

## Original Article

# Pathological accumulation of atrophin-1 in dentatorubral-pallidoluysian atrophy

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**Abstract:** Dentatorubral-pallidoluysian atrophy (DRPLA) is caused by the expansion of polyglutamine (polyQ) in atrophin-1 (ATN1), also known as DRPLA protein. ATN1 is ubiquitously expressed in the central nervous system (CNS), although selective regions of CNS are degenerated in DRPLA, and this selective neuronal damage gives rise to the specific clinical features of DRPLA. Accumulation of mutant ATN1 that carries an expanded polyQ tract seems to be the primary cause of DRPLA neurodegeneration, but it is still unclear how the accumulation of ATN1 leads to neurodegeneration. Recently, cleaved fragments of ATN1 were shown to accumulate in the disease models and the brain tissues of patients with DRPLA. Furthermore, proteolytic processing of ATN1 may regulate the intracellular localization of ATN1 and its fragments. Therefore, proteolytic processing of ATN1 may provide clues to disease pathogenesis and hopefully aid in the determination of molecular targets for effective therapeutic approaches for DRPLA.

**Keywords:** Dentatorubral-pallidoluysian atrophy (DRPLA), atrophin-1 (ATN1), polyglutamine (polyQ) disease, neurodegeneration

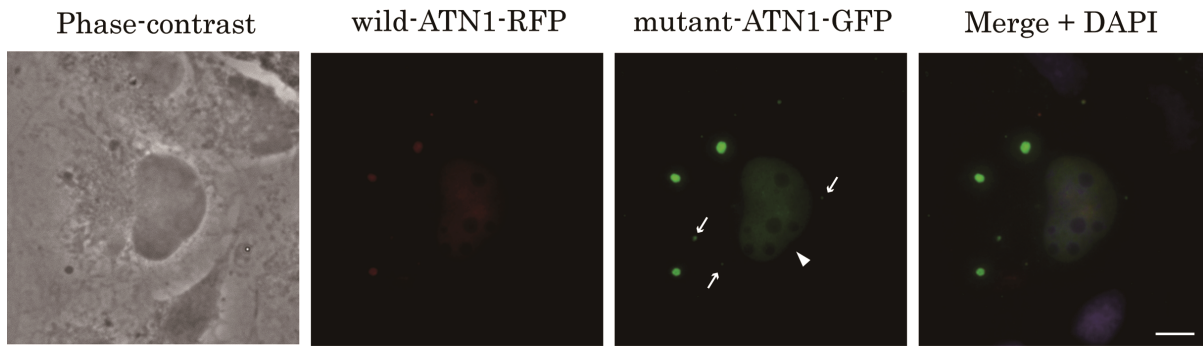
## Introduction

The polyglutamine (polyQ) diseases are a group of hereditary neurodegenerative disorders that include Huntington's disease (HD), dentatorubral-pallidoluysian atrophy (DRPLA), spinal and bulbar muscular atrophy, and several forms of spinocerebellar ataxia (SCA) [1-3]. These diseases are caused by expansion of CAG trinucleotide repeats that encode a polyQ tract in the responsible genes. In addition, the diseases are characterized by a universal paternal inheritance, termed genetic anticipation, where the disease severity increases in successive generations while the age at disease onset decreases. The age at onset is inversely correlated with the length of the abnormal CAG expansion [4]. Because the polyQ diseases have some properties in common, they might have a common pathological mechanism leading to neuronal cytotoxicity.

Neuronal intranuclear inclusions containing polyQ were first observed in HD transgenic mice and the brain tissues of patients with HD [5, 6]. The HD gene product huntingtin was surmised

to aggregate in HD neurons. Similar neuronal intranuclear inclusions showing immunoreactivity with the gene product have also been reported in the brain tissues of patients with other polyQ diseases and of the mice in other polyQ disease models [7-11]. These findings suggest that the mechanism of pathogenesis is derived from aggregation of proteins or peptides with the expanded polyQ tract. Beside the CAG trinucleotide repeat, the genes responsible for causing the various polyQ diseases have no homology with one another. Therefore, speculation concerning pathogenesis has been focused on the expanded polyQ tract itself, which appears to cause the gene products to undergo a conformational change that makes them aggregate in neurons [12]. Aggregation of the gene products that carry an expanded glutamine repeat is believed to be a primary pathological mechanism in polyQ diseases. However, the onset of a neurological phenotype or cell dysfunction mediated by the expanded polyQ tract in the responsible gene product was independent of the formation of inclusions [11, 13, 14]. In fact, one study showed that the presence of inclusion bodies reduced the risk of neuronal death due

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**Figure 1.** Expression of the ATN1 gene in COS-7 cells. The ATN1 gene with a normal CAG repeat was fused to the red fluorescent protein (RFP) gene at the 3'-end, and the mutant ATN1 gene that carries an expanded CAG repeat was fused to the green fluorescent protein (GFP) gene. The COS-7 cells were transiently co-transfected with wild ATN1-RFP and mutant ATN1-GFP. Twenty-four hours after co-transfection, the COS-7 cells were visualized by immunofluorescence microscopy. Immunocytochemistry using anti-RFP (red) and anti-GFP antibodies (green) showed that the two antibodies were labeled in the cytoplasm and nucleus. Note that the anti-GFP antibody labeled additional aggregates in the cytoplasm (white arrows) and showed stronger immunoreactivity in the nucleus (white arrowhead). The nuclei of cells were visualized by staining with DAPI (blue). Scale bar = 10 $\mu$ m.

to polyQ expansion [15]. Thus, the relationship between inclusions and neurotoxicity remains controversial [16]. Soluble polyQ oligomers have been proposed as the cytotoxic structures of the proteins with expanded polyQ tract [17, 18].

The polyQ diseases show progressive and refractory neurological symptoms that are caused by neuronal cell loss in selective regions of the central nervous system (CNS). This selective neuronal damage gives rise to the specific features of each disease. Over the past few years, studies have focused on the specific regions of an individual disease in combination with expanded polyQ tract [19-22]. It is still unclear how a gene product with an expanded polyQ tract directly causes neurodegeneration.

In this review, we will discuss the accumulation of the DRPLA gene product in neurodegeneration. We will focus on proteolytic processing in the gene product of DRPLA but will also refer to apoptosis and autophagy in the disease.

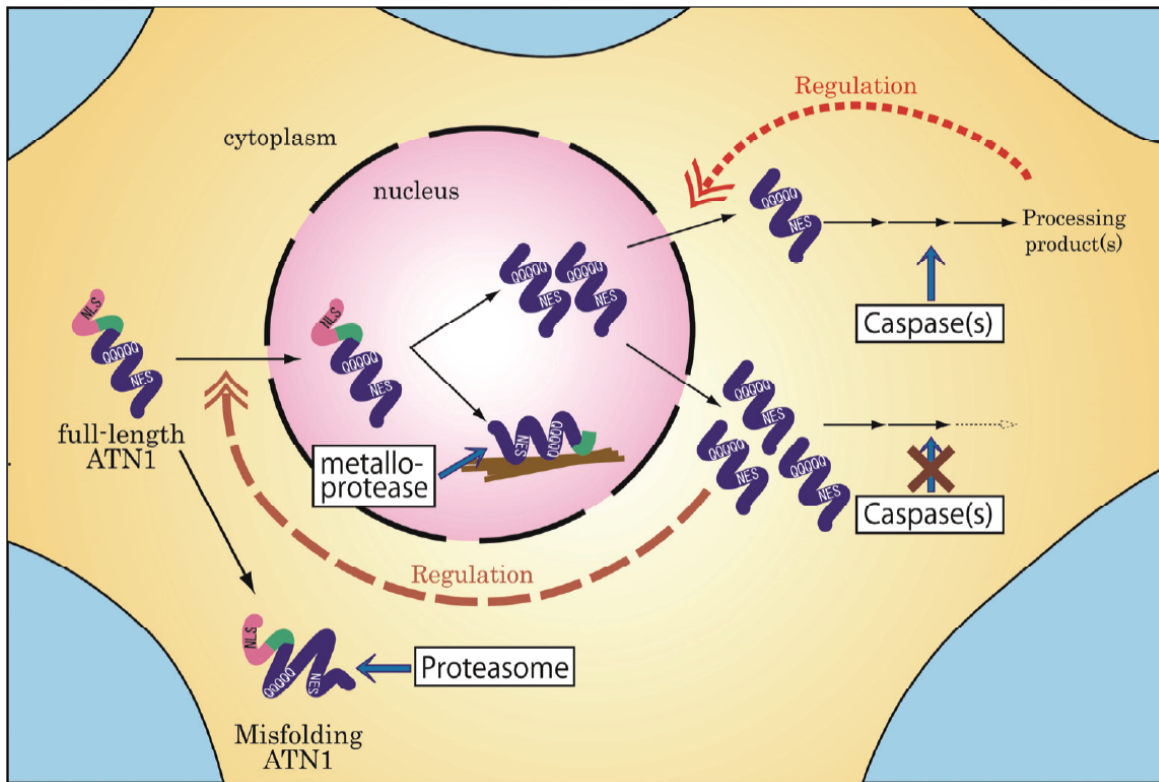
### *Characterization of the DRPLA protein, atrophin-1 (ATN1)*

DRPLA is an autosomal dominant neurodegenerative disorder characterized clinically by progressive dementia, epilepsy, gait disturbance, and involuntary movement (chorea and myoclonus). Pathologically, DRPLA shows combined degeneration of the dentatorubral and pallidoluysian systems [23, 24]. DRPLA pedigrees

show genetic anticipation and phenotypic heterogeneity [25-27]. The DRPLA gene is located on human chromosome 12p13.31, and the genetic defect that underlies DRPLA is expansion of a CAG repeat [28, 29]. The DRPLA gene product is ubiquitously expressed in CNS, although selective regions of CNS are involved in the neuronal degeneration in DRPLA [30]. DRPLA is caused by expansion of the polyQ tract within atrophin-1 (ATN1), also known as DRPLA protein.

A number of *in vitro* studies have investigated the effects of polyQ expansion on the intracellular localization of ATN1. These studies have demonstrated that ATN1 is localized in both the nucleus and cytoplasm of neurons in human CNS [30-32]. Our recent data from biochemical and immunocytochemical analyses demonstrated that full-length ATN1 and C-terminal fragments are localized in the nucleus and cytoplasm of COS-7 cells (**Figure 1**). The sequence of ATN1 contains a nuclear localizing signal (NLS) in the N-terminal and a nuclear export signal (NES) in the C-terminal domains. Mutation assays have demonstrated that these signals are functional in ATN1 and that deletion or mutation of NES in ATN1 changed its localization, whereby it accumulated in the nucleus and increased cellular toxicity [33].

Previously, in cultured cells expressing ATN1, it has been demonstrated that truncated ATN1 with an expanded polyQ tract formed peri- and



**Figure 2.** Schematic of the proteolytic pathways of ATN1. ATN1 is synthesized in the cytoplasm and translocates to the nucleus where it is proteolytically cleaved. Misfolded ATN1s are targets of proteasomal degradation. After importation into the nucleus, the full-length ATN1 was independently cleaved into two fragments. One fragment stayed within the nuclear matrix and executed its function on the nuclear matrix and was regulated by zinc-dependent metalloprotease [22]. The other was exported to the cytoplasm and assembled in the cytoplasmic organelle. Caspases were directly involved in the cleavage of the latter fragment and regulated the accumulation of the fragment in the cytoplasm. Z-VAD-FMK, an inhibitor of caspase activity, accelerated the accumulation of the fragment in the cytoplasm.

intranuclear aggregates and caused apoptotic cell death [9]. Cleavage of ATN1 may be a factor in disease pathogenesis, although the nature of the relevant cleavage product is uncertain. A C-terminal fragment of ATN1 was first found in the brain tissues of human patients with DRPLA [30]. Studies in transgenic mice have shown that a 120-kDa N-terminal fragment of mutant ATN1 accumulated within the nuclei of neurons, and the presence of the N-terminal fragment in the brain tissues of DRPLA patients was also demonstrated [33, 34]. Thus, it is still unclear which fragments contribute to neuronal degeneration in DRPLA. Our recent study revealed that proteolytic processing of ATN1 regulated the intracellular localization of the cleaved fragments. Biochemical examination of subcellular localization demonstrated that one of the C-terminal fragments with an expanded polyQ

tract was preferentially localized in the membrane/organelle, nuclear, and insoluble fractions, whereas the other was localized in the nuclear and insoluble fractions. The cleavage products of ATN1 represent a failure of N-terminal NLS to import the proteins into the nucleus. Thus, it is predicted that the complete, full-length ATN1 is imported into the nucleus and is subsequently cleaved into two C-terminal fragments (Figure 2). Then, one of these fragments is exported to the cytoplasm as a nucleocytoplasmic shuttling protein where it then functions in the cytoplasm, while the other remains in the nucleus and executes its function on the nuclear matrix. Furthermore, proteolytic processing of ATN1 plays an important role in the intracellular localization of ATN1. We have shown that degradation of ATN1 directly affected the formation of aggregates, presumably

because of regulation by remaining ATN1 [22]. A cellular model of DRPLA, in which ATN1 with expanded polyQ tract was expressed, was used to demonstrate that ATN1 and its associated fragments were accumulated in the nucleus and cytoplasm. A major cause of the accumulation was that mutant ATN1 was degraded more slowly than ATN1 with a normal polyQ tract in the cells, especially in the cytoplasmic C-terminal fragment. In addition, the cytoplasmic C-terminal fragment was detected only in patient brains and not in normal brains [22]. Thus, the cytoplasmic C-terminal fragment was selectively accumulated by the expansion of the polyQ tract of ATN1. These findings suggested that the cytoplasmic C-terminal fragment plays an important role in the accumulation of ATN1, ultimately leading to neurodegeneration in DRPLA.

### *Proteolytic regulation of ATN1 by caspases*

Proteolytic processing of gene products responsible for polyQ diseases has been shown to generate toxic fragments containing expanded polyQ tracts *in vitro* [9, 35, 36], although whether all of the proteins undergo cleavage *in vivo* remains unclear. Caspases appear to act as catabolic enzymes that target proteins with a polyQ tract. For instance, Wellington et al. [37] predicted that cleavage sites for caspases were contained in huntingtin, ATN1, ataxin-3, and the androgen receptor and showed that the cleavage of all these four proteins could be inhibited by treatment with caspase inhibitors. In HD, an N-terminal huntingtin fragment that contained the polyQ tract was cleaved by caspase-3 *in vitro* and in the human brain tissues [38]. Another study demonstrated that cleavage at the caspase-6 site in huntingtin was essential for the HD-related behavioral and neuropathological features in the YAC128 model of HD [21]. Previous studies on DRPLA have also shown that caspase-3 generated a C-terminal fragment containing the polyQ tract by cleavage at Asp<sup>109</sup> *in vitro*, and that blocking the cleavage at Asp<sup>109</sup> reduced aggregation of mutant ATN1 with expanded polyQ tract in 293T cells [39, 40]. Our recent *in vitro* study demonstrated that an inhibitor of caspase-3 activity did not reduce the accumulation of C-terminal fragments of ATN1, but the general caspase inhibitor Z-VAD-FMK increased the accumulation of the cytoplasmic C-terminal fragment [22].

Caspases, a family of cysteine proteases, are

mostly activated in the cytoplasm. Recent studies have shown that caspases may have functions beyond their apparent role in apoptosis, including cell differentiation, proliferation, axon guidance, synaptic plasticity, neuroprotection, and other nonlethal processes [41, 42]. One study demonstrated that caspase-3 directly cleaved AMPA receptor subunit GluR1 and modulated neuronal excitability [43]. It is speculated that the cleavage of ATN1 by caspases may be involved in the regulatory mechanism of ATN1. A pan-caspase inhibitor, Z-VAD-FMK, selectively increased the accumulation of the C-terminal fragment in the cytoplasm, which recapitulated the cytoplasmic inclusion observed in the DRPLA brain. In particular, decelerated cleavage of ATN1 might induce disruption of signal transduction and consequently result in neurodegeneration.

### *Ubiquitin proteasome system and neurodegeneration in DRPLA*

The ubiquitin-proteasome system suppresses the potentially toxic effects of misfolded, unassembled, or damaged proteins by degrading these proteins, a mechanism known as quality control machinery. Growing evidence shows that the ubiquitin-proteasome system is involved in pathogenesis of many neurodegenerative diseases, including the polyQ diseases [44]. For example, in SCAs, mutant ataxin-1, -3, and -7 with an expanded polyQ tract are susceptible to ubiquitination and targeted by the proteasome for degradation and clearance [45-47]. In DRPLA, ATN1 was colocalized with ubiquitin in DRPLA-affected neurons [9, 48]. The significant neuropathological features characterizing DRPLA include both cytoplasmic and nuclear inclusions, which were shown to be immunoreactive to anti-ubiquitin and anti-ATN1 antibodies in DRPLA brains [2, 47]. Our recent study determined that the inhibition of proteasome increased the accumulation of full-length ATN1 in the cellular model [22]. Moreover, a previous report indicated that the ubiquitin-proteasome system is impaired by a long chain of polyQ [44]. Together, it is speculated that the ubiquitin-proteasome system may be related to the abnormal accumulation of ATN1.

### *Accumulation of ATN1 and autophagy*

Another protein quality control system is the lysosomal pathway, termed autophagy. Recent studies have shown that degradation of the dis-

ease-related mutant protein was highly dependent on autophagy [49, 50]. For example, the amount of huntingtin that accumulated in autophagic compartments was in proportion to the length of the polyQ tract in the protein [51], and inhibition of autophagy increased huntingtin aggregates [52]. In contrast, the accumulation of ATN1 was not caused by inhibition of lysosomal proteases in the DRPLA cellular model [22], and induction of autophagy had no effect on the toxicity of mutant ATN1 in a fly model of DRPLA [53]. Thus, it is still unclear whether autophagy plays an important role in neuronal degeneration in DRPLA.

### Conclusions

Although the polyQ diseases are caused by the expansion of a CAG repeat in the responsible gene, it is still unclear that abnormal accumulation of the gene product leads to neuronal degeneration. A number of polyQ disease models were generated to clarify the cellular mechanisms of neuronal degeneration in polyQ disease. Cellular and animal models of the diseases have also shown abnormal accumulation of mutant proteins, and the presence of intranuclear inclusion was one of the pivotal findings in polyQ disease [7-11]. However, the studies discussed above have shown that identification of molecular targets that could suppress the abnormal accumulation remains a major challenge at present. In DRPLA, it is speculated that proteolytic processing of ATN1 may provide clues to pathogenesis of DRPLA disease. Since previous studies have demonstrated that N-terminal and C-terminal fragments were found in the DRPLA brain tissues [22, 34, 35], it is necessary to clarify the relationship between proteolytic processing of ATN1 and intracellular localization (**Figure 2**). We need to study further the effect of polyQ expansion on proteolytic processing to determine the molecular targets for effective therapeutic approaches in DRPLA.

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