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BRIEF COMMUNICATION

Identification of a novel missense mutation in Friedreich's ataxia –FXN^{W168R}

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Abstract

Friedreich's ataxia, characterized by decreased expression of frataxin protein, is caused by GAA trinucleotide repeats within intron 1 in 98% of patients. Two percent of patients carry GAA repeats in conjunction with a point mutation. In this work, we find that frataxin^{W168R}, a novel disease-causing missense mutation, is expressed predominantly as the intermediate frataxin$^{42\text{-}210}$ form, with very little expression of mature frataxin$^{81\text{-}210}$ form. Its localization to mitochondria is not impaired. Additionally, increasing frataxin^{W168R} precursor levels do not lead to an increase in mature frataxin levels, suggesting these patients will require alternative approaches to repair frataxin processing in order to treat the disorder in a disease-modifying manner.

Introduction

Friedreich's Ataxia (FRDA), which affects about 1 in every 50,000 people in the United States, is a slowly progressive ataxia with symptoms including dysarthria, spasticity in the lower limbs, cardiomyopathy, scoliosis, absent lower limb reflexes, and loss of position and vibration sense.[1–3] At present there is no cure or effective treatment for FRDA. Typical FRDA is characterized by decreased expression of the frataxin (FXN) protein, from the *FXN* gene on chromosome 9, caused by the presence of expanded GAA trinucleotide repeats within intron 1. Frataxin is crucial for proper mitochondria function and iron-sulfur cluster biogenesis, but the mechanism by which decreased protein expression leads to disease pathology is not fully known. Ninety percent of patients carry GAA expansions on both alleles in which the length of the allele with the shortest GAA expansion correlates with disease severity; longer alleles result in earlier onset and a faster progression.[4–6] In contrast, 2–4% of patients carry expanded GAA repeats on one allele, and a point mutation on the other allele. These patients generally have lower FXN levels compared to typical FRDA patients.[7,8] As intronic, nonsense, and frame shift point mutations lead to absence of functional frataxin,[9–14] the phenotype in such patients is usually severe. In contrast patients with missense mutations can have a mild or severe clinical outcome depending on the exact mutation and the length of the GAA repeat on the opposite allele.[9–11,15,16]

The full-length precursor (FXN$^{1\text{-}210}$) is processed by mitochondrial processing peptidase to intermediate (FXN$^{42\text{-}210}$) and mature (FXN$^{81\text{-}210}$) forms. Many different missense mutations have been described in patients with FRDA, and some of which lead to the absence of functional protein. In contrast, the G130V mutation, generally associated with a milder phenotype, is associated with incomplete processing of frataxin to its mature form.[8] In vitro studies allow one to distinguish between those affecting frataxin processing and those altering overall levels (due to folding, RNA splicing or other severely pathogenic processes), and show that the specific location of missense mutations within the protein structure affects folding of frataxin into a native conformation (L106S)[9,12–14] and decreases participation in iron-sulfur cluster biogenesis (R165C, W155R), which is hypothesized to be the primary function of frataxin[17,18] The nonconservative W168R mutation would change an aromatic, nonpolar tryptophan to a basic, electrically charged arginine at amino acid position 168, on Beta-sheet 5 of the human FXN crystal structure. Here, we present a patient who carries the novel

W168R missense mutation and 1133 expanded GAA repeats, leading to an especially severe phenotype.

Methods

Transfection and immunostaining

FXN^{W168R} was created using Addgene XL Site-Directed Mutagenesis Kit and primers to pcDNA3.1-hFrataxin-HA (Plasmid #31895). Human Embryonic Kidney (HEK) cells were co-transfected with 4 μg of FXN and mitoGFP cDNA via Lipofectamine 2000 reagent. Twenty-four hours after transfection, cells were fixed with 4% Paraformaldehyde followed by treatment with blocking buffer containing 5% normal goat serum, 3% Triton X-100, and 1% BSA. Primary antibody to the HA epitope was added at a 1:1000 dilution overnight. Alexa Fluora 568 secondary antibody was added at a dilution of 1:1000 and cells were imaged by confocal microscopy.

Transfection, subcellular fractionation, and western blot

Following transfection of FXN mutants, HEK cells were centrifuged at 150*g* to collect whole cell lysates. The soluble mitochondria fraction and insoluble mitochondria pellet were collected using Thermo Scientific Mitochondria Isolation Kit for Mammalian Cells (#89874). Protein concentration of each fraction was determined using BCA Protein Assay and each fraction was loaded on a 12% NuPage Gel for electrophoresis, followed by transfer to nitrocellulose membranes. Membranes were blocked with 3% Milk for 1 h and incubated with primary HA-antibody overnight at 4°C. Membranes were then incubated with secondary HRP-conjugated antibody for 1 h and immunoreactive bands were visualized using luminol-enhanced chemiluminescence (ECL) HRP substrate.

MG132 treatment

HEK cells were transfected with FXN^{WT} and FXN^{W168R} mutants via Lipofectamine 2000 reagent. Twenty-four hours after transfection cells were treated with 10 μmol/L MG132 for 5 h followed by cell lysis. Equal amounts of total lysate were loaded on a 12% NuPage gel.

Quantification and statistical analysis

Image J Software was used to quantify FXN levels on western blots and is represented as mean ± SEM. Two-tailed student's *t*-test was used to compare FXN^{W168R} to FXN^{WT}. Significance was set at $P < 0.05$. Image J software was also used to calculate Pearson's correlation coefficient for quantification of co-localization in immunofluorescence images.

Results

Case history

A 6-year-old boy evaluated for ataxia, was historically smaller in size and slower to progress developmentally than his fraternal twin brother. His height and weight were consistently below the first percentile since birth. Hypotonia, decreased stamina, clumsiness, and balance difficulties were noted around age two. When symptoms failed to improve by age three, orthotics were prescribed, and physical therapy was initiated. At age four, he was diagnosed with mild concentric left ventricular hypertrophy, diastolic dysfunction as well as a scoliosis of 14°. Initial testing included standard blood work, a brain MRI, and ophthalmologic evaluation. Whole exome sequencing performed to identify mitochondrial diseases and other neurodegenerative disorders ultimately rendered a diagnosis of Friedreich ataxia with GAA repeats of 19 and 1133 and a novel W168R missense mutation. The subject's father carried this mutation and his mother carried an expanded GAA repeat. With time, he continued to progress and has failed to gain fully independent walking. At age 8 he developed new onset type I diabetes. His echo cardiogram reveals thickened ventricular septum (*Z* score >9), and his scoliosis progressed to a curvature... to, and his ambulatory ability decreased as he became wheel chair bound.

W168R impairs FXN processing from intermediate to mature form, but does not impair FXN association with mitochondria

Based on western blotting from transfected cells, FXN^{W168R} is expressed predominantly as the $FXN^{42\text{-}210}$ form with nearly no detectable $FXN^{81\text{-}210}$ immunoreactivity compared to FXN^{WT}, which produces both $FXN^{42\text{-}210}$ and $FXN^{81\text{-}210}$ immunoreactivity (Fig. 1A). To determine the effects of the W168R missense mutation on FXN import into the mitochondria, the FXN^{W168R} variant containing a C-terminal HA tag was co-transfected with mitoGFP in Human Embryonic Kidney (HEK 293) cells. Confocal microscopy images with an antibody to the hemagglutinin epitope, to detect exogenous FXN only, show FXN^{W168R} co-localization with mitoGFP, but with lower levels of FXN immunoreactivity compared to FXN^{WT} (Fig. 2). These data suggests that although W168R decreases mature FXN levels, the immunoreactivity that remains still can associate with mitochondria. In addition, FXNW168R co-immunoprecipitates more readily with mitochondrial processing peptidase (MPP) compared to FXN^{WT} showing enhanced

interactions of with the $FXN^{W168R\text{-}42\text{-}210}$ form (Fig. 1B). Similarly, the $FXN^{42\text{-}210}$ form of W168R is present in both soluble and insoluble mitochondria fractions (Fig. 1C), suggesting that W168R impairs FXN processing from the $FXN^{42\text{-}210}$ to $FXN^{81\text{-}210}$ form; thus it fails to dissociate from MPP within the inner mitochondria membrane.

Increasing FXN^{W168R} precursor levels does not lead to an increase in mature FXN levels

Traditional therapies for FRDA involve increasing FXN levels. To model this, transfected cells were treated with 10 μmol/L MG132 protease inhibitor to increase precursor FXN levels in an effort to overcome the impaired FXN processing by FXN^{W168R}. FXN^{W168R} precursor levels increased, as did $FXN^{42\text{-}210}$ levels, but not $FXN^{81\text{-}210}$ levels (Fig. 1D). This suggests that increasing mature FXN levels for FXN^{W168R} will require alternative approaches to repair FXN processing from intermediate to mature form.

Discussion

This work identifies W168R as a particularly severe pathogenic mutation in FRDA. This patient presented

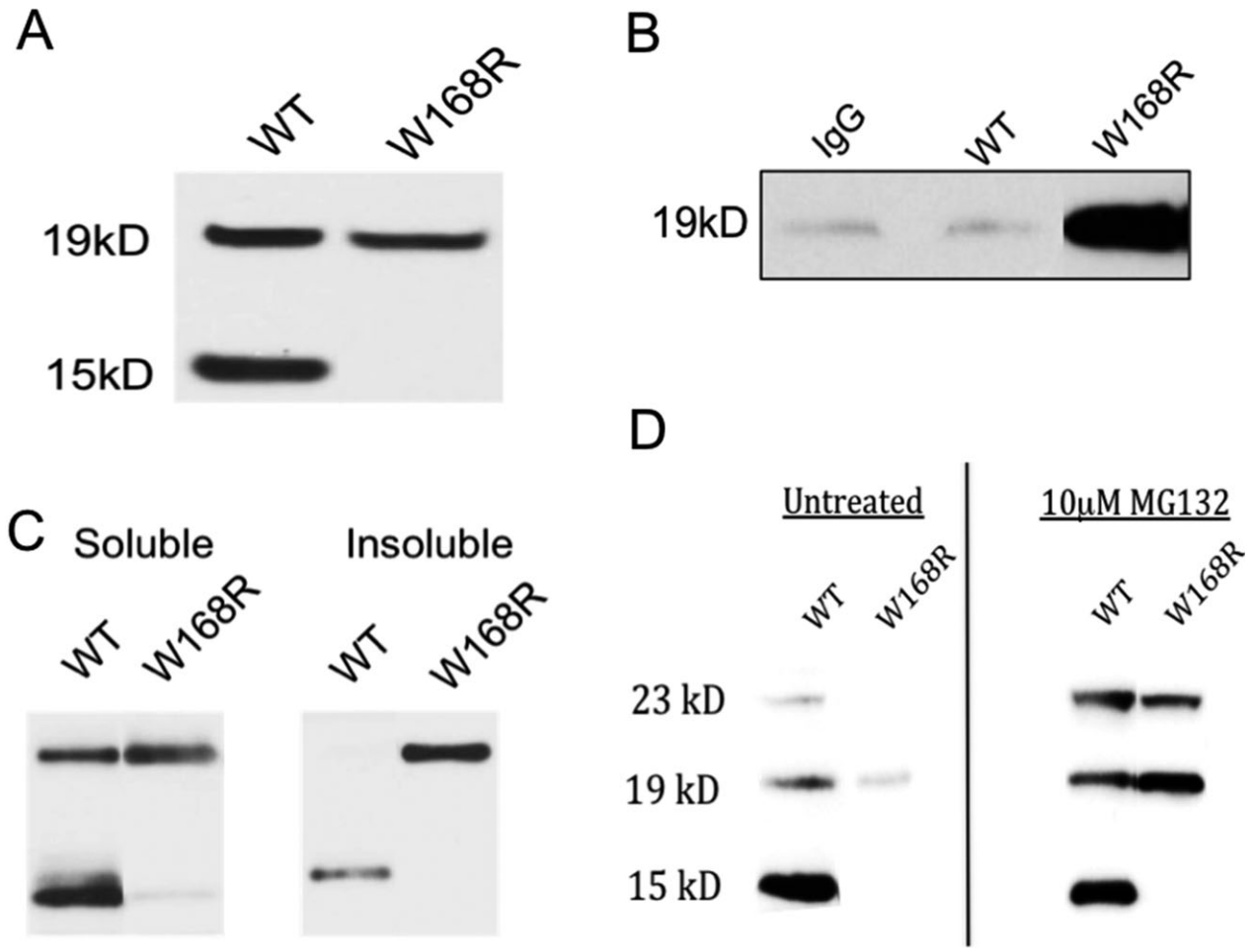

Figure 1. (A) W168R lead to decreased $FXN^{81–210}$ levels. Western blot of whole cell lysates collected from HEK 293 cells transfected with FXNWT and FXNW168R. An anti-HA antibody was used to detect exogenous FXN81–210 (15 kD) FXN81–210 levels after transfection. (B) W168R enhances the association of $FXN^{42–210}$ with MPP. Whole cell lysates from transfected HEK 293 cells were immunoprecipitated with anti-MPP antibody and immunoblotted with primary anti-HA antibody. Western blot was used to detect FXN pulled down by anti- MPP. The Co-IP 19 kD blot represents immunoprecipitated FXN42-210. (C) W168R impairs processing from $FXN^{42–210}$ to $FXN^{81–210}$. Following transfection of mutant constructs in HEK 293 cells, whole cell lysates were centrifuged to perform subcellular fractionation of soluble mitochondria fraction and insoluble mitochondrial pellet. FXN levels were detected by western blot using an anti-HA antibody. (D) Increasing W168R $FXN^{1–210}$ levels does not increase $FXN^{81–210}$ levels. Following transfection of HEK 293 cells with mutant FXN constructs, cells were treated with 10 lmol/L MG132 proteasome inhibitor for 5 h followed by cell lysis. Exogenous FXN1–210 (23 kD), FXN42–210 (19 kD), and FXN81–210 (15 kD) levels, before and after treatment, were detected by western blot using a primary anti-HA antibody.

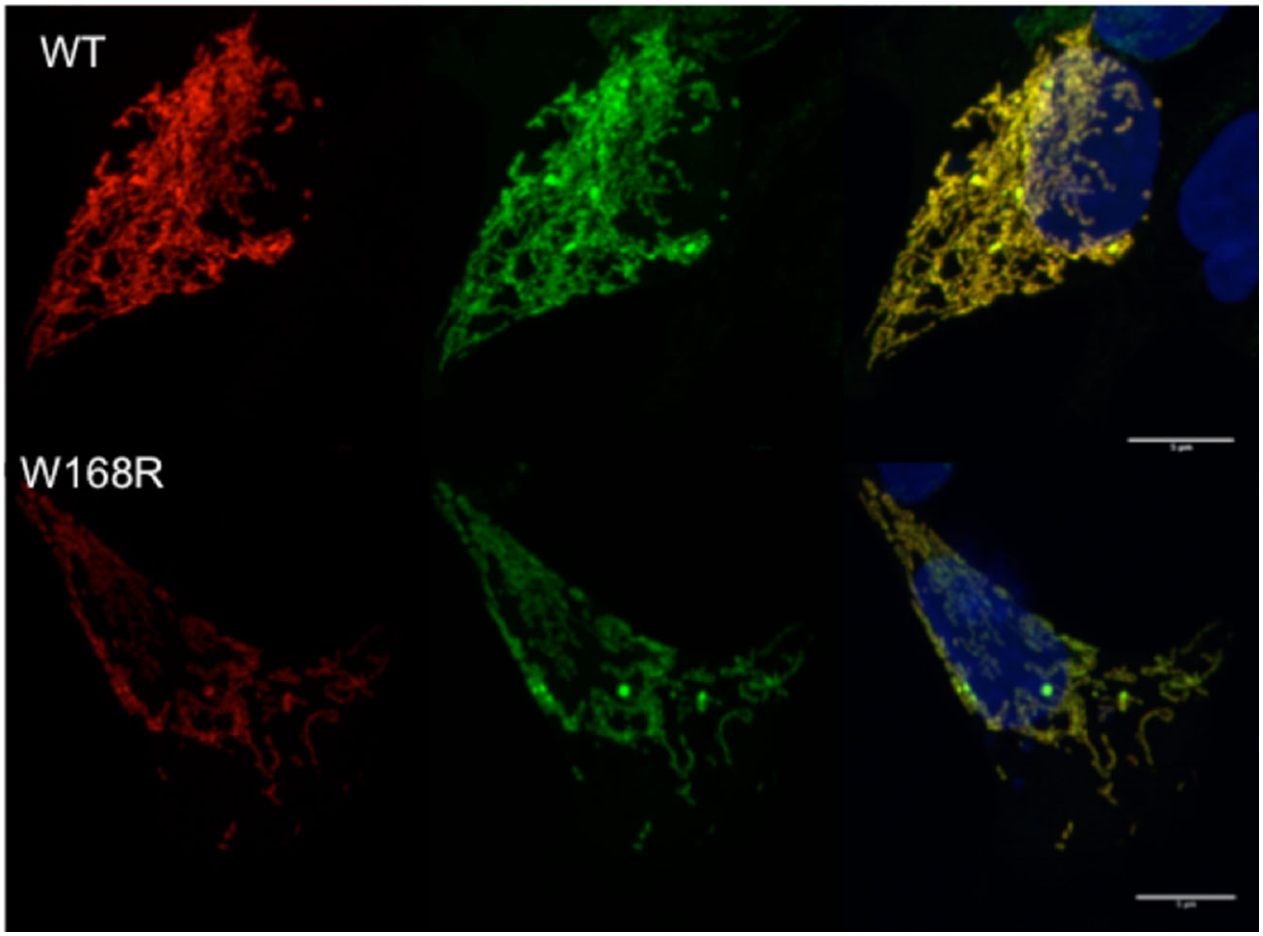

Figure 2. W168R does not impair FXN association with mitochondria. Confocal microscopy images of HEK 293 cells cotransfected with mutant FXN constructs and mito-GFP, fixed, and stained using a primary anti-HA antibody to detect exogenous FXN only and secondary antibody Alexa Fluor 568 (FXN). DAPI was also used as a nuclear stain.

with an age of onset (2) much earlier than most childhood patients. Other individuals presenting at this age have sometimes had end stage cardiomyopathy in the absence of ataxia, distinct from the presentation of the present patient.[19] While the patient described here had a single long GAA repeat, the extremely early onset and significant progression demonstrates that the second mutation (W168R) also must lead to a severely deficient amount of functional frataxin. FXN^{42-210} is the dominant form of FXN made from W168R, with production of almost no detectable amounts of $FXN^{81-210.}$ G130V also predominantly leads to production of FXN^{42-210} with very low levels of FXN^{81-210} but is associated with a mild phenotype. The small amount of fully processed FXN^{81-210} produced from G130V may be sufficient to ameliorate the phenotype in a way that the extreme processing defect of W168R does not. Alternatively, W168R may also have another dysfunctional feature beyond abnormal processing that leads to a more severe phenotype. The W168R mutation occurs one alpha helix turn away from R165C, a mutation associated with abnormalities in the cysteine desulturase binding and iron – sulfur chaperoning activity of frataxin. Conceivably, W168R may also have impaired functionality, and phenotypic differences related to W168R mutations may also reflect abnormalities in addition to processing differences from wild type frataxin. Thus amelioration of the dysfunction associated with W168R will require approaches to overcome abnormal processing (e.g., enhancement of MPP binding) and potentially methods for restoring function to W168R once its activity is able to be defined.

Conflict of Interest

All authors declare no conflict of interest.

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