·Original Article·

PolyQ-expanded ataxin-3 interacts with full-length ataxin-3 in a polyQ length-dependent manner

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Abstract: Objective Machado-Joseph disease (MJD), also known as spinocerebellar ataxia type 3 (SCA3), is a dominant neurodegenerative disorder caused by an expansion of the polyglutamine (polyQ) tract in *MJD-1* gene product, ataxin-3 (AT3). This disease is characterized by the formation of intraneuronal inclusions, but the mechanism underlying their formation is still poorly understood. The present study is to explore the relationship between wild type (WT) AT3 and polyQ expanded AT3. **Methods** Mouse neuroblastoma (N2a) cells or HEK293 cells were co-transfected with WT AT3 and different truncated forms of expanded AT3. The expressions of WT AT3 and the truncated forms of expanded AT3 were detected by Western blotting, and observed by an inverted fluorescent microscope. The interactions between AT3 and different truncated forms of expanded AT3 were detected by immunoprecipitation and GST pull-down assays. **Results** Using fluorescent microscope, we observed that the truncated forms of expanded AT3 aggregate in transfected cells, and the full-length WT AT3 is recruited onto the aggregates. However, no aggregates were observed in cells transfected with the truncated forms of WT AT3. Immunoprecipitation and GST pull-down analyses indicate that WT AT3 interacts with the truncated AT3 in a polyQ length-dependent manner. **Conclusion** WT AT3 deposits in the aggregation that was formed by polyQ expanded AT3, which suggests that the formation of AT3 aggregation may affect the normal function of WT AT3 and increase polyQ protein toxicity in MJD.

Keywords: Machado-Joseph disease/spinocerebellar ataxia type 3; ataxin-3; polyglutamine

1 Introduction

Machado-Joseph Disease (MJD), also called spinocerebellar ataxia type 3 (SCA3), is an autosomal dominant neurodegenerative disease caused by an expansion of a polyglutamine (polyQ) stretch near the C-terminus of the *MJD-1* gene product, ataxin-3 (AT3)[1,2]. Ataxin-3 is widely expressed in the brains of normal and affected individuals, preferentially in neurons. Compared with other known polyQ disease proteins, the expanded glutamine segment of AT3 is located at the C-terminus, and the repeat length threshold for disease development is about 50 glutamine residues[3]. Both wild type (WT) and expanded AT3 proteins are expressed ubiquitously, although the neurodegeneration in MJD has been described in particular brain regions such as the cerebellum (dentate nuclei; the Purkinje cells are hardly affected), substantia nigra, and pontine nuclei[4-6]. It was reported that AT3 is an ubiquitin protease to be involved in the ubiquitin proteasome system, meanwhile, it may be important for aggresome formation and transcription regulation[7-9].

An important feature of polyQ diseases is the accumulation of insoluble intracellular deposits containing the aggregated disease protein[10-12]. Although the precise mechanism of the formation of the inclusions and the process of aggregation are still not well illuminated, these inclusions are as-

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sociated with pathogenesis clearly. PolyQ toxicity could be, at least partly, caused by recruiting WT polyQ disease proteins onto aggregates formed by expanded polyQ proteins[13]. The previous studies showed that expressing expanded AT3 fragments in cultured cell resulted in inclusion formation. What's more, intranuclear inclusions and fragmentation of expanded AT3 in the brain were associated with neurodegenerative phenotype that was found in a transgenic mouse model for full-length AT3 (71Q)[14], and transgenic mice for AT3 (79Q) fragments also displayed ataxic phenotypes[14]. More recently, it was reported that proteolytic cleavage of expanded AT3 was critical for the aggregation and sequestration of non-expanded AT3 in neuroblastoma (N2a) cells[15]. In present study, we measured the interaction between WT AT3 and the truncated forms of expanded AT3 to explore the relationship between WT AT3 and polyQ expanded AT3.

2 Materials and methods

2.1 Plasmid constructs Full length MJD constructs containing 20 CAG repeats were excised from the pAS2-1 vectors[16] and subcloned into pGEX-5X-1 (Amersham Biosciences, Peapack, NJ, USA) vectors via *Bam*HI/*Sal*I sites. The pDsRED-N1-MJD-CAG20, pEGFP-N1-MJD-CAG20, pEGFP-N1-MJD-CAG20-truncated 20Q, pEGFP-N1-MJD-CAG80-truncated 80Q and pEGFP-N1-MJD-CAG130-truncated 130Q constructs were described previously[16]. The p3×Flag-myc-CMV-24-MJD-CAG20Q construct was generated by subcloning the PCR product, amplified using primers 5'-GCGTCGACATGGAGTCCATCTTCCAC-3'/5'-GCGGATCCTTATGTCAGATAAAGTGTGAAGG-3' into p3×Flag-myc-CMV-24 (Sigma, Saint Louis, MO, USA) at *Sal*I/*Bam*HI sites[17].

2.2 Cell culture and transfection Mouse N2a cells or HEK293 cells were cultured overnight in Dulbecco's modified Eagle's medium (DMEM) (GIBCO, Los Angeles, CA, USA), containing 10% calf serum (GIBCO, Los Angeles, CA, USA), 100 U/mL penicillin and 100 μg/mL streptomycin (GIBCO, Los Angeles, CA, USA). They were washed with Opti-MEM and then transfected with expressing vectors using Lipofectamine™ 2000 reagent (Invitrogen, Carlsbad, CA, USA) in Opti-MEM medium without serum. The same volume of DMEM containing 10% calf serum was added to the culture medium 6 h after transfection. After 48 h, the transfected cells were observed using an inverted system microscope IX71 (Olympus, Tokyo, Japan), or used for immunoblot (IB) analysis or immunoprecipitation (IP). For fluorescent observation, we transfected pDsRED-N1 or pDsRED-N1-MJD-CAG20, along with pEGFP-N1, pEGFP-N1-MJD-CAG20-truncated 20Q, pEGFP-N1-MJD-CAG80-truncated 80Q or pEGFP-N1-MJD-CAG130-truncated 130Q into N2a cells. And we transfected p3×Flag-myc-CMV-24-MJD-CAG20Q along with pEGFP-N1, pEGFP-N1-MJD-CAG20-truncated 20Q, pEGFP-N1-MJD-CAG80-truncated 80Q or pEGFP-N1-MJD-CAG130-truncated 130Q into HEK293 cells. After 48 h, the transfected cells were observed using fluorescent microscope.

2.3 Immunoblot analysis Proteins were separated by 12% SDS-PAGE and then transferred onto polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). The following primary antibodies were used: MJ2-5-3 antibody[18], and monoclonal anti-Flag antibody (Sigma, Saint Louis, MO, USA), monoclonal anti-GFP-antibody (Santa Cruz, Santa Cruz, CA, USA). Sheep anti-mouse IgG-HRP antibodies or anti-rabbit IgG-HRP antibodies (Amersham Pharmacia Biotech, Peapack, NJ, USA) were used as the secondary antibodies. The proteins were visualized using an ECL detection kit (Amersham Pharmacia Biotech, Peapack, NJ, USA).

2.4 Immunoprecipitation HEK293 cells co-transfected with p3×Flag-myc-CMV-24-MJD-CAG20Q along with pEGFP-N1, pEGFP-N1-MJD-CAG20-truncated 20Q, pEGFP-N1-MJD-CAG80-truncated 80Q or pEGFP-N1-MJD-CAG130-truncated 130Q were collected 48 h after transfection. The cells were sonicated in TSPI buffer [50 mmol/L Tris-HCl (PH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 μg/mL of aprotinin, 10 μg/mL of leupeptin, 0.5 μmol/L Pefabloc SC, and 10 μg/mL of pepstain] containing 1% NP-40. Cellular debris was removed by centrifugation at 12,000 g for 15 min at 4 ºC. The supernatants were incubated with monoclonal anti-GFP antibody (Roche, Basel, Switzerland) for 1 h at 4 ºC. After incubation, protein G agarose (Roche, Basel, Switzerland) was used for precipitation. The beads were washed with TSPI buffer four times and then eluted with SDS sample buffer for IB analysis.

2.5 GST pull-down assay An aliquot containing 20 μg of protein from the soluble fraction of *Eschericha coli* cell lysates expressing glutathione S-transferase (GST) or GST-ataxin3-20Q was incubated with 20 μL of glutathione agarose beads (Amersham Biosciences, Peapack, NJ, USA) for 20 min at 25 ºC. After washing three times with 1×PBS, beads bound with GST, and GST-ataxin3-20Q were incubated with 50 μg of protein from the supernatants of N2a cells transfected with pEGFP-N1, pEGFP-N1-MJD-CAG20-truncated

20Q, pEGFP-N1-MJD-CAG80-truncated 80Q or pEGFP-N1-MJD-CAG130-truncated 130Q in 0.25 mL HNTG-buffer [20 mmol/L HEPES-KOH (pH 7.5), 100 mmol/L NaCl, 0.1% Triton X-100, and 10% glycerol] for 1 h at 4 ºC. After incubation, the beads were washed four times with 1 mL HNTG buffer to remove unbound proteins. Bound proteins were eluted from the beads by boiling in sodium dodecyl sulfate (SDS) sample buffer and detected by immunoblot analysis using monoclonal anti-GFP-antibody.

3 Results

3.1 Recruitment of WT AT3 onto aggregates formed by the truncated forms of expanded AT3 in N2a cells To observe the aggregation properties of the truncated forms of expanded AT3 in cells and its influence to WT AT3, we constructed plasmids of pDsRED-N1-MJD-CAG20, pEGFP-N1-MJD-CAG20-truncated 20Q, pEGFP-N1-MJD-CAG80-truncated 80Q and pEGFP-N1-MJD-CAG130-truncated 130Q. We

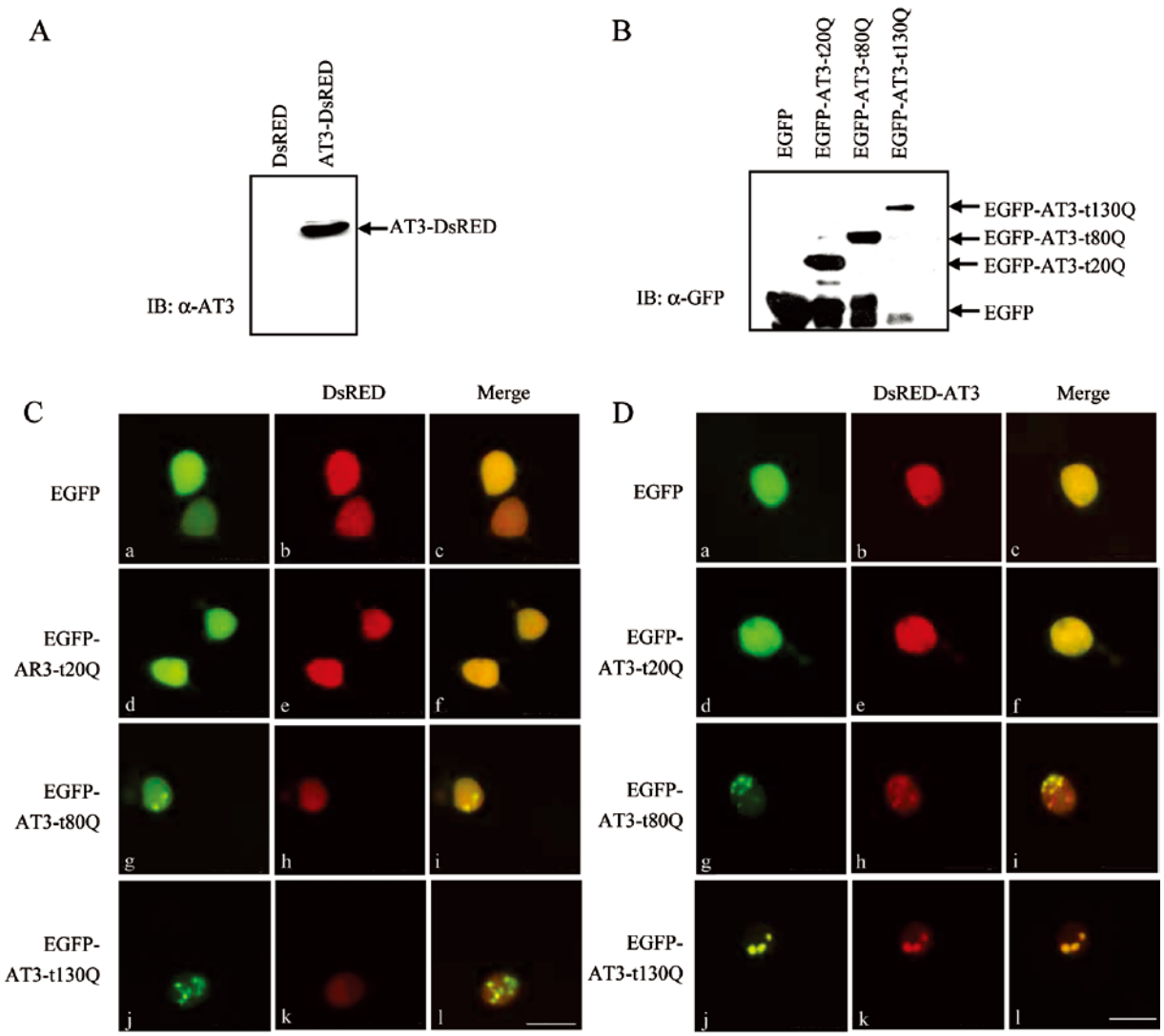

Fig. 1 WT AT3 co-localizes onto aggregates formed by the truncated forms of expanded AT3 in N2a cells. N2a cells cultured in 10-cm dish were transfected with pDsRED-N1-MJD-CAG20 (DsRED-AT3), pEGFP-N1-MJD-CAG20-truncated 20Q (EGFP-AT3-t20Q), pEGFP-N1-MJD-CAG80-truncated 80Q (EGFP-AT3-t80Q) or pEGFP-N1-MJD-CAG130-truncated 130Q (EGFP-AT3-t130Q), respectively. At 48 h after tansfection, cells were harvested and IB analysis was performed using anti-AT3 (MJ2-5-3) (A) and anti-GFP monoclonal antibodies (B). N2a cells were transfected with pDsRED-N1 (C) or pDsRED-N1-MJD-CAG20 (D), along with pEGFP-N1, pEGFP-N1-MJD-CAG20-truncated 20Q, pEGFP-N1-MJD-CAG80-truncated 80Q or pEGFP-N1-MJD-CAG130-truncated 130Q. Cells were observed via fluorescence microscopy 48 h after transfection. Scale bar, 28 μm for C and D.

transfected pDsRED-N1 or pDsRED-N1-MJD-CAG20, along with pEGFP-N1-MJD-CAG20-truncated 20Q, pEGFP-N1-MJD-CAG80-truncated 80Q or pEGFP-N1-MJD-CAG130-truncated 130Q into mouse N2a cells, respectively. Protein expressions of transfected cells were detected by IB analysis using MJ2-5-3 antibody and monoclonal anti-GFP-antibody (Fig. 1A and B). The transfected cells were observed using an inverted fluorescent microscope. In cells co-expressing RFP (red color) and the truncated forms of AT3 (green color), the truncated forms of expanded AT3 (EGFP-AT3-t80Q or EGFP-AT3-t130Q) formed aggregates in almost all transfected cells, but the truncated forms of WT AT3 (EGFP-AT3-t20Q) were distributed diffusively in transfected cells (Fig. 1C d, g, j). Meanwhile, the RFP kept diffusively distribution that is not affected by EGFP or any truncated forms of AT3 (Fig. 1C b, e, h and k). In cells co-expressing WT AT3 (RFP-AT3) and EGFP-AT3-t20Q, both red and green colored proteins were distributed diffusely (Fig. 1D a, b, d, e). While in the cells co-expressing RFP-AT3 and EGFP-AT3-t80Q or EGFP-AT3-t130Q, RFP-AT3 was recruited onto the aggregates formed by EGFP-AT3-t80Q or EGFP-AT3-t130Q (Fig.1D g, h, i, j, k, l). Thus data suggest that WT AT3 could be recruited onto the aggregates formed by expanded AT3.

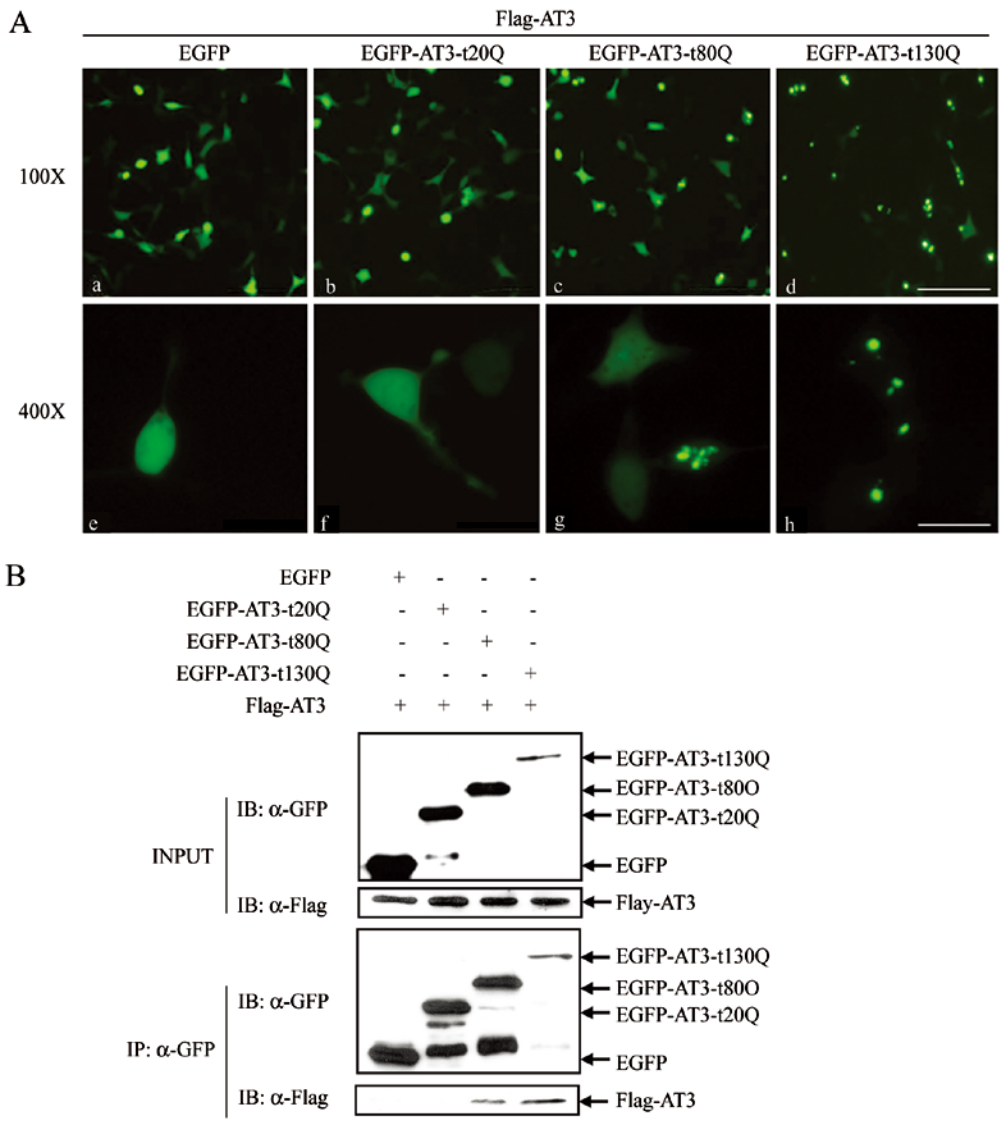

Fig. 2 WT AT3 interacts with the truncated forms of expanded AT3 in transfected HEK293 cells. HEK293 cells were co-transfected with p3×Flag-myc-CMV-24-MJD-CAG20Q (Flag-AT3) along with plasmids expressing EGFP, EGFP-AT3-t20Q, EGFP-AT3-t80Q or EGFP-AT3-t130Q. Forty-eight hours after transfection, cells were observed using inverted fluorescent microscope (A). Scale bar, 7 μm for a, b, c and d; 28 μm for e, f, g and h. These cells were harvested and subjected to IP assays. Proteins were immunoprecipitated with anti-GFP antibody and then detected with anti-GFP or anti-Flag antibody. Flag-AT3 was co-immunoprecipitated with EGFP-AT3-t80Q and EGFP-AT3-t130Q, but not with EGFP or EGFP-AT3-t20Q (B).

3.2 WT AT3 interacts with the truncated forms of expanded AT3 in transfected cells To further examine the possible interactions between WT AT3 and the truncated forms of expanded AT3, we performed co-immunoprecipitation experiments. HEK293 cells were co-transfected with p3×Flag-myc-CMV-24-MJD-CAG20Q (Flag-AT3) along with plasmids expressing EGFP, EGFP-AT3-t20Q, EGFP-AT3-t80Q or EGFP-AT3-t130Q. Forty-eight hours after transfection, cells expressing EGFP-tagged proteins were observed using inverted fluorescent microscope (Fig. 2A). And then, the cells were collected and subjected to IP assays with anti-GFP antibody. The coimmunoprecipitants were analyzed using immunobloting. Expressions of EGFP, EGFP-AT3-t20Q, EGFP-AT3-t80Q, or EGFP-AT3-t130Q were detected with anti-GFP antibody. Co-immunoprecipitated full-length WT AT3 was detected with anti-Flag antibody. As shown in Fig. 2, when EGFP-AT3-t80Q or EGFP-AT3-t130Q was immunoprecipitated, the full length Flag-AT3 was also co-immunoprecipitated (Fig. 2B). However, when EGFP-AT3-t20Q was immunoprecipitated, the full length WT AT3 was not co-immunoprecipitated (Fig. 2B). Thus data suggest that WT AT3 interacts with the truncated forms of expanded AT3 (80Q or 130Q), but not with the truncated forms of WT AT3 (20Q). Interestingly, the binding ability of full length WT AT3 to the truncated forms of expanded AT3 is dependent on polyQ length in the truncated forms of expanded AT3. That is, the longer the polyQ is, the stronger the binding ability is (Fig. 2B).

3.3 WT AT3 interacts with the truncated forms of expanded AT3 in a polyQ length-dependent manner *in vitro* To investigate if there is a physical interaction between WT AT3 and expanded AT3, we purified GST-fused proteins. We evaluated the binding of AT3 with the truncated forms of expanded AT3 using an *in vitro* GST pull-down assay. WT AT3 expressed by pGEX-5X-1 vector encoding GST-AT3 and coupled to glutathione-agarose beads was incubated with the extracts of N2a cells transfected with pEGFP-N1, pEGFP-N1-MJD-CAG20-truncated 20Q, pEGFP-N1-MJD-CAG80-truncated 80Q and pEGFP-N1-MJD-CAG130-truncated 130Q, respectively. As shown in Fig. 3, GST-AT3 did not pull down EGFP or EGFP-AT3-t20Q (Fig. 3A, B). However, it pulled down

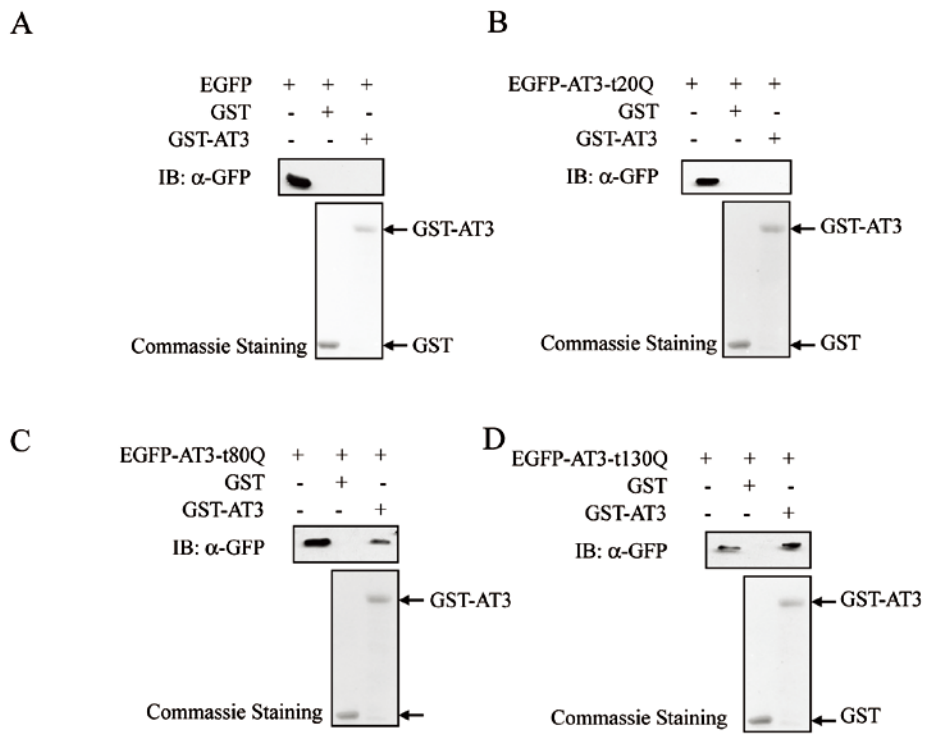

Fig. 3 WT AT3 interacts with the truncated forms of expanded AT3 in a polyQ length-dependent manner *in vitro*. Plasmids expressing EGFP, EGFP-AT3-t20Q, EGFP-AT3-t80Q or EGFP-AT3-t130Q were transfected into N2a cells respectively. At 48 h after tansfection, cells were harvested and they were incubated with GST or GST-AT3 coupled to glutathione-agarose beads. After incubation, the beads were washed with HNTG buffer and bound proteins were detected using anti-GFP antibody by immunoblot analysis. Expressions of these proteins were observed by commassie blue staining. EGFP-fused proteins were incubated with GST or GST-AT3 coupled to glutathione-agarose beads. Input represents 10% of cell extracts containing EGFP (A), EGFP-AT3-t20Q (B), EGFP-AT3-t80Q (C) and EGFP-AT3-t130Q (D) incubated with GST or GST-AT3, respectively.

EGFP-AT3-t80Q and EGFP-AT3-t130Q (Fig. 3C, D). These data suggest that WT AT3 interacts with the truncated forms of expanded AT3, but not the truncated forms of WT AT3 *in vitro*. Consistent with our observations in IP assays, WT AT3 interacts more strongly with Q130 than with Q80, showing a polyQ length-dependent manner.

4 Discussion

Mutated protein aggregate formation is the hallmark of many neurodegenerative diseases including polyQ diseases[13]. Numerous reports have provided evidence that polyQ disease proteins with the expanded polyQ tract are misfolded and tend to self-aggregation[13,19-24]. The aggregation of these proteins is associated with the cellular toxicity[13,24-26], suggesting that the aggregation of these proteins contributes to neurodegeneration. Specially, several studies showed that the polyQ neurodegenerative protein, AT3, might regulate aggresome formation by destabilizing the protein structure and full-length AT3 might be recruited into SDS-insoluble aggregates[15,27,28]. Using fluorescence microscopy, we here showed that full-length WT AT3 was co-localized onto the aggregates formed by the truncated forms of expanded AT3 in N2a cells. These results are consistent with previous report that cleaved expanded AT3 sequestrates full length AT3 to aggregate[15,29].

In our study, we showed that WT AT3 interacts with the truncated forms of expanded AT3, but not the truncated forms of WT AT3 in transfected cells as well as in GST pulldown assay. These results are compatible with our fluorescent observations. Furthermore, IP and GST pulldown assays also show that WT AT3 interacts with the truncated forms of expanded AT3 depending on the length of polyQ expansion. These results match to the pathological characteristics of polyQ diseases in which these pathologic proteins form aggregates more easily when the numbers of polyQ of these protein increase[11,13]. Among polyQ diseases, SCA3 has several distinguishing features. In most disorders, including Huntington's disease (HD), the repeat threshold for pathogenesis is close to 40 residues. In contrast, the repeat threshold for SCA3 disease is greater than 50[30]. AT3 has been identified as an ubiquitin protease to edit the length of polyubiquitin chains and regulate protein degradation[8,31]. It is also reported that AT3 represses transcription via chromatin binding, interaction with histone deacetylase 3, and histone deacetylation[32]. Recently, it was reported that ataxin-3 suppresses polyQ neurodegeneration in *Drosophila*, and the normal activity of ataxin-3 strikingly protects against polyQ neurotoxicity *in vivo*[33], suggesting that WT AT3 has protective effects against cellular toxicity induced by expanded polyQ. Our results indicate that WT AT3 interacts with the truncated forms of expanded AT3 in a polyQ length-dependent manner both in GST pulldown and IP assays, and is recruited onto the aggregates formed by the truncated forms of expanded AT3. Taken together, dysfunction of WT ataxin-3 may result in an increase of expanded AT3 toxicity in MJD patients.

In certain neurodegenerative disease states, including polyQ diseases, mutant proteins are abnormally accumulated in disease brains. Expanded ataxin-3, as well as other polyQ disease proteins, is prone to aggregation both *in vivo* and *in vitro*[13,27,31]. Although many polyQ diseases occur, at least in part, because of a dominant gain-of-function associated with polyQ-expanded gene products, recruitment of wild type protein onto the aggregates formed by mutant protein was also observed[34], suggesting that impairment of normal protein functions may be involved in the pathogenesis of polyQ diseases. Furthermore, clinical data suggest that a partial loss of AT3 function may also play a role in pathogenesis in SCA3[3].

Our present study showed that WT AT3 interacts with the truncated forms of expanded AT3 in a polyQ length-dependent manner, which provide a clue to investigate both the physiological and the potential pathological functions of AT3.

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多聚谷氨酰胺长度依赖性的突变型ataxin-3片段募集野生型ataxin-3

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摘要：目的 脊髓小脑共济失调III型(spinocerebellar ataxia type 3, SCA3)/马查多-约瑟夫病(Machado-Joseph disease, MJD)是*MJD-1*基因中编码谷氨酰胺的密码子CAG的数量非正常扩增引起的一种多聚谷氨酰胺疾病，也是神经退行性疾病的一种。该病的主要病理特征为突变的ataxin-3在患者易感脑区的神经元胞核内聚集形成核内包涵体，但其致病机制和突变蛋白在核内聚集的机制仍不清楚。本研究是为了探讨突变型ataxin-3的病理学特性以及其聚集特性。**方法** 将野生型ataxin-3及不同多聚谷氨酰胺长度的突变型ataxin-3片段共转染到人胚胎肾细胞(HEK293 cells)和鼠成神经母细胞(neuroblastoma cells)中，用荧光显微镜观察。共转染48小时后，收集在细胞中表达的蛋白，用免疫印迹、免疫共沉淀和GST-pull down实验检测蛋白的表达及结合。**结果** 用荧光显微镜观察到突变型ataxin-3片段在细胞内形成聚集，其聚集体上募集有野生型全长ataxin-3；突变型和野生型ataxin-3在细胞内聚集体上有共定位现象。野生型ataxin-3片段并不形成聚集体，与野生型全长ataxin-3在细胞内无聚集现象发生。免疫共沉淀技术及GST-pulldown实验显示，突变型ataxin-3片段与野生型全长ataxin-3存在相互作用，且该作用强度呈现出多聚谷氨酰胺长度依赖性。**结论** 结果提示，野生型ataxin-3通过与突变型ataxin-3片段的相互作用沉积于突变片段形成的聚集体中，这可能影响野生型ataxin-3的正常功能，进而可能对脊髓小脑共济失调III型(SCA3)/马查多-约瑟夫病(MJD)的发病产生影响。

关键词：脊髓小脑共济失调III型/马查多-约瑟夫病；ataxin-3；多聚谷氨酰胺