an Alexa Fluor 488 goat anti-rabbit IgG secondary antibody. To quantify the percentage of elongated mitochondria, images from 8 different fields of cells were obtained and the percentage of elongated mitochondria relative to the total mitochondria scored was determined in each field. These percentages were then averaged and statistical significance calculated using a **Student's t-test.**

Results

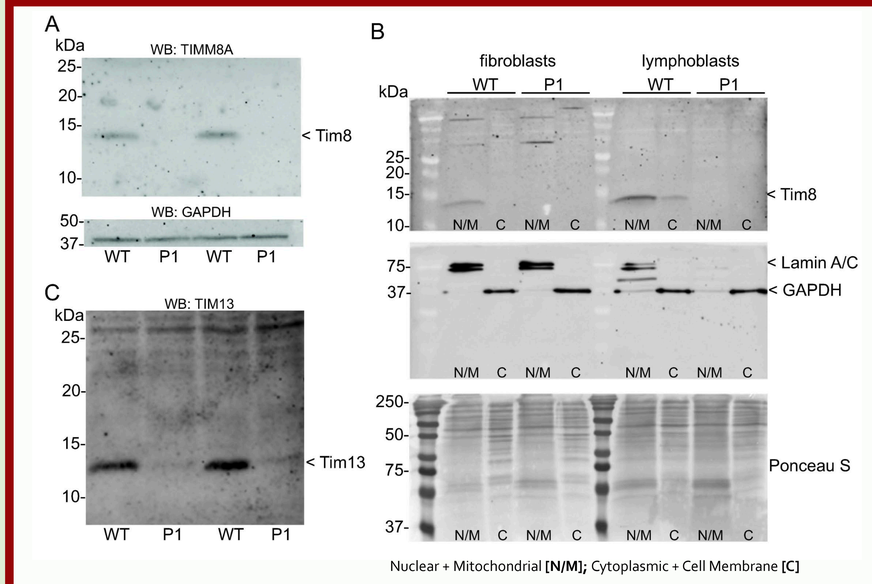

Figure 1: **(A)** Western blot analysis of the *TIMM8A* gene product in control (WT) and patient (P1) fibroblast lysates. A representative blot of three independent experiments is shown. GAPDH was used as a loading control. **(B)** Subcellular fractionation and Western blot analysis of fibroblast and lymphoblast lysates. Lamin A/C and GAPDH were used as controls for the fidelity of the fractionation of nuclear/mitochondria and cytosolic pools, respectively. **(C)** Western blot analysis of the *TIM13* gene product in control (WT) and patient (P1) fibroblast lysates. Tim13 protein is greatly reduced but still detectable in patient cells.

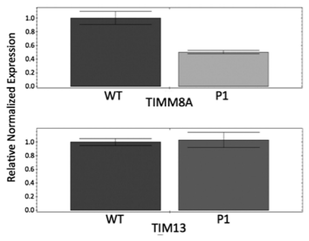

Figure 2: Quantitative PCR analysis of *TIMM8A* and *TIM13* transcript abundance in WT and P1 fibroblasts, normalized to *RPL4* transcript abundance. Data represents the average of three independent analyses.

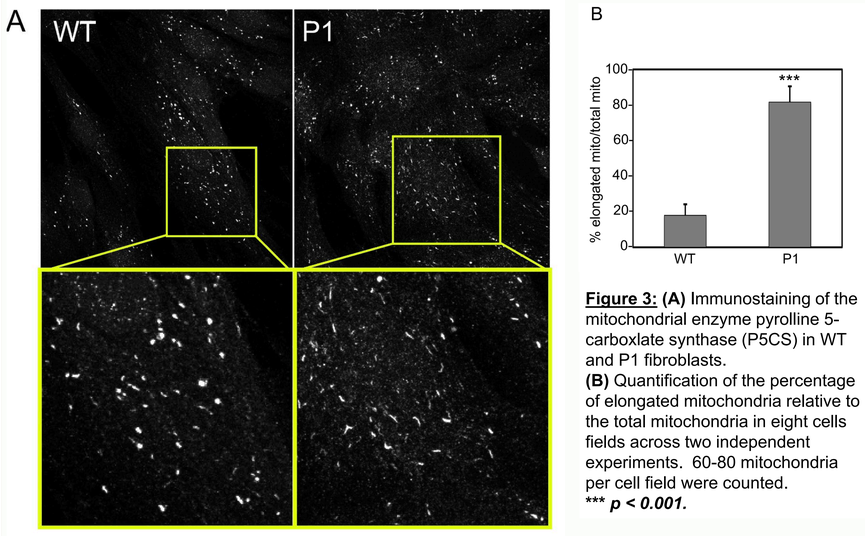

Figure 3: **(A)** Immunostaining of the mitochondrial enzyme pyrolline 5-carboxlate synthase (P5CS) in WT and P1 fibroblasts.
(B) Quantification of the percentage of elongated mitochondria relative to the total mitochondria in eight cells fields across two independent experiments. 60-80 mitochondria per cell field were counted.
*** ***p < 0.001.***

Discussion

- The variant described in this report of a c.1A>T start loss, the methionine required to initiate protein translation is replaced leading to a complete absence of the Tim8a/DDP protein. The absence or variant forms of the *TIMM8A* gene product, appears to only significantly influence the function of neurons within specific neuronal populations.
- Rates of mitochondrial fusion and fission directly dictate the organelle's morphology and function. The elongated mitochondria demonstrated in the present work, which have a cigar-like (as opposed to a globular) appearance in the patient cells, may arise due to increased fusion—a phenotype that could reflect the failure to import specific inner membrane proteins or another Tim8-dependent function.
- One theory to account for DDON syndrome pathogenesis would be that the primary neurons of visual and audiological sensory pathways along with other cortical tracts require an advanced level of regulation of their mitochondrial morphology via the Tim8 and DRP1 interaction to ensure the appropriate distribution of mitochondria as these primary neurons further differentiate. Failure of proper localization of DRP1 to the mitochondria due to the absence of the Tim8 protein leading to subsequent loss of mitochondrial fission would cause an accumulation of elongated mitochondria. Further differentiation of these primary neurons as the brain matures would not allow for proper recycling of mitochondria so that accumulation of damaged mitochondria would lead to death of these primary neurons. This Tim8/DRP1 interaction must play a central role in the primary neurons of the auditory and visual sensory pathways so that in its absence, degeneration occurs to produce the hallmark DDON symptomology and histopathologic findings.
- Optic atrophy protein 1 (Opa1) is a well-characterized mediator of mitochondrial fusion that is localized to the mitochondrial inner membrane. Its function is in direct opposition to that of DRP1 and contains splice variants that are specific to tissues located in the retina and cochlea. Mutations in Opa1 resulting in autosomal-dominant optic atrophy plus syndrome (ADOA+) bear a striking resemblance to that of DDON (27-29). Here however, optic atrophy with visual impairment is the initial manifestation during childhood, followed by sensorineural hearing loss in late childhood to young adulthood. Mitochondria in ADOA+ display morphological abnormalities characterized by increased fragmentation resulting in smaller, more punctuated mitochondria. This morphological difference is attributed to increased rates of mitochondrial fission due to the lack of Opa1 mediated fusion.
- DDON syndrome results from mutations of a single gene, *TIMM8A*, and the novel variant described in this case report as a start loss located at the first codon may be a candidate for read through pharmaceuticals (30-32). These drugs are characterized by binding to ribosomes to induce a translational 'readthrough' of premature stop codons to generate a full-length protein. The drug ELX-02 is an investigational synthetic eukaryotic ribosome-selective glycoside currently undergoing clinical trials to assess its use in CF patients currently within the US (33). Whether this compound, or other drugs being considered for the treatment of optic neuropathy, will prove effective requires further investigation.

References

1. Tranebjaerg, L. (1993) Deafness-Dystonia-Optic Neuronopathy Syndrome. in *GeneReviews((R))* (Adam, M. P., Ardinger, H. H., Pagon, R. A., Wallace, S. E., Bean, L. J. H., Stephens, K., and Amemiya, A. eds.), Seattle (WA).
2. Tranebjaerg, L., Schwartz, C., Eriksen, H., Andreasson, S., Ponjavic, V., Dahl, A., Stevenson, R. E., May, M., Arena, F., Barker, D., and et al. (1995) A new X linked recessive deafness syndrome with blindness, dystonia, fractures, and mental deficiency is linked to Xq22. *Journal of Medical Genetics* **32**, 257-263
3. Jin, H., May, M., Tranebjaerg, L., Kendall, E., Fontan, G., Jackson, J., Subramony, S. H., Arena, F., Lubs, H., Smith, S., Stevenson, R., Schwartz, C., and Vetrie, D. (1996) A novel X-linked gene, DDP, shows mutations in families with deafness (DFN-1), dystonia, mental deficiency and blindness. *Nature Genetics* **14**, 177-180
4. Bahmad, F., Jr., Merchant, S. N., Nadol, J. B., Jr., and Tranebjaerg, L. (2007) Otopathology in Mohr-Tranebjaerg syndrome. *The Laryngoscope* **117**, 1202-1208
6. Kojovic, M., Parees, I., Lampreia, T., Pienczk-Reclawowicz, K., Xiromerisiou, G., Rubio-Agusti, I., Kramberger, M., Carecchio, M., Alazami, A. M., Brancati, F., Slawek, J., Pirtosek, Z., Valente, E. M., Alkuraya, F. S., Edwards, M. J., and Bhatia, K. P. (2013) The syndrome of deafness-dystonia: clinical and genetic heterogeneity. *Movement Disorders* **28**, 795-803
8. Beverly, K. N., Sawaya, M. R., Schmid, E., and Koehler, C. M. (2008) The Tim8-Tim13 complex has multiple substrate binding sites and binds cooperatively to Tim23. *Journal of Molecular Biology* **382**, 1144-1156
9. Hasson, S. A., Damoiseaux, R., Glavin, J. D., Dabir, D. V., Walker, S. S., and Koehler, C. M. (2010) Substrate specificity of the TIM22 mitochondrial import pathway revealed with small molecule inhibitor of protein translocation. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 9578-9583
10. Rothbauer, U., Hofmann, S., Muhlenbein, N., Paschen, S. A., Gerbitz, K. D., Neupert, W., Brunner, M., and Bauer, M. F. (2001) Role of the deafness dystonia peptide 1 (DDP1) in import of human Tim23 into the inner membrane of mitochondria. *The Journal of Biological Chemistry* **276**, 37327-37334
27. Davies, V. J., Hollins, A. J., Piechota, M. J., Yip, W., Davies, J. R., White, K. E., Nicols, P. P., Boulton, M. E., and Votruba, M. (2007) Opa1 deficiency in a mouse model of autosomal dominant optic atrophy impairs mitochondrial morphology, optic nerve structure and visual function. *Human Molecular Genetics* **16**, 1307-1318
28. Yu-Wai-Man, P., Griffiths, P. G., and Chinnery, P. F. (2011) Mitochondrial optic neuropathies - disease mechanisms and therapeutic strategies. *Progress in Retinal and Eye Research* **30**, 81-114
29. Yu-Wai-Man, P., Griffiths, P. G., Gorman, G. S., Lourenco, C. M., Wright, A. F., Auer-Grumbach, M., Toscano, A., Musumeci, O., Valentino, M. L., Caporali, L., Lamperti, C., Tallaksen, C. M., Duffey, P., Miller, J., Whittaker, R. G., Baker, M. R., Jackson, M. J., Clarke, M. P., Dhillon, B., Czermin, B., Stewart, J. D., Hudson, G., Reynier, P., Bonneau, D., Marques, W., Jr., Lenaers, G., McFarland, R., Taylor, R. W., Turnbull, D. M., Votruba, M., Zeviani, M., Carelli, V., Bindoff, L. A., Horvath, R., Amati-Bonneau, P., and Chinnery, P. F. (2010) Multi-system neurological disease is common in patients with OPA1 mutations. *Brain* **133**, 771-786
30. Bello, L., and Pegoraro, E. (2016) Genetic diagnosis as a tool for personalized treatment of Duchenne muscular dystrophy. *Acta Myologica : Myopathies and Cardiomyopathies* **35**, 122-127
31. Keeling, K. M., Xue, X., Gunn, G., and Bedwell, D. M. (2014) Therapeutics based on stop codon readthrough. *Annual Review of Genomics and Human Genetics* **15**, 371-394
32. Rowe, S. M., and Clancy, J. P. (2009) Pharmaceuticals targeting nonsense mutations in genetic diseases: progress in development. *BioDrugs*: *Clinical Immunotherapeutics, Biopharmaceuticals and Gene Therapy* **23**, 165-174
33. Leubitz, A., Frydman-Marom, A., Sharpe, N., van Duzer, J., Campbell, K. C. M., and Vanhoutte, F. (2019) Safety, Tolerability, and Pharmacokinetics of Single Ascending Doses of ELX-02, a Potential Treatment for Genetic Disorders Caused by Nonsense Mutations, in Healthy Volunteers. *Clinical Pharmacology in Drug Development* **8**, 984-994