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Brief report

ATM, the Mre11/Rad50/Nbs1 complex, and topoisomerase I are concentrated in the nucleus of Purkinje neurons in the juvenile human brain

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ABSTRACT

The genetic disease ataxia telangiectasia (AT) results from mutations in the ataxia telangiectasia mutated (*ATM*) gene. AT patients develop a progressive degeneration of cerebellar Purkinje neurons. Surprisingly, while ATM plays a criticial role in the cellular reponse to DNA damage, previous studies have localized ATM to the cytoplasm of rodent and human Purkinje neurons. Here we show that ATM is primarily localized to the nucleus in cerebellar Purkinje neurons in postmortem human brain tissue samples, although some light cytoplasmic ATM staining was also observed. No ATM staining was observed in brain tissue samples from AT patients, verifying the specificity of the antibody. We also found that antibodies against components of the Mre11/Rad50/Nbs1 (MRN) complex showed strong staining in Purkinje cell nuclei. However, while ATM is present in both the nucleoplasm and nucleolus, MRN proteins are excluded from the nucleolus. We also observed very high levels of topoisomerase 1 (TOP1) in the nucleus, and specifically the nucleolus, of human Purkinje neurons. Our results have direct implications for understanding the mechanisms of neurodegeneration in AT and AT-like disorder.

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1. Introduction

The genetic disease ataxia telangiectasia (AT) results from mutations in the ataxia-telangiectasia mutated (*ATM*) gene [1], which encodes a protein kinase that is activated in response to DNA damage [2]. Patients with AT develop a progressive ataxia as a result of degeneration of cerebellar Purkinje neurons, though other brain regions may be affected as well [3,4].

There is no doubt that ATM plays a crucial role in response to DNA damage in eukaryotic cells [2,5–8]. However, the question of whether the neurodegeneration observed in AT patients results from a loss of this DNA damage response function of ATM has been controversial, since several authors have found that ATM protein is located in the cytoplasm of Purkinje neurons in the human [9] and rodent [10,11] brain. Recently however, Shiloh and co-workers [12,13] found that the ATM protein is present in the nucleus of neuronal derived human cells maintained in tissue culture. Also, Barzilai and co-workers [14] showed that nuclear ATM protein is phosphorylated in Purkinje neurons of mice exposed to ionizing radiation.

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Table 1 – Background information on the donors of the tissue samples used in this work

Case #	Age	Postmortem interval (h)
1465 Normal	17 years 189 days	4
917 Normal	14 years 227 days	10
1459 AT	19 years 342 days	2
836 AT	28 years 271 days	4

The numbers are the case numbers at the University of Maryland Brain Bank. Age is at time of death. Postmortem interval in the time between death and tissue collection in hours. For additional information see http://medschool.umaryland.edu/BTBank/.

In light of these findings, we have reinvestigated the intracellular localization of the ATM protein in Purkinje neurons of the human brain. Here, using tissue from both normal and AT patients to verify antibody specificity, we show that the ATM protein is in fact predominantly localized to the nucleus in human Purkinje neurons. Components of the Mre11/Rad50/Nbs1 (MRN) complex are also concentrated in the nucleus of Purkinje neurons, though the distribution of MRN proteins was not identical to that of ATM. Specifically, ATM was detected in both the nucleolus and nucleoplasm, while the MRN proteins were excluded from the nucleolus. We also found that topoisomerase 1 (TOP1) was concentrated in Purkinje cell nuclei, particularly in the nucleolus. We discuss the implications of these results for neurodegeneration in AT patients.

2. Materials and methods

2.1. *Tissue samples*

The formalin-fixed postmortem brain tissue samples were obtained from the University of Maryland Brain Bank for Developmental Disorders. Information on the donors is listed in Table 1.

2.2. *Antibodies*

The following antibodies were used in this work, at the indicated dilutions: ATM rabbit monoclonal Y170 (Epitomics) 1:100; Histone H1 mouse monoclonal (Santa Cruz) 1:100; Mre11 rabbit polyclonal PC388 (Oncogene/Calbiochem) 1:500; Rad50 mouse monoclonal NB 100–147 (Novus) 1:500; Calbindin mouse monoclonal (Sigma) 1:100 and Calbindin rabbit polyclonal (Sigma) 1:100; TOP1 mouse monoclonal (Topogen) 1:50 and mouse monoclonal antibody against TOP1 (kindly provided by Dr. Igor Bronstein; see [15]) 1:25; S100β rabbit polyclonal (DakoCytomation) 1:50.

2.3. *Immunofluorescence*

Formalin-fixed tissue blocks were cryoprotected in phosphate buffered saline containing 25% sucrose for >24 h prior to sectioning. Cryoprotected tissues were carefully frozen and then sectioned at 30 μm thickness. Sections were then processed through a heat-induced antigen retrieval protocol (autoclaving in 10 mM Sodium citrate buffer, pH 6.0, at 121 °C for 15 min), cooled to room temperature, then processed as free-floating sections.

Sections were washed with buffer containing PBST (PBS containing 0.1% Tween-20 or 0.1% Triton X-100), then blocked with 10% normal goat serum (Jackson Immunoresearch lab) prepared in PBST for 2 h at room temperature and incubated with primary antibodies overnight at 4 °C. The next day, the sections were washed 3 times for 10 min each with PBST, then incubated for 2 h at room temperature with secondary antibodies (goat anti-mouse or anti-rabbit IgG labeled with Alexa Fluor 488, or 594, Invitrogen) diluted 1:200. For RNA and DNA staining, some sections were incubated in propidium iodide (PI) diluted 1:2000 in PBS.

For confocal microscopy, following primary and secondary antibody incubation and washings, sections were incubated for 10 min in 0.3% Sudan Black in 70% ethanol to reduce endogenous autofluorescence in the tissues, then washed with PBS, mounted in PBS, and coverslipped with Prolong Gold Antifade reagent (Invitrogen). Sections were examined and images prepared using a Zeiss LSM 5 Pascal confocal microscope.

3. Results

Biton et al. [12] found that the antibody Y170 could be used to specifically localize the ATM protein in the nucleus of cultured human neuronal cells. Therefore, we utilized this antibody to detect the ATM protein in the cerebellum of human postmortem brain samples from two normal donors. For a negative control, we also used postmortem cerebellar tissue from AT patients (see Table 1). As shown in Fig. 1A and B, the Y170 antibody strongly stained Purkinje neurons in postmortem cerebellar samples from the brains of normal donors. In contrast, no specific staining was observed when the antibody was used on brain sections from two AT patients (Fig. 1A and B) demonstrating the specificity of staining. The absence of ATM staining in the AT patient samples cannot be explained by postmortem delay, since the time between death and tissue collection was either equivalent or shorter in the AT patient samples than in controls (see Table 1). The pattern of ATM staining in the cerebellum was consistent in samples from different normal donors (Fig. 1C and data not shown). We therefore conclude that the Y170 antibody specifically detects the ATM protein in formalin-fixed human postmortem brain tissue samples.

ATM staining in Purkinje neurons appeared to be predominantly nuclear (Fig. 1A–C), but to verify this double-labeling studies were carried out using an antibody against histone H1. As shown in Fig. 2A, the strongest ATM staining in Purkinje neurons colocalized with histone H1 staining, verifying nuclear localization.

However, in addition to the intense ATM staining in Purkinje cell nuclei, it is important to stress that we also observed a light but detectable ATM staining in the cytoplasm of Purkinje neurons (Fig. 2B).