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Disruption of spermatogenesis and infertility in Ataxia with Oculomotor Apraxia Type 2 (AOA2)

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Abstract

Ataxia with oculomotor apraxia type 2 (AOA2) is a rare autosomal recessive cerebellar ataxia characterized by onset between 10–20 years of age and a range of neurological features that include progressive cerebellar atrophy, axonal sensorimotor neuropathy, oculomotor apraxia in a majority of patients, and elevated serum alpha-fetoprotein (AFP). AOA2 is caused by mutation of the *SETX* gene which encodes senataxin, a DNA/RNA helicase involved in transcription regulation, RNA processing, and DNA maintenance. Disruption of senataxin in rodents led to defective spermatogenesis and sterility in males uncovering a key role for senataxin in male germ cell survival. Here, we report the first clinical and cellular evidence of impaired spermatogenesis in AOA2 patients. We assessed sperm production in 3 AOA2 patients and testicular pathology in one patient and compared the findings to those of *Setx* knockout mice. Sperm production was impaired in all patients assessed (3/3, 100%). Analyses of testicular biopsies from an AOA2 patient recapitulate features of the histology seen in *Setx* knockout mice, strongly suggesting an underlying mechanism centering on DNA-damage-mediated germ cell apoptosis. These findings

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support a role for senataxin in human reproductive function and highlight a novel clinical feature of AOA2 that extends the extra-neurological roles of senataxin. This raises an important reproductive counselling issue for clinicians, and fertility specialists should be aware of *SETX* mutations as a possible diagnosis in young male patients presenting with oligospermia or azoospermia since infertility may presage the later onset of neurological manifestations in some individuals.

Keywords

Gait disorders/ataxia; Cerebellum; Genetics; Fertility; Sperm

Introduction

Ataxia oculomotor apraxia type 2 (AOA2, also known as SCAR1, MIM# 606002) is a member of the autosomal recessive spinocerebellar ataxias (SCAR, SCA recessive), a class of progressive neurological disorders that result from cerebellar atrophy¹. First described in 2000, AOA2 was subsequently mapped to chromosome 9q34 and the gene mutated in AOA2, *SETX*, was found to encode the protein senataxin, a DNA/RNA helicase involved in transcription regulation, RNA processing and in protecting the genome against oxidative damage^{2–13}. The major clinical features of AOA2 include onset in the second decade, progressive cerebellar ataxia with axonal sensorimotor peripheral neuropathy, diffuse cerebellar atrophy, frequent oculomotor apraxia, early loss of reflexes, loss of Purkinje cells in the cerebellar cortex, and elevated alpha-fetoprotein (AFP) serum level^{14, 15}. Disruption of the *SETX* gene in mice failed to recapitulate the neurological phenotype seen in human patients¹⁶, however, it uncovered a new aspect of senataxin function in germ cell maturation by showing that *Setx*^{-/-} male mice were infertile¹⁶. Senataxin is essential for spermatogenesis since in its absence, germ cells failed to progress past the pachytene stage of meiosis prophase I¹⁶. Furthermore, inefficient repair of Spo11-mediated DNA double strand breaks (DSB) was observed in *Setx*^{-/-} spermatocytes resulting in the accumulation of Rad51 protein on autosomes at the pachytene stage and a failure to complete meiotic recombination and initiate crossovers¹⁶. A defect in meiotic sex chromosome inactivation (MSCI) was also found in *Setx*^{-/-} germ cells due to reduced recruitment of the chromatin remodeler CHD4 to the XY chromosomes, preventing the silencing of XY-linked genes at pachytene stage¹⁷. Finally senataxin has been shown to attenuate the activity of RNA polymerase II at genes stimulated by viral infection to regulate the magnitude of the host response¹⁶. Altogether, these animal studies revealed an important role for senataxin at the interface of transcription regulation, gene silencing, chromatin remodelling, RNA processing, and protection of the genome during germ cell maturation.

Several ataxic disorders have been associated with reproductive conditions.¹⁸ Gordon Holmes syndrome, for example, due to mutations in the *PNPLA6*, *RNF216*, or *STUB1* genes, causes hypogonadotropic hypogonadism through effects on the endocrine system.¹⁸ Women with expansions in the *FMR1* gene associated with Fragile X associated tremor ataxia syndrome may experience primary ovarian insufficiency.¹⁹ While there is currently no information on male fertility in AOA2, there are a few reports of problems in females. In

two studies, two AOA2 females patients were reported to have entered menopause in their mid-twenties providing evidence of premature ovarian failure^{15, 20}. In addition, ovarian failure^{21, 22}, polycystic ovarian syndrome²³, and amenorrhoea secondary to hypogonadotrophic hypogonadism¹⁴ have also been reported in patients with AOA2. These findings suggest a role for senataxin in reproductive function and perhaps, as shown in the *Setx*^{-/-} mouse model, in germ cell maturation and survival. However, cellular gonadal investigation was not performed in these patients so it is unknown whether there was a primary ovarian defect. Moreover, the contrasting clinical features ranging from high ovarian reserve to suspected ovarian failure leave it unclear as to whether loss of senataxin compromises germ cell integrity.

Here, we report the first clinical and cellular evidence of impaired spermatogenesis and the first cellular evidence of impaired gonadal function in AOA2 patients. Analyses of testicular biopsies from an AOA2 patient recapitulate features of the histology seen in *Setx*^{-/-} mice. These biopsies revealed the presence of immature germ cells with high levels of DNA damage, an increased number of R-loop-containing germ cells, and elevated germ cell apoptosis. Together, this provides the first clinical and cellular evidence that an infertility phenotype exists in male AOA2 patients similar to that observed in *Setx*^{-/-} male mice.

Patients and Methods

Patient recruitment

All patients were initially seen at a tertiary referral ataxia center. For enrollment in this study, patients were required to have positive *SETX* genetic testing and/or a clinical phenotype consistent with a diagnosis of AOA2. Patients were provided genetic counselling both before and after completion of the study. For semen analysis men were advised to abstain from ejaculation for 48 hours prior to testing, to provide the sample to the lab within 30–45 min of production, and to keep the sample warm. Written informed consent was obtained to collect biological samples for further analysis. All study methods were approved by the Institutional Review Board of the University of California, Los Angeles (UCLA).

Mouse tissue samples.

All animal work and experiments have been approved by The University of Queensland Animal Ethics Committee (HMRC/UQCCR/155/15/ARC). Testes from adult (35-day-old) mice were collected and fixed in PBS buffered 10% formalin, embedded in paraffin block and sectioned at 4 µm as previously described^{16, 24}.

Molecular analysis.

Testicular tissue was collected using standard procedures and immediately processed for paraffin embedding followed by histological sectioning and immunostaining. Briefly, testis biopsies were collected and fixed in PBS-buffered 10% formalin, embedded in paraffin block and sectioned at 4µm. Control testes sample was obtained from a tissue bank repository (Aquesta Pathology, QLD, Australia). Sections were stained with Hematoxylin & Eosin (H&E). Slides were examined under light microscope (Olympus CKX41 and

objective CAChN 10X/0.25 PhP) and images captured by QIMAGING MicroPublisher 3.3 RTV camera and QCapture Pro 6.0 software.

Apoptosis (TUNEL) and Immunostaining

Paraffin sections from control, AOA2 patient, and *Setx*^{+/+} and *Setx*^{-/-} testes were dewaxed and rehydrated with Shandon Varistain Gemini ES (Thermo Scientific, USA). Apoptotic cells were detected by Terminal deoxynucleotidyl transferase UTP Nick End Labelling (TUNEL) assay using the Fluorescence in situ Cell Death Detection Kit (Roche, Switzerland) following the manufacturer's instructions. TUNEL is a method for detecting DNA fragmentation by labelling the terminal end of nucleic acids and a common method for detecting DNA fragmentation that results from apoptotic signalling cascades. DNA was stained with DAPI (1:10,000; Sigma-Aldrich) for 10 min at room temperature, and slides were mounted in Prolong Gold medium (Life Technologies, USA). Images were captured at room temperature using a digital camera (AxioCam 503 mono, Carl Zeiss Microimaging Inc., Germany) attached to a fluorescent microscope (AXIO Imager.M1, Carl Zeiss Microimaging Inc., Germany) and the Zen 2012 (Blue Edition) software (Carl Zeiss, Microimaging Inc. Germany). The objectives employed were Zeiss APOCHROMAT 10x/0.45 Ph1; Zeiss EC Neofluar 20x/0.5 and Zeiss EC Neofluar 40x/0.75 (Carl Zeiss, Germany). Images were subsequently assembled in Adobe Photoshop 10 (Adobe Systems Inc, USA), and contrast and brightness were adjusted on the whole image panel at the same time. For immunostaining, slides with tissue sections were dewaxed and enzymatic antigen retrieval was performed by incubating the sections with 1:10 Trypsin dilution in PBS for 20 min at 37°C. Slides were washed 3 times for 5 min with PBS at room temperature for 5 min each. Tissues sections were blocked in (20% FCS, 2% BSA, 0.2% Triton X-100) for 1 h at room temperature. Slides were incubated with anti-R-loop (1:100, S9.6), anti- γ H2AX (1:100, Y-P1016, Millipore) or anti-VASA (DDX4/MVH, 1:100, ab13840, Abcam) antibodies overnight at 4°C in a humidified chamber. Slides were washed 5 times with 1xPBS containing 0.5% Triton X-100 for 5 min each at room temperature. Alexa-Dye488 or Alexa-Dye594-conjugated secondary antibody (Molecular Probes, Life technologies) was added for 1 h at 37°C in a humidified chamber. Subsequently, slides were washed 3 times as before and DAPI (1:10,000; Sigma-Aldrich) was added for 10 min to staining nuclei. Slides were finally washed twice and glass coverslips were mounted in Prolong Gold (Life Technologies) medium for imaging. Images were subsequently assembled in Adobe Photoshop 10 (Adobe Systems, San Jose, CA, USA), and contrast and brightness were adjusted on the whole image panel at the same time. R-loop and apoptosis quantification was performed by examining a random field of view under the microscope and counting all the seminiferous tubules within it. The presence of one or more positive cells within a tubule defined that tubule as positive.

Results

Sperm abnormalities are observed in AOA2 patients.

To investigate possible abnormalities in spermatogenesis and fertility in AOA2 patients we assessed 3 subjects (Table I). The first patient (AOA2-P1), reported in more detail below, demonstrated a complete absence of sperm production. Another adult subject (AOA2-P2)

was found to have oligospermia with 100% of their sperm non-motile. A third subject, AOA2-P3, also showed oligospermia with 73% non-motility. In total, sperm abnormalities were seen in all three subjects assessed (3/3, 100%) (Table I). We assessed hormonal levels in these 3 individuals as well as an additional 2 other male AOA2 patients and no clear correlation was observed (data not shown). Overall these findings are consistent with a reproductive defect in a subset of male patients with AOA2. In order to pursue these abnormalities in greater detail we obtained a testicular biopsy for analysis from patient AOA2-P1.

Patient AOA2-P1 clinical presentation.

The patient is a 34 year old man of Eastern European descent with no other past medical history who developed slowly progressive ataxia starting at age 15. Neurological examination was notable for cerebellar ataxia characterized by abnormal eye movements (saccadic smooth pursuit, direction-changing nystagmus, overshoot and ocular dysmetria on tracking, but no oculomotor apraxia), moderately impaired limb coordination, severe gait and truncal ataxia, distal sensorimotor polyneuropathy with amyotrophy, and severe impairment of posterior column function. At the time of evaluation, his Scale for the Assessment and Rating of Ataxia (SARA-5) score was 22/40. Genetic testing demonstrated two novel compound heterozygous variations in the *SETX* gene; c.2747_2748insAT (p.Met917Leufs*2) and c.6689T>C(p.Met2230Thr). His sister is also affected with a similar clinical presentation and carries the identical mutations. The patient and his wife underwent genetic counselling and decided to proceed with childbearing. Unfortunately pregnancy did not occur so a fertility workup was initiated during which the patient was noted to have absence of sperm in the ejaculate (azoospermia) which prompted our further evaluation including a testicular biopsy.

Sterility in an AOA2 patient due to the absence of mature germ cells in seminiferous tubules.

We have previously shown that *Setx*^{-/-} male mice have severe disruption of seminiferous tubules and lack mature germ cells due to a block at the pachytene stage of meiotic prophase I¹⁶. To investigate the effect of senataxin deficiency on reproductive function in AOA2, we obtained a testicular biopsy from the infertile AOA2 man described above. Semen analysis performed on this patient revealed azoospermia. A small testicular biopsy was subsequently collected, fixed and embedded in paraffin to further characterize the histology of the germ cells or lack thereof defect in this patient. H&E staining of testicular sections showed the presence of immature germ cells ranging from spermatogonia to spermatocytes in the seminiferous tubules but an absence of mature sperm in the AOA2 patient compared to a control sample (Figure 1A). These findings are reminiscent of those obtained in adult *Setx*^{-/-} mice which show a total absence of mature germ cells in contrast to wildtype littermates, which exhibit all stages of spermatogenic development (Figure 1B). Vacuolated seminiferous tubules in which both spermatozoa and mature spermatids are absent are observed in both AOA2 and *Setx*^{-/-}. Taken together, this patient's infertility is consistent with early maturation arrest. To confirm the presence of germ cells in the seminiferous tubules, sections were immunostained for the VASA (DDX4/MVH) protein (Figure 2), an RNA-dependent helicase essential for germ cell development that is expressed in fetal and

adult gonadal germ cells in both males and females^{25, 26}. A normal cytoplasmic staining pattern was observed in both human (control and AOA2 patient) (Figure 2A) and in mouse seminiferous tubules (Figure 2B) as previously described²⁶.

Elevated levels of DNA damage, R-loops and apoptosis in AOA2 germ cells consistent with infertility secondary to maturation arrest.

We have previously shown that the persistence of unrepaired DNA damage in *Setx*^{-/-} mouse spermatocytes prevented cross-over formation and triggered the mid-pachytene checkpoint resulting in the elimination of DNA-damage-containing cells^{16, 24}. To assess whether DNA repair is also compromised in AOA2 patient germ cells, we carried out immunostaining for pSer139 of histone H2AX (γ H2AX), a well-characterized maker of DNA double strand breaks²⁷. AOA2 spermatocytes exhibited increased levels of γ H2AX staining compared to the spermatocytes from a healthy control patient (Figure 3) suggesting that defective senataxin function hinders meiotic DSB repair and leads to the accumulation of unrepaired DNA breaks that can threaten genomic integrity in these cells. These findings mirror those of *Setx*^{-/-} mice (Figure 3) confirming that senataxin is essential for the effective repair of programmed meiotic DSBs¹⁶.

Recent studies have demonstrated that senataxin, through its DNA/RNA helicase activity, resolves DNA:RNA hybrid structures known as R-loops that form during normal DNA transcription when the RNA transcription machinery encounters a GC rich region and stalls, allowing the nascent RNA strand to pair with the DNA template¹². Both *in vitro* and *in vivo* studies have provided compelling evidence for the formation and/or accumulation of these structures in *Setx*-defective proliferating cells^{4, 14, 16, 24}. Indeed, increased levels of R-loops were detected in *Setx*^{-/-} spermatocytes compared to their wild type counterpart, and this was not only limited to germ cells but was also found in other tissues such as the spleen and intestine of *Setx*^{-/-} mice, two tissues characterized by a high proportion of proliferating cells²⁴. To determine whether this would also be the case in AOA2 male patient germ cells, we looked for the presence of R-loops by immunostaining using the well-characterized R-loop-specific (S9.6) antibody^{28, 29}. A greater proportion of tubules containing R-loop positive cells (57%) were detected in the AOA2 patient testis compared to that of the control (8%) in agreement with findings in the *Setx*^{-/-} mouse model^{16, 24} (Figure 4A). However, the number of R-loop positive cells per tubule in the AOA2 patient was less than that observed in *Setx*^{-/-} mouse testis (Figure 4B and 4C). For each positive seminiferous tubule, only 1 to 2 R-loop positive cells were detected in the AOA2 patient, while multiple R-loop positive cells (4 to 40; mean 18±9) were detected in *Setx*^{-/-} mouse tubules (Figure 4B and 4C). This difference in the number of R-loop positive germ cells per tubule may possibly reflect differences in phenotypic expressivity in patients, disease variance across tissues, a milder phenotype of the patient's AOA2 mutation(s) as compared to the complete ablation of the *Setx* gene in the mouse, or an inherent difference between humans and rodents.

The accumulation of DNA breaks, the increased number of R-loop-containing cells, and the absence of mature germ cells in the AOA2 patient testis prompted us to next investigate the level of germ cell apoptosis. As expected, elevated levels of apoptosis were detected in the AOA2 patient (29%) compared to the control (5%) using TUNEL assay (Figure 5A, Figure

5B, **upper panel**) and these levels were comparable to those observed in *Setx*^{-/-} (34%) and *Setx*^{+/-} (6%) mice (Figure 5B, **lower panel**). Overall, these data suggest that in humans, as in mice, persistent DNA breaks secondary to defective senataxin function leads to the induction of a stage IV mid-pachytene checkpoint that results in male germ-cell depletion and severe infertility.

Discussion

Our earlier work identified a severe male infertility phenotype in transgenic mice lacking the *Setx* gene and characterised its underlying mechanism. In this report, we describe the observation of a similar phenotype characterised by defective spermatogenesis and lack of mature germ cells in male AOA2 patients, confirming the essential role of senataxin in male reproductive function. The similarities between the mouse model and the AOA2 patient phenotype are striking and strongly suggest that similar molecular mechanisms apply to both.

The absence of mature germ cells in the AOA2 patient suggests a blockage in meiosis and perhaps the elimination of aberrant germ cells at the mid-pachytene checkpoint as previously observed in the *Setx*^{-/-} mouse model¹⁶. This observation identifies a protein with a significant role in protecting the brain as also playing a role in spermatogenesis. This is consistent with the association of gonadal dysfunction with other genes involved in protecting the genome, such as premature ovarian failure seen with mutation of *ATM* in ataxia-telangiectasia,³⁰ another ataxia disorder, or *NBN* in Nijmegen breakage syndrome.³¹ Our observation is also reminiscent of how the generation of a mouse model of pantothenate kinase deficiency revealed the presence of azoospermia, which was not previously appreciated in the human disorder,³² emphasizing the importance of such models in identifying associated phenotypes or pathologies in rare neurological disorders such as cerebellar ataxia. Furthermore, this is one of the few examples of male infertility in humans in which the cellular defect underpinning spermatogenic failure has been elucidated. These findings are the first evidence of male infertility in male patients with AOA2 and support essential extra-neurological roles for senataxin in germ cell maturation and survival in humans. As indicated in Table I, the phenotype may be variable among male AOA2 patients so future studies will be necessary to examine the role of senataxin in normal human spermatogenesis and connections to this and potentially other forms of azoospermia. Interestingly, during this study, we identified one male AOA2 patient who had successfully fathered a child at age 30, suggesting either variability to the phenotype, similar to what has been reported with female fertility issues^{15, 20–23}, or a successful pregnancy in the setting of oligospermia. This raises an important reproductive counselling issue for clinicians to address in their male patients with AOA2.

These findings have major implications for the investigation and management of male infertility due to spermatogenic failure. Genetic causes of severe spermatogenic defects have typically been linked with sex chromosome abnormalities such as Y chromosome micro-deletions or sex chromosome aneuploidies such as Klinefelter syndrome (47, XXY males). Our data uncover a novel cause of spermatogenic impairment in humans that can be ascribed to an autosomal mutation. It is possible that other *SETX* variants that do not incur significant

neurological sequelae could affect male infertility and future study will be needed to address this.

Conclusion

In this study we report the first clinical and cellular evidence of impaired spermatogenesis in AOA2 patients, a finding not previously observed in this patient population, and consistent with the phenotype seen in *Setx* knockout mice, both clinically and histologically. This supports a novel role for senataxin in human germ cell development and reproductive function and extends its extra-neurological roles. Fertility specialists should be made aware of *SETX* mutations as a possible diagnosis in young male patients who present with severe oligozoospermia or azoospermia since infertility may potentially presage the later onset of neurological manifestations in some individuals.

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Figure 1. Spermatogenesis is disrupted in an AOA2 patient as shown by the absence of mature germ cells in seminiferous tubules.

Hematoxylin and eosin (H&E)-stained sections of testis from **A.** control and AOA2 patient sample, and **B.** adult *Setx*^{+/+} and *Setx*^{-/-} mice. Black arrows indicate mature germ cells in the control and *Setx*^{+/+} while vacuolated seminiferous tubules in which both spermatozoa and mature spermatids are absent are observed in both AOA2 patient and *Setx*^{-/-} seminiferous tubules. Scale bar, 20 μ m.

Figure 2. Presence of germ cells in control, AOA2, and *Setx* mice seminiferous tubules.
A. Immunostaining of germ cells in human AOA2 and control testis biopsy sections, and **B.** mouse *Setx*^{+/+} and *Setx*^{-/-} seminiferous tubules. Germ cells were stained for VASA (DDX4/MVH), an RNA binding protein with an RNA-dependent helicase that is essential for germ cell development²⁵. Nuclei were counterstained with DAPI. Scale bar, 20 μm.

Figure 3. Elevated levels of DNA damage in AOA2 patient germ cells compared to control. Increased levels of DNA breaks are shown by immunostaining with anti- γ H2AX (pSer139 H2AX) antibody were detected in AOA2 patient germ cells and *Setx*^{-/-} seminiferous tubules as compared to Control and *Setx*^{+/+}. Both low and high magnification images are shown with scale bars of 100 μ m and 20 μ m, respectively. Nuclei were stained with DAPI.

Figure 4. Detection of R-loops (DNA:RNA hybrid structures) in AOA2, control, *Setx*^{+/+} and *Setx*^{-/-} testis.

A. Percentage of R-loop-positive tubules in Control, AOA2, *Setx*^{+/+} and *Setx*^{-/-} samples. Tubules containing ≥ 1 R-loop-positive germ cell were scored as R-loop-positive tubule. **B.** Example of R-loop staining in control and AOA2 patient testis biopsy sections. **C.** R-loop staining in *Setx*^{+/+} and *Setx*^{-/-} mouse testis seminiferous tubules. R-loops were detected in germ cells as shown by the white arrow. While many R-loop-positive germ cells were detected per tubule in *Setx*^{-/-} mice as shown in (C), only one to two R-loop-positive germ cells were detected per tubule in the AOA2 patient sample (B).

Figure 5. Elevated levels of germ cells apoptosis in an AOA2 patient.

A. TUNEL-stained testis sections from control, AOA2 patient and adult *Setx*^{+/+} and *Setx*^{-/-} mice. White arrow indicates TUNEL-positive germ cells. DAPI was used to stain nuclei. Scale bar, 20 μ m. **B.** Quantitation of the number of apoptotic tubules (tubules containing 1 TUNEL positive cell). More than 200 tubules were scored for each sample.

Table I.

Semen Testing in AOA2 Patients.

	Normal Values	AOA2-P1*	AOA2-P2	AOA2-P3
Age at examination	Years	34	35	17
Sperm Concentration	> 15 million/mL	None	2.0	3.5
Sperm Morphology	> 4% normal	-	0%	7%
Sperm Progressive Motility	> 32%	-	0%	20%
Sperm Non-motility	< 60%	-	100%	73%

*, subject described in this report