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Laboratory Studies

Ataxia with oculomotor apraxia type 2 fibroblasts exhibit increased susceptibility to oxidative DNA damage

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ABSTRACT

Ataxia with oculomotor apraxia type 2 (AOA2) is an autosomal recessive cerebellar ataxia associated with mutations in *SETX*, which encodes the senataxin protein, a DNA/RNA helicase. We describe the clinical phenotype and molecular characterization of a Colombian AOA2 patient who is compound heterozygous for a c.994 C>T (p.R332W) missense mutation in exon 7 and a c.6848_6851delCAGA (p.T2283KfsX32) frameshift deletion in *SETX* exon 21. Immunocytochemistry of patient-derived fibroblasts revealed a normal cellular distribution of the senataxin protein, suggesting that these mutations do not lead to loss or mis-localization of the protein, but rather that aberrant function of senataxin underlies the disease pathogenesis. Furthermore, we used the alkaline comet assay to demonstrate that patient-derived fibroblast cells exhibit an increased susceptibility to oxidative DNA damage. This assay provides a novel and additional means to establish pathogenicity of *SETX* mutations.

1. Introduction

Ataxia with oculomotor apraxia type 2 (AOA2) (Mendelian Inheritance in Man [MIM] #606002) is an autosomal recessive cerebellar ataxia (ARCA) characterized clinically by progressive cerebellar ataxia, sensorimotor peripheral neuropathy, oculomotor apraxia, as well as chorea and/or dystonia. Symptom onset is usually noted in adolescence or early adulthood [1–9]. Laboratory testing of AOA2 patients frequently reveals elevated serum α-fetoprotein (AFP) [10–12] and creatine kinase levels [12].

ARCA with oculomotor apraxia [13,14] also includes ataxia with oculomotor apraxia type 1 (AOA1) (MIM #208920), associated with mutations in the *aprataxin* (*APTX*) gene [15–18], ataxia-telangiectasia (AT) (MIM #208900) due to mutations in the *AT mutated* (*ATM*) gene [19,20] and ataxia-telangiectasia-like disorder (MIM #604391) caused by mutations in the *MRE11A* gene [21,22]. Compared with AOA2, AOA1 usually manifests earlier in childhood [16] and may be more distinctively associated with hypoalbuminemia and hypercholesterolemia. AFP levels are also elevated in AT patients, but in contrast to AT, individuals with AOA2 do not show increased sensitivity to ionizing radiation or susceptibility to cancer [19]. Senataxin, the protein encoded by *SETX*, is a large 2677 amino acid DNA/RNA helicase [17,22–24] functioning in the processing of non-coding RNA and in defense against DNA damage [17,25].

Various missense, nonsense and frameshift mutations in *SETX* have been described in families mostly within Europe, North America, Japan, and North Africa (Supp. Table 1). Exonic or multiexonic deletions and duplications have also been reported [3,5]. In general, missense mutations in the helicase domain (HD) of *SETX* seem to result in less severe phenotypes than deletions, truncation mutations, or missense mutations outside of the HD. Mutations in *SETX* are also responsible for a rare autosomal dominant form of juvenile amyotrophic lateral sclerosis (ALS), ALS4 [26].

In this study, we report a patient from Colombia who is compound heterozygous for two known AOA2 mutations in *SETX*. We used skin fibroblasts and the comet assay to demonstrate an alteration in recovery from DNA damage, providing a novel way to investigate the pathogenicity of senataxin mutations as well as providing support for oxidative DNA damage as the underlying mechanism for disease in AOA2.

2. Materials and methods

2.1. Patient and genetic testing

The patient provided informed consent to participate in this study under a clinical research protocol approved by the USA

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National Institutes of Health (NIH) Combined Neuroscience Institutional Review Board, and was evaluated at the NIH Clinical Research Center by board-certified neurologists. Mutations were identified in DNA isolated from peripheral blood through Clinical Laboratory Improvement Amendments-certified molecular genetic testing (Athena Diagnostics, Worcester, MA, USA).

2.2. Cell culture and immunocytochemistry

Fibroblasts from a forearm skin punch biopsy were prepared using standard procedures and maintained in Dulbecco's modified Eagle's medium (DMEM) media supplemented with 20% fetal bovine serum at 37 °C in 5% CO_2. For immunostaining, cells grown on a coverglass were washed three times with ice-cold phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde for 5 minutes at room temperature, permeabilized for 15 minutes in 0.1% NP-40, and blocked for 30 minutes in 5% normal goat serum. Slides where then incubated overnight with 1:200 anti-senataxin antibody (ab56984; Abcam, Cambridge, UK) at 4 °C, washed three times with ice-cold PBS, incubated for 2 hours at room temperature with 1:500 goat anti-mouse Alexa Fluor 488 (A-11001; Life Technologies Molecular Probes, Carlsbad, CA, USA), washed once with ice-cold PBS, incubated for 10 minutes with 1:100 Alexa Fluor 555 Phalloidin (Invitrogen, Carlsbad, CA, USA) and washed once more, incubated for 10 minutes with 4′,6-diamidino-2-phenylindole (DAPI), washed three times with PBS, and finally mounted for imaging using Fluoromount-G (SouthernBiotech, Birmingham, AL, USA). Cells were imaged using a Zeiss LSM 710 confocal microscope with a 63 × 1.4 NA Plan-Apochromat oil objective, and image acquisition was performed using LSM 710 version 3.2SP2 software (all Carl Zeiss Microscopy GmbH, Jena, Germany).

2.3. Comet assay

We used the OxiSelect Comet Assay Kit (Cell Biolabs, San Diego, CA, USA). Fibroblasts (both AOA2 patient cells and normal controls) were treated with 2 mM H_2O_2 in serum-free DMEM for 45 minutes. Cells were then washed with PBS, and fresh medium (DMEM with serum) was added to the culture plates for different times (no recovery, 4 hours, and 24 hours); the "no recovery" set did not receive fresh media. After the recovery period, cells were trypsinized, washed, counted, and finally diluted to 10^5/ml in PBS. Cells were then added to low-melting temperature agarose (10^4/ml final). This cell suspension was plated (80 μl) on comet assay glass slides (Cell Biolabs) coated with normal-melting temperature agarose. After lysis, slides were placed in a horizontal gel electrophoresis chamber and covered with an alkaline buffer (5 mM NaOH and 200 mM Na_2EDTA; pH >13). Following a 20 minute DNA "unwinding" period, electrophoresis was performed under standard conditions (20 V, 300 mA; distance between electrodes = 20 cm) for 25 minutes. Following neutralization to pH 7.5 using Trizma base (Sigma-Aldrich, St Louis, MO, USA), gels were stained with Vista Green DNA dye and stored at 4 °C until analysis. Images were acquired with a Zeiss LSM 710 confocal microscope and LSM 710 version 3.2SP2 software. DNA damage was quantified per the manufacturer's instructions by calculating the extent tail moment: Extent Tail Moment = Tail DNA% × Length of Tail; where Tail DNA% = 100 × Tail DNA Intensity/Cell DNA intensity. For each time point, means ± standard error of the mean were calculated. Statistical analysis was performed using an unpaired Student's *t*-test.

3. Results

3.1. Identification of a compound heterozygous AOA2 patient from South America

The proband is a 47-year-old woman of Spanish ancestry originally from Colombia who started to develop difficulty walking in her early twenties. Her early motor development as a child was normal, and she walked at an appropriate age. She attended grade school and had no cognitive difficulties. Although she was not particularly athletic, she developed normally physically. Starting in her early twenties, she experienced a "sense of weakness," with difficulty lifting objects as well as occasional falls. Concurrently, she developed paresthesias in her feet, "enveloping" her lower

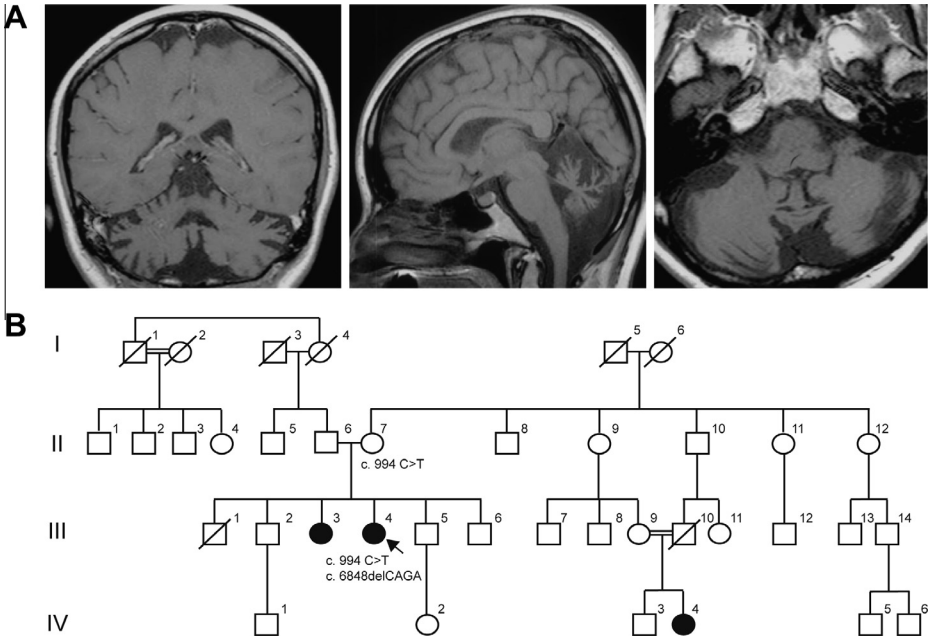

Fig. 1. Identification of ataxia with oculomotor apraxia type 2 in a South American woman. (A) Coronal (left), sagittal (middle) and axial (right) T1-weighted brain MRI of III.4 showing severe cerebellar (particularly vermal) atrophy. (B) Pedigree of the reported family. The proband patient III.4 carries both c.994 C>T and c.6848delCAGA mutations. II.7 is a known carrier for the c.994 C>T mutation.

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