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NEUROLOGY

Evaluation of corticospinal tracts in ALS with diffusion tensor MRI and brainstem stimulation

N. K. Iwata, S. Aoki, S. Okabe, N. Arai, Y. Terao, S. Kwak, O. Abe, I. Kanazawa, S. Tsuji and Y. Ugawa

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Evaluation of corticospinal tracts in ALS with diffusion tensor MRI and brainstem stimulation

N.K. Iwata, MD, PhD
S. Aoki, MD, PhD
S. Okabe, MD, PhD
N. Arai, MD, PhD
Y. Terao, MD, PhD
S. Kwak, MD, PhD
O. Abe, MD, PhD
I. Kanazawa, MD, PhD
S. Tsuji, MD, PhD
Y. Ugawa, MD, PhD

Address correspondence and reprint requests to Dr. Nobue K. Iwata, Human Motor Control Section, Medical Neurology Branch, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Building 10, Room 5N226, 10 Center Drive, Bethesda, MD 20892-1428
iwatan@ninds.nih.gov

ABSTRACT

Objective: To assess corticospinal tract involvement in patients with amyotrophic lateral sclerosis (ALS) by correlating diffusion tensor imaging (DTI) measures with intra- and extracranial central motor conduction time (CMCT) and clinical features of the patients.

Methods: We investigated 31 patients with ALS and 31 normal volunteers by DTI and measured fractional anisotropy (FA) within the corticospinal tracts and in the extramotor white matter. We measured CMCT for the first dorsal interosseous muscle and segmented it into cortical-brainstem (CTX-BS CT) and brainstem-cervical root (BS-CV CT) conduction times by magnetic brainstem stimulation at the foramen magnum level. Clinical status of each patient was evaluated with the ALS Functional Rating Scale-Revised (ALSFRS-R) and upper motor neuron (UMN) score devised for this study.

Results: We found a significant decrease of mean FA in all regions of the corticospinal tracts in patients with ALS as compared with controls. We found that FA along the corticospinal tract decreased significantly with higher UMN scores. There was no significant correlation between FA and ALSFRS-R, to which both upper and lower motoneuron involvements contribute. FA showed a significant correlation with the intracranial part of the central motor conduction (CTX-BS CT) but not with the extracranial conduction time.

Conclusions: Fractional anisotropy reflects functional abnormality of intracranial corticospinal tracts and can be used for objective evaluation of upper motor neuron impairment in amyotrophic lateral sclerosis. *Neurology* 2008;70:528–532

GLOSSARY

ALS = amyotrophic lateral sclerosis; **ALSFRS-R** = ALS Functional Rating Scale-Revised; **BS-CV CT** = brainstem-cervical root conduction time; **CMCT** = central motor conduction time; **CTX-BS CT** = cortical-brainstem conduction time; **FA** = fractional anisotropy; **FDI** = first dorsal interosseous; **LMN** = lower motor neuron; **ROI** = region of interest; **UMN** = upper motor neuron.

Amyotrophic lateral sclerosis (ALS) is clinically diagnosed by lower motor neuron (LMN) signs of limb and bulbar muscles associated with upper motor neuron (UMN) signs. Subclinical LMN involvement is detectable by needle electromyographic findings of denervation, which are incorporated in revised El Escorial criteria.[1] UMN involvement can be evaluated by physiologic measures or neuroimaging techniques,[2] although these have not been sufficiently well established to be incorporated into diagnostic criteria. Diffusion tensor MRI visualizes the overall orientation of the fiber tracts and their integrity in the white matter by measuring anisotropic water diffusion.[3] Decreased fractional anisotropy (FA) along the corticospinal tract has recently been reported in patients with ALS.[4,5] However, the pathophysiology of such reduced FA remains unclear. The present

From the Department of Neurology, Division of Neuroscience (N.K.I., S.O., N.A., Y.T., S.K., I.K., S.T., Y.U.), and Department of Radiology and Bioengineering (S.A., O.A.), Graduate School of Medicine, University of Tokyo; National Institute of Neuroscience (I.K.), National Center of Neurology and Psychiatry, Tokyo, Japan; and Human Motor Control Section (N.K.I.), Medical Neurology Branch, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, MD.

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investigation was undertaken with the intent of clarifying the mechanism for reduced FA in ALS by studying correlations of the FA value with central motor conduction time segmented into intracranial and extracranial conduction times using brainstem stimulation.

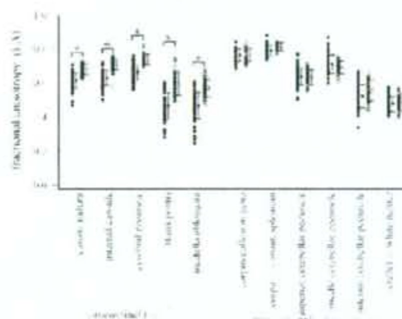
METHODS **Subjects.** We recruited 31 patients with ALS and 31 age-matched normal subjects (ALS 60.7 ± 12.9 years, normal 57.1 ± 13.0 years, $p = 0.166$). The Ethical Review Committee of the University of Tokyo approved this study. All subjects gave their written informed consent to participate in the study. All patients were enrolled if they met definite, probable, or possible categories of revised El Escorial criteria.[1] The degree of abnormality was quantified using the ALS Functional Rating Scale-Revised (ALSFRS-R). The UMN score was designed to assess UMN impairment. The following neurologic signs were rated on a 0 to 2 scale according to their severity (0 = absent or normal, 1 = moderately impaired, and 2 = greatly impaired): jaw jerk, other pathologic reflexes of the cranial regions, overactive tendon reflexes in upper limbs, overactive finger flexor reflexes, overactive tendon reflexes in lower limbs, pathologic reflexes in lower limbs, spasticity, and presence of clonus. The scale generates a score from 0 to 16.

Transcranial magnetic stimulation of corticospinal pathways. Central motor conduction time (CMCT) was measured with methods described previously,[4] recorded from the first dorsal interosseous (FDI) muscles. A round coil was used for motor cortical and spinal motor root stimulation, and a double cone coil was used for brainstem stimulation. CMCT (motor-evoked potential latency difference between motor cortical and cervical root simulation), cortical-brainstem conduction time (CTX-BS CT, latency difference between motor cortical and brainstem stimulation), and brainstem-cervical root conduction time (BS-CV CT, latency difference between brainstem and cervical root stimulation) were calculated and evaluated by neurophysiologists blinded to MRI results.

Diffusion tensor MRI scanning protocol. Diffusion tensor images were acquired with 1.5-tesla Signa Horizon LX MRI system (GE Medical Systems), using single-shot spin-echo echoplanar sequences (repeat time 6,000 msec, echo time 78 msec, field of view 24 cm, NEX 4, 128 × 128-pixel matrix, diffusion gradients [b-value of 1,000 sec/mm^2], 3-mm slice thickness). Diffusion properties were measured along 13 noncollinear directions. FA was measured using a region-of-interest (ROI) method. Elliptical ROIs were placed along bilateral corticospinal tracts (corona radiata, internal capsule, cerebral peduncle, basis pontis, and medulla oblongata) and extramotor white matters (genu and splenium of the corpus callosum, superior, middle, and inferior cerebellar peduncle, and cerebellar white matter) on FA maps by one author blinded to subject clinical status, based on empirical anatomic knowledge and reference to pertinent literature.

Statistical analyses. We used a two-way analysis of variance (ANOVA) (factors of subject group and region). We used Scheffe analysis as post hoc multiple comparisons (significance level 0.05). Linear regression analyses were applied for all correlations (significance level 0.05) using StatView software (version 5; SAS Institute). Because FA is reported to decline with advancing age,[7] correlations between FA and clinical or physiologic measures are examined by using ratio of FA at each ROI to that of the splenium of the corpus callosum, to compensate for interindividual variability of absolute FA values. In evaluation of correlations between FA and CMCT as well as CTX-BS CT and BS-CV CT, we used all FA values and compatible physiologic measures, such as a FA on one side and a physiologic measure for the contralateral FDI. When a correlation between anisotropy data and ALSFRS-R or UMN score was analyzed, we averaged values from right and left sides to provide a single mean FA at a site for each individual.

Figure 1 Individual plots of fractional anisotropy (FA) at each region of interest (ROI) for patients with amyotrophic lateral sclerosis (filled circles) and controls (open circles) (mean ± SD)



RESULTS **Fractional anisotropy.** Individual plots of FA at each ROI for patients with ALS and controls are shown in figure 1. Two-way ANOVA showed an effect of the subject group (patient and control) and region (effect of subject group: F = 205.763, $p < 0.0001$; effect of region: F = 395.421, $p < 0.0001$). It also showed an interaction between the subject group and region (F = 25.457, $p < 0.0001$). Post hoc analyses showed that the mean FA was lower in patients with ALS than in controls in all ROI within the corticospinal tracts (the corona radiata, posterior limb of the internal capsule, cerebral peduncle, basis pontis, pyramid of the medulla oblongata) ($p < 0.0005$). No significant differences were found within extramotor white matter. FA decreased significantly with higher UMN scores at corona radiata, internal capsule, and pyramids of medulla oblongata. No correlation was apparent between UMN scores and FA in extramotor white matter. FA showed no significant correlation with ALSFRS-R in any

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Table 1 Correlation between fractional anisotropy (FA) and clinical/physiologic measures

Site	Correlation between FA and clinical measures		Correlation between FA and physiologic measures		
	ALSFRS-R	UMN score	CMCT	CTX-BS CT	BS-CV CT
FA of corticospinal tract					
Corona radiata	0.070 (0.707)	0.397 (0.027*)	0.432 (0.004*)	0.409 (0.020*)	0.236 (0.201)
Internal capsule	0.292 (0.111)	0.440 (0.013*)	0.547 (<0.0001*)	0.535 (0.002*)	0.249 (0.176)
Cerebral peduncle	0.307 (0.093)	0.173 (0.353)	0.284 (0.065)	0.178 (0.330)	0.264 (0.152)
Basis pontis	0.037 (0.843)	0.210 (0.258)	0.306 (0.046*)	0.378 (0.033*)	0.073 (0.696)
Medulla oblongata	0.215 (0.245)	0.436 (0.014*)	0.408 (0.007*)	0.349 (0.051)	0.239 (0.195)
FA of extracorticospinal tract					
Corpus callosum genu	0.136 (0.465)	0.147 (0.429)	0.072 (0.751)	0.085 (0.763)	0.093 (0.741)
Superior cerebellar peduncle	0.120 (0.520)	0.071 (0.704)	0.213 (0.171)	0.083 (0.652)	0.106 (0.571)
Middle cerebellar peduncle	0.105 (0.574)	0.035 (0.853)	0.249 (0.108)	0.183 (0.315)	0.173 (0.353)
Inferior cerebellar peduncle	0.105 (0.575)	0.044 (0.814)	0.005 (0.974)	0.064 (0.727)	0.037 (0.843)
Cerebellar white matter	0.321 (0.078)	0.100 (0.591)	0.061 (0.698)	0.092 (0.616)	0.149 (0.425)

Correlation coefficient and the respective p value are shown.
*Significant.
ALSFRS-R = ALS Functional Rating Scale-Revised; BS-CV CT = brainstem-cervical root conduction time; CMCT = central motor conduction time; CTX-BS CT = cortical-brainstem conduction time; UMN = upper motor neuron.

ROI (table 1).

Transcranial magnetic stimulation of corticospinal pathways. We examined 47 limbs of 25 patients. In 16 limbs of 10 patients, no responses were obtained with motor cortical, brainstem, or motor root stimulation. The averaged ALSFRS-R of these patients was 29.7 ± 11.7, which was worse than that of the rest of the patients (36.7 ± 8.2, $p < 0.05$). There was no difference in the averaged UMN scores between the two patient groups (6.3 ± 3.8 and 6.2 ± 4.1, $p > 0.05$). Theoretically, unobtainable responses are attributable to cortical inexcitability resulting from motor cortical cell loss, severe peripheral involvement, or a combination of both. However, in the patients studied here, based on the above results of correlations, we can infer that dysfunction of LMN contributes more than that of the UMN to the lack of responses. These absent responses were excluded from the following correlation analyses because there were no measurable latencies.

In all, we obtained 43 CMCTs and 31 CTX-BS CTs as well as BS-CV CTs. The averaged CMCT of FDI of the patients was 8.5 ± 3.4 msec (the average $\pm$ SD of the normal subjects at our facility was 7.0 ± 0.4 msec). Seventeen of 43 CMCTs were abnormally delayed (above the average + 2SD of the normal values). The average of CTX-BS CTs from the patients was 4.4 ± 3.0 msec (the normal average was 3.3 ± 0.3 msec). Twelve of 31 CTX-BS CTs were delayed. The average of BS-CV CTs of the patients was 4.3 ± 2.9 (the normal average was 3.7 ± 0.5), and 12 of 31 BS-CV CTs were abnormally prolonged. We found overall abnormal results including absent responses to either cortical or root stimulation, and delayed responses, in 44.7% of all the limbs studied. Delayed CMCT, CTX-BS CT, and BS-CV CT were found in 39.5%, 38.7%, and 38.7% of recorded responses. CMCT and CTX-BS CT correlated significantly with both ALSFRS-R and UMN scores, but BS-CV CT correlated only with ALSFRS-R (table 2).

Table 2 Correlations between central motor conduction times and clinical indices

Measures	Correlation	p Value
CMCT vs		
ALSFRS-R	0.482	0.0011*
UMN score	0.598	<0.0001*
CTX-BS CT vs		
ALSFRS-R	0.457	0.0098*
UMN score	0.595	0.0004*
BS-CV CT vs		
ALSFRS-R	0.559	0.0011*
UMN score	0.234	0.2048

*Significant.
CMCT = central motor conduction time; ALSFRS-R = ALS Functional Rating Scale-Revised; BS-CV CT = brainstem-cervical root conduction time; CTX-BS CT = cortical-brainstem conduction time; UMN = upper motor neuron.

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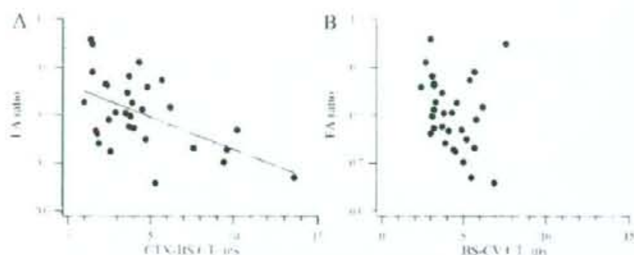
Figure 2 Regression plots of fractional anisotropy at the internal capsule as a function of cortical-brainstem conduction time (CTX-BS CT) (A) and brainstem-cervical root conduction time (BS-CV CT) (B)



Correlation was only apparent between FA and cortical-brainstem conduction time (|r| = 0.535, p = 0.002). No correlation was observed between FA and the extracranial conduction time (brainstem-cervical root conduction time) (|r| = 0.249, p = 0.176).

Correlation between FA and CMCTs. FAs at most regions along the corticospinal tract decreased significantly with delayed CMCT and CTX-BS CT (table 1, figure 2A). However, BS-CV CT did not correlate with FA in any ROI (figure 2B). No significant correlation was found between FA of extramotor regions and CMCT or CTX-BS CT.

DISCUSSION In ALS, we have demonstrated reduced FA restricted to the corticospinal tracts. We also found that FA along the corticospinal tract decreased with higher UMN scores or delayed CTX-BS CT. The brainstem stimulation is considered to differentiate the CMCT delay due to an intracranial lesion from that due to an extracranial spinal lesion.[8] The CTX-BS CT must purely reflect UMN function, whereas the BS-CV CT must mostly reflect LMN function, and CMCT both LMN and UMN functions. Based on this theory, our present results suggest that FA measurement can evaluate UMN function in patients.

We showed that CTX-BS CT, but not BS-CV CT, delayed significantly with decreased FA at sites of the corticospinal tracts other than the cerebral peduncle where CSF has a greater partial volume effect on images. Decreased FA suggests tract degeneration that engenders the loss of organized coherent structures. FA changes are explainable by both intracellular water diffusion changes and extracellular matrix changes. Previous histopathologic studies of ALS suggest that the former corresponds to degeneration of the corticospinal tract axon itself with associated astrocytosis and accumulation of axonal spheroids, whereas the latter corresponds to extracellular matrix expansion and astrocytosis within interaxonal spaces. Both processes can cause reduced anisotropy of water diffusion. Meanwhile, slowing of the conduction time is considered to result from the loss of larger and faster conducting neurons and reduction of functioning rapidly conducting axons following corticospinal cell loss.[9] From animal experiments, the magnitude of latency delay by failed firing attributable to functioning fiber loss is estimated to be a few milliseconds at maximum from the cortex to cervical spinal cord. A striking increase of the conduction time in excess of this range would suggest slowing of conduction itself, which might be attributable to conduction through slowly conducting fibers due to degeneration of rapidly conducting fibers, or secondary demyelination when cortical neuronal loss is severe. These inferences are supported by white matter histopathology of the corticospinal tract: Myelin loss is commonly observed, especially in advanced patients, and the severity of that loss is generally related to neuronal loss of the motor cortex.[10] With the finding that the FA along the corticospinal tract decreased with intracranial motor conduction delay in ALS, we can infer that impaired axonal function, rather than extracellular factors, mainly contributes to the reduced FA. Demyelination secondary to motor axonal loss may add water diffusion changes in patients with excessive delayed motor conduction. This idea is consistent with the current view of determinants of anisotropy that the primary contributor is axonal membrane function, whereas other microstructures such as the myelin sheath, the neurofibrils (microtubules, neurofilaments), and axonal transport can play a secondary modulating role.[11] For more precise elucidation on potential determinants of anisotropic changes in ALS, thorough comparative studies of diffusion tensor imaging and postmortem specimens are necessary.

We demonstrated a significant correlation of FA with other clinical or physiologic indices. Potential applications of this method for patients with ALS include its use as an objective marker in following the natural course of the disease or modified course in therapeutic trials, or detecting a mild lesion of the corticospinal tracts at early stages.

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Evaluation of corticospinal tracts in ALS with diffusion tensor MRI and brainstem stimulation

N. K. Iwata, S. Aoki, S. Okabe, N. Arai, Y. Terao, S. Kwak, O. Abe, I. Kanazawa, S. Tsuji and Y. Ugawa

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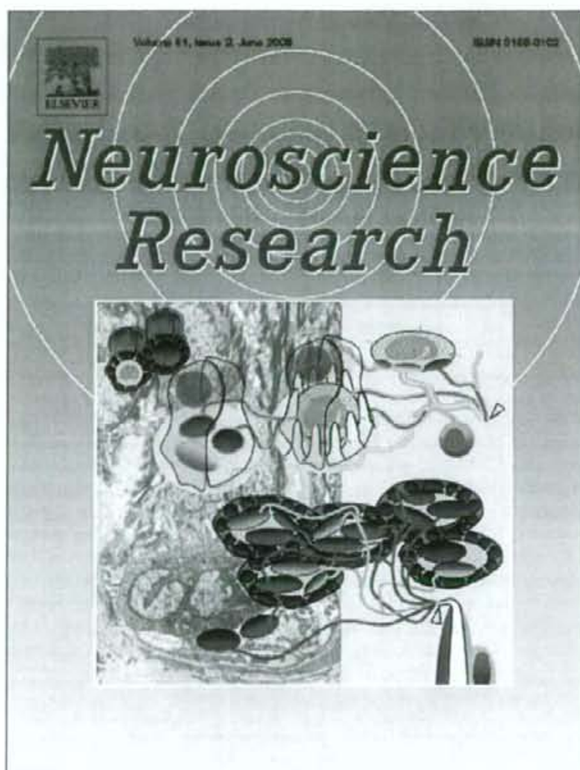

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Determination of editors at the novel A-to-I editing positions

Yoshinori Nishimoto [a,b,1], Takenari Yamashita [a,1], Takuto Hideyama [a], Shoji Tsuji [a], Norihiro Suzuki [b], Shin Kwak [a,*]

[a] Department of Neurology, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan
[b] Department of Neurology, Graduate School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan

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Abstract

A-to-I RNA editing modifies a variety of biologically important mRNAs, and is specifically catalyzed by either adenosine deaminase acting on RNA type 1 (ADAR1) or type 2 (ADAR2) in mammals including human. Recently several novel A-to-I editing sites were identified in mRNAs abundantly expressed in mammalian organs by means of computational genomic analysis, but which enzyme catalyzes these editing sites has not been determined. Using RNA interference (RNAi) knockdowns, we found that cytoplasmic fragile X mental retardation protein interacting protein 2 (CYFIP2) mRNA had an ADAR2-mediated editing position and bladder cancer associated protein (BLCAP) mRNA had an ADAR1-mediated editing position. In addition, we found that ADAR2 forms a complex with mRNAs with ADAR2-mediated editing positions including GluR2, kv1.1 and CYFIP2 mRNAs, particularly when the editing sites were edited in human cerebellum by means of immunoprecipitation (IP) method. CYFIP2 mRNA was ubiquitously expressed in human tissues with variable extents of K/E site editing. Because ADAR2 underactivity may be a causative molecular change of death of motor neurons in sporadic amyotrophic lateral sclerosis (ALS), this newly identified ADAR2-mediated editing position may become a useful tool for ALS research.
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Keywords: RNA editing; Adenosine deaminase acting on RNA (ADAR); Immunoprecipitation (IP); RNA interference (RNAi); Cytoplasmic fragile X mental retardation protein interacting protein 2 (CYFIP2); Bladder cancer associated protein (BLCAP)

1. Introduction

Adenosine deaminases acting on RNA (ADARs) catalyze A-to-I RNA editing in a wide range of organisms including human. Among three structurally related ADARs (Keegan et al., 2001; Bass, 2002; Maas et al., 2003), ADAR1 is indispensable for normal development (Wang et al., 2000) and ADAR2 plays a key role in the regulation of neuronal excitability in mice (Brusa et al., 1995; Higuchi et al., 2000), and presumably in the pathogenesis of sporadic amyotrophic lateral sclerosis (ALS) in humans, by specifically editing the Q/R site of GluR2, a subunit of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor (Takuma et al., 1999; Kawahara et al., 2004; Kwak and Kawahara, 2005). Extensive A-to-I conversion occurs in the large numbers of mRNAs (Burns et al., 1997; Higuchi et al., 2000; Wang et al., 2000; Bhalla et al., 2004), and studies using a computational genomic approach have recently demonstrated several novel A-to-I editing sites in mRNAs abundantly expressed in peripheral as well as neuronal tissues (E.Y. Levanon et al., 2005). Using immunoprecipitation (IP) and the RNA interference (RNAi) knockdown system *in vitro*, we investigated whether the recently reported A-to-I editing sites in cytoplasmic fragile X mental retardation protein interacting protein 2 (CYFIP2), filamin A (FLNA), bladder cancer associated protein (BLCAP), and insulin-like growth factor binding protein 7 (IGFBP7) mRNAs (E.Y. Levanon et al., 2005) are the substrates of ADAR1 or ADAR2 in humans. Furthermore, we also investigated whether these mRNAs in humans form complex with ADAR2 by means of ADAR2-immunoprecipitation method on nuclear extracts of human cerebellum.

* Corresponding author.
E-mail address: kwak-tky@umin.ac.jp (S. Kwak).
[1] Both these authors equally contributed to this study.

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2. Materials and methods

2.1. Isolation of RNA–protein complexes

The nuclear pellet was extracted from 6 g of frozen human cerebella. Briefly, after homogenizing the tissue in ten-volumes of cold 0.25 M sucrose in buffer A (Tris–saline-HCl buffer (pH 7.5) containing 25 mM KCl, 5 mM $MgCl_2$ and 1 mM dithiothreitol), the nuclear pellet was obtained by centrifuging the P1-homogenate in 1.6 M sucrose in buffer A at 130,000 × g for 1 h. The RNA–protein complex was isolated from the nuclear pellet according to previously described methods (Ohlson et al., 2005). Briefly, the nuclear pellet was sonicated in 8 ml of ice-cold buffer solution containing 0.1% sodium dodecylsulphate (SDS), 0.5% sodium deoxycholate, 0.5% Igepal CA-630 (Sigma Chemicals, St. Louis, MO) and 1 mM ribonucleoside vanadyl complex (Sigma), and then treated with 800 units of DNase I (Sigma). The resultant solution was centrifuged at 10,000 × g, 4 °C for 20 min, and RNA–protein complexes were obtained in the supernatant. All studies were carried out in accordance with the Declaration of Helsinki and the Ethics Committee of the University of Tokyo has approved the experimental procedures used.

2.2. Immunoprecipitation of ADAR2-RNA complexes

Stock Sepharose G was prepared by suspending Protein G Sepharose 4 Fast Flow beads (swollen Sepharose G beads; GE healthcare Bioscience, Piscataway, NJ) treated with tRNA (1 mg/ml) and bovine serum albumin (1 mg/ml) in two volumes of phosphate buffered saline (PBS) containing 0.05% of NaN_3. First, after pre-clearing once with 50 μl of the untreated Sepharose G suspension in PBS, recombinant ADAR2a and FLAG-ADAR2a proteins which were prepared with TNT T7 Quick for PCR DNA kit (Promega, Madison, WI) were incubated with 50 μl of the stock Sepharose G at 4 °C for 2 h in the presence of 2 μg of either E-20 or C-15 (Santa Cruz Biotechnology, Santa Cruz, CA). Antibody–bead complex was collected by centrifugation and eluted with PBS containing 1% SDS at 65 °C for 10 min. Presence of recombinant ADAR2a and FLAG-ADAR2a proteins in the eluate were verified with western blotting.

Because E-20 more effectively bound to recombinant ADAR2a and Flag-ADAR2a proteins than C-15 (Fig. 1a), we used only E-20 for the immunoprecipitation of nuclear eluate. One ml of the nuclear eluate obtained from 0.75 g of human cerebellum was incubated at 4 °C for 2 h in the presence of 2 μg of E-20 or control goat anti-human IgG (H + L) (Jackson ImmunoResearch, West Grove, PA) after pre-clearing once with 50 μl of the untreated Sepharose G suspension in PBS, and then for another h with additional 50 μl of stock Sepharose G beads. The nuclear eluate–antibody–bead complex was collected by centrifugation and eluted with 50 μl of PBS containing 1% SDS at 65 °C for 10 min and then the eluate was treated with proteinase-K at 37 °C for 60 min. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA).

2.3. RNAi of ADAR1 and ADAR2

HeLa cells were cultured in MEMα (Wako, Osaka, Japan), and then in Opti-MEM I Reduced-Serum Medium (GIBCO, Langley, OK) without fetal bovine serum (FBS) or antibiotics, containing 30 nM of one of the small interference RNA (siRNA) listed in Supplementary Table S1 (Qiagen HP GenomeWide siRNAs; Qiagen, Valencia, CA) and Lipofectamine RNAiMAX (1:600; Invitrogen) (Forward Transfection). The following siRNAs were used: siR1a and siR1b were used to target human ADAR1, and siR2a and siR2b to target human ADAR2. Cells cultured in Opti-MEM containing 30 nM ALLStars Negative Control siRNA (siR n/c; Qiagen) were used as the negative control. After 5 h of incubation, the medium was switched back to the original MEMα. Total RNA was extracted 96 h after the administration of siRNAs using an RNA spin Mini RNA Isolation kit (Qiagen), and reverse transcription (RT)-polymerase chain reactions (PCRs) were carried out (Supplementary Tables S1 and S2).

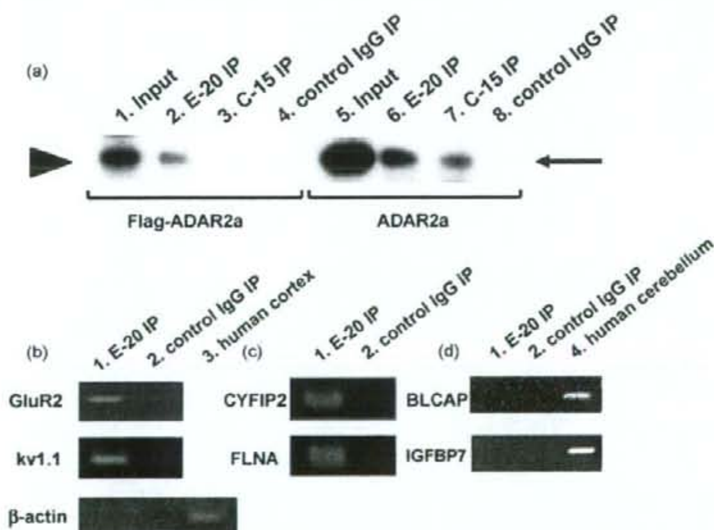

Fig. 1. Immunoprecipitated ADAR2 protein complex specifically contains substrates with selective editing sites. (a) Western blot analysis of eluates immunoprecipitated with anti-human ADAR2 polyclonal antibodies, E-20 (lanes 2 and 6) or C-15 (lanes 3 and 7), containing recombinant Flag-ADAR2a (lanes 2–4) or ADAR2a proteins (lanes 6–8) synthesized *in vitro*. Goat anti-human IgG (H + L) was used as a precipitating control (lanes 4 and 8). Untreated recombinant Flag-ADAR2a (lane 1) and ADAR2a proteins (lane 5) are also shown. Flag-ADAR2a (arrowhead) and ADAR2a proteins (arrow) were immunoprecipitated more effectively by E-20 than by C-15 or control goat anti-human IgG. Total RNA was extracted from the eluate of immunoprecipitate (IP) with E-20 and that of control IgG. (b–d) RT-PCR conducted on these eluates demonstrated mRNAs of GluR2 and kv1.1 (b) (known to have ADAR2-mediated editing sites) as well as those of CYFIP2 (cytoplasmic fragile X mental retardation protein interacting protein 2) and FLNA (filamin A) (c) in the IP with E-20 (lane 1) but not that with control goat anti-human IgG (lane 2). The mRNA of β-actin demonstrated in total RNA extracted from the human cortex (b, lane 3) was not detected in either of the eluates (b). Moreover, both the mRNAs of BLCAP (bladder cancer-associated protein) and IGFBP7 (insulin-like growth factor binding protein 7) demonstrated in total RNA extracted from the human cerebellum (d, lane 4) were not detected in either of the eluates (d).

2.4. Analyses for extents of A-to-I editing sites

Editing efficiencies at the A-to-I editing sites in GluR2, kv1.1 and CYFIP2 mRNAs were calculated by quantitative analyses of the digests of PCR products with restriction enzymes (Kawahara et al., 2003, 2004; Bhalla et al., 2004). The PCR products for GluR2 and kv1.1 were cleaved by BbvI (New England Biolabs, Ipswich, MA) and MfeI (New England Biolabs), respectively, and those for CYFIP2 were cleaved by MseI (New England Biolabs). Relative amounts of the resulting digests of PCR products were analyzed with a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA). The extent of GluR2 Q/R site editing was calculated as the ratio of the molarity of the 129-bp band (derived from edited GluR2 mRNA) to the sum of the 129- and 91-bp bands (derived from unedited GluR2 mRNA). Similarly, the extent of kv1.1 I/V site editing was calculated as the ratio of the molarity of the 117-bp band (derived from edited kv1.1 mRNA) to the sum of the 117- and 66-bp bands (derived from unedited kv1.1 mRNA). The extent of CYFIP2 K/E site editing was calculated as the ratio of the molarity of the 216-bp band (derived from edited CYFIP2 mRNA) to the sum of those of both the 216- and 60-bp bands (derived from unedited CYFIP2 mRNA). Editing efficiencies at the A-to-I(G) editing sites in FLNA and BLCAP mRNAs were evaluated by sequencing the PCR products with a 3100 Genetic Analyzer sequencer (Applied Biosystems, Faster City, CA). Expression level of CYFIP2 mRNA was measured using a LightCycler System (Roche Diagnostics, Mannheim, Germany). The internal standards, primers, and probes for quantitative PCR for ADAR1, ADAR2 and CYFIP2 were designed as previously described (Kawahara et al., 2003) (Supplementary Table S1).

2.5. ADAR2 mRNA expression in human tissues

Quantitative PCR was performed using a LightCycler System (Roche Diagnostics, Indianapolis, IN), as described previously (Kawahara et al., 2003). Each cDNA human tissue sample was amplified in a reaction mixture (20 μl total volume) composed of 10 μl of 2× LightCycler 480 Probes Master Roche (Roche Diagnostics), 0.5 μM each primer, 0.1 μM probes (Universal ProbeLibrary Set, Human, #42 for ADAR2 and #64 for β-actin, Roche Diagnostics) (Supplementary Table S1).

2.6. Statistics

Differences between groups were evaluated using Student's *t*-tests. Differences were considered statistically significant at $P < 0.05$.

3. Results and discussion

3.1. mRNAs co-precipitated with ADAR2 in human brain

To identify mRNAs with ADAR2-mediated editing sites, we analyzed the ADAR2-immunoprecipitates of human cerebellum. First we confirmed that recombinant ADAR2 and Flag-ADAR2 proteins could be successfully immunoprecipitated with the E-20, and to a lesser extent with the C-15 antibodies against human ADAR2 peptides (Fig. 1a). As a second control of the method, immunoprecipitation using the E-20 antibodies was performed on nuclear extract of human cerebellum, and GluR2 and kv1.1 mRNAs, which were already known to have ADAR2-mediated editing sites, were selectively amplified from the RNA fraction of the complexes with RT-PCR (Fig. 1b; upper, middle). Thus, the natural substrates for ADAR2 should also be immunoprecipitable from human brain.

We found that CYFIP2 and FLNA mRNAs, but not BLCAP or IGFBP7 mRNAs, were specifically recovered from the ADAR2-precipitate (Fig. 1c and d), suggesting that CYFIP2 and FLNA mRNAs should have ADAR2-mediated editing sites. Then, we analyzed the A-to-I editing sites in kv1.1, CYFIP2, FLNA and GluR2 mRNAs extracted from cerebellar tissues and their ADAR2-immunoprecipitates. All CYFIP2 mRNAs recovered from the immunoprecipitates had U(G) instead of A at the predicted A-to-I editing position (K/E site) in genomic DNA (E.Y. Levanon et al., 2005), whereas 84% of the mRNA in the human cerebellar tissue was edited at this site (Fig. 2c and d). On the other hand, the previously reported A-to-I editing position (Q/R site) of FLNA mRNA (E.Y. Levanon et al., 2005) was edited to only 51% in the immunoprecipitate, and not to a detectable level in human cerebellum (Fig. 2e and f). In addition, the I/V site of kv1.1 mRNA was edited to a greater extent (36%) in the immunoprecipitate than in the cerebellar tissue (20%) (Fig. 2a and b), although the Q/R site of GluR2 mRNA was fully edited in both the immunoprecipitate (data not shown) and the human cerebellum (Kawahara et al., 2003). These results suggest that ADAR2 seems to bind predominantly to these mRNAs with ADAR2-mediated editing positions, more efficiently when these sites were edited than when unedited (Fig. 2a, c, e vs. b, d, f).

3.2. Editors at A-to-I positions in ADAR2-associated mRNAs

To determine whether ADAR2 specifically edits the putative editing sites of CYFIP2 and FLNA mRNAs but not BLCAP or IGFBP7 mRNAs, we investigated changes in the extent of RNA editing at these sites after knockdown of ADAR1 and ADAR2 using siRNAs (Supplementary Table S1) in HeLa cells. However, because the Q/R site in FLNA mRNA was not edited at all in HeLa cells (data not shown), and because the base sequence around the K/R site of IGFBP7-derived cDNA was GC-rich and was not suitable for sequencing analysis, we could determine the effects of siRNAs only at the K/E site of CYFIP2 and at the Y/C site of BLCAP mRNAs. Applications of siR1a and siR1b, to target ADAR1, and siR2a and siR2b, to target ADAR2, specifically and significantly decreased the copy numbers of ADAR1 and ADAR2 mRNAs, respectively (Fig. 3a and b). In addition, extent of GluR2 Q/R site editing was markedly decreased to nearly 0% after ADAR2 knockdown, but not significantly decreased after ADAR1 knockdown (data not shown), indicating that the knockdown of ADAR2 by this method sufficiently suppressed ADAR2 activity but not ADAR1 activity. Although the effects of ADAR1 knockdown could not be tested on natural substrates, previous studies have demonstrated that ADAR1 activity was significantly decreased with a similar method (Wong and Lazinski, 2002). The editing efficiencies at the CYFIP2 K/E site in HeLa cells were significantly reduced after applications of siR2a and siR2b (both $0.0 \pm 0.0\%$), and also, but to a lesser extent, after siR1a and siR1b, as compared to those after the application of siR n/c or normal culture medium alone (Fig. 3c). The results suggested that ADAR2 played a major role in RNA editing at this site, and ADAR1 may also have taken a part (Fig. 3b and c). On the other hand, sequence analysis of BLCAP mRNA-derived cDNA indicated that editing extent of the Y/C site of BLCAP mRNA was significantly lower after the application of

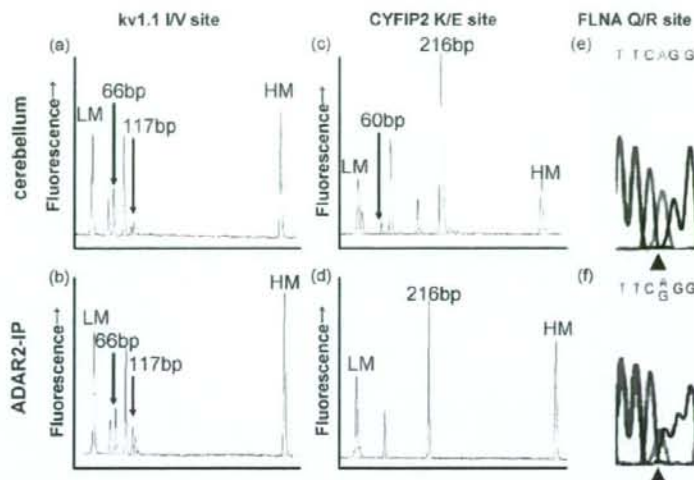

Fig. 2. Extent of editing in situ and in ADAR2-immunoprecipitates. The editing efficiency at the I/V site of kv1.1 mRNA was calculated as the ratio of the molarity of the 117-bp band (derived from edited kv1.1 mRNA) to the sum of those of both 117- and 66-bp bands (derived from unedited kv1.1 mRNA) after digestion of the PCR products with *MfeI*. Similarly, the editing efficiency at the K/E site of CYFIP2 mRNA was calculated as the ratio of the molarity of the 216-bp band (derived from edited CYFIP2 mRNA) to the sum of those of both the 216- and 60-bp bands (derived from unedited CYFIP2 mRNA) after digestion of the PCR products with *MseI*. (a, c and e) In the cerebellar tissue, extent of RNA editing was 20% at the kv1.1 I/V site (a), 84% at the CYFIP2 K/E site (c), and 0% at the FLNA Q/R site (G in e; arrowhead). (b, d and f) In the ADAR2-immunoprecipitates, on the other hand, extent of RNA editing was 36% at the kv1.1 I/V site (b), 100% at the CYFIP2 K/E site (d) and 51% at the FLNA Q/R site (G in f; arrowhead). LM, lower marker; HM, higher marker. Each value is the mean of two samples.

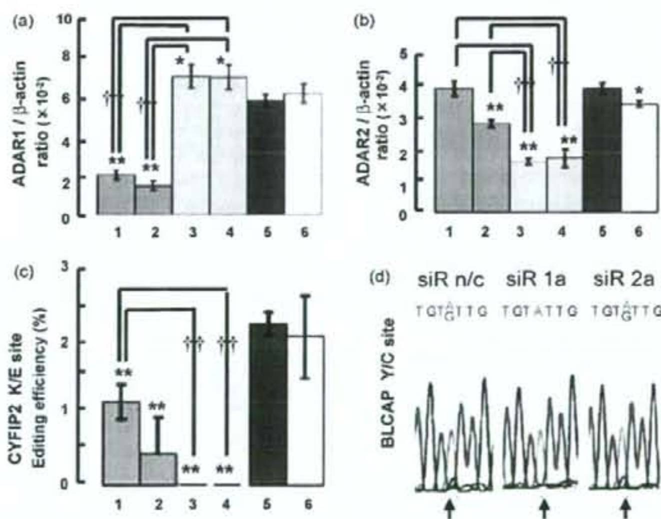

Fig. 3. Knockdown of ADAR1 and ADAR2. (a) The amount of ADAR1 mRNA relative to that of β-actin mRNA in HeLa cells was significantly decreased following knockdown treatment with siR1a and siR1b (1 and 2) but not with siR2a or siR2b (3 and 4). (b) The amount of ADAR2 mRNA relative to that of β-actin mRNA in HeLa cells was significantly decreased following knockdown treatment with siR2a and siR2b (3 and 4), compared to with siR1a or siR1b (1 and 2). (c) The editing efficiencies at the K/E site of CYFIP2 mRNA in HeLa cells were markedly decreased after ADAR2 knockdown (siR2a and siR2b, both $0.0 \pm 0.0\%$), and to a lesser extent after ADAR1 knockdown (siR1a, $1.1 \pm 0.2\%$; siR1b, $0.4 \pm 0.5\%$), than control siRNA (siR n/c, $2.1 \pm 0.2\%$; normal medium alone, $1.9 \pm 0.5\%$). Each bar represents the value after administration of siRNA (mean ± S.D.; $n = 3$). Bar 1, siR1a; Bar 2, siR1b; Bar 3, siR2a; Bar 4, siR2b; Bar 5, siR negative control (n/c); Bar 6, no siR (Student's *t*-test, $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. siR n/c and $^{\dagger\dagger}P < 0.01$ between bars as shown). (d) The sequence chromatography of the peaks at the Y/C site of BLCAP mRNA after the application of siR n/c (negative control, left), siR1a (ADAR1 knockdown, middle) or siR2a (ADAR2 knockdown, right). These demonstrate the lower peak of G at the Y/C site of BLCAP mRNA (arrows) after ADAR1 knockdown (siR1a, 2%; siR1b, 0%) than after ADAR2 knockdown (siR2a, 18%; siR2b, 16%) or control siRNA (siR n/c, 17%; normal medium alone, 13%). Each value is calculated as the mean of two samples.

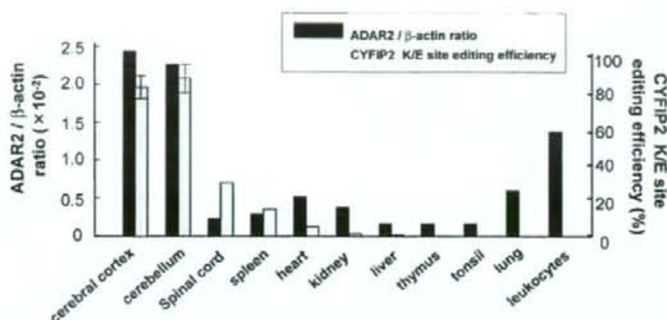

Fig. 4. CYFIP2 mRNA-editing extent and ADAR2 mRNA expression level in human tissues. Tissues with high expression levels of ADAR2 mRNA in the β-actin mRNA base (black columns) tend to show higher extent of RNA editing at the CYFIP2 K/E site (gray columns) than those with low ADAR2 mRNA expression level, whereas some regions with high ADAR2 mRNA expression level (e.g. leukocytes) showed very low extents of CYFIP2 mRNA editing. (Value represents the mean ± S.D. for multiple samples of cerebral cortex ($n = 4$), cerebellum ($n = 5$) and leukocytes ($n = 7$).)

ADAR1 siRNAs than after ADAR2 siRNAs or control siRNA (siR n/c and normal culture medium alone) (Fig. 3d). These results suggest that the Y/C site of BLCAP mRNA was edited predominantly with ADAR1 (Fig. 3d). Together with the results of immunoprecipitation (Fig. 1b–d), it seems likely that only mRNAs that have ADAR2-mediated editing positions form a complex with ADAR2.

The majority of A-to-I editing sites were localized in the non-coding regions particularly in the repetitive sequences within *Alu* and long interspersed element (LINE) sequences (K. Levanon et al., 2005) but the editors responsible for these sites have been only sporadically demonstrated (Kawahara et al., 2007). BLCAP mRNA has a long dsRNA hairpin structure (E.Y. Levanon et al., 2005) similar to *Alu* sequence, which suggests ADAR1 as a possible editor of A-to-I editing sites frequently seen in *Alu* sequence.

ADAR2 specifically catalyzes GluR2 Q/R site editing in mammalian and human brains, and the reduction of its editing activity may play a causative role in death of motor neurons in sporadic ALS patients (Kwak and Kawahara, 2005). Therefore knowledge about the regulatory mechanism of ADAR2 activity may promote our understanding of ALS pathogenesis. However, GluR2 Q/R site editing is always complete in neurons, and is somewhat reduced only when the expression of ADAR2 mRNA is reduced to a certain threshold level in human white matter (Kawahara et al., 2003), hence editing extents at this site may remain 100% when the reduction of ADAR2 activity was mild. On the other hand, CYFIP2 mRNA is ubiquitously expressed with the K/E site edited to variable extents among human tissues, especially abundant in the nervous system and lymphocytes (Mayne et al., 2004; Affymetrix HG-U133A: 215785_s_at). In this study, editing efficiencies at the CYFIP2 K/E site were $80.3 \pm 6.0\%$ in the cerebral cortex, $85.0 \pm 8.4\%$ in the cerebellum, 28.8% in the spinal cord, 14.5% in the spleen, 5.1% in the heart, and less than 1.5% in the other tissues. In addition, we found that the expression level of ADAR2 mRNA relative to that of β-actin mRNA was higher in the cerebral cortex (2.49×10^{-2}) and the cerebellum (2.30×10^{-2}) with high CYFIP2 K/E site-editing efficiency than in tissues with low CYFIP2 K/E site-editing efficiency (from 6.12×10^{-3} in the lung to 1.66×10^{-3} in the tonsil) (Fig. 4). However, CYFIP2 K/E site editing was inactive in the leukocytes and the lung in which ADAR2 mRNA expression was higher than in the spinal cord and the spleen where CYFIP2 K/E site editing was active (Fig. 4).

Consistent with the results of the knockdown experiment, there was a rough correlation between the CYFIP2 K/E site editing and ADAR2 mRNA expression. However, this correlation was less clear in some tissues such as leukocyte, suggesting the participation of certain unidentified tissue-specific ADAR2-regulatory factor(s) other than the expression level of ADAR2 mRNA. Indeed, ADAR2 mRNA-self-editing produced an mRNA isoform with premature stop codon in rat and mouse (Rueter et al., 1999; Feng et al., 2006), and protein-coding ADAR2 mRNA variants accounted for a small proportion among more than 48 splicing variants in human cerebellum (Kawahara et al., 2005). These results indicate that the total amount of ADAR2 mRNA may not directly correlate with that of active ADAR2 proteins. Furthermore expression level of ADAR1 may influence the ADAR2 activity by forming inactive heterodimers with ADAR2 (Centi et al., 2008).

Extent of editing at the CYFIP2 mRNA K/E site, a novel ADAR2-mediated A-to-I editing site, might become a potential marker for ADAR2 activity and hence a potential tool for ALS research.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.neures.2008.02.009.

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OBSERVATION

Lymphomatoid Granulomatosis Involving Central Nervous System Successfully Treated With Rituximab Alone

Hiroyuki Ishiura, MD; Masato Morikawa, MD; Masashi Hamada, MD; Takuro Watanabe, MD, PhD; Shinichi Kako, MD; Shigeru Chiba, MD, PhD; Toru Motokura, MD, PhD; Akira Hangaishi, MD, PhD; Junji Shibahara, MD, PhD; Masaaki Akahane, MD, PhD; Jun Goto, MD, PhD; Shin Kwak, MD, PhD; Mineo Kurokawa, MD, PhD; Shoji Tsuji, MD, PhD

Objective: To report the successful treatment of a patient with lymphomatoid granulomatosis (LYG), a rare Epstein-Barr virus–positive lymphoproliferative disorder, using rituximab (anti-CD20 monoclonal antibody). The prognosis for LYG has been reported to be poor, and no satisfactory treatment has been established. Because central nervous system (CNS) involvement of LYG has been known to show poor prognosis, the establishment of an effective treatment for CNS LYG with mild adverse effects is desired.

Design: Case report.

Setting: University hospital.

Patient: A 48-year old Japanese man presenting with slowly progressive spastic paraparesis diagnosed as LYG involving the CNS and lungs.

Interventions: The patient was treated with rituximab (375 mg/m^2, once weekly for 1 month) alone.

Main Outcome Measure: Improvement of the lesions on imaging.

Results: The neurological signs resolved and the lesions in the CNS and lungs were mostly diminished after the rituximab monotherapy without any adverse effects. The patient stayed in remission for 18 months.

Conclusion: Rituximab monotherapy was effective in treating the patient; hence, rituximab should be considered as the initial treatment against LYG involving the CNS.

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LYMPHOMATOID GRANULOMATOSIS (LYG) is a rare lymphoproliferative disorder characterized by angiocentric and angiodestructive Epstein-Barr virus (EBV)–positive B-cell proliferation associated with extensive reactive T-cell infiltration.[1] The common sites of involvement include the lungs, skin, and central nervous system (CNS). To date, no satisfactory treatment has been established and treatments using corticosteroids, chemotherapy, interferon alfa-2b, radiotherapy, and stem cell transplantation have been selected according to patients' conditions.[1] The prognosis of LYG is generally poor, because some may evolve to EBV positive diffuse large B-cell lymphoma. After the involvement of EBV positive B cells had been established as essential in LYG development, a few reports on treatment with rituximab (a chimeric anti-CD20 monoclonal antibody) were published[1-4]; however, the outcome of this treatment for LYG involving the CNS is still undetermined. We herein report a case of LYG involving the CNS and lungs successfully treated with rituximab alone.

REPORT OF A CASE

A 48-year-old man with well controlled asthma and atopic dermatitis felt pain in both his shoulders 2 years and 9 months before his admission to our hospital. The pain gradually extended to his back and knees. Two years before his admission, he felt weakness in his knees. Eight months before his admission, he started to show gait disturbance. Numbness appeared in his toes on both feet and subsequently spread to his knees. Four months before his admission, he developed bladder in-

Author Affiliations: Departments of Neurology (Drs Ishiura, Hamada, Goto, Kwak, and Tsuji), Hematology and Oncology (Drs Morikawa, Watanabe, Kako, Chiba, Motokura, Hangaishi, and Kurokawa), Pathology (Dr Shibahara), and Radiology (Dr Akahane), Graduate School of Medicine, The University of Tokyo, Tokyo, Japan.

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