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Amyotrophic Lateral Sclerosis Associated with Genetic Abnormalities in the Gene Encoding Cu/Zn Superoxide Dismutase: Molecular Pathology of Five New Cases, and Comparison with Previous Reports and 73 Sporadic Cases of ALS

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Abstract. Molecular pathology has identified 2 distinct forms of neuronal inclusion body in Amyotrophic Lateral Sclerosis (ALS). ALS-type inclusions are skeins or small dense filamentous aggregates which can only be demonstrated by ubiquitin immunocytochemistry (ICC). In contrast hyaline conglomerates (HC) are large multifocal accumulations of neurofilaments. Previous reports have failed to clarify the distinction and relationship between these inclusions. Correlation of molecular pathology with sporadic and familial cases of ALS will detect specific associations between molecular lesions and defined genetic abnormalities; and determine the relevance of molecular events in familial cases to the pathogenesis of sporadic disease. We describe the molecular pathology of 5 ALS cases linked to abnormalities of the SOD1 gene, in comparison with a series of 73 sporadic cases in which SOD1-gene abnormalities were excluded. Hyaline conglomerate inclusions were detected only in the 2 cases with the SOD1 I113T mutation and showed a widespread multisystem distribution. In contrast ALS-type inclusions characterized sporadic cases (70/73) and were restricted to lower motor neurons. Hyaline conglomerates were not seen in sproadic cases. Confocal microscopic analysis and ICC shows that HC contain equally abundant phosphorylated and nonphosphorylated neurofilament epitopes, indicating that phosphorylation is not essential for their formation. In contrast neurofilament immunoreactivity is virtually absent from typical ALS-type inclusions. The SOD1-related cases all had marked corticospinal tract and dorsal column myelin loss. In 4 cases the motor cortex was normal or only minimally affected. This further illustrates the extent to which upper motor neuron damage in ALS is usually a distal axonopathy. Previously reported pathological accounts of SOD1-related familial ALS (FALS) are reviewed. Hyaline conglomerates are so far described in cases with mutations A4V, I113T and H48Q. In only 1 of 12 cases (H48Q) reported were both HC and ALS-type inclusions present in the same case. These findings suggest the possibility that the molecular pathology of neuronal inclusions in ALS indicates 2 distinct pathogenetic cascades.

Key Words: ALS; Familial ALS; Molecular pathology; Neurofilament; SOD1; Sporadic ALS, Ubiquitin.

INTRODUCTION

Autosomal dominant inheritance is present in approximately 10% of patients presenting with amyotrophic lateral sclerosis (ALS)(1). The clinical and pathological phenotypes of such familial ALS (FALS) cases is described as indistinguishable from sporadic ALS (1–3). In one fifth of these families mutations in the gene encoding Cu/Zn superoxide dismutase (SOD1) are found in affected individuals (4). There are more than 50 such abnormalities reported from affected kindreds, the great majority being point mutations in exons 1, 2, 4 and 5 (5, 6). A few exon 3 and intronic mutations and deletions are described, and most recently we have identified deletions in the 3′ untranslated region of the gene, although the significance of this latter abnormality awaits clarification (7). The exonic mutations that cause disease seem to be associated with abnormal protein folding or protein-protein interaction, leading to increased exposure of the active site, or reduced dimer stability. The possibility that disease is caused by a reduction in the normal function of SOD1 has been proposed (8–11), but most evidence currently supports an unknown 'toxic-gain-of-function' conferred upon mutant SOD1 molecules (11–13).

Clinical studies in FALS have emphasized that most parameters (e.g. age of onset) are not correlated with either specific mutations, or groups of mutations having similar predicted effects on SOD1 structure, nor with the degree of inactivation of the enzyme (14). Within individual families there can be considerable variation in the age of onset and clinical features of the disease (15). An exception is duration of disease, such that certain mutations are characteristically associated with rapidly (e.g. A4V) and slowly (e.g. G37R, H46R) progressive phenotypes (5). These data have lead to working hypotheses that: 1) mutations can consistently affect the severity of disease once it emerges but not other clinical parameters; 2) additional, probably genetic, factors act to modify disease phenotype and onset; 3) the toxicity of the mutant SOD1 molecules is due to a toxic 'gain of function,' and that the different mutations act via similar intracellular targets.

Pathological accounts of cases of FALS in which a mutation in the SOD1 gene is defined are few (15–20). Of the 7 cases reported, 3 had a duration of less than 2 years and showed lower motor neuron inclusions of the

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TABLE 1
Clinical Features

Case no.	Sex	Age (y)	Onset	Progression			UMN signs	LMN signs	Duration (m)	Family history	SOD1 mutation
				B	UL	LL					
1	m	65	UL	~		√	√	√	10	×	I113T
2	f	61	UL	~		√	√	√	13	√	I113T
3	f	79	B		√	√	√	√	33	×	del bp726 3'UTR
4	f	63	LL	√	√		√	√	17	×	del bp816-819 3'UTR
5	m	64	UL	√		√	√	√	12	×	intron 1 T→A 108bp upstream of exon2

B = bulbar; UL = upper limb; LL = lower limb; √ = present; × = absent; ~ = equivocal; bp = base pair; del = deletion; 3'UTR = 3 prime untranslated region

'hyaline conglomerate' (HC) type (16, 18, 19) and these may be the inclusions described by Takahashi et al (20). Such inclusions differ from the classical ubiquitinated inclusions first described in sporadic ALS which were present in a patient with an SOD1 mutation at E100G dying after 39 months of ALS (17). In the present report we describe the conventional and molecular neuropathological features of 5 new cases associated with SOD1-gene abnormalities. Two of these cases showed motor neuron inclusions of 'HC type,' and the others have typical ALS-type ubiquitinated inclusions. Molecular pathology suggests that these inclusions are distinct in terms of immunoreactivity to neurofilament antibodies, electron microscopical appearances and argyrophilia (18, 19, 21). The findings do not support the hypothesis that neurofilament phosphorylation is a key factor in the pathogenesis of HC. The multisystem nature of the neurodegenerative process in these cases is emphasized with particular reference to long projecting pathways.

MATERIALS AND METHODS

Patients

Five cases of ALS from the Newcastle Brain Tissue Bank were found to have abnormalities of the SOD1 gene on screening of brain-derived DNA samples. Clinical details of the disease presentation and progression are given in Table 1. All the cases were extensively investigated, including confirmatory electrophysiology, and correspond to the El Escorial category 'definite ALS.'

Genetic Analysis

The genetic analysis of these 5 cases, and of a further 73 cases in which the SOD1 gene was normal, is reported in detail elsewhere (7). In brief, genomic DNA was extracted from snap frozen cerebral cortex and screened for abnormalities in the SOD1 gene using both single strand conformation analysis (SSCA) and heteroduplex analysis. Primers for PCR were used which amplify intronic and exonic sequences associated with exons 1 to 5 across the whole gene. DNA extracted from blood samples of 209 unrelated Caucasian normal control cases were similarly screened for SOD1 gene abnormalities. For the 5 cases the SOD1 gene abnormalities are shown in Table 1. One control patient, a 22-year-old female, showed the 4bp deletion present in case 4. No follow-up information is available for this individual whose DNA was made available anonymously (7).

Pathology and Immunocytochemistry

At autopsy the brain, spinal cord, sympathetic and dorsal root ganglia, and multiple skeletal muscle samples were retained. The brain and spinal cord were subdissected while fresh to provide samples for freezing and for fixation. The spinal cord was exposed over its ventral surface by incision of the dura. The ventral roots were carefully identified with reference to the T1 root which was located prior to removal. In all cases tissue from the following spinal cord levels was available for examination as paraffin processed blocks: C4, C6, C8, T2, T6, T8, L2, L4. In addition blocks of levels T2 and C3 were retained for Marchi impregnation. The remaining segments from the limb enlargements and representative thoracic levels were snap frozen for long-term storage.

The brain was dissected as follows. The caudal pons with the medulla and cerebellum were removed by horizontal incision. The left hemibrainstem was frozen and the right fixed in formalin. The midbrain and cerebral hemispheres were separated by a midline sagittal incision. The left hemisphere was sliced for freezing and the right hemisphere fixed in formalin prior to paraffin embedding. Serial levels through the midbrain and brainstem were prepared for histology together with samples of the cerebellar cortex and dentate nucleus, neocortical lobes, hippocampus and entorhinal cortex, and motor cortex (4 levels). The basal ganglia from the right hemisphere were sampled as consecutive blocks to include all major structures.

Routine sections were prepared from all of these blocks and stained with conventional stains and immunocytochemistry. The panel of antisera employed in the study are shown in Table 2. An avidin biotin complex system was used for the secondary antibody step (Vectastain Elite, Vector Labs) and microwave antigen retrieval was used for GFAP and SMI32.

Sporadic ALS Cases

In addition to the cases described with SOD1 gene abnormalities the same pathological examination protocol was carried out on another 73 cases of sporadic ALS.

Confocal Microscopy

Double labeling immunofluoresence with FITC and Rhodamine was used to colocalise ubiquitin with neurofilaments in

TABLE 2
Antibodies

Antibody	m/p	Specificity	Dilution	Source
CD68	m	macrophages	1:50	Dako
Ubiquitin	p	ubiquitin conjugates	1:1,000	Dako
GFAP	p	astrocytes	1:4,000	Dako
SMI31	m	phosphorylated neurofilament	1:10,000	Sternberger
SMI32	m	non-phosphorylated neurofilament	1:8,000	Sternberger
SOD1	p	Cu/Zn superoxide dismutase	1:200	Binding Site
1A6	m	nitrotyrosine	1:10	Dr Beckman
1/91	p	neurofilament 200	1:25	Sigma

p = polyclonal antiserum
m = monoclonal antibody

spinal motor neuron inclusion bodies in ALS. The I113T cases were studied together with a group of 8 sporadic cases. The I113T cases had inclusions of HC type (see below) compared with typical ubiquitinated inclusions in the sporadic cases. The method used employed a comparison of rabbit polyclonal antisera with mouse monoclonal antibodies using species specific secondary antibodies. The antibodies were used in the combinations: SMI31 or SMI32 (Sternberger Monoclonals) vs polyclonal anti-ubiquitin (Dako); polyclonal anti-neurofilament heavy chain (Dako) vs monoclonal anti-ubiquitin (Novocastra). The staining for neurofilaments was amplified using avidin-biotin complex and biotinylated rhodamine. Ubiquitin was demonstrated using indirect labeling with FITC-conjugated secondary antibody. Multiple sections were screened from each case with a minimum of 10 inclusion bodies per case examined. Colocalisation was confirmed by optical sectioning using an MRC600 laser scanning confocal microscope (Biorad).

RESULTS

The pathological findings in the cases 1–5 in relation to ALS are shown in Table 3. Molecular pathology of the neuronal inclusion bodies in these cases is summarized in Table 4.

Long Tract Changes

All 5 cases showed a typical pattern of long tract degeneration which can be summarized as 'ALS with dorsal column involvement.' The corticospinal tract degeneration was most prominent in the cervical cord region and was not predictive of discernible Betz cell depletion in the motor cortex (Fig. 1a, b). Only 1 case (case 3) showed severe neuronal loss in the motor cortex. In another 3 cases there was some increase in gliosis in the perirolandic subcortical white matter. These changes clearly demonstrate that the corticospinal tract degeneration in these cases is predominantly an axonopathy, and frequently occurs in the absence of evidence for neuronopathy. In both the affected corticospinal tracts and in the motor cortex of cases 3 and 5 the sections stained for CD68 showed an increased population of dendritic macrophages. These changes were consistently present in mildly affected areas and this stain was regarded as a useful marker of early degenerative changes.

The dorsal column involvement was also most marked in the proximal cord and was more prominent in the funiculus cuneatus (Fig. 1b). There was evidence of "transneuronal degeneration" in the medullary relay nuclei of the proprioceptive pathway. In the 2 cases with the I113T mutation (cases 1, 2), occasional large dorsal horn neurons contained HC inclusions (see below). The appearances suggest that degeneration in these fibre tracts may also represent a predominant axonopathy. Pallor of all the lateral and ventral funiculi was apparent in all these cases. This included the regions of the ascending dorsal and ventral spinocerebellar pathways. In the 2 cases with the I113T mutation, Clarke's nucleus in the dorsal cord was severely depleted of neurons and the few survivors contained HC inclusions. Subjective assessment indicated that there were lesser degrees of neuronal loss from this nucleus in the other 3 cases. These 2 severely affected cases showed marked loss of neurons from the inferior olivary nucleus (and corresponding myelin pallor of olivary efferent tracts) due to "transneuronal degeneration."

Lower Motor Neuron Degeneration and Molecular Pathology of Neuronal Inclusions

All 5 cases showed marked loss of lower motor neurons from the spinal ventral horn and the hypoglossal nucleus. The motor nuclei of the facial and trigeminal nerves were more variably affected. These affected regions showed variable astrocytic gliosis but a consistent increase in CD68 immunoreactive macrophages. Onuf's nucleus did not appear depleted of neurons in any of the cases but was affected by HC inclusions in cases 1 and 2. Motor neuron inclusion bodies were present in all cases and comprised 2 separate categories. The first type of inclusion corresponded to the ubiquitinated skein and Lewy-like inclusions typical of sporadic ALS (Fig. 1c). They were identified in 3 cases (cases 3, 4, 5). These

TABLE 3
Pathological Appearances in 5 ALS Cases with *SOD1*-gene Abnormalities

CNS region/neuron populations		Case 1	Case 2	Case 3	Case 4	Case 5
SOD1 mutation		I113T	I113T	delA726	del816TTTC819	intron 1 T108A
Ventral horn cells	lumbar	depleted (HC)	depleted (HC)	depleted (Sk,L1)	depleted (Sk,L1)	depleted (Sk,L1)
	cervical	depleted (HC)	depleted (HC)	depleted (Sk,L1)	depleted (L1)	depleted (Sk,L1)
	bulbar	severe depletion N.XII	severe depletion N.XII, VII, V	severe depletion N.XII	severe depletion N.XII, VII	severe depletion N.XII, VII
	oculomotor	preserved	preserved	preserved	preserved	preserved
Motor cortex	Betz cells	preserved HC infrequent	preserved HC infrequent	preserved no inclusions	depleted/shrunken no inclusions	preserved no inclusions
WM pallor	pyramids	mild	mild	severe	moderate	severe
	LCST	severe	severe	severe	severe	severe
Hippocampus	dentate granule cells	normal	normal	normal	normal	ubiquitin inclusions
Substantia nigra		preserved	preserved	preserved	mild depletion	preserved
Clarke's nucleus/spinocerebellar tr		severe depletion HC	severe depletion HC	preserved no inclusions	preserved no inclusions	preserved no inclusions
Dorsal column pallor		severe	severe	severe	severe	severe
Frontal neocortex/white matter		normal	normal	normal	normal	WM gliosis

HC = hyaline conglomerates; Sk = skein-like ubiquitinated inclusions; L1 = lewy body-like ubiquitinated inclusions; LCST = cervical/thoracic lateral corticospinal tract; N.XII = hypoglossal nucleus; N.VII = motor nucleus of facial nerve; N.V = motor nucleus of trigeminal nerve

inclusion bodies did not stain with the neurofilament markers used. In contrast to some previous reports there was no discrete staining of these inclusions using the antibodies either to SOD1 or nitrotyrosine, which showed diffuse reactivity over the whole somato-dendritic cytoplasm. Skeins or Lewy-like inclusions were both present in variable proportions in different spinal levels from all 4 cases. One of these cases (case 5) had hippocampal inclusions in dentate granule cells typical of "ALS-dementia" as described above (Fig. 1d) although there was no clinical history of intellectual impairment.

The second type of lesion comprised so-called HC type inclusions and were present in cases 1 and 2. In contrast to the classical inclusions of ALS these were large multifocal patches within the soma which were immunoreactive for both SMI31 and SMI32 (Fig. 1e–h). They were confined to neurons where they frequently occupied at least 50%–70% of the cytoplasm of affected cells. Immunoreactivity for SMI32 (Fig. 1e) was usually stronger than SMI31 (Fig 1f) indicating that the lesions were independent of the phosphorylation-specific epitopes identified by these antibodies. Staining with ubiquitin antibodies was quite different from classical ALS-type inclusions and comprised a weak diffuse granular reactivity over the whole inclusion (Fig. 1g). These inclusions were not immunoreactive for SOD1 or for nitrotyrosine. They were readily observed in Cresyl violet stained sections as irregular hyaline objects displacing normal structure such as nissl substance, lipofuscin and the nucleus (Fig. 1h). In the silver-staining method used (Cross-modification of Palmgren) the inclusions were markedly argyrophilic (Fig. 1i–m) and were shown to be widely distributed throughout spinal neuronal groups including: spinal and bulbar lower motor neurons (Fig 1i), Clarke's nucleus (Fig. 1j), nucleus ambiguus (Fig. 1k), Onuf's nucleus (Fig 1l), and larger dorsal horn neurons. They were also present in Betz cells of the motor cortex (Fig. 1m). The molecular pathology of inclusions present in previous reports of ALS together with the present cases are shown in Tables 4 and 5.

Colocalisation Studies

The HC inclusions in cases 1 and 2 showed strong neurofilament reactivity throughout (Fig. 1n) but only diffuse faint ubiquitin staining (Fig. 1o). In contrast skein type inclusions in the sporadic cases showed strong ubiquitin staining but were uniformly negative for all 3 neurofilament antibodies used. Lewy-like inclusions occasionally showed strong ubiquitin immunoreactivity (Fig. 1q) and only weak peripheral colocalisation with the polyclonal anti-neurofilament antiserum (Fig. 1p) which may not represent a major constituent of the lesion. These findings emphasize the fundamental difference between these HC and ALS-type inclusions at the molecular level.

TABLE 4
Molecular Pathology of 5 ALS Cases with *SOD1*-gene Abnormalities

SOD1 mutation	Age (y) at onset	Disease duration (m)	Inclusion type	Location	Ubiquitin ICC	Neurofilament ICC	Tau ICC	Argyrophilia	Case
I113T	64	10	HC	LMN	+	+++ (SMI31)	–	+++	case 1
				(+ others see text)	(weak diffuse)	+++ (SMI32)			
I113T	64	13	HC	LMN	+	+++ (SMI31)	–	+++	case 2
				(+ others see text)	(weak diffuse)	+++ (SMI32)			
delA726	78	26	ALS	LMN	+++	–	–	–	case 3
del 816TTC819	63	17	ALS	LMN	+++	–	–	–	case 4
intron1 T→A 108bp upstream of exon 2	63	12	ALS	LMN	+++	–	–	–	case 5

HC = hyaline conglomerates, ALS = typical ubiquitinated inclusions; LMN = lower motor neurons

Other Pathology in SOD1-related FALS

Pathology associated with other neurodegenerative diseases were minimal. Neuronal loss from the substantia nigra was pronounced in cases 1–3 but was not associated with inclusion bodies. There were scanty neurofibrillary tangles in the entorhinal cortex (pre-α clusters) and pyramidal sector CA1 of the hippocampus in 3 of the 5 cases. In all of these the finding was compatible with the patients age. There was no evidence of amyloid plaque formation even in the oldest cases and Lewy bodies were not identified.

Pathology of Sporadic Cases

Comparative examination of the 73 sporadic cases in whom the same methods were used showed the following major observations. Seventy cases had lower motor neuron inclusion bodies of the typical 'skein' and 'Lewy-like' appearance immuoreactive for ubiquitin and negative for all other markers used and virtually undetectable in conventionally stained sections. The frequency of LMN containing these inclusions varied markedly from case to case and at different levels of the spinal cord and brain stem. In some cases they were infrequent and their identification required extensive sampling. This was especially so in cases with few surviving motor neurons. In 4 cases of ALS with dementia ubiquitinated inclusions were also present in dentate granule cells of the hippocampus. Inclusions were not demonstrated in upper motor neurons, neocortex or in any other neuronal or glial population except for 1 case who had Lewy-body Parkinson's disease simultaneously with ALS (22). Three cases did not show ubiquitinated inclusions in LMN. All were clinical examples of progressive muscular atrophy corresponding to the El Escorial category of 'suspected ALS.' Two had a brief illnesses (24 months (23), and 15 months (24)) and the third a more prolonged disease duration (240 months). Long tract degeneration in the dorsal columns and the spinocerebellar pathways was also highly variable and present in many cases. Substantia nigra neuron loss was marked in approximately 10% of cases, especially those with a clinical history of dementia, and was not associated with Lewy bodies except for the case previously mentioned (22). Alzheimer type pathology was present in some older cases (> 70 yr) but below densities associated with dementia. No cases showed neuritic plaque scores above CERAD 3 (moderate frequency (25) or neurofibrillary tangle formation above Braak stage 2 (26).

DISCUSSION

Details of the pathology of 7 cases of FALS related to known mutations in the SOD1 gene have been reported previously (Table 5). All of these cases had point mutations in exons of the SOD1 gene. The present report adds

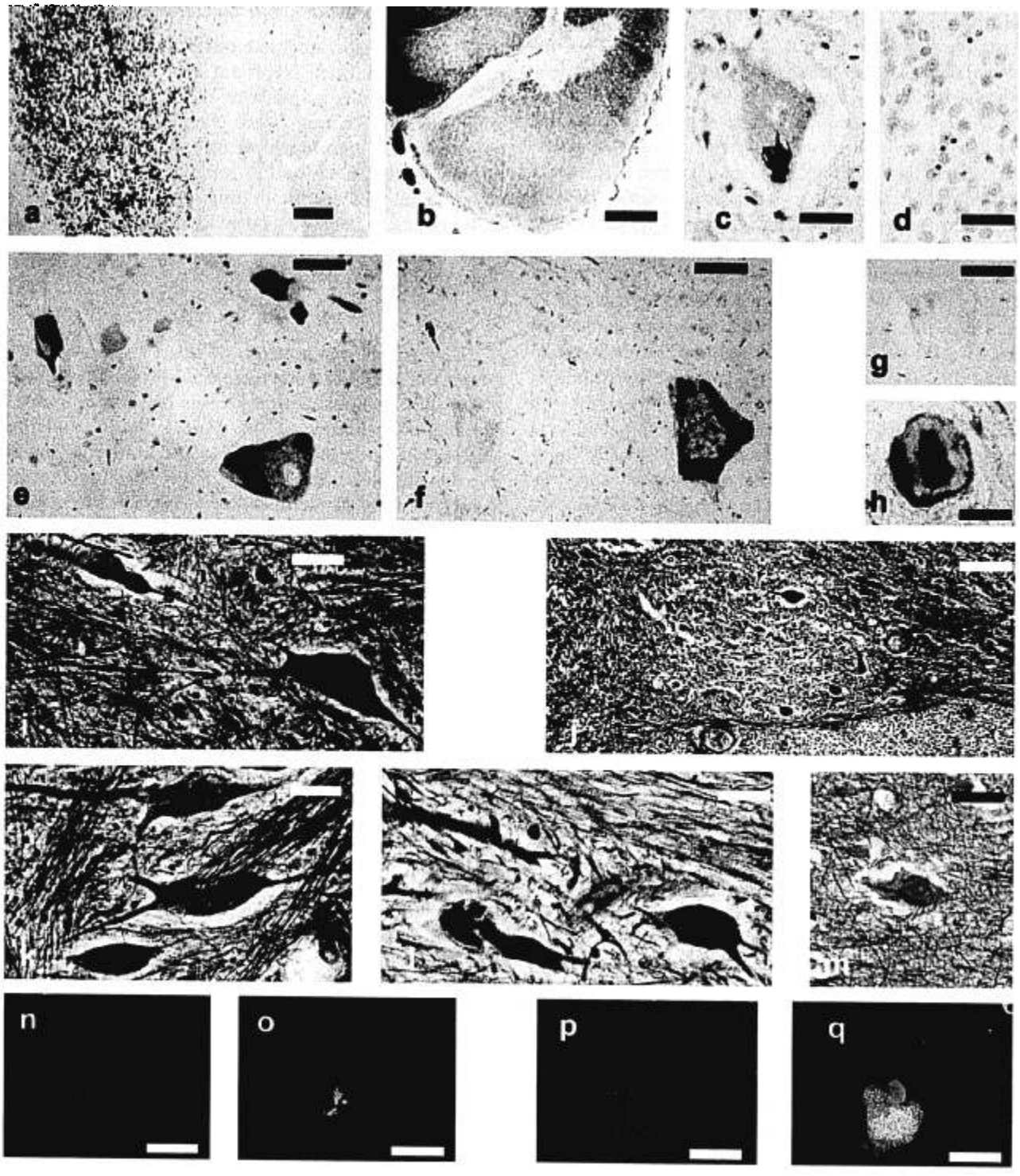

Fig. 1. Photomicrographs and confocal microscopy of changes in familial and sporadic ALS. Marchi reaction product (myelin breakdown) in FALS cases 1–5 was largely restricted to the corticospinal tract region (a) although myelin pallor was present more diffusely in the ventral, lateral and dorsal white matter (b). In cases 3–5 typical ubiquitinated skein inclusions of ALS were present in lower motor neurons (c). In case 5 the dentate granule cells showed ubiquitinated inclusions typical of ALS-dementia (d). Hyaline conglomerate inclusions characterized cases 1 and 2 (SOD1 mutation I113T). These large irregular inclusions are equally reactive using antibodies to nonphosphorylated (e; SMI32) and phophorylated (f; SMI31) neurofilaments, and show only weak diffuse ubiquitin immunoreactivity (g). In contrast to conventional ALS-type inclusions (e.g. 1c above), HC are readily detected by conventional stains e.g. cresyl violet (h) and are argyrophilic. In Palmgren stained sections they can be found in lower motor neurons (i; somatic inclusion and large axonal torpedo), Clarke's nucleus (j), nucleus ambiguus (k), Onuf's nucleus (l) and Betz cells (m). Confocal microscopic analysis of HC (n, o) shows extensive SMI32 immunoreactivity (n; red) associated

5 new cases, of which 2 unrelated patients had a previously described point mutation (I113T). The other 3 had abnormalities either in the 3'UTR or in an intron. Details of the characterization of these genetic abnormalities are presented elsewhere (7) but several points need to be emphasized. Firstly, the significance of the abnormalities requires confirmation: One of the 3'UTR deletions (3'UTR del816TTTC819) was found in 1 of 209 normal control samples screened. Since the control individual carrying the abnormality was 22-years-of-age when sampled we do not know if she is predisposed to the disease later in life. Secondly, if these abnormalities are pathogenic, then they can only operate via a loss of function mechanism since the coding sequences are not affected. Current evidence regarding the more commonly described exonic point mutations supports the concept of a toxic gain of function hypothesis (27). However, it is not established that all mutations act via the same pathogenetic pathway and the possibility remains of toxicity due to loss of function hypothesis (28). It is possible that some abnormalities of the SOD1 gene may act as highly penetrant dominant mutations whilst others are better regarded as 'risk factors' requiring additional predisposing influences to operate before the disease is manifest.

Many features of the clinical phenotype of SOD1-related FALS do not correlate with particular mutations and there can be considerable heterogeneity within families (5, 15). The most consistent correlate is disease duration (i.e. rate of progression) where the known mutations can be grouped into 3 approximate groups (5) using cut-off points of 24 and 60 months duration; the I113T mutation, present in 2 of our cases, can be included in both the rapidly progressive and classical groups. The case of Orrell et al (15) shows that this mutation can also be associated with a very long disease duration indicating that even in autosomal dominant cases there must be other strong modifiers of phenotype (either genetic or environmental).

There is much less data available to correlate specific pathological features of SOD1-related FALS to particular genotypes. Previously reported cases, where sufficient detail was presented, have corresponded to "ALS with posterior column changes." The 5 cases presented here all show posterior column myelin loss predominantly affecting the funiculus cuneatus and most prominent at cervical levels. These changes are consistent with axonal degeneration of long projecting neurons which also occurs in the descending motor fibres in cases with "lateral sclerosis" in the absence of Betz cell loss (cases 1, 2, 3, 5). These abnormalities in the sensory system highlight the multisystem distribution of pathology in FALS. It is also not infrequent in the sporadic case series which we examined. Pathological lesions of spinal somatosensory neurons are not previously described in ALS but were unequivocally demonstrated in the 2 cases with mutation I113T in whom these neurons were shown to contain HC inclusions. These 2 cases also had inclusions in surviving neurons of Clarke's nucleus and in Onuf's nucleus, confirming the presence of similar molecular pathology in these neuronal groups, both of which have previously been shown to be involved in sporadic ALS (29, 30).

An important finding of the present study is variation in the type of neuronal inclusion bodies encountered in the SOD1-related cases. Given that the 2 cases with I113T (cases 1, 2) are unrelated, the similarity in the type of inclusion (HC) and their anatomical distribution is remarkable and contrasts with the other cases (cases 3–5). Morphologically their difference from the "classical" ubiquitinated inclusions of ALS should be emphasized: They are large somatic argyrophilic conglomerates. They are found as irregular multifocal masses occupying a large proportion of the neuronal soma. They are strongly immunoreactive for neurofilament epitopes but not for other cytoskeletal proteins and show only weak, diffuse, granular ubiquitin staining. Electron microscopy also confirms the neurofilamentous nature of these lesions (19). It has been suggested (18) that they arise from abnormal neurofilament phosphorylation, but this conclusion is not supported by the current observations. In the study of Rouleau et al. (18) the only neurofilament antibody used was directed at phosphorylated epitopes with no attempt to demonstrate nonphosphorylated neurofilaments. The antibodies used in the present study (SMI31 and SMI32), and in the 2 cases of A4V-related ALS (Table 5) reported by Chou (16), recognize phosphorylated and nonphosphorylated residues respectively within the same epitope and clearly demonstrate that the formation of HC is not phosphorylation dependent. Stronger staining was consistently present with SMI32 and these lesions probably represent partial collapse or condensation of the normal somatic framework of nonphosphorylated neurofilaments into disorganized aggregates. The abnormal phosphorylation observed could then arise, together with ubiquitination, as a secondary phenomenon due to cytoskeletal dysfunction. In the previously reported cases with SOD1 gene mutations in Table 5, there are 4 of 7

←

with low level immunoreactivity for ubiquitin (o; green). In contrast a compact ALS-type inclusion illustrated from a sporadic case (p, q) is strongly reactive for ubiquitin (q; green) with peripheral low level staining for SMI32 (p; red). The neurons in these confocal images contain small (n, o) and large (p, q) areas of lipofuscin autofluorescence in both channels conforming to the yellow/orange regions in o and q. Scale bars: a = 0.5 mm; b = 1 mm; c–i = 40 μm; j = 100 μm; k–q = 40 μm.

TABLE 5
Morphology and Molecular Pathology (Immunocytochemistry) of Neuronal Inclusions in Previously Reported Cases of *SOD1*-related FALS

SOD1 mutation	Age (y) at onset	Disease duration (m)	Inclusion type	Location	Ubiquitin ICC	Neurofilament ICC (antibody)	Tau ICC	Argyrophilia	Case or ref
I113T	63	24	HC	LMN (others ns)	ns	+++ (ns)	ns	+++	18
I113T	48	252	"tangles"	LMN	−	−(BF10)	−	−	15
				Globus pallidus	−	−	+	+++	
				Substantia nigra	−	−	+	+++	
				Locus ceruleus	−	−	+	+++	
				Inferior olivary n.	−	−	+	+++	
E100G	37	38	ALS	LMN, DGC	+++	−	−	−	17
A4T	40	9	?ALS	LMN med. ret. form.	+++	halo(SMI31)	ns	−	20
A4V	50	ns	HC	LMN	+	+++(SMI31) +++(SMI32)	ns	ns	16
	60	ns	HC	LMN	+	+++(SMI31)	ns	ns	11
					+	+++(SMI31) +++(SMI32)			
H48Q	54	9	HC	LMN/UMN	+	+++(RT97; mAb147)	−	+++	19
			ALS	hypoglossal	+++	−			

HC = hyaline conglomerates; ALS = typical ubiquitinated inclusions; LMN = lower motor neurons; ns = not stated in paper; DGC = dentate granule cells; UMN = upper motor neurons; BF10; RT97; mAb147 = anti-neurofilament monoclonal antibodies; ICC = immunocytochemistry; med. ret. form. = medullary reticular formation; frequency/prominence of lesions described as: frequent = +++, infrequent = +.

cases with HC inclusions. These lesions contrast markedly with the ubiquitinated inclusions regarded as typical of ALS (31, 32) which comprise variable skeins and Lewy body-like structures, including intermediate forms, in which the underlying protein substrate for ubiquitination has not been identified. In our careful study of ubiquitin/neurofilament colocalisation we were only able to demonstrate weak peripheral reactivity to a polyclonal anti-neurofilament antibody in occasional dense Lewy body-like inclusions. No "ubiquitinated" inclusions were ever reactive to SMI31 or SMI32. This type of inclusion is much more common in sporadic ALS as a proportion of all patients. In the present study ubiquitinated inclusions characterized 70 of 73 sporadic cases of ALS and none of the cases had HC. In the 12 cases related to SOD1 gene abnormalities in Tables 4 and 5 these inclusions were present in half. The molecular pathology of these 2 types of inclusion body therefore seems to be separate. In only 1 case (case 19, Table 5) HC inclusions were present in spinal motor neurons (and some cerebral neurons) but typical ALS-type ubiquitinated inclusions were present in the hypoglossal nucleus.

The significance of these distinctive lesions is uncertain. Data from this and other studies does not support the hypothesis that the formation of HC may be a function of rapid progression. An alternative hypothesis is that the type of inclusion body relates to the particular gene mutation in SOD1. In this respect the I113T mutation is especially interesting because it can be associated both with HC and with atypical "tangle" pathology (15). This strongly indicates that other genetic or environmental modifiers affect pathological phenotype, even when the disease is "caused" by a specific mutation in the SOD1 gene.

Cytoskeletal abnormalities are central to the pathogenesis of neuronal and axonal degeneration in ALS. If HC inclusions and ubiqutinated inclusions are markers of different pathogenetic pathways, then it is likely that there are differences in the biochemical lesions associated with the various SOD1 gene mutations now characterized. The current observations need to be confirmed and extended in larger series of familial and SOD1-linked cases.

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