Molecular analysis and biochemical classification of TDP-43 proteinopathy

Hiroshi Tsuji,1,2 Tetsuaki Arai,3,4 Fuyuki Kametani,1 Takashi Nonaka,1 Makiko Yamashita,1 Masami Suzukake,1 Masato Hosokawa,3 Mari Yoshida,5 Hiroyuki Hatsuta,6 Masaki Takao,6 Yuko Saito,7 Shigeo Murayama,6 Haruhiko Akiyama,3 Masato Hasegawa,1 David M. A. Mann8 and Akira Tamaoka2

1 Department of Neuropathology and Cell Biology, Tokyo Metropolitan Institute of Medical Science, Tokyo 156-8585, Japan
2 Department of Neurology, Graduate School of Comprehensive Human Sciences, University of Tsukuba, Tsukuba-shi 305-8576, Japan
3 Department of Dementia and Higher Brain Function, Tokyo Metropolitan Institute of Medical Science, Tokyo 156-8585, Japan
4 Department of Psychiatry, Graduate School of Comprehensive Human Sciences, University of Tsukuba, Tsukuba-shi 305-8576, Japan
5 Department of Neuropathology, Institute for Medical Science of Aging, Aichi Medical University, Aichi 480-1195, Japan
6 Department of Neuropathology, Tokyo Metropolitan Institute of Gerontology, Tokyo 173-0015, Japan
7 Department of Pathology and Laboratory Medicine, National Center Hospital of Neurology and Psychiatry, Tokyo 187-8551, Japan
8 Mental Health and Neurodegeneration Research Group, Greater Manchester Neuroscience Centre, University of Manchester, Manchester M13 9PT, UK

Correspondence to: Masato Hasegawa,
Department of Neuropathology and Cell Biology,
Tokyo Metropolitan Institute of Medical Science,
2-1-6 Kamikitazawa,
Setagaya-ku,
Tokyo 156-8506,
Japan
E-mail: hasegawa-ms@igakuken.or.jp

Amyotrophic lateral sclerosis and frontotemporal lobar degeneration with TAR DNA-binding protein of 43 kDa pathology are progressive neurodegenerative diseases that are characterized by intracytoplasmic aggregates of hyperphosphorylated TAR DNA-binding protein of 43 kDa. These TAR DNA-binding protein 43 proteinopathies can be classified into subtypes, which are closely correlated with clinicopathological phenotypes, although the differences in the molecular species of TAR DNA-binding protein 43 in these diseases and the biological significance thereof, remain to be clarified. Here, we have shown that although the banding patterns of abnormally phosphorylated C-terminal fragments of TAR DNA-binding protein 43 differ between the neuropathological subtypes, these are indistinguishable between multiple brain regions and spinal cord in individual patients. Immunoblot analysis of protease-resistant TAR DNA-binding protein 43 demonstrated that the fragment patterns represent different conformations of TAR DNA-binding protein 43 molecular species in the diseases. These results suggest a new clinicopathological classification of TAR DNA-binding protein 43 proteinopathies based on their molecular properties.

Keywords: amyotrophic lateral sclerosis; frontotemporal lobar degeneration; TDP-43; classification

Abbreviations: ALS = amyotrophic lateral sclerosis; FTLD = frontotemporal lobar degeneration; FTLD-TDP = frontotemporal lobar degeneration with TAR DNA-binding protein of 43 kDa pathology; TDP-43 = TAR DNA-binding protein of 43 kDa

© The Author (2012). Published by Oxford University Press on behalf of the Guarantors of Brain. All rights reserved.
For Permissions, please email: journals.permissions@oup.com
Introduction

Amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration with TDP-43 pathology (FTLD-TDP) are sporadic and familial neurodegenerative diseases characterized neuropathologically by intracytoplasmic aggregates of TAR DNA-binding protein of 43 kDa (TDP-43) (Arai et al., 2006; Neumann et al., 2006). In ALS, upper and lower motor neurons progressively degenerate. Neuropathologically, the TDP-43-positive structures appear as rounded or skein-like inclusions in the lower motor neurons. Similar TDP-43-positive inclusions are also observed in the prefrontal gyrus that contains the upper motor neurons. Moreover, TDP-43-positive glial cytoplasmic inclusions are found close to the upper and lower motor neurons in ALS (Tan et al., 2007). In FTLD-TDP, TDP-43 pathology is distinguished into four histological subtypes (types A–D) based on the predominant type of TDP-43-positive structures present (Mackenzie et al., 2011). Type A is characterized by numerous short dystrophic neurites and crescentic or oval neuronal cytoplasmic inclusions; type B has moderate numbers of neuronal cytoplasmic inclusions, throughout all cortical layers, but few dystrophic neurites; type C has a predominance of elongated dystrophic neurites in upper cortical layers, with few neuronal cytoplasmic inclusions; and type D refers to the pathology associated with inclusion body myopathy with early onset Paget disease and frontotemporal dementia caused by VCP mutations, characterized by numerous short dystrophic neurites and frequent lentiform neuronal intranuclear inclusions. There is a relationship between subtypes of TDP-43 pathology and clinical phenotype, and many cases of ALS and frontotemporal lobar degeneration (FTLD) are readily distinguished by each clinical symptom. However, some cases have symptoms of both ALS and FTLD. ALS with dementia refers to cases initially presenting with motor neuron disease becoming demented, whereas FTLD-motor neuron disease refers to cases presenting with cognitive impairment and subsequently developing motor neuron disease.

TDP-43 pathology is also present in a subset of familial ALS and FTLD due to mutations in TARDBP (Kabashi et al., 2008; Sreedharan et al., 2008), progranulin (GRN; Baker et al., 2006) and C9ORF72 (DeJesus-Hernandez et al., 2011; Renton et al., 2011) genes. Although most patients with mutations in TARDBP present with ALS, some present with FTLD (Gitcho et al., 2009; Kovacs et al., 2009). Cases with FTLD-TDP with GRN mutation often show type A pathology (Mackenzie et al., 2006b; Cairns et al., 2007b; Josephs et al., 2007). The pathology of ALS and FTLD due to mutations in C9ORF72 is heterogeneous: TDP-43 pathology overlaps between ALS and FTLD-TDP types A and B (Murray et al., 2011). One large multicentre study of sporadic and familial FTLD-TDP showed broad overlap between the TDP-43 subtyping, especially between types A and B (Armstrong et al., 2010). These overlaps might occur because current pathological classification may be inadequate, as it is based solely on the morphological assessment of certain subjective cortical regions. A more objective and unbiased classification is needed.

In this study, we have investigated a wide range of patients with various TDP-43 proteinopathies to investigate whether patterns of protease-resistant TDP-43 might indicate different TDP-43 strain types, and characterize the TDP-43 C-terminal banding patterns in multiple regions of the CNS, basing our approach on the method used for demonstration of prion strain variation and the aetiology of new variant Creutzfeldt-Jakob disease (Collinge et al., 1996). We show at least three C-terminal banding patterns that distinguish diseases with TDP-43 proteinopathy and report that the banding pattern in individual patients is indistinguishable in different brain regions and spinal cord. Corresponding patterns of protease-resistant phosphorylated TDP-43 are also seen between the pathological phenotypes. As with the prion diseases, the present results suggest that the different conformation of abnormal TDP-43 deposits in the CNS in patients corresponding with various subtypes of TDP-43 proteinopathy, and that the conformation state of the abnormal TDP-43 protein may determine the pathological phenotype.

Materials and methods

Patients

Human brain tissues were obtained from the Brain Donation Programme at the University of Tsukuba (Japan), Tokyo Metropolitan Institute of Gerontology (Japan), National Shimofusa Mental Hospital (Japan) and the University of Manchester (UK). This study was approved by the local Research Ethics Committee. The subjects in this study included eight patients with ALS, five patients with FTLD-TDP type A, eight patients with FTLD-TDP type B, six patients with FTLD-TDP type C and two patients with Alzheimer’s disease without TDP-43 pathology. All cases with ALS met the revised El Escorial criteria for ALS (Brooks, 1994) without dementia. All cases with FTLD-TDP fulfilled clinical diagnostic criteria of FTLD (Neary et al., 1998), and classifications of TDP-43 subtype were made in accordance with published guidelines (Cairns et al., 2007a; Mackenzie et al., 2011). Four patients with FTLD-TDP type A were cases of familial FTLD-U with GRN mutations. One familial ALS case, one with type A, and two with type B had the GGGCC repeat expansion in C9ORF72. The age, gender, brain regions examined and clinical diagnosis are given in Table 1.

A fresh frozen tissue sample was taken and cut into two pieces. One piece was fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 days and was used for immunohistochemical analysis. The other piece was homogenized and used for immunoblot analysis. In principle, we took the precentral gyrus and lumbar part of the spinal cord in the ALS cases, and the frontal lobe in the FTLD-TDP cases, because TDP-43 pathology is always known to be prevalent in these regions (Tan et al., 2007; Geser et al., 2008, 2009). However, the spinal cord was not available in four cases with ALS, and both motor regions in two cases were not available. In these cases, the frontal lobe was examined instead. For ALS Cases 1, 3, 5 and ALS and FTLD-TDP type C Case 22, the whole of the cerebral hemisphere and brainstem were available as fresh frozen tissues. In these four cases, we took the multiple regions, as described in Table 1. Every tissue sample was examined immunohistochemically for TDP-43-positive lesions. All samples, except some from the cerebellar cortex, showed an accumulation of abnormal TDP-43-positive structures.

Immunoblotting

Sarkosyl-insoluble, urea-soluble fractions were extracted from each region as previously described (Arai et al., 2006; Hasegawa et al., 2008).
The samples were loaded on 15% SDS–PAGE gels. Proteins in the gel were then transferred onto a polyvinylidene difluoride membrane (Millipore). After blocking with 3% gelatine in 0.01 M PBS (pH 7.4), membranes were incubated overnight with phosphorylation dependent anti-TDP-43 rabbit polyclonal antibody (pS409/410, 1:1000; Hasegawa et al., 2008), phosphorylation independent TDP-43 polyclonal antibody 10782-1-AP (TDP-43 pAb, 1:3000) and TDP-43 monoclonal antibody, 60019-2-Ig (TDP-43 mAb, 1:3000) (ProteinTech Group). After incubation with the appropriate biotinylated secondary antibody, immunolabelling was detected using the VECTASTAIN® ABC system (Vector Laboratories) coupled with a 3,3′-diaminobenzidine reaction intensified with nickel chloride. The blot membranes were digitally analysed, and densitometric analyses were performed with ImageJ version 1.44p (NIH, http://rsbweb.nih.gov/ij/index.html). The densitometry data were averaged for all cases in each group to illustrate the different patterns.

### Immunohistochemistry

After cryoprotection in 15% sucrose in 0.01M PBS (pH 7.4), paraformaldehyde-fixed tissue blocks were cut on a freezing microtome at 30-μm thickness. The free-floating sections were immunostained with phosphorylation-dependent TDP-43 monoclonal antibody (pS409/410, 1:10 000) (Inukai et al., 2008) for 72 h in the cold. After treatment with mouse secondary antibody, immunolabelling was detected using the VECTASTAIN® ABC system coupled with a 3,3′-diaminobenzidine reaction to yield a brown precipitate. Sections were lightly counterstained with haematoxylin.

---

**Table 1 Description of the patients**

<table>
<thead>
<tr>
<th>Case number</th>
<th>Age at death (year)</th>
<th>Age at onset (year)</th>
<th>Sex</th>
<th>Family history</th>
<th>Brain weight (g)</th>
<th>Clinical diagnosis</th>
<th>Region</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>62</td>
<td>61</td>
<td>M</td>
<td>N</td>
<td>1150</td>
<td>ALS</td>
<td>Prec, L and other regions&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>72</td>
<td>71</td>
<td>F</td>
<td>N</td>
<td>1390</td>
<td>ALS</td>
<td>Prec and L</td>
</tr>
<tr>
<td>3</td>
<td>42</td>
<td>40</td>
<td>F</td>
<td>N</td>
<td>1140</td>
<td>ALS</td>
<td>Prec and L</td>
</tr>
<tr>
<td>4</td>
<td>76</td>
<td>75</td>
<td>F</td>
<td>N</td>
<td>1230</td>
<td>ALS</td>
<td>Prec and L</td>
</tr>
<tr>
<td>5</td>
<td>62</td>
<td>54</td>
<td>M</td>
<td>N</td>
<td>1230</td>
<td>ALS</td>
<td>Prec and other regions&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>6</td>
<td>77</td>
<td>76</td>
<td>F</td>
<td>N</td>
<td>1414</td>
<td>ALS</td>
<td>Fr</td>
</tr>
<tr>
<td>7</td>
<td>67</td>
<td>65</td>
<td>M</td>
<td>N</td>
<td>1250</td>
<td>ALS</td>
<td>Fr</td>
</tr>
<tr>
<td>8</td>
<td>55</td>
<td>53</td>
<td>M</td>
<td>Y(mC9ORF72)</td>
<td>1050</td>
<td>FTD</td>
<td>Fr</td>
</tr>
<tr>
<td>FTD-TDP type A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>58</td>
<td>49</td>
<td>M</td>
<td>Y(mC9ORF72)</td>
<td>1210</td>
<td>FTD</td>
<td>Fr</td>
</tr>
<tr>
<td>10</td>
<td>67</td>
<td>54</td>
<td>F</td>
<td>Y(mGRN)</td>
<td>1280</td>
<td>FTD + MND</td>
<td>Fr</td>
</tr>
<tr>
<td>11</td>
<td>71</td>
<td>63</td>
<td>F</td>
<td>Y(mGRN)</td>
<td>1150</td>
<td>FTD + MND</td>
<td>Fr</td>
</tr>
<tr>
<td>12</td>
<td>66</td>
<td>56</td>
<td>F</td>
<td>Y(mGRN)</td>
<td>1210</td>
<td>FTD + MND</td>
<td>Fr</td>
</tr>
<tr>
<td>13</td>
<td>68</td>
<td>60</td>
<td>M</td>
<td>Y(mGRN)</td>
<td>1050</td>
<td>FTD + MND</td>
<td>Fr</td>
</tr>
<tr>
<td>FTD-TDP type B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>45</td>
<td>43</td>
<td>M</td>
<td>N</td>
<td>1260</td>
<td>FTD + MND</td>
<td>Fr</td>
</tr>
<tr>
<td>15</td>
<td>59</td>
<td>57</td>
<td>M</td>
<td>Y(mC9ORF72)</td>
<td>1210</td>
<td>FTD + MND</td>
<td>Fr</td>
</tr>
<tr>
<td>16</td>
<td>67</td>
<td>65</td>
<td>M</td>
<td>Y(mC9ORF72)</td>
<td>1280</td>
<td>FTD + MND</td>
<td>Fr</td>
</tr>
<tr>
<td>17</td>
<td>76</td>
<td>74</td>
<td>M</td>
<td>Y(mC9ORF72)</td>
<td>1150</td>
<td>FTD + MND</td>
<td>Fr</td>
</tr>
<tr>
<td>18</td>
<td>69</td>
<td>58</td>
<td>M</td>
<td>Y(mC9ORF72)</td>
<td>1050</td>
<td>FTD + MND</td>
<td>Fr</td>
</tr>
<tr>
<td>19</td>
<td>52</td>
<td>50</td>
<td>F</td>
<td>Y(mC9ORF72)</td>
<td>1210</td>
<td>FTD + MND</td>
<td>Fr</td>
</tr>
<tr>
<td>20</td>
<td>65</td>
<td>61</td>
<td>M</td>
<td>Y(mC9ORF72)</td>
<td>1530</td>
<td>FTD + MND</td>
<td>Fr</td>
</tr>
<tr>
<td>21</td>
<td>68</td>
<td>64</td>
<td>M</td>
<td>Y(mC9ORF72)</td>
<td>1213</td>
<td>FTD + MND</td>
<td>Fr</td>
</tr>
<tr>
<td>FTD-TDP type C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>82</td>
<td>NA</td>
<td>M</td>
<td>N</td>
<td>1200</td>
<td>SD</td>
<td>Fr, Te and other regions&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>23</td>
<td>67</td>
<td>65</td>
<td>M</td>
<td>N</td>
<td>1200</td>
<td>SD</td>
<td>Fr</td>
</tr>
<tr>
<td>24</td>
<td>59</td>
<td>53</td>
<td>M</td>
<td>N</td>
<td>1200</td>
<td>SD</td>
<td>Fr</td>
</tr>
<tr>
<td>25</td>
<td>63</td>
<td>58</td>
<td>M</td>
<td>N</td>
<td>1200</td>
<td>SD</td>
<td>Fr</td>
</tr>
<tr>
<td>26</td>
<td>66</td>
<td>55</td>
<td>F</td>
<td>N</td>
<td>1035</td>
<td>SD</td>
<td>Fr</td>
</tr>
<tr>
<td>27</td>
<td>75</td>
<td>60</td>
<td>M</td>
<td>N</td>
<td>1174</td>
<td>SD</td>
<td>Fr</td>
</tr>
<tr>
<td>AD</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>65</td>
<td>56</td>
<td>F</td>
<td>N</td>
<td>1165</td>
<td>AD</td>
<td>Fr</td>
</tr>
<tr>
<td>29</td>
<td>70</td>
<td>NA</td>
<td>F</td>
<td>N</td>
<td>1126</td>
<td>AD</td>
<td>Fr</td>
</tr>
</tbody>
</table>

<sup>a</sup> Other regions contained striatum, thalamus, hippocampus dentate gyrus, substantia nigra, pons, medulla and cerebellum cortex. In these cases, the grey and white matter of precentral gyrus were separated from each other macroscopically and examined.

<sup>b</sup> Other regions contain striatum, thalamus, hippocampus dentate gyrus, substantia nigra, pons, medulla and cerebellum cortex. FTLD-TDP type B without MND and type D are not analysed in this study.

AD = Alzheimer’s disease; Fr = frontal cortex; FTD = frontotemporal dementia; L = lumbar part of spinal cord; mC9ORF72 = mutation of chromosome 9 open-reading frame 72 gene; mGRN = mutation of progranulin gene; MND = motor neuron disease; NA = not available; PNFA = progressive non-fluent aphasia; Prec = precentral gyrus; SD = semantic dementia; Y = yes; N = no.
Protease treatment of phosphorylated TDP-43

Sarkosyl-insoluble fractions extracted from the neocortical regions of patients with ALS or FTLD-TDP were treated with final concentration of 100 μg/ml trypsin (Promega) or 10 μg/ml chymotrypsin (Sigma-Aldrich) at 37°C for 30 min. The reaction was stopped by boiling for 5 min. After centrifuging at 15,000 rpm for 1 min, the samples were analysed by immunoblotting as described earlier.

Mass spectrometry

Sarkosyl-insoluble, trypsin-resistant fractions were loaded on 15% SDS–PAGE gels. The pS409/410-positive ~16kDa bands were dissected and digested in-gel with chymotrypsin. The digests were applied to the Paradigm MS4 high-performance liquid chromatography system (Microm BioResources). A reversed phase capillary column (Develosil ODS-HG5, 0.075 × 150 mm, Nomura Chemical) was used at a flow rate of 300 n/L/min with a 4–80% linear gradient of acetonitrile in 0.1% formic acid. Eluted peptides were directly detected with an ion trap mass spectrometer, LXQ (Thermo Fisher Scientific). The obtained spectra were analysed with Mascot (Matrix Science).

Statistical analysis

The P-values for the description of the statistical significance of differences were calculated by means of the paired, two-tailed t-test using Prism 5.04 software (GraphPad Software, Inc).

Results

Banding patterns of phosphorylated C-terminal TDP-43 in ALS and FTLD with TDP-43 pathology

Immunoblot analysis using an antibody specific for abnormal TDP-43, pS409/410, showed high-molecular-weight smearing substances, phosphorylated full-length TDP-43 at 45 kDa and several C-terminal fragments at 18–26 kDa to be present in affected brain regions in all cases (Fig. 1). Three major bands at 23, 24 and 26 kDa, and two minor bands at 18 and 19 kDa were seen in the precentral gyrus and frontal cortex of cases with ALS, while the 24 kDa band being the most intense (Fig. 1A and F). In the lumbar spinal cord, the two minor bands at 18 and 19 kDa were barely present, but the banding pattern of the three major bands at 23, 24 and 26 kDa was similar to that in the cerebral cortex (Fig. 1A). No such pS409/410-positive TDP-43 bands were detected in control cases with Alzheimer’s disease with no TDP-43 pathology (Fig. 1B). In the FTLD-TDP cases, the banding pattern could be distinguished into three types according to the FTLD-TDP histological subtype (Fig. 1C–E). In FTLD-TDP type A, three major bands at 23, 24 and 26 kDa, and two minor bands at 18 and 19 kDa were detected, with the 23 kDa band being the most intense (Fig. 1C and F). In FTLD-TDP type B cases, the banding pattern was the same as that in the ALS cases (Fig. 1D and F). In FTLD-TDP type C cases, two major bands at 23 and 24 kDa, and two minor bands at 18 and 19 kDa were detected, with the 24 kDa band being the most intense (Fig. 1E and F). Densitometric analyses of the immunoblots for all cases are shown in Supplementary Fig. 1. Each component of the C-terminal fragments was significantly different (Fig. 1F).

Immunoblot analysis using phosphorylation independent TDP-43 polyclonal and monoclonal antibodies detected phosphorylated full-length TDP-43 at 45 kDa, two bands ~25 kDa and high-molecular-weight smears, in addition to the normal TDP-43 band at 43 kDa in ALS and various subtypes of FTLD-TDP. The banding patterns between ALS and various subtypes of FTLD-TDP could not be distinguished with these antibodies. In the cases with Alzheimer’s disease, the normal TDP-43 band at 43 kDa was detected, but neither the phosphorylated 45 kDa band nor the ~25 kDa fragments were observed (Supplementary Fig. 2). Immunoblot analysis of α-tubulin in Tris saline-soluble fractions from cases with types A, B and C pathology showed no correlation between the banding pattern of α-tubulin and that of TDP-43 (Supplementary Fig. 3), indicating that the differences in the banding patterns are not because of protein degradation caused by a long post-mortem interval or unfavourable agonal status.

Immunohistochemistry and immunoblot analyses of phosphorylated TDP-43 in multiple regions of ALS and FTLD with TDP-43 pathology

In ALS cases, the neuronal cytoplasmic pathology, which included skein-like inclusions, irregularly shaped TDP-immunoreactive neuronal cytoplasmic inclusions and densely staining granules, was confirmed in multiple regions by immunohistochemistry analysis using pS409/410 (Fig. 2A–G). Glial cytoplasmic inclusions were also present in many regions. Glial cytoplasmic inclusions were more frequent in the white matter than in the grey matter (Fig. 2H). A few neuronal cytoplasmic inclusions were found in the cerebellar cortex granule cells (Fig. 2G). In FTLD-TDP type C, dystrophic neurites were seen in multiple regions except for the cerebellar cortex (Fig. 2I–L), whereas neuronal cytoplasmic inclusions were also present in the striatum and hippocampus dentate gyrus granule cells (Fig. 2J and L). No abnormal structures were found in the cerebellar cortex (data not shown). These observations show that pathological TDP-43 is present throughout many CNS areas in ALS, suggesting that ALS does not selectively affect only the motor system, but it is rather a multisystem neurodegenerative TDP-43 proteinopathy.

Immunoblot analyses of three ALS cases confirmed that phosphorylated TDP-43 and the C-terminal fragments are deposited in multiple brain regions in ALS (Fig. 3A). Relatively strong immunoreactivities were detected in the striatum (in Cases 3 and 5) and substantia nigra (in Cases 1 and 5), although this varied between cases (Fig. 3A). Importantly, the banding pattern for the TDP-43 C-terminal fragments in these three cases was basically the same in all brain regions examined (Fig. 3A). In FTLD-TDP type C, a C-terminal banding pattern, clearly distinct from that
Figure 1 Immunoblot analyses of sarkosyl-insoluble TDP-43 in the brains or spinal cords of ALS (Cases 1–8) (A), Alzheimer’s disease (Cases 28–29) (B), FTLD-TDP type A (Cases 9–13) (C), FTLD-TDP type B (Cases 14–21) (D) and FTLD-TDP type C (Cases 22–27) (E), using a phosphorylation-dependent anti-TDP-43 antibody (pS409/410). In all cases, high-molecular-weight smearing substances, phosphorylated full-length TDP-43 at 45 kDa and several C-terminal fragments at 18–26 kDa are detected. In ALS (A) and FTLD-TDP type B (D) cases, three major bands at 23, 24 and 26 kDa and two minor bands at 18 and 19 kDa are detected, whereas in the FTLD-TDP Type C (E) cases, two major bands at 23 and 24 and two minor bands at 18 and 19 kDa. A 24 kDa band is the most intense in ALS (A) and FTLD-TDP type B (D), whereas a 23 kDa band is the most intense in FTLD-TDP type C (D). The band pattern of the cases with type A (C) is an intermediate between FTLD-TDP type B (D) and FTLD-TDP type C (E). In spinal cords of cases with ALS, the 18 and 19 kDa bands are hardly detectable, but the same banding pattern of the 23–26 kDa bands as in precentral gyrus is detected. No such TDP-43 fragments are detected in brains of patients with Alzheimer’s disease (AD) (B). The intensity of each C-terminal band was analysed using the ImageJ software and each component was statistically analysed by Student’s t-test (F). Data indicate mean (SEM). ****P < 0.0001, ***P < 0.001, **P < 0.01, *P < 0.05. F = frontal cortex; L = lumbar part of spinal cord; P = precentral cortex.
of ALS, was detected in the temporal cortex, striatum and hippocampus, but was barely detected in the thalamus, substantia nigra, pons and medulla, and not at all in the cerebellar cortex (Fig. 3B). The banding pattern observed in these brain regions was indistinguishable (Fig. 3B). These results suggest that the same abnormal TDP-43 molecular species is deposited in different brain regions and different cell types, although the morphology of the TDP-43 inclusions may be different in the brain regions. Densitometric analyses of the immunoblots for all cases are shown in Supplementary Fig. 4.
Protease-resistant TDP-43 in ALS and FTLD with frontotemporal dementia-43 pathology

These different banding patterns in TDP-43 proteinopathies may represent different conformations of abnormal TDP-43 or their aggregates. To test this hypothesis, we subjected the abnormal TDP-43 recovered in the sarkosyl-insoluble pellets to protease treatment and analysed the protease-resistant bands. Proteins can be easily cleaved by proteases if they are denatured or unstructured, but domains that have rigid structures, such as a β-sheet conformation or that are structurally buried or interacting with other molecules, are highly resistant to proteases. On trypsin or chymotrypsin treatment, the full-length 45-kDa band and the smearing substance of TDP-43 disappeared, leaving protease-resistant fragments at 16–25 kDa (Figs 4 and 5). As expected, the protease-resistant banding patterns were different and distinguishable into three patterns (Figs 4 and 5). In ALS, trypsin-resistant doublet bands at 16 and 15 kDa, and two minor bands at ~24 kDa were detected, whereas a single band at 16 kDa and some additional bands at ~24 kDa were detected in FTLD-TDP type A (Fig. 4A, Lanes 1 and 2). In FTLD-TDP type B, the same banding pattern as that in ALS was observed (Fig. 4A, Lane 3). In FTLD-TDP type C, a broad single band at 16 kDa and some additional bands at ~24 kDa were detected (Fig. 4A, Lane 4). No such bands were detected in Alzheimer’s disease (Fig. 4A, Lane 5).

Similarly, on chymotrypsin treatment, multiple protease-resistant bands were detected at 16–25 kDa and the chymotrypsin-resistant band patterns were also different between the three disease subtypes (Fig. 4B). Doublet bands were seen in ALS and FTLD-TDP type B, but only a single band in FTLD-TDP type C was detected at ~16 kDa (Fig. 4B). In FTLD-TDP type A, the lower band (15 kDa) of the ~16 kDa doublet was more intense than the upper one (16 kDa).

Figure 3 Immunoblot analyses of the C-terminal fragments of phosphorylated TDP-43 in the different brain regions of cases with ALS (Cases 1, 3 and 5, as shown in Fig. 1) (A) and FTLD-type C (Case 22, as shown in Fig. 1) (B). (A) Immunoblots of insoluble TDP-43 in the grey or white matter of precentral cortex, striatum, thalamus, hippocampus, substantia nigra, pons and medulla of ALS cases. (B) Immunoblot of TDP-43 in temporal cortex, striatum, hippocampus, thalamus, substantia nigra, pons and cerebellar cortex of the case with FTLD-TDP type C. Ce = cerebellar cortex; Gr = grey matter of precentral gyrus; Hip = hippocampus; M = medulla; Po = pons; Sn = substantia nigra; St = striatum; Tha = thalamus; Te = temporal cortex; Wt = white matter of precentral gyrus. Immunoblots of spinal cords of cases with ALS are shown in Fig. 1.

Figure 4 Immunoblot analysis of phosphorylated TDP-43 from representative ALS and FTLD-TDP cases after protease treatment. (A) Immunoblot of insoluble TDP-43 from cases with ALS, FTLD-TDP type A, type B, type C and Alzheimer’s disease (AD) after trypsin treatment. Doublet bands at ~16 kDa (arrow) and some minor 23–24 kDa bands are detected in ALS and FTLD-TDP type B, whereas a single band at ~16 kDa and several bands at 23 and 24 kDa are detected in FTLD-TDP type A and type C. No such bands are detected in the Alzheimer’s disease case. (B) Immunoblot of insoluble TDP-43 from cases with ALS, FTLD-TDP type A, type B, type C and Alzheimer’s disease after chymotrypsin treatment. Multiple protease-resistant TDP-43 bands are detected at 16–25 kDa. Doublet bands at ~16 kDa (arrow) are detected in ALS and FTLD-TDP type A and B, whereas a single band at ~16 kDa (arrow) is detected in the case with FTLD-TDP type C. In FTLD-TDP type A, the lower band of the doublet at 16 kDa is more intense. No such bands are detected in the Alzheimer’s disease case.
In all cases examined, the trypsin-resistant banding patterns were clearly distinguishable between the disease subtypes in accordance with the three different types of banding pattern of TDP-43 C-terminal fragments, although it is difficult to distinguish the trypsin band pattern of type A from that of type C (Figs 5A, 6A and Supplementary Fig. 5). The chymotrypsin-resistant banding patterns were distinguishable and could be differentiated into three types (Figs 5B, 6B and Supplementary Fig. 6), also in accordance with the banding pattern of the TDP-43 C-terminal fragment. The banding patterns of ALS and FTLD-TDP type B were the same, whereas the banding pattern of FTLD-TDP type A was distinguishable from those of type C and type B (Figs 4 and 5). The combination analyses of trypsin and chymotrypsin-resistant banding patterns confirmed that TDP-43 proteinopathies can also be biochemically distinguishable into three types according to TDP-43 subtypes. These results strongly suggest that the different C-terminal banding patterns represent different conformations of TDP-43 aggregates and that the distinct types of TDP-43 are deposited in association with distinct pathological phenotypes of TDP-43 proteinopathies.

Immunoblot analysis using phosphorylation independent TDP-43 polyclonal and monoclonal antibodies detected some TDP-43 fragments in the ALS and FTLD-TDP cases after trypsin or chymotrypsin treatment, although no clear difference was observed in the banding patterns between ALS and other subtypes of FTLD-TDP (Supplementary Fig. 7). The distinctive
protease-resistant bands at ~16 kDa of ALS were not detected with both phosphorylation independent antibodies (Supplementary Fig. 8).

We also analysed the banding pattern of phosphorylated TDP-43 in another series of five sporadic cases with TDP-43 path-ology (Alzheimer’s disease, Alzheimer’s disease/dementia with Lewy bodies and Alzheimer’s disease/argyrophilic grain disease) (Supplementary Table 1). The banding pattern of the C-terminal fragments, and trypsin- or chymotrypsin-resistant fragments, in these were same as those of FTLD-TDP type A with GRN mutation (Supplementary Fig. 9).

**Mass spectrometric analysis of protease-resistant bands of TDP-43 in ALS and FTLD-TDP type C**

To further investigate the differences in the abnormal TDP-43 protein species at a molecular level, we analysed the ~16 kDa trypsin-resistant bands by mass spectrometry. Mass analysis of chymotrypsin digests of ~16 kDa trypsin-resistant fragments identified 4 peptides, amino acid residues 277–289, 290–299, 294–333 and 300–316, suggesting these peptides are derived from trypsin-resistant fragments 276–414 and 294–414. Mass spectrometric analysis of the single broad band from FTLD-TDP type C identified the peptides of amino acids 273–283, 277–289, 290–313 and 317–330, strongly suggesting that the trypsin-resistant fragments from FTLD-TDP type C are derived from peptides 273–414 and 276–414. These analyses clearly indicate that trypsin-resistant core regions of the abnormal TDP-43 accumulated in the brain are not necessarily the same between ALS and FTLD (Supplementary Fig. 10).

**Discussion**

In this study, we have shown that the banding patterns for TDP-43 C-terminal fragments in ALS and FTLD are distinguishable and classifiable into at least three types. This difference was consistently demonstrated in 27 cases, eight with ALS, five with FTLD-TDP type A, eight with FTLD-TDP type B and six with FTLD-TDP type C. These results strongly suggest that distinct
types of TDP-43 molecules constitute the distinct types of pathologies of TDP-43 and determine the clinicopathological phenotypes of TDP-43 proteinopathies. In TDP-43 histopathology, ALS is considered to represent a distinct pathological subtype because the distribution of TDP-43 inclusions is different from that of FTLD-TDP (Mackenzie et al., 2006a). However, as shown in this study, the TDP-43 accumulations in ALS and FTLD-TDP type B are biochemically indistinguishable. In fact, clinical and histopathological motor neuron disease is often present in cases with FTLD-TDP type B histology. In the three types of phosphorylated C-terminal TDP-43 banding pattern, the pattern seen in FTLD-TDP type C is the most distinctive, lacking the 26 kDa band detected in ALS, FTLD-TDP type A and type B cases (Fig. 1). The clinical diagnosis of the FTLD-TDP type C cases was semantic dementia in every instance, consistent with other studies showing this type of histology to be associated with semantic dementia (Mackenzie et al., 2006a). FTLD is clinically classified into frontotemporal dementia, demantic dementia and progressive non-fluent aphasia, based on topographical distributions of degeneration (Neary et al., 1998). In frontotemporal dementia, the bilateral frontal and temporal lobes are affected, whereas the bilateral temporal lobes are affected in semantic dementia and the left hemisphere in progressive non-fluent aphasia. Present data showing the most distinctive pattern of abnormal TDP-43 in type C indicate that semantic dementia may be biochemically different from frontotemporal dementia. Similar differences in tau fragment banding patterns have been shown between progressive supranuclear palsy and corticobasal degeneration (Arari et al., 2004). Progressive supranuclear palsy and corticobasal degeneration are neurodegenerative diseases that are characterized by intracytoplasmic aggregates of hyperphosphorylated tau with four microtubule-binding repeats, with distinctive pathological features. Immunoblot analysis of Sarkosyl-insoluble tau demonstrated that a 33 kDa C-terminal fragment of tau band predominated in progressive supranuclear palsy, whereas two closely related bands of ~37 kDa predominated in corticobasal degeneration. The clinicopathological subtypes of these diseases may be explained by different formations of protein aggregates or species of abnormal proteins.

Unfortunately, we were unable to obtain brain tissue samples from patients with FTLD-TDP type D (associated with VCP mutation; Cairns et al., 2007b; Neumann et al., 2007). However, because the deposition of abnormal TDP-43 in this disorder is mostly within neuronal nuclei, it is possible that the conformation of abnormal TDP-43 in FTLD-TDP type D may also differ from that in FTLD-TDP types A–C. Familial ALS and FTLD-TDP cases in which known mutations [GRN (Baker et al., 2006) or C9ORF72 (DeJesus-Hernandez et al., 2011; Renton et al., 2011)] were examined in this study. In FTLD-TDP due to GRN mutations, type A pathology is exclusively seen (Mackenzie et al., 2006b; Cairns et al., 2007b; Josephs et al., 2007). All our cases with FTLD with GRN mutation showed the same C-terminal banding patterns of phosphorylated TDP-43 corresponding to type A histology. Some recent studies describing the clinical and pathological features of cases of FTLD-TDP with hexanucleotide repeat expansions in C9ORF72 reported that many of the ‘pure’ frontotemporal dementia cases had type A pathology, whereas many of the combined frontotemporal dementia and motor neuron disease cases had type B pathology (Murray et al., 2011; Boeve et al., 2012; Hsiung et al., 2012; Mahoney et al., 2012; Simon-Sanchez et al., 2012; Snowden et al., 2012). Present cases with C9ORF72 expansions included one case of ALS, one case of pure frontotemporal dementia with type A pathology, and two cases of frontotemporal dementia with motor neuron disease and type B pathology. The C-terminal banding pattern of these cases with familial ALS and frontotemporal dementia with motor neuron disease was not different from that in the sporadic ALS and FTLD-TDP type B cases, and that of the frontotemporal dementia case was not different from that in the cases with GRN mutation. Therefore, expansions in C9ORF72 do not seem to influence the various types of TDP-43 C-terminal banding pattern or histological type of TDP-43 pathology.

Immunohistochemical studies using TDP-43 antibodies have shown that pathological TDP-43 is present throughout many CNS areas in ALS, suggesting that ALS does not selectively affect only the motor system, but is rather a multisystem neurodegenerative TDP-43 proteinopathy (Geser et al., 2008). We also confirmed this viewpoint, immunohistochemically and biochemically, finding the same disease characteristic C-terminal fragment (banding) patterns of phosphorylated TDP-43 within the cerebral cortex, spinal cord and the other different brain regions in ALS. Although the types of pathological structures or their morphologies detected on immunohistochemistry analysis appeared different, the banding patterns for the C-terminal fragments were the same in all regions examined in three patients with ALS. This was also true for the one case with FTLD-TDP type C, where the same banding pattern of the C-terminal fragments was detected in several different brain regions beyond the frontal cortex (Fig. 3). These results strongly suggest that the same abnormal TDP-43 molecule is deposited in different brain regions in ALS (and probably also in FTLD-TDP type B) and FTLD-TDP type C, although we need to examine whether this is also true for cases with FTLD-TDP type A. Importantly, the extent of the abnormal protein pathology is closely correlated with the disease progression, such as Alzheimer’s disease in tauopathies (Braak and Braak, 1991), and Parkinson’s disease in α-synucleinopathies (Braak et al., 2003; Saito et al., 2003). However, the molecular mechanisms governing different clinicopathological phenotypes of these neurodegenerative diseases and their progression are poorly understood. Recent studies using cellular or animal models have suggested that aggregation-prone proteins, such as tau and α-synuclein, can spread to other cells and brain regions like prion disorders (Claveru et al., 2009; Frost et al., 2009; Nonaka et al., 2010). The spreading of α-synuclein lesions to the grafts is also observed in Parkinson’s disease brains after transplantation (Li et al., 2008). However, it remains to be clarified whether the ‘propagating’ abnormal protein species represents a distinct ‘strain type’ that can be differentiated by molecular criteria in human patients or whether the species are the same in different brain regions.

We have also shown that the banding patterns of protease-resistant fragments of phosphorylated TDP-43 are similarly different in accordance with the banding patterns seen in untreated C-terminal fragments, confirming the direct link between neuro-pathological subtypes and biochemical banding patterns. The mass spectrometric analysis indicated that the protease resistant regions
of abnormal TDP-43 are different between the diseases. As abnormally phosphorylated TDP-43 has been shown to accumulate in a filamentous form in ALS spinal cords (Hasegawa et al., 2008), the filament core regions may be different between the diseases. Protease-resistant bands, and differences in banding patterns, have been reported in the prion diseases, Creutzfeldt–Jakob disease and bovine spongiform encephalopathy (Collinge et al., 1996). Protease-resistant prion protein extracted from cases with new-variant Creutzfeldt–Jakob disease showed a different and characteristic pattern from that in cases with sporadic Creutzfeldt–Jakob disease, with the banding pattern being indistinguishable from that of mice infected with bovine spongiform encephalopathy prion. Protease-treated prion protein species are thought to have different mobilities because of different conformations. These observations in prion disease suggest that the different banding patterns to the abnormal TDP-43 fragments in ALS and FTLD might represent different TDP-43 strains with different conformations.

Recently, TDP-43 pathology has been detected in some cases with Alzheimer’s disease (Arai et al., 2009). We have shown here that the banding patterns of TDP-43 in cases of Alzheimer’s disease with TDP-43 pathology are the same as those in FTLD-TDP type A. These novel observations suggest a biochemical commonality between FTLD and Alzheimer’s disease with respect to TDP-43 pathology.

The results shown in this study also suggest a molecular basis for the clinicopathological classification of TDP-43 proteinopathies, which complements the histological classifications (Mackenzie et al., 2011).

**Funding**

This work was supported by a Grant-in-Aid for Scientific Research (A) (to M.H., 11000624) from Ministry of Education, Culture, Sports, Science and Technology of Japan, and grants from Ministry of Health, Labor and Welfare of Japan (to M.H.).

**Supplementary material**

Supplementary material is available at Brain online.

**References**


