Diffusion tensor imaging analysis of sequential spreading of disease in amyotrophic lateral sclerosis confirms patterns of TDP-43 pathology

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Diffusion tensor imaging can identify amyotrophic lateral sclerosis-associated patterns of brain alterations at the group level. Recently, a neuropathological staging system for amyotrophic lateral sclerosis has shown that amyotrophic lateral sclerosis may disseminate in a sequential regional pattern during four disease stages. The objective of the present study was to apply a new methodological diffusion tensor imaging-based approach to automatically analyse in vivo the fibre tracts that are prone to be involved at each neuropathological stage of amyotrophic lateral sclerosis. Two data samples, consisting of 130 diffusion tensor imaging data sets acquired at 1.5 T from 78 patients with amyotrophic lateral sclerosis and 52 control subjects; and 55 diffusion-tensor imaging data sets at 3.0 T from 33 patients with amyotrophic lateral sclerosis and 22 control subjects, were analysed by a tract of interest-based fibre tracking approach to analyse five tracts that become involved during the course of amyotrophic lateral sclerosis: the corticospinal tract (stage 1); the corticorubral and the corticopontine tracts (stage 2); the corticostriatal pathway (stage 3); the proximal portion of the perforant path (stage 4); and two reference pathways. The statistical analyses of tracts of interest showed differences between patients with amyotrophic lateral sclerosis and control subjects for all tracts. The significance level of the comparisons at the group level was lower, the higher the disease stage with corresponding involved fibre tracts. Both the clinical phenotype as assessed by the amyotrophic lateral sclerosis functional rating scale-revised and disease duration correlated significantly with the resulting staging scheme. In summary, the tract of interest-based technique allowed for individual analysis of predefined tract structures, thus making it possible to image in vivo the disease stages in amyotrophic lateral sclerosis. This approach can be used not only for individual clinical work-up purposes, but enlarges the spectrum of potential non-invasive surrogate markers as a neuroimaging-based read-out for amyotrophic lateral sclerosis studies within a clinical context.

Keywords: amyotrophic lateral sclerosis; diffusion tensor imaging; fractional anisotropy; magnetic resonance imaging; motor neuron disease; staging

Abbreviations: ALS = amyotrophic lateral sclerosis; ALS-FRS-R = ALS functional rating scale-revised; DTI = diffusion tensor imaging; TFAS = tract-wise fractional anisotropy statistics
Introduction

Amyotrophic lateral sclerosis (ALS) is the most frequent adult-onset motor neuron disease and is characterized by rapidly progressive paresis leading to death with a mean survival of ~3 years (Kiernan et al., 2011). Brettschneider et al. (2013) defined four neuropathological stages of ALS based upon the distribution patterns of phosphorylated 43 kDa TAR DNA-binding protein in 76 autopsy cases (Brettschneider et al., 2013). They found that ALS progresses according to a sequential regional pattern. Briefly, stage 1 lesions were observed in the agranular motor cortex, brainstem motor nuclei of cranial nerves V, VII, and XII, and in spinal cord α-motor neurons, with beginning involvement of the prefrontal neocortex (middle frontal gyrus), brainstem reticular formation, precerebellar nuclei, and the red nucleus in stage 2. In stage 3, pathology developed in the prefrontal (gyrus rectus and orbital gyrus), postcentral neocortex, and striatum, followed by changes in anteromedial portions of the temporal lobe, including the hippocampal formation, during stage 4. The task of the current study was the transfer of this ex vivo staging system into the non-invasive in vivo diagnostics of patients with ALS by using MRI-based techniques.

Diffusion tensor imaging (DTI) is recognized as a robust MRI tool for the in vivo analysis of white matter neuronal tracts (Smith et al., 2006) and has been applied recently with success to ALS in cross-sectional (Turner et al., 2010; Müller et al., 2012; Bede et al., 2013; Foerster et al., 2013) and longitudinal studies (Keil et al., 2012). White matter imaging was successfully applied in frontotemporal lobar degeneration (McMillan et al., 2012, 2013) and in the analysis of brain microstructural damage and in corpus callosum involvement in ALS (Filippini et al., 2010; Canu et al., 2011). DTI data derive neural tract directional information on the basis of the local properties of water diffusion, and regional reductions of DTI metrics (such as fractional anisotropy) may be indicative of axonal degeneration (i.e. reduced water diffusion parallel to axonal tracts) as well as to myelin degradation (i.e. increased water diffusion perpendicular to axonal tracts; Le Bihan et al., 2001). Thus, by using DTI, diffusivity in human brain white matter can be non-invasively mapped to quantify (i) the directional dependence by way of fractional anisotropy mapping; and (ii) the reconstruction of fibre tracts by fibre tracking techniques (Mori et al., 2002).

Here, we performed DTI-based fibre tracking, applying a tract of interest-based approach, in a large cohort of patients with ALS and control individuals to automatically analyse major involved white matter pathways corresponding to those seen at autopsy during staging.

Materials and methods

Subjects and clinical characterization

All participating patients provided written informed consent for the MRI protocol according to institutional guidelines. The study was approved by the Ethical Committee of the University of Ulm.

Patients underwent standardized clinical-neurological and routine laboratory examinations. One-hundred and eleven patients with ALS (n = 68 male, n = 43 female, mean age 62 ± 11 years) were included who presented with a sporadic form of clinically definite or probable ALS according to the revised El Escorial diagnostic criteria (Brooks et al., 2000). Seventy-eight patients were in the sub-study in which MRI data were acquired on a 1.5T scanner, the other 33 patients were investigated on a 3.0T scanner. Severity of physical symptoms as measured with the revised ALS functional rating scale (ALS-FRS-R) (Cedarbaum et al., 1999) was in the range of mild to moderate (mean 39.7 ± 7.4; range 17–48); disease duration was 16 ± 14 months. None of the patients with ALS had a history of other neurological or psychiatric disorders or additional conditions.

A normal database consisting of 74 age-matched healthy controls (n = 41 male, n = 33 female, mean age 59 ± 11 years), i.e. 52 control subjects at 1.5T and 22 control subjects at 3.0T, was used for comparison. None had a history of neurological-psychiatric disease or other medical conditions. In patients and controls, vascular brain alterations or inflammatory or neoplastic brain processes had been excluded by conventional MRI.

Magnetic resonance imaging acquisition

Two scanning protocols (A and B) were performed. Seventy-eight patients with ALS and 52 control subjects underwent Protocol A on a 1.5T clinical scanner (Magnetom Symphony, Siemens Medical). The DTI study protocol was identical for the patient and control groups and consisted of 2 × 31 gradient directions, including two b = 0 gradient directions (64 slices, 64 × 64 pixels). The slice thickness was 3.0 mm, in-plane pixel size was 3.3 mm × 3.3 mm. The echo time and repetition time were 28 ms and 3080 ms, respectively; b was 1000 s/mm².

Thirty-three patients with ALS and 22 control subjects underwent Protocol B on a 3.0T head scanner (Allegra, Siemens Medical). The 3.0T DTI study protocol was also identical for both groups and consisted of 49 gradient directions, including one b = 0 gradient direction (52 slices, 96 × 128 pixels; slice thickness was 2.2 mm, in-plane pixel size was 2.2 mm × 2.2 mm). The echo time and repetition time were 85 ms and 7600 ms, respectively; b was 1000 s/mm².

Postprocessing

The DTI analysis software Tensor Imaging and Fiber Tracking (TIFT) (Müller et al., 2007a) was used for postprocessing and statistical analysis. In a first step, non-linear spatial normalization to the Montreal Neurological Institute (MNI) stereotactic standard space (Brett et al., 2002) was performed using study-specific templates (Müller et al., 2007b). Directional information during the normalization process was preserved. For details of the normalization procedure, see Müller et al. (2012).

First, for the comparison with previous studies in which differences between patients with ALS and control subjects along the corticospinal tract had been reported, fractional anisotropy values in two regions of interest localized along the corticospinal tract in both hemispheres were compared (Supplementary Fig. 1).

In the main analysis procedure, averaged DTI data sets were calculated from control data sets (from 1.5T and 3.0T data separately) by arithmetic averaging of the MNI transformed data. In this manner, two averaged DTI data sets, one at 1.5T and one at 3.0T, were calculated while preserving directional information of individual data...
Postprocessing and statistical analysis

The group comparison for single subject results for each of the pathways was performed by t-test for tract of interest-based fractional anisotropy values. Because the corticospinal tract is affected in ALS-stage 1 (Brettschneider et al., 2013) it can be reasoned that all subjects with a clinical diagnosis of ALS should show pathological involvement of the corticospinal tract, i.e. reduced fractional anisotropy values as compared to controls at the group level. Therefore, the tracts of interest in the corticospinal tract were used as reference for defining a threshold for the group separation between patients with ALS and controls by calculation of the Youden-index (i.e. sensitivity + specificity – 1) to obtain a good balance between sensitivity and specificity. The result of a fractional anisotropy threshold of $\mu - 0.47\sigma$ fits to a normal distribution with a probability of 68% of the values being above the threshold.

After z-transformation of tract of interest fractional anisotropy results to the respective fractional anisotropy threshold (defined separately for each tract of interest structure), a staging categorization was performed using the decision algorithmus shown in Supplementary Fig. 2B: from all patients with ALS, those were further analysed who had z-transformed tract of interest fractional anisotropy values < 0, i.e. tract of interest-fractional anisotropy values below the fractional anisotropy-threshold defined for the corticospinal tract (‘ALS-stage 1’). Out of this group, those were defined as ‘ALS-stage 1’ who had z-transformed tract of interest fractional anisotropy values > 0 in the corticospinal and corticorubral tracts, and those were defined as ‘ALS-stage 2’ with z-transformed tract of interest fractional anisotropy values > 0 in the corticostriatal pathway. The remaining individuals were categorized into ‘ALS-stage 3’ or ‘ALS-stage 4’, depending on whether their z-transformed tract of interest fractional anisotropy values were > 0 (or < 0) in the proximal portion of the perforant path. To show that the ‘stages’ defined by imaging correspond to a clinical phenotype, the individual staging data were correlated with the individual ALS-FRS-R values and with disease duration (Pearson correlation).

For additional analysis, whole brain-based spatial statistics was calculated for fractional anisotropy maps by use of techniques as previously published (Müller et al., 2012). In addition to fractional anisotropy analysis, calculation of axial diffusivity, which is related to axonal injury, and calculation of radial diffusivity, which represents myelin degeneration (Song et al., 2003), were performed to complement the results. Thus, axial diffusivity and radial diffusivity maps were calculated by the whole brain-based spatial statistics approach. After smoothing with a Gaussian kernel of full 8 mm width at half-maximum, maps were compared at the group level and false discovery rate (FDR) corrected at $P < 0.05$.

Results

Data analysis was performed both at the group and individual levels. Region of interest analysis (Supplementary Fig. 1) allowed for a separation between patients with ALS and controls with a sensitivity of 78% ($\mu - 0.47\sigma$) and a specificity of 69% in the 1.5 T data set. In the data acquired at 3.0 T, the separation was similar, with a sensitivity of 82% and a specificity of 68%. An improvement in sensitivity and specificity, again with similar results for 1.5 T and 3.0 T data, could be obtained by using TFAS instead.

### Table 1 Seed and target MNI coordinates for fibre tracking

<table>
<thead>
<tr>
<th>Tract</th>
<th>Seed x/y/z, mm</th>
<th>Target x/y/z, mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corticospinal tract</td>
<td>±17/−19/6</td>
<td>±17/−37/63</td>
</tr>
<tr>
<td>Corticopontine tract</td>
<td>±8/−24/−11</td>
<td>±29/21/43</td>
</tr>
<tr>
<td>Corticorubral tract</td>
<td>±5/−23/−4</td>
<td>±28/39/29</td>
</tr>
<tr>
<td>Corticostriatal pathway</td>
<td>±18/6/16</td>
<td>±32/37/35</td>
</tr>
<tr>
<td>Proximal part perforant path</td>
<td>±25/−17/−24</td>
<td>±22/−29/−18</td>
</tr>
<tr>
<td>Corpus callosum</td>
<td>0/−44/16</td>
<td>±23/−90/6</td>
</tr>
<tr>
<td>(area V)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Optic tract</td>
<td>0/−13/−32</td>
<td>±20/−34/−40</td>
</tr>
</tbody>
</table>
of region of interest analysis: for the 1.5 T data, a sensitivity of 79% and a specificity of 71%, for the 3.0 T data a sensitivity of 79% and a specificity of 73% were obtained. The Youden index for fractional anisotropy group separation in the corticospinal tract showed a plateau around 0.37 for 1.5 T fractional anisotropy thresholds and for 3.0 T fractional anisotropy thresholds, thereby indicating that for both 1.5 T and 3.0 T data, a specificity of ~70% led to a sensitivity of 79% (Fig. 2). These values are in accordance with previous DTI studies in ALS (for a meta-analysis see Foerster et al., 2012). Receiver operating characteristics (ROC) curves confirmed these findings, indicating high values at a fractional anisotropy threshold of ~0.37 (\( \mu = 0.47n \)) for both 1.5 T and 3.0 T data (Supplementary Fig. 3A).

In the TFAS analysis of the other tracts (corticopontine and corticorubral tracts, corticostriatal pathway, proximal portion of the perforant path, and reference paths), a plausible pattern with a decreasing sensitivity in the separation of patients with ALS from controls was observed. In the 1.5 T and 3.0 T samples, the sensitivity decreased with the analysis of the tracts of interest which are related to increasing neuropathological ALS stages. The \( P \)-values for the differences between the ALS and the control sample (1.5 T and 3.0 T) were \( P < 0.0000001 \) and < 0.00001 for the corticospinal tract, \( P < 0.0001 \), \( P < 0.001 \), \( P = 0.03 \) and \( P = 0.0003 \) for the corticopontine/corticorubral tracts, \( P = 0.24 \) and \( P = 0.52 \) for the proximal portion of the perforant path,

\[ \text{Figure 1 (A) Schematic representation of the pathways analysed. (B) Three-dimensional images of the corticospinal tract (CST, red) corresponding to ALS stage 1, corticopontine tract (dark blue) and corticorubral tract (light blue) corresponding to ALS stage 2, corticostriatal pathway (yellow) corresponding to ALS stage 3, and proximal portion of the perforant path (green) corresponding to ALS stage 4. (C) Reference paths (magenta) show starting points in the corpus callosum (area V) and starting points in the optic tract. (D) Sagittal slice for the illustration of the differences between the corticopontine tract (dark blue), corticorubral tract (light blue), and corticostriatal pathway (yellow).} \]
respectively (Supplementary Table 1 and Supplementary Fig. 3B). In both data sets, the reference path did not differ between the patient and control groups.

These results at the group level allowed for data analysis at the individual level in a second step to categorize each single patient. By application of analogous thresholds \( \frac{m}{C_0} = 0.47 \) for TFAS, this categorization into the staging scheme (‘ALS-stage 1’ to ‘ALS-stage 4’) was performed using the decision algorithm depicted in Supplementary Fig. 2B. It should be noted that the resulting thresholds \( \frac{m}{C_0} = 0.47 \) for 1.5 T and 3.0 T were nearly identical (Supplementary Table 1). For demonstration, examples of individual staging categorizations in individual patients are shown in Fig. 3. A summary of ALS-patient categorization into ALS stages is provided in Table 2.

Whole brain-based spatial statistics for fractional anisotropy maps demonstrated major significant differences bihemispherically along the corticospinal tract (Supplementary Fig. 4A). The axial diffusivity and radial diffusivity analysis demonstrated significant changes between the patients and controls with the largest changes along the corticospinal tract, as shown in Supplementary Fig. 4B and C. For the staging scheme, owing to the higher significance levels, fractional anisotropy was considered to be the most useful parameter.

Correlation of ALS stages to ALS-FRS-R and to disease duration for categorized data sets is displayed in Fig. 4. Both the clinical phenotype as assessed by ALS-FRS-R and disease duration correlated significantly with the resulting staging scheme (\( P = 0.0017 \) and \( P = 0.0019 \)).

**Discussion**

The findings by Brettschneider *et al.* (2013) in a large cohort of clinically characterized ALS autopsy cases indicate that the disease pathology in ALS progresses in a regional and sequential pattern that permits recognition of successive neuropathological disease stages. Such staging protocols can help to improve our understanding of disease progression in ALS (Braak *et al*., 2013), and a transfer of these neuropathological data to *in vivo* techniques is not only desirable, but necessary, as these could allow *in vivo* monitoring of disease progression in ALS. Neuroimaging was considered a possible candidate, and a DTI-based approach to analyse...
the ALS-associated fibre tracts was chosen inasmuch as no direct imaging marker of TDP-43 pathology is currently available and DTI has been shown to be capable of identifying ALS-associated patterns of brain alterations in previous studies (Agosta et al., 2010; Canu et al., 2011; Kassubek et al., 2012). Importantly, several studies analysing the neuropathology of ALS showed a close relation between the extent of TDP-43 pathology and the severity of neuro-axonal loss (Geser et al., 2008; Brettschneider et al., 2012). Although the causes of TDP-43-mediated neuronal degeneration remain to be elucidated (recently reviewed by Lee et al., 2012; Ling et al., 2013), measurement of neuro-axonal loss in white matter fibre tracts seems to be a valid surrogate to assess the spreading of TDP-43 pathology in vivo. The objective of the present study was to apply the fibre tract-based approach to automatically analyse the pathways that are prone to be involved in ALS at different stages by DTI-based fibre tracking.

In accordance with this working hypothesis, the statistical analyses of TFAS-based tracts of interest showed significant differences between patients with ALS and controls for those tracts which are characteristically involved in a sequential progression, i.e. the corticospinal tract (stage 1), the corticorubral and corticopontine tracts (stage 2), the corticostriatal pathway (stage 3), and the proximal portion of the perforant path (stage 4). Data were separately analysed for MRI acquisition protocols both at 1.5 T and at 3.0 T and showed similar results, i.e. the results obtained in the 1.5 T data were confirmed in the data acquired at the higher field strength.

For the complete sample, the highest significance in discriminating between patients with ALS and controls was observed for ALS-stage 1 (the corticospinal tract), whereas the significance level of the comparisons at the group level was lower the higher the disease stage was with the corresponding tracts involved in. Only for stage 4 structures the group comparison did not demonstrate any statistical difference from controls. These results mirror the recently proposed neuropathological propagation pattern (Brettschneider et al., 2013). It should be noted, however, that acquisition and postprocessing-related factors (i.e. signal-to-noise ratio, registration accuracy as well as variable quality of tractography across tracts) could also be responsible for this decrease of significance.
In the subsequent analysis at the individual single patient level, the tract of interest analysis technique by TFAS was used for the individual analysis of alterations in the predefined tract structures, followed by an individual categorization into the ALS-staging scheme in vivo. The advantage of the used DTI technique is that the specific fibre tract structures could be analysed at individual subject level and that this analysis is hypothesis-driven, on the one hand, but performed in an automatic and unbiased way without observer effect on the other. In favour of the technique chosen, the results seen at the two different field strengths were nearly identical, although the smaller 3.0 T sample has to be viewed as a limiting factor. Our hypothesis-driven approach explains why the results pattern of the current study differed from previous DTI studies in ALS in which mainly corticospinal tract-associated structures were observed to be altered in directionality, and the anatomical tracts defined by the tract of interest approach in this study were not specifically addressed. The correlation to clinical phenotype is an indicator for the usefulness of the proposed ALS staging scheme. Further analysis geared to linking the results to pathological results (Brettschneider et al., 2013) is in progress.

Our study has several limitations. The major limitation is the conceptional design of the neuroimaging approach in which the categorization into the four ALS stages was not autopsy-confirmed. As such, by the advanced tract of interest-based analysis approach, only a plausible pattern of the in vivo ‘staging’ could be provided, which was the case for both patient samples. A direct comparison of the observed frequencies of the single stages between this study and the previous neuropathological study does not seem appropriate owing to the different spectra of general condition in the patient samples, given that the patients in the present study were not only living but also capable of compliance with an MRI investigation that exerts some physical demands on the participants. A minor drawback of the technique is, in general, that grey matter contamination could not be excluded. Whole brain-based spatial statistics analysis could also detect areas with significant differences (areas that were crossed by the fibre tract under observation). Nevertheless, the tract-based analysis allows for a more specific focus along the entire tract owing to its hypothesis-guided approach.

An additional limitation is that a number of individual patients (n = 16 for 1.5 T data and n = 7 for 3.0 T data) could not be assigned to a given stage by the selected approach and, thus, did not ‘fit’ into the individual analysis. However, this is technique-immanent because thresholds had to be defined for the differentiation between patients and controls in a data-driven approach, which implied that not all patients would be classifiable owing to an incomplete separation between patients and controls. Finally, cross-sectional imaging data were used to analyse a progression scheme in analogy to the neuropathological study.

In summary, the results of this DTI-based analysis of the neuropathologically-defined progression pattern in ALS provide a tool for imaging in vivo the stage of disease in correlation to the clinical phenotype. This approach can be used not only in the clinical work-up of individuals but also enlarges the spectrum of potential non-invasive surrogate markers as a neuroimaging-based read-out for clinical ALS studies. Future longitudinal data might provide progression markers by intraindividual shifts to higher stages and could provide rates of progression at the individual and at the group levels.

**Funding**

This study was supported by the German Research Foundation (Deutsche Forschungsgemeinschaft, DFG Grant Number LU 336/15-1) and the German Network for Motor Neuron Diseases (BMBF 01GM1103A).

**Supplementary material**

Supplementary material is available at *Brain* online.

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