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CASE REPORT

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An immunohistochemical study of Purkinje cells in a case of hereditary cerebellar cortical atrophy

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Abstract We report an immunohistochemical study of Purkinje cells located in the molecular layer (ectopic Purkinje cells) and in the Purkinje cell layer (orthotopic Purkinje cells) of a patient who died young and had been diagnosed with hereditary cerebellar cortical atrophy from both clinical and neuropathological findings. All the ectopic and orthotopic Purkinje cells were immunoreactive with an anti-calbindin-D antibody, confirming that these stained cells were Purkinje cells. The perikarya of some ectopic and orthotopic Purkinje cells were stained by an antibody to phosphorylated neurofilament. In contrast, Purkinje cells of three normal controls did not react with this antibody. This finding of an abnormal accumulation of phosphorylated neurofilaments in the perikarya may be due to abnormal phosphorylation. Moreover, the regions around the cell bodies of some ectopic and orthotopic Purkinje cells were intensely immunoreactive with an antibody to synaptophysin, which suggests an abnormal increase in presynaptic terminals. It is suggested that ectopia of Purkinje cells, accumulation of phosphorylated neurofilament in the perikarya and an abnormal increase in presynaptic terminals around the soma of the Purkinje cells may be relevant to the pathophysiology of Purkinje cell degeneration in this case. In addition, the relationship between phosphorylated neurofilament and synaptophysin reactivity is discussed.

Key words Hereditary cortical cerebellar atrophy · Ectopic Purkinje cell · Calbindin-D · Phosphorylated neurofilament · Synaptophysin

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Introduction

Hereditary cortical cerebellar atrophy (HCCA) is a hereditary disorder characterized clinically by "pure" cerebellar ataxia. However, its pathogenesis remains unknown. The pathological findings of HCCA include a loss of Purkinje cells, proliferation of Bergmann's glia and fibrillary gliosis in the Purkinje cell layer with or without loss of neurons in the inferior olivary complex [3]. However, ectopia of surviving Purkinje cells in the molecular layer has rarely been reported in human degenerative cerebellar disorders, and the mechanism of the ectopia as well as its relevance to neuronal degeneration remains to be elucidated. On the other hand, the accumulation of phosphorylated neurofilament (pNF) in the neuronal perikarya has often been recognized in several neurodegenerative disorders [2, 6, 11, 12, 14, 18], and may be relevant to the pathophysiology of neuronal death. In addition, an abnormal increase in presynaptic terminals around the neuronal cell body has been reported in some neurological disorders [7, 10, 13, 15, 22]. In the present study, we applied immunohistochemical techniques to investigate the pathophysiology of the surviving Purkinje cells in a patient with HCCA.

Case report and methods

This investigation was carried out on the cerebella obtained at autopsy of a patient with HCCA and of three patients who had died of carcinoma of the body with no brain lesions. The clinical characteristics of the HCCA patient are as follows. His parents, who were cousins, were in good health. His younger brother had dysarthria and ataxia. The patient began to notice dysarthria at around the age of 15 years and ataxic gait at about 25. When he was admitted to our hospital at the age of 34, neurological examination revealed limb and truncal ataxia, intention tremor, nystagmus and diplopia. Neither pyramidal tract signs, sensory disturbance nor Romberg's sign were observed. A CT scan of his brain showed a "pure" form of cerebellar atrophy. His limb and truncal ataxia progressed gradually. At the age of 35, he committed suicide by taking paraquat, dying 11 days later. Genetic examination of peripheral leukocyte DNA ruled out the diagnosis of SCA1,

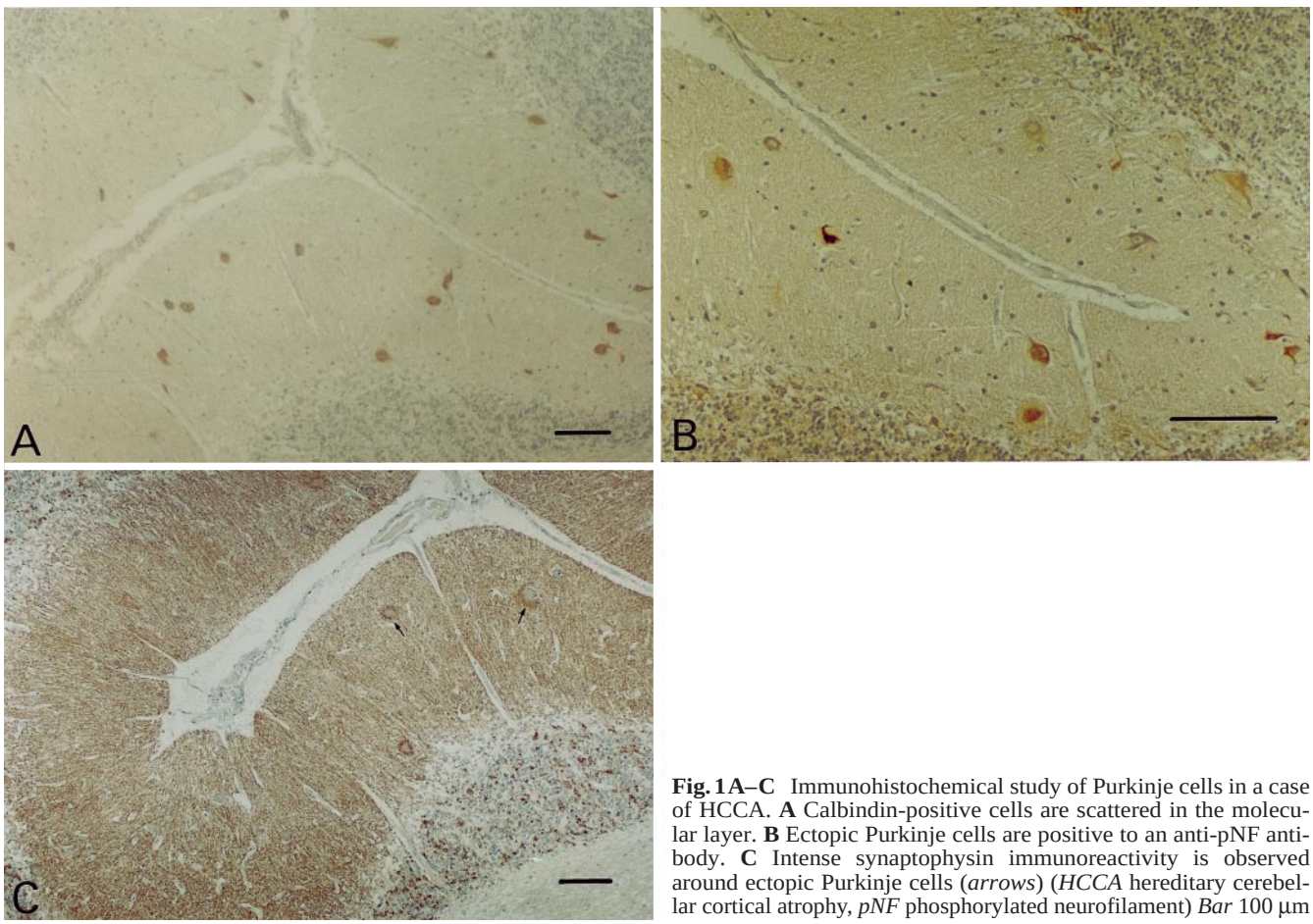

Fig. 1 A–C Immunohistochemical study of Purkinje cells in a case of HCCA. **A** Calbindin-positive cells are scattered in the molecular layer. **B** Ectopic Purkinje cells are positive to an anti-pNF antibody. **C** Intense synaptophysin immunoreactivity is observed around ectopic Purkinje cells (*arrows*) (*HCCA* hereditary cerebellar cortical atrophy, *pNF* phosphorylated neurofilament) *Bar* 100 μm

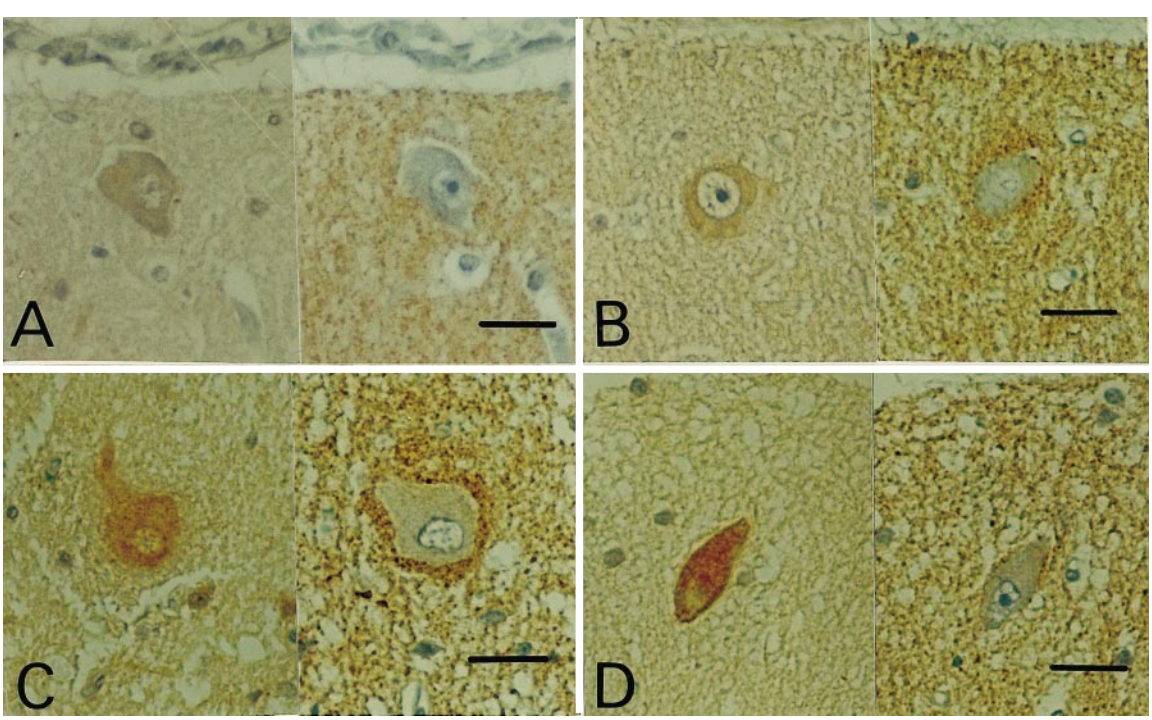

Fig. 2 A–D The association of staining patterns of pNF and SYP in identical Purkinje cells in a case of HCCA. Immunohistochemical staining with anti-pNF antibody is shown on the *left* of each panel, and with anti-SYP antibody on the *right*. **A** An ectopic Purkinje cell that is pNF-negative and SYP-normostained, **B** pNF-negative and SYP hyperstained, **C** pNF-positive and SYP-hyperstained, **D** and pNF-positive and SYP normostained (*SYP* synaptophysin). *Bars* 25 μm

SCA2, SCA3 (Machado-Joseph disease), SCA6, and dentatorubro-pallido-luisian atrophy (DRPLA).

Serial 4-μm-thick sections of 10% formalin-fixed, paraffin-embedded cerebellar tissues were used for immunohistochemical studies. The primary antibodies used were an anti-pNF antibody that reacts with pNF proteins (160 and 200 kDa components) [27], and an anti-synaptophysin (SYP) antibody (Dako) that recognizes the 28-kDa synaptic vesicle protein, synaptophysin [31]. In addition, a monoclonal anti-calbindin-D antibody (Sigma) that specifically reacts with Purkinje cells in the cerebellum [4] was used to verify that the large neurons in the molecular layer were Purkinje cells. Serial sections were used to investigate the association between SYP staining around the cell bodies and pNF staining in the neuronal perikarya in identical Purkinje cells. The sections were deparaffinized, quenched for 30 min with 3% hydrogen peroxide, rehydrated, rinsed in TRIS-buffered saline (pH 7.6), and incubated with the antibodies overnight at room temperature. Antibody binding was visualized with the avidin-biotin-immunoperoxidase complex method (Vectastain ABC kit, Vector Laboratories, Burlingame, Calif.) following the manufacturers' protocol: 3,3′-diaminobenzidine tetrahydrochloride was used as chromogen. Some immunostained sections were counterstained with hematoxylin.

Results

A neuropathological examination of the patient's brain showed no neuronal cell loss in the inferior olivary complex, pontine nuclei, substantia nigra, basal ganglia (caudate nucleus, putamen, globus pallidus, and subthalamic nucleus), or any other regions of the cerebrum and brainstem. In the upper cervical cord, no pathological changes were observed in the posterior column nuclei, spinocerebellar tracts, or corticospinal tracts. In the cerebellum, loss of Purkinje cells with mild proliferation of Bergmann's glia was observed. The cerebellar white matter and granule cells were well preserved and the thickness of the molecular layer seemed to be normal. Overall, the degree of Purkinje cell loss was moderate to severe. In the flocculus, however, a considerable number of Purkinje cells were preserved. There were some large neurons in the molecular layer of the flocculus which were considered to be Purkinje cells from their size and shape. We defined these Purkinje cells in the molecular layer as *ectopic* Purkinje cells. Purkinje cells in the Purkinje cell layer are defined as *orthotopic* Purkinje cells. Some of the ectopic Purkinje cells were abnormally arborized, showing invertion or lying of the Purkinje cell body. Since many ectopic Purkinje cells were observed especially at the flocculus, we used a tissue section of the flocculus for an immunohistochemical study. In the control cases, virtually all Purkinje cells were located in the Purkinje cell layer and no ectopic Purkinje cells were detectable in the flocculus.

Anti-calbindin-D antibody recognized all large neurons in the molecular layer and the Purkinje cell layer, which indicates that these stained neurons are all Purkinje cells and that the large neurons in the molecular layer are really ectopic Purkinje cells (Fig. 1 A).

Immunostaining for pNF revealed that many of the ectopic Purkinje cells reacted with the antibody, but control Purkinje cells did not. A considerable number of the orthotopic Purkinje cells of the HCCA patient also reacted with the antibody. However, these labeled Purkinje cells were not ballooned (Fig. 1 B).

Table 1 Association of immunohistochemical findings of pNF and SYP staining on identical Purkinje cells (*pNF* phosphorylated neurofilament, *SYP* synaptophysin, + positively stained, – not stained, *HS* hyperstained, *NrS* normostained, *orthotopic* orthotopic Purkinje cell, *ectopic* ectopic Purkinje cell)

pNF	SYP	Number			%
		Orthotopic	Ectopic	Total	
+	HS	4	14	18	8.5
+	NrS	11	56	67	32.0
–	HS	4	14	18	8.5
–	NrS	8	100	108	51.0
Total		27	184	211	100.0

SYP immunoreactivity was normally expressed in the neuropil of the molecular layer. The regions surrounding the soma of some ectopic and orthotopic Purkinje cells were more intensely immunoreactive with the anti-SYP antibody than those of control Purkinje cells (Fig. 1 C). This antibody also recognized the glomeruli in the granular layer in both HCCA and control cases.

The results of the association of SYP and pNF staining are shown in Table 1 and Fig. 2. In the control sections, Purkinje cell bodies showed no pNF immunoreactivity (pNF-negative), and no or only few granules with SYP immunoreactivity were observed around Purkinje cell bodies (SYP-normostained). In the present case, a total of 211 Purkinje cells (orthotopic Purkinje cells: 27, ectopic Purkinje cells: 184) were examined to investigate the association between SYP and pNF staining. Four patterns of staining were observed in both orthotopic and ectopic Purkinje cells as follows. pNF-positive and SYP-hyperstained Purkinje cells accounted for 8.5% (18/211), pNF-positive and SYP-normostained 32.0% (67/211), pNF-negative and SYP-hyperstained 8.5% (18/211), and pNF-negative and SYP-normostained 51.0% (108/211).

Discussion

Although SCA5 and SCA6 seem to be a part of autosomal dominant HCCA [21, 33], a complete method for the genetic diagnosis of HCCA has not yet been developed. Therefore, diagnosis depends largely on clinical and pathological findings. Accordingly, the present case was diagnosed as having HCCA with autosomal recessive inheritance from clinical and pathological findings. However, the clinico-pathological findings in the present case would have changed with time if the patient had lived longer. Had the patient died older, we might not have seen so many ectopic Purkinje cells, which constitutes a remarkable pathological feature of the present case. In other words, the pathological findings of the present case may be halfway in the disease progression. Therefore, the immunohistochemical findings observed in the present case

may not always reflect the pathophysiology of cases previously reported as HCCA.

In animals, Purkinje cells ectopically situated in the molecular layer have been described in studies of mice treated with cytosine arabinoside and in NZB/BINJ and reeler mice [23, 32]. In the case of humans, on the other hand, ectopic Purkinje cells in the molecular layer have been reported in cases with acquired conditions such as Hunter-Russel syndrome and crossed cerebellar atrophy [9, 25], and in hereditary disorders such as granule cell type of cerebellar degeneration [20] and Menkes' kinky hair disease. Abnormal migration and arborization of Purkinje cells may arise from lack of synaptic contact, such as deafferentation and loss of dendritic tree [28]. In the present case, since there were no abnormal findings in the granular layer and inferior olivary nucleus, the abnormal rotation and dislocation of Purkinje cells may be due to the degeneration of Purkinje cells per se or to an abnormality of basket cells, as has been pointed out by several authors [9, 20, 25, 28]. The possibility that paraquat intoxication can induce such abnormal migration of Purkinje cells is unlikely, because similar changes have not been reported in human cases of paraquat poisoning [5, 8, 16, 24].

The accumulation of pNF in the neuronal perikarya has been reported in ballooned neurons in various neurodegenerative diseases, including Creutzfeldt-Jakob disease [11, 18]. Pick's disease [2, 26], amyotrophic lateral sclerosis [11, 14], corticobasal degeneration [6] and Alzheimer's disease [2]. In Menkes' kinky hair disease, on the other hand, the accumulation of pNF has been seen in non-ballooned neurons [12]. Likewise, in the present case, pNF accumulation was demonstrated in non-ballooned Purkinje cells. Therefore, it is likely that the accumulation of pNF in this case is due to an abnormal phosphorylation of NF proteins in the perikaria of the Purkinje cells rather than to an impairment of axoplasmic transport. The disappearance of afferent signals might have induced phosphorylation of NF in the neuronal perikarya in the present case, as suggested by Torack et al. [26]. In addition, the accumulation of pNF observed in both ectopic and orthotopic Purkinje cells suggests that the ectopia of Purkinje cells may be an independent phenomenon from abnormal phosphorylation of NF. It is unlikely that abnormal pNF staining in Purkinje cells is caused by paraquat intoxication, because such abnormal staining was observed only in Purkinje cells, and not in other neurons of the brain in the present case. The blood-brain barrier has been reported to impede paraquat entry into the brain in adult rats, thus causing no primary lesions in the brain [17, 19, 29, 30]. Therefore, it is most likely that abnormal staining of pNF in Purkinje cells is due to the disease process of HCCA itself, and not to paraquat intoxication.

The regions around some ectopic and orthotopic Purkinje cell bodies were intensely stained with the anti-SYP antibody. This finding suggests that presynaptic terminals around some Purkinje cell bodies may increase abnormally, although the origin of the presynaptic terminals is unknown. Abnormally increased presynaptic terminals on neuronal cell bodies have been reported in cases of progressive supranuclear palsy [15], amyotrophic lateral sclerosis [22], Werdnig-Hoffmann disease [10], pseudohypertrophy of olivary nucleus [13], and temporal lobe epilepsy [7]. Although the precise, molecular mechanism that regulates synaptic formation remains to be clarified, an increase in presynaptic terminals might be a compensatory phenomenon in response to the derangement of postsynaptic neurons. Possibly, the dysfunction of the postsynpatic site on Purkinje cell dendrites might lead to a failure of regulation of presynaptic formation and might induce axonal sprout to and presynaptic formation on neuronal cell bodies, resulting in an abnormal increase in presynaptic terminals around neuronal cell bodies. Moreover, the increase in presynaptic terminals observed in both orthotopic and ectopic Purkinje cells suggests that ectopia and increased presynaptic terminals may be independent phenomena.

The association between pNF accumulation in the perikaryon and SYP increase around the neuronal cell body has not been reported. From the present results, we can speculate the sequence of the Purkinje cell changes in the course of degeneration. If the accumulation of pNF had occurred progressively with time and an increase in the density of presynaptic terminals had occurred

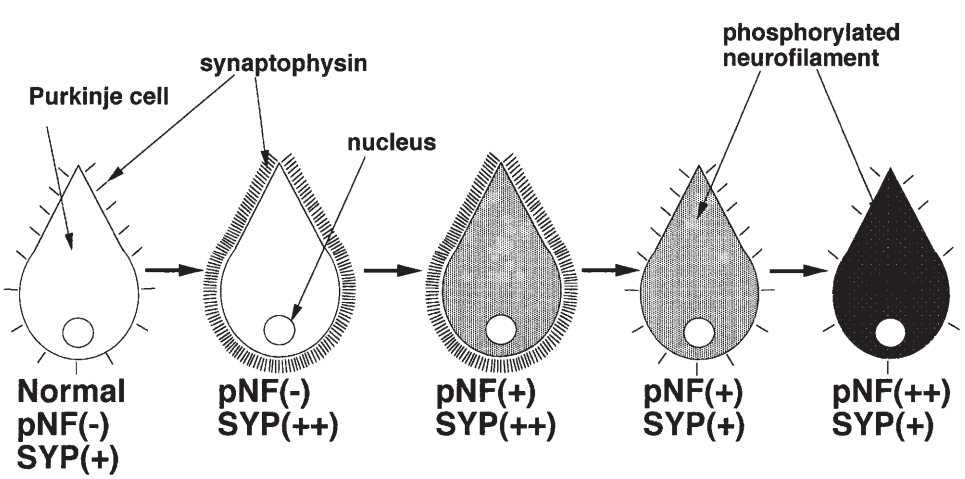

Fig. 3 A speculation on the changes in the staining pattern of Purkinje cells for anti-pNF antibody and anti-SYP antibody. pNF immunoreactivity increases progressively with time, but SYP immunoreactivity decreases progressively after a transient increase at the early stage

monophasically, the sequence of Purkinje cell changes would be as shown in Fig. 3. Initially, the presynaptic terminals might increase compensatively around the Purkinje cell body in response to the loss of synaptic contact with dendrites. On the other hand, abnormal phosphorylation of NF might begin in association with the neuronal deafferentation. Some deafferented Purkinje cells might migrate in the molecular layer. Then, the increased presynaptic terminals might decrease and disappear gradually as accumulation of pNF advances. Finally, Purkinje cell death might occur.

The elucidation of the molecular mechanisms of NF phosphorylation, presynaptic formation, and migration of Purkinje cells may be the key to the mystery of neuronal degeneration in this disorder.

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