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Ubiquitinated filamentous inclusions in cerebellar dentate nucleus neurons in dentatorubral-pallidoluysian atrophy contain expanded polyglutamine stretches

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Abstract We have recently reported that, in addition to the widespread occurrence of ubiquitinated neuronal intranuclear inclusions (NIIs), the restricted occurrence of ubiquitinated intracytoplasmic filamentous inclusions in the neurons of the cerebellar dentate nucleus (CDN) is a characteristic feature of dentatorubral-pallidoluysian atrophy (DRPLA). Interestingly, these neuronal intracytoplasmic filamentous inclusions (NIFIs) were morphologically indistinguishable from the skein-like inclusions (SLIs) described previously in the spinal anterior horn cells in amyotrophic lateral sclerosis (ALS). In the present study, we examined immunohistochemically the CDN in ten patients with clinicopathologically and genetically confirmed DRPLA and the spinal anterior horns in five patients with sporadic ALS, using a monoclonal antibody (1C2) directed against long polyglutamine stretches. In all of the patients with DRPLA, both the NIFIs and the NIIs were visualized clearly with 1C2. Conversely, in the patients with ALS all structures, including the SLIs, were completely negative. These findings indicate that in DRPLA, the NIFIs in the CDN are an alteration that is directly related to the causative gene abnormality (an expanded CAG repeat encoding polyglutamine) and that, from the molecular point of view, they are distinct from the SLIs in ALS.

Key words Dentatorubral-pallidoluysian atrophy · Cerebellar dentate nucleus neuron · Skein-like inclusion · Polyglutamine · Immunohistochemistry

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Introduction

Dentatorubral-pallidoluysian atrophy (DRPLA) [9] is one of the hereditary neurodegenerative diseases caused by the expansion of a CAG repeat encoding a polyglutamine tract in the disease protein [12, 21]. The combined degeneration of the dentatorubral and pallidoluysian systems of the central nervous system is a major pathological feature of this disorder [9, 17, 19]. Recently, neuronal intranuclear inclusions (NIIs) have been identified in DRPLA [4, 6] as well as in the other CAG repeat diseases, such as Huntington's disease [2] and several forms of spinocerebellar ataxia [5, 10, 16]. Their presence is thought to be related to a common pathogenic mechanism [6, 13].

In a previous study of DRPLA, we demonstrated the presence of ubiquitinated filamentous intracytoplasmic inclusions in neurons of the cerebellar dentate nucleus (CDN), one of the most severely affected brain regions in this disease [3]. Although these neuronal intracytoplasmic filamentous inclusions (NIFIs) appear to be a significant feature in this disorder, the constituent proteins that are ubiquitinated for proteolysis still remain to be established [3]. In the present study, we conducted an immunohistochemical examination to determine whether the NIFIs contain expanded polyglutamine stretches, a pathogenic abnormality common to all disease proteins in CAG repeat diseases.

Materials and methods

Ten patients with clinicopathologically confirmed DRPLA (age 18–79 years, mean 44.4 years), five with sporadic amyotrophic lateral sclerosis (ALS; age 68–75 years, mean 71.0 years) and seven controls (age 65–83 years, mean 73.4 years) served as the subjects of the present study. In all ten DRPLA cases, five with juvenile (age at onset less than 20 years) and five with adult onset, the diagnosis was also confirmed genetically (Table 1; [18] and unpublished data). We prepared 4-μm-thick, formalin-fixed, paraffin-embedded sections of the CDN tissue from the DRPLA and control subjects, and of the spinal cord from the subjects with ALS. We also prepared sections of the temporal lobe from an ALS patient who exhibited eosinophilic intranuclear inclusions in the hip-

Table 1 Summary of clinical findings of patients with DRPLA in this study [*DRPLA* dentatorubral-pallidoluysian atrophy, *(CAG)n* number of expanded CAG repeats in the DRPLA allele]

Patient	Age/sex (years)	Age at onset (years)	Disease duration		Brain weight (g)
			(years)	(CAG)n	
1	26/F	13	13	66[a]	1010
2	18/M	8	10	68[a]	920
3	28/F	10	18	66[a]	950
4	32/F	14	18	65[b]	940
5	29/F	16	13	67[b]	780
6	37/F	30	7	64[a]	1020
7	79/F	63	16	60[a]	870
8	76/F	61	15	60[a]	960
9	63/F	56	7	59[b]	1040
10	56/M	42	14	65[b]	1025

[a] Frontal cortices were examined
[b] Peripheral leukocytes were examined. Patients 1–3, and 6–8 were reported in the previous study [18]

pocampal pyramidal neurons [7]. The sections were immunostained by the avidin-biotin-peroxidase complex (ABC) method with a Vectastain ABC kit (Vector Laboratories, Burlingame, Calif.), using a mouse monoclonal antibody against expanded polyglutamine stretches (1C2 [20], Chemicon, Temecula, Calif.; 1:16,000) and a rabbit polyclonal antibody against ubiquitin (Dakopatts, Glostrup, Denmark; 1:800). For 1C2 immunohistochemistry, the sections were pretreated with 99% formic acid for 5 min. We used diaminobenzidine as the chromogen.

Results

In all ten cases of DRPLA, occasional NIFIs in the CDN were visualized clearly with 1C2 (Fig. 1 a–c). Serial sections immunostained with anti-ubiquitin antibody revealed that these were identical to the ubiquitinated neuronal intracytoplasmic inclusions reported in our previous study [3]. In addition, polyglutamine-immunoreactive intracytoplasmic punctate staining was observed in the neu-

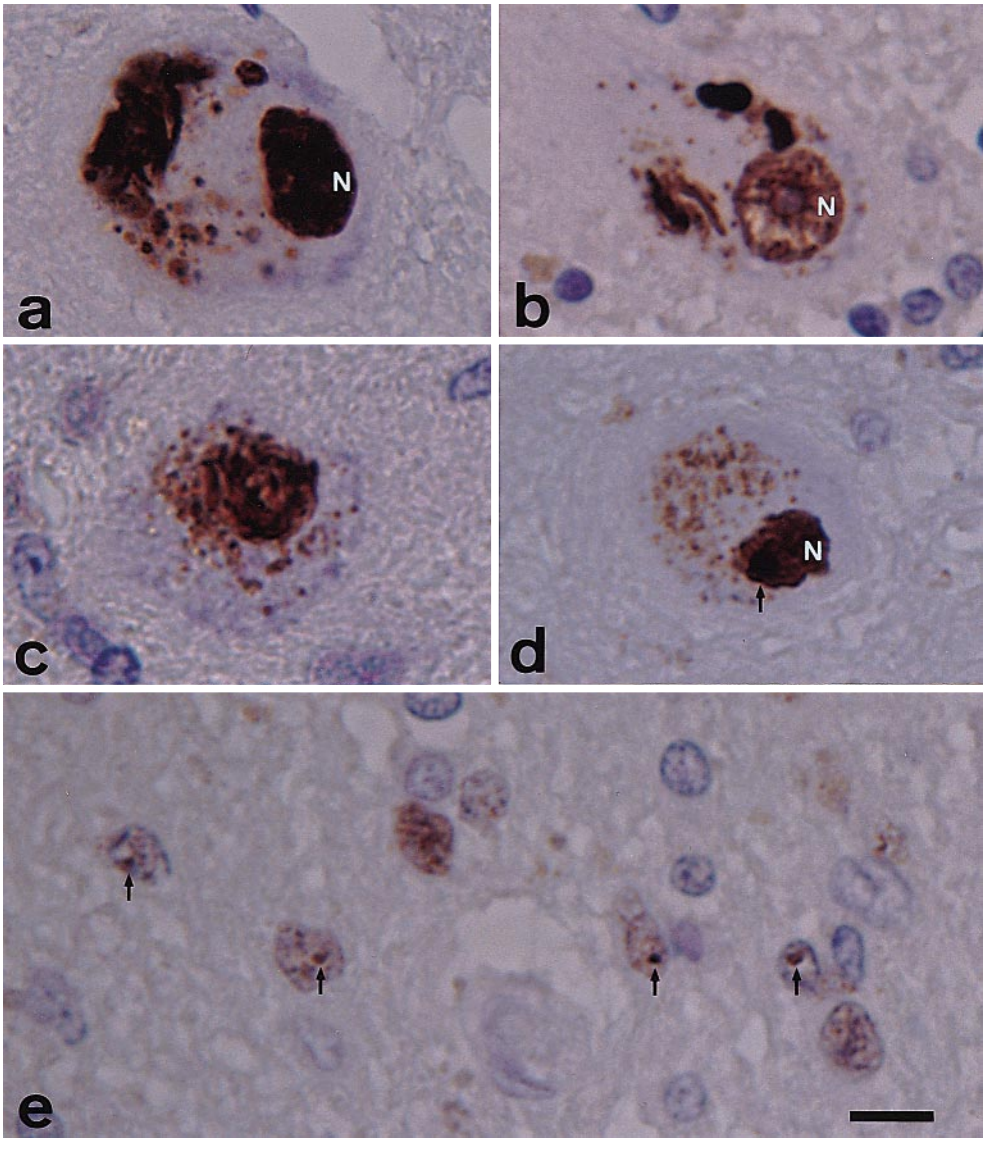

Fig. 1 a–e Immunohistochemistry of the cerebellar dentate nucleus in dentatorubral-pallidoluysian atrophy, stained with a monoclonal antibody against expanded polyglutamine stretches (1C2). Immunoreactive filamentous (**a–c**) and granular (**a–d**) structures are evident in the cytoplasm of neurons. Neuronal nuclei (**a**, **b**, **d**), as well as intranuclear inclusions (*arrows*) of a neuron (**d**) and astrocytes (**e**), are also intensely labeled (*N* nucleus). Sections are counterstained with hematoxylin. *Bar* 10 μm

rons with NIFIs and in many other neurons without obvious NIFIs (Fig. 1 a–d). Moreover, most of the neuronal nuclei (Fig. 1 a, b, d), as well as occasional NIIs (Fig. 1 d) and inclusions in the astrocytic nuclei (Fig. 1 e), were intensely labeled with 1C2. There was no polyglutamine immunoreactivity in the CDN tissues of control cases or in the spinal cords of ALS patients. The eosinophilic intranuclear inclusions in the hippocampal pyramidal neurons in the previously reported ALS patient [1] were also negative for 1C2.

Discussion

Our previous immunohistochemical study of DRPLA failed to demonstrate the presence of the disease protein in the ubiquitinated NIFIs [4]. This result did not necessarily imply the absence of the disease protein containing an expanded polyglutamine in these ubiquitinated inclusions; as suggested by the case in Huntington's disease [2], truncation may occur in the mutant DRPLA protein during the process of NIFI formation, resulting in a partial loss of antigenicity. In the study described here, we studied the participation of the DRPLA protein in the formation of the NIFIs using an expanded polyglutamine stretch as a marker. The present results indicate that these inclusions in the CDN neurons contain expanded polyglutamine stretches and that this alteration is directly related to the causative gene abnormality (an expanded CAG repeat encoding polyglutamine). The occurrence of NIFIs in the CDN in DRPLA may correspond to that of the huntingtin-containing intraneuritic inclusions that have been well studied in Huntington's disease [2, 14].

In previous studies of DRPLA, we have demonstrated the presence of NIIs in various brain regions including the CDN, which were immunoreactive for both ubiquitin and DRPLA protein [4, 6]. In the present study, we have shown that the NIIs were also immunolabeled with 1C2. In addition, 1C2 immunohistochemistry revealed that diffuse and intense labeling occurred in most nuclei of the CDN neurons in DRPLA, including those that did not contain NII. This diffuse and intense labeling of the nucleoplasm was not encountered in ubiquitin-immunostaining. These findings suggest that in the nuclei, the pathological process is not restricted to NIIs, but extends to almost the entire nuclear region, and that the accumulation of expanded polyglutamine stretches in the nuclei precedes the formation of NIIs. Developmental analysis of DRPLA transgenic mouse brains [15] may elucidate the chronological process of cell degeneration.

The formation of both intranuclear and intracytoplasmic inclusions by mutant DRPLA proteins with an expanded polyglutamine stretch was demonstrated in an in vitro experiment [6]. In human DRPLA, however, these intracytoplasmic filamentous inclusions occur exclusively in the CDN neurons [3], in contrast to the widespread occurrence of NIIs [4]. The reason for this selective appearance is uncertain. However, there could be at least two explanations: (1) their appearance might be dependent upon a particular peculiar characteristic of the CDN neurons, since the DRPLA protein is not localized exclusively in the nucleus, but is expressed widely among the CNS neurons [22], and (2) their appearance could be caused by the mutant DRPLA protein itself rather than by the CAG repeat expansion, since no intracytoplasmic inclusions have been demonstrated in the CDN neurons of SCA3/Machado-Joseph disease [10], another CAG repeat expansion disorder [8] with severe involvement of this nucleus [11, 19]. Alternatively, normal DRPLA protein may play a peculiar and important role in the cellular metabolism in the CDN neurons.

The NIFIs in the CDN in DRPLA are identical to skein-like inclusions (SLIs) in the lower motor neurons in sporadic ALS in terms of their light and electron microscopic morphology, ubiquitin-immunostaining pattern and mode of appearance [3]. However, in the present study, the SLIs in ALS were immunonegative for the 1C2 antibody. The NIIs encountered in the hippocampal pyramidal neurons of our previously reported ALS patient [7] were also negative. These results suggest little or no association of expanded polyglutamine aggregation with the pathogenesis of ALS.

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