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ORIGINAL PAPER

TDP-43 pathology in sporadic ALS occurs in motor neurons lacking the RNA editing enzyme ADAR2

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Abstract Both the appearance of cytoplasmic inclusions containing phosphorylated TAR DNA-binding protein (TDP-43) and inefficient RNA editing at the GluR2 Q/R site are molecular abnormalities observed specifically in motor neurons of patients with sporadic amyotrophic lateral sclerosis (ALS). The purpose of this study is to determine whether a link exists between these two specific molecular changes in ALS spinal motor neurons. We immunohistochemically examined the expression of adenosine deaminase acting on RNA 2 (ADAR2), the enzyme that specifically catalyzes GluR2 Q/R site-editing, and the expression of phosphorylated and non-phosphorylated TDP-43 in the spinal motor neurons of patients with sporadic ALS. We found that all motor neurons were ADAR2-positive in the control cases, whereas more than half of them were ADAR2-negative in the ALS cases. All ADAR2-negative neurons had cytoplasmic inclusions that were immunoreactive to phosphorylated TDP-43, but lacked non-phosphorylated TDP-43 in the nucleus. Our results suggest a molecular link between reduced ADAR2 activity and TDP-43 pathology.

Keywords Amyotrophic lateral sclerosis · Adenosine deaminase acting on RNA 2 · TDP-43 · RNA editing · Motor neuron

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Introduction

Amyotrophic lateral sclerosis (ALS) is a devastating disease characterized by a progressive deterioration of motor function resulting from the degeneration of motor neurons. More than 90% of ALS cases are sporadic and approximately 5–10% are familial. Although at least six causal genes have been identified so far in individuals affected with familial ALS, SOD1 [30], ALS2 (alsin) [10, 39], senataxin (ALS4) [7], vesicle-trafficking protein/synaptobrevin-associated membrane protein [27], TAR DNA-binding protein (TDP-43) [9, 15, 32, 37, 40] and FUS/TLS [22, 38], the pathogenesis of sporadic ALS remains largely unexplored.

One hypothesis for selective neuronal death in sporadic ALS is excitotoxicity mediated by abnormally Ca^{2+}-permeable α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors, which are a subtype of the ionotropic glutamate receptor [6, 20, 21]. The recent finding of reduced RNA editing of the AMPA receptor subunit GluR2 at the Q/R site provides a plausible pathogenic mechanism underlying motor neuron death in sporadic ALS [16, 21, 34]. Reduced GluR2 Q/R site-editing, catalyzed by an

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enzyme called adenosine deaminase acting on RNA 2 (ADAR2), appears to be specific to sporadic ALS among several neurodegenerative diseases [1, 19, 20, 29, 33].

TDP-43 was identified as a component of ubiquitin-positive, but tau-negative cytoplasmic inclusions in cortical neurons in patients with frontotemporal lobar degeneration (FTD) and spinal motor neurons in patients with sporadic ALS [3]. The TDP-43 found in these inclusions was demonstrated to be abnormally phosphorylated [12, 26].

Because both reduced GluR2 Q/R site-editing and formation of TDP-43-containing inclusions occur specifically in sporadic ALS motor neurons, we used immunohistochemistry to examine the expression of TDP-43 and ADAR2 in ALS motor neurons and elucidate a link between these two molecules.

Materials and methods

Subjects

This study was conducted using lumbar spinal cords from seven cases of sporadic ALS and six disease-free control cases. Consent for autopsy and approval for the use of human tissue specimens for research purposes was approved by appropriate institutional human ethics committees. Clinical information is given in Table 1.

Western blot analysis using the anti-ADAR2 antibody (RED1)

To examine the specificity of the polyclonal anti-ADAR2 antibody (RED 1) (Exalpha Biologicals, Watertown, MA) in the human brain, Western blot analysis was performed as reported previously [17]. From 100 mg of human frontal cortex, nuclear and cytoplasmic fractions were separated with the PARIS Protein and RNA Isolation System (TAKARA, Tokyo) according to the manufacturer's instructions. Nuclear and cytoplasmic proteins as well as those containing recombinant ADAR2a (rADAR2a) and recombinant ADAR2b (rADAR2b) proteins synthesized by in vitro translation were suspended in 500 μl of cold Cell Fraction Buffer provided with the PARIS Protein and RNA Isolation System (TAKARA). Samples were then boiled with 500 μl of 2 × SDS gel-loading buffer and subjected to SDS-PAGE. After electrophoresis, proteins were transferred to an Immobilon-P transfer membrane (Millipore, Bedford, MA). Blots were blocked in a buffer containing Tween/PBS and 1% bovine serum albumin (BSA). Then immunoblotting for histone protein (MAB052; CHEMICON, Temecula, CA, 1:2,000), glyceraldehyde-3 phosphate dehydrogenase (GAPDH) (MAB374; CHEMICON, 1:2,000) or ADAR2 (RED1; Exalpha Biologicals, Watertown, MA, 1:4,000) was conducted overnight at 4°C. For secondary antibodies, peroxidase-conjugated AffiniPure goat anti-mouse IgG (H + L) (Jackson ImmunoResearch, West Grove, PA; 1:5,000) or peroxidase-conjugated AffiniPure rabbit anti-sheep IgG (H + L) (Jackson ImmunoResearch; 1:5,000) was used. Visualization was carried out using ECL plus Western blotting detection reagents (GE Healthcare Bioscience, Piscataway, NJ). Specific bands were detected with an LAS 3000 system (Fujifilm, Tokyo).

Immunohistochemical analysis

The human spinal cords were fixed in 10% neutral buffered formalin for about 7 days and then embedded in paraffin. Serial 7 μm sections were cut for immunohistochemical analysis. The immunoreactive features of the anti-ADAR2 antibody on frozen sections were also evaluated. To examine the localization of ADAR2 and TDP-43 in a single neuron, a pair of adjacent sections was used for immunohistochemistry. The sections were mounted on slides and then deparaffinized in xylene, hydrated with an ethanol series and heated at 120°C for 2 min for antigen retrieval. The sections were then washed with phosphate-buffered saline (PBS) and incubated with the primary antibody overnight at 4°C. Polyclonal anti-ADAR2 (RED1, 1:100), monoclonal anti-phosphorylated TDP-43 (pTDP-43) (pS409/410) (Cosmo Bio Co., Ltd., Tokyo, Japan; 1:3,000) and rabbit polyclonal phosphorylation-independent anti-TARDBP (piTDP-43) (Protein-Tech Group, Inc.; 1:3,000) were used. Bound antibodies were detected using an avidin–biotin–peroxidase complex kit (Vector Laboratories, Burlingame, CA, USA). Diaminobenzidine tetrahydrochloride was used as the chromogen, and the sections were lightly counterstained with hematoxylin. The RED1 antibody was incubated with 5 μg/μl of recombinant ADAR2 (Abnova Corp., Taiwan) at 4°C overnight. These samples were then subjected to immunohistochemistry for the preabsorption test.

To test the effects of fixation, the paraffin-embedding procedure and the postmortem delay on ADAR2 immunoreactivity, rat spinal cord samples were processed either immediately after removal (PMI-0) or after the spinal cords were held at room temperature for 6 h (PMI-6) or 24 h (PMI-24). Some samples were quickly frozen on dry ice, and the others were fixed in 10% buffered formalin after each treatment. The immunohistochemical process was the same as that used for the paraffin-embedded human sections, except that frozen sections were incubated with the primary antibody for 1 h at room temperature after washing with PBS.

Frozen sections of lumbar spinal cords from $SOD1^{G93A}$ transgenic mice at 24 and 35 weeks of age were also used for immunohistochemistry with anti-ADAR2 and anti-pTDP-43 antibodies.

Table 1 Profiles of ALS cases and disease controls

Case	Age	Sex	PMI (h)	Brain weight (g)	Diagnosis	Duration of ALS	Onset of ALS	Number of MN/AH mean ± SD	ADAR2 (+) pTDP-43 (−) MN/AH mean ± SD (%)	ADAR2 (−) pTDP-43 (+) MN/AH mean ± SD (%)	ADAR2 (+) pTDP-43 (+) MN/AH *n* (%)	Number of AH examined
1	67	M	3	1,390	ALS	1 year 1 month	UE	6.0 ± 2.6	1.3 ± 1.5 (20.8)	4.8 ± 2.4 (79.2)	0	4
2	59	F	5	1,290	ALS	1 year 9 months	UE	2.3 ± 1.2	0 ± 0 (0.0)	2.3 ± 0.5 (100)	0	4
3	69	M	3	1,430	ALS	2 years	UE	3.3 ± 1.5	2.3 ± 1.9 (69.6)	1.0 ± 0.8 (30.4)	0	7
4	75	F	2	1,080	ALS	2 years 6 months	LE	8.7 ± 3.1	5.7 ± 2.8 (65.4)	3.0 ± 3.0 (34.6)	1 (0.2)	6
5	72	M	1.5	1,290	ALS	3 years 1 month	Bulb	3.5 ± 1.6	0.5 ± 0.6 (14.3)	3.0 ± 1.2 (85.7)	0	4
6	62	M	3	1,390	ALS	3 years 7 months	UE	4.9 ± 1.7	1.3 ± 0.8 (26.5)	3.6 ± 1.7 (73.5)	0	7
7	69	M	1.5	1,460	ALS	12 years	UMN	2.2 ± 0.7	0.8 ± 0.4 (38.5)	1.3 ± 0.3 (61.5)	0	6
8	64	M	2	1,200	Cerebellar tumor			15.8 ± 8.6	15.8 ± 8.6 (100)	0	0	4
9	58	M	7	1,420	Myotonic dystrophy			13.3 ± 7.2	13.3 ± 7.2 (100)	0	0	4
10	70	F	2	1,040	Limb-girdle muscular dystrophy			10.2 ± 5.4	10.2 ± 5.4 (100)	0	0	6
11	73	M	3	1,170	Theophylline intoxication			8.2 ± 4.9	8.2 ± 4.9 (100)	0	0	6
12	77	M	1.5	1,200	Limb-girdle muscular dystrophy			10.9 ± 6.1	10.9 ± 6.1 (100)	0	0	8
13	78	M	1	1,310	Meningitis			8.4 ± 4.6	8.4 ± 4.6 (100)	0	0	8

PMI postmortem interval, *AH* anterior horn, *MN* motor neuron, *UE* upper extremities, *LE* lower extremities, *Bulb* bulbar symptoms, *UMN* upper motor neuron sign

Double immunofluorescence study using anti-ADAR2 antibody and anti-phosphorylation-independent TDP-43 antibody

Formalin-fixed paraffin-embedded spinal cord sections from an ALS patient (case 6 in Table 1) were double-immunostained with RED1 antibody (×100) and anti-TDP-43 monoclonal antibody (Abnova, ×1,000). Labeled goat anti-rabbit IgG antibody (Molecular Probes, Alexa 488) and labeled goat anti-mouse IgG (Molecular Probes, Alexa 594) were used as secondary antibodies (×1,000).

Quantification of motor neurons in ALS and control spinal cords

Serial sections of both ALS and control cases were immunostained with piTDP-43, ADAR2, or pTDP-43. Large ADAR2-positive and -negative neurons with nucleoli in the anterior horns on each section were counted separately. In addition, we examined whether each of the motor neurons was immunostained with pTDP-43 or piTDP-43 in the respective adjacent section.

Statistics

The Mann–Whitney *U* test was used to compare the number of anterior horn cells (AHC) in ALS samples compared to controls.

Results

Nuclear localization of ADAR2

The Western blot analysis of the human cortex showed that the anti-ADAR2 antibody (RED1) recognized two isoforms of active ADAR2 protein, ADAR2a and ADAR2b, in the nuclear fraction, but not in the cytoplasmic fraction. It is reasonable for ADAR2 to be localized in the nuclear fraction because ADAR2 primarily acts on RNA. The validity of this fractionation was verified by the presence of histone in the nuclear fraction and of GAPDH in the cytoplasmic fraction (Fig. 1a). ADAR2 immunoreactivity is observed in the nuclei of motor neurons from frozen sections of rat (Fig. 1b) and human spinal cords (Fig. 1c).

ADAR2 expression in motor neurons of normal rat and SOD1 transgenic mouse

Intense ADAR2 immunoreactivity was observed in the nucleolus of the nuclei from all motor neurons examined in the frozen rat sections (Fig. 2a, c), whereas both the nucleus and cytoplasm were immunoreactive for ADAR2

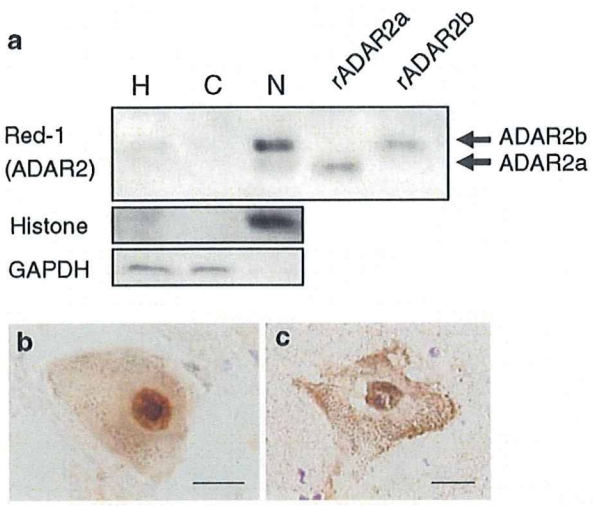

Fig. 1 Nuclear localization of the ADAR2 protein in the human brain. Western blot analysis of the human cortex demonstrates that ADAR2 protein is localized in the nuclear fraction, but not in the cytoplasmic fraction. The validity of this fractionation was verified by the presence of histone in the nuclear fraction and of GAPDH in the cytoplasmic fraction. ADAR2 immunoreactivity is demonstrated in the nuclei of large neurons in the anterior horn of the rat spinal cord (**b**) and the human spinal cord (**c**). *Bar* indicates 20 μm. *N* nuclear fraction, *C* cytoplasmic fraction, *H* brain homogenate, *rADAR2a* recombinant human ADAR2a, *rADAR2b* recombinant human ADAR2b

in the paraffin-embedded sections of both PMI-0 and PMI-6 tissues (Fig. 2b, d, e, g, h). The intensity of ADAR2 immunoreactivity varied markedly among the nuclei (even on the same section) and was uniformly low in the cytoplasm of the motor neurons in the paraffin-embedded sections. ADAR2 immunoreactivity in the nuclei of motor neurons on the frozen and paraffin-embedded sections of PMI-24 tissue was less intense than in PMI-0 and PMI-6 tissues (Fig. 2). ADAR2 immunoreactivity was observed in the nucleus of all the motor neurons examined in the spinal cords of $SOD1^{G93A}$ transgenic mice (Fig. 3b).

ADAR2, phosphorylation-dependent TDP-43 and phosphorylation-independent TDP-43 expression in human control spinal motor neurons

In the spinal cords of human control cases, all motor neurons examined ($n = 380$ from 6 cases) showed ADAR2 immunoreactivity, typically in the cytoplasm with slight or no apparent immunoreactivity observed in the nuclei (Table 1; Figs. 4a, b, 5a). Similar ADAR2 immunoreactivity was observed in all the neurons in the pontine nuclei, including atrophic neurons in patients with multiple system atrophy and spinocerebellar atrophy type 1 (Supplementary Figure 1). Phosphorylation-independent TDP-43 (piTDP-43) stained the nuclei of the same motor

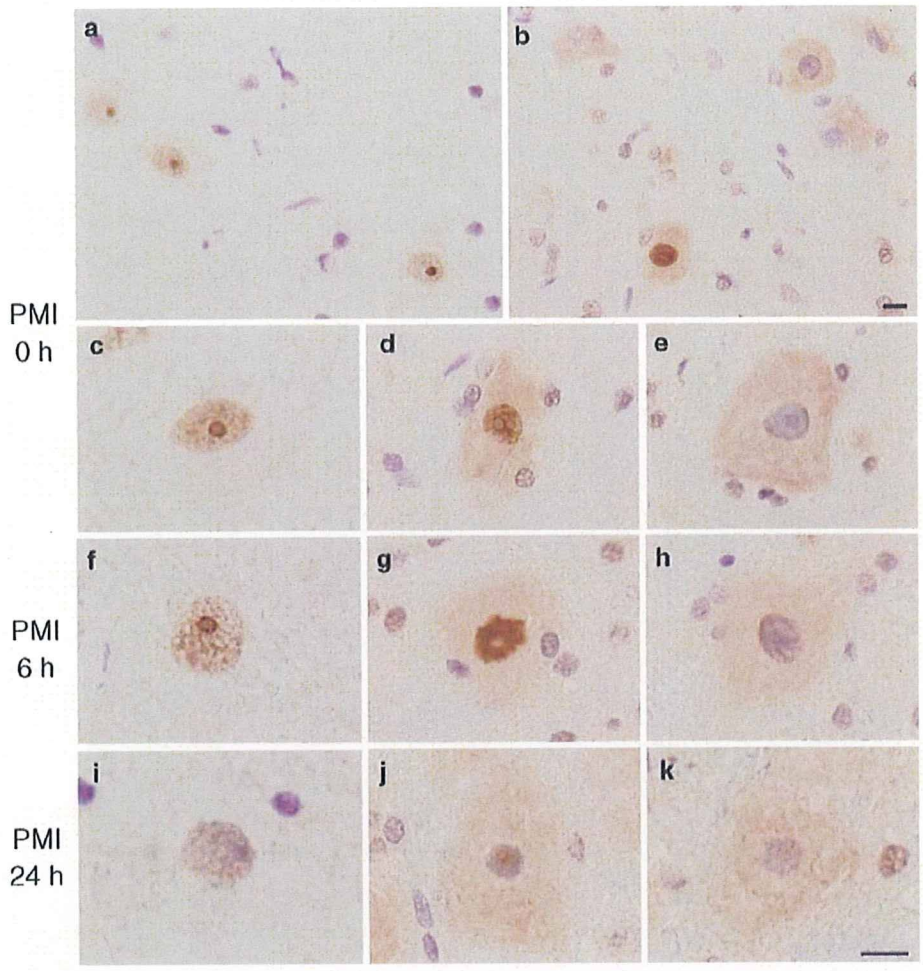

Fig. 2 ADAR2 immunohistochemistry of motor neurons in the rat lumbar spinal cord. ADAR2 is expressed in the nuclei of neurons in frozen sections created at 0 h postmortem (**a**, **c**). On the contrary, ADAR2 is always positive in the cytoplasm of neurons in formalin-fixed, paraffin-embedded sections from different rats, with a variable intensity of nuclear immunoreactivity among neurons (**b**, **d**, **e**). Frozen (**f**) and paraffin-embedded sections (**g**, **h**) with a 6-h postmortem interval display immunoreactivity similar to those with a 0-h postmortem interval. Nuclear immunoreactivity was less intense on both frozen sections (**i**) and paraffin-embedded sections (**j**, **k**) created at 24 h postmortem compared with those prepared at 0 h. *PM* postmortem interval. *Bar* indicates 20 μm

neurons (Figs. 4a′, a″, b′, 5b, c), while phosphorylation-dependent TDP-43 (pTDP-43) did not stain either the nucleus or cytoplasm on the adjacent section (Fig. 4b″). Two different anti-ADAR2 antibodies exhibited immunoreactivity in the cytoplasm of motor neurons (Fig. 4a, b, g, Supplementary Figure 2), and preabsorbed anti-ADAR2 antibody did not show any immunoreactivity (Fig. 4h).

ADAR2, pTDP-43 and piTDP-43 expression in ALS spinal motor neurons

Both ADAR2-positive (Fig. 4c open arrow) and -negative motor neurons (Fig. 4c closed arrow) were observed in the ALS spinal cords. The immunoreactivity in the ADAR2-positive neurons was observed in the cytoplasm, but apparently not in the nuclei (Figs. 4c open arrow, e, f, 5d, f), as observed in the control motor neurons. These ADAR2-positive neurons showed normal piTDP-43 immunoreactivity in the nucleus (Figs. 4e′, f′, 5e, f,), but exhibited no pTDP-43-positive inclusions (Fig. 4c, c′). In contrast, all ADAR2-negative neurons showed pTDP-43-positive inclusions in the cytoplasm (Fig. 4c′, d′).

Cell count of anterior horn motor neurons

The number of anterior horn cells (motor neurons) (AHC) in 7 sporadic ALS cases was reduced to 39 ± 21% (mean ± SD) of the number in control cases ($p < 0.0001$, Mann–Whitney's U test, Fig. 6). A significant proportion of motor neurons (98 out of 170 anterior horn cells; 58%) in the spinal cords obtained from patients with ALS were ADAR2-negative (Table 1; Fig. 6). ADAR2-negative motor neurons were observed in all ALS cases examined, but the proportions varied from 30% in case 3 to 100% in case 2 (Table 1). Notably, all the ADAR2-negative motor neurons had pTDP-43-positive inclusions in the cytoplasm. Conversely, virtually all the ADAR2-positive motor neurons had piTDP-43 immunoreactivity in their nuclei, but did not exhibit pTDP-43-positive cytoplasmic inclusions (Table 1; Fig. 6). Only one motor neuron was stained with

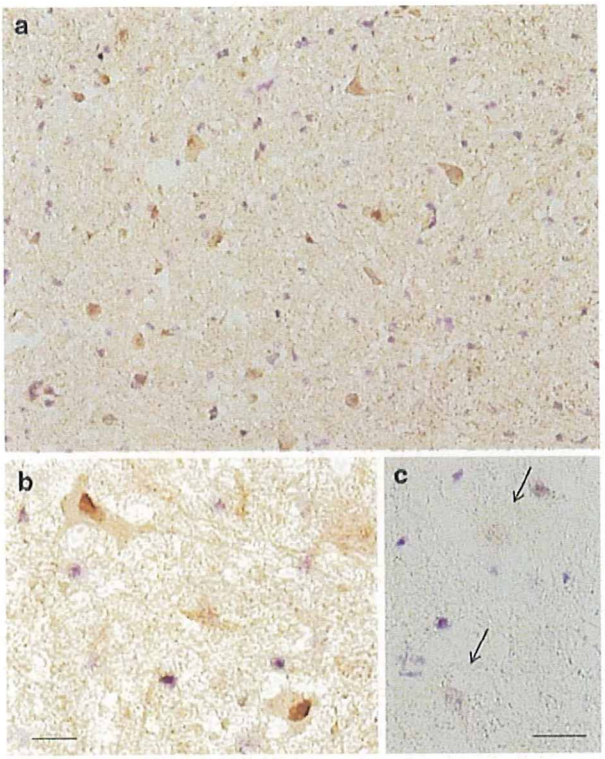

Fig. 3 Expression of ADAR2 and phosphorylation-dependent TDP-43 in spinal motor neurons of SOD1 transgenic mice. A low-magnification view of the lumbar spinal anterior horn in a $SOD1^{G93A}$ transgenic mouse, at 34 weeks of age showing that all the motor neurons are ADAR2 positive (**a**). A high-magnification view shows predominant nuclear immunoreactivity (**b**). There is no phosphorylation-dependent TDP-43 immunoreactive inclusion in the motor neurons (**c**, *arrow*). *Bar* indicates 20 μm

both ADAR2 and pTDP-43 ($n = 1$ in case 4), but none of the motor neurons lacked immunoreactivity to both ADAR2 and pTDP-43. These results indicate a strong association between ADAR2-deficiency and the development of pTDP-43-positive inclusions in the motor neurons of patients with sporadic ALS. However, there is no apparent relationship between the duration of disease and the number of remaining motor neurons with ADAR2 positivity and/or pTDP-43 positivity.

Discussion

ADAR2 expression was observed in all 380 spinal motor neurons examined in the control cases in this study, and in a portion of the spinal motor neurons from ALS cases (approximately 42% of 170 neurons). The nuclei of these ADAR2-positive neurons were also immunoreactive for piTDP-43. Notably, more than half the motor neurons in ALS cases lacked immunoreactivity to both ADAR2 and piTDP-43, but these double-negative neurons always displayed pTDP-43-positive inclusions in the cytoplasm. Therefore, normal motor neurons express ADAR2 without forming phosphorylated TDP-43-positive cytoplasmic inclusions, whereas motor neurons lacking ADAR2 in sporadic ALS formed inclusions.

ADAR2 immunoreactivity was exclusively observed in the nuclei of rat motor neurons on the frozen sections created 0–6-h postmortem. Because the density of motor neurons in the spinal ventral gray matter is too low to detect ADAR2 by Western blotting analysis, we used frozen human brain for the analysis, which demonstrated that RED1 specifically recognized two isoforms of active ADAR2 protein in the nuclear fraction [17]. The nuclear localization of the ADAR2 protein in the motor neurons was demonstrated immunohistochemically in frozen human spinal cord sections. In addition, expression of ADAR2 mRNA in the human spinal cord was demonstrated [18]. These results are consistent with the function of ADAR2 in the cell nucleus, which is catalysis of the conversion of adenosine to inosine (A-to-I) at various pre-mRNA positions including the Q/R site of GluR2. We also observed ADAR2 immunoreactivity in the cytoplasm of motor neurons in human paraffin-embedded sections. Importantly, the nuclei of motor neurons were predominantly immunoreactive to ADAR2 in frozen sections of the same control subject. In paraffin-embedded sections and frozen sections from the spinal cord after a 24-h postmortem interval, there was a reduction in ADAR2 immunoreactivity in the nucleus with the concomitant appearance of immunoreactivity in the cytoplasm of rat motor neurons. Therefore, ADAR2 immunoreactivity in the cytoplasm likely represented ADAR2 protein translocated from the nucleus to the cytoplasm resulting from the procedure of paraffin embedding and/or the delay between death and tissue fixation, which are unavoidable during routine neuropathological examinations of human autopsy materials. Because all the control motor neurons demonstrated cytoplasmic ADAR2 immunoreactivity, it is likely that ADAR2-negative motor neurons in ALS spinal cords lacked ADAR2 protein localized to the nucleus.

ADAR2 is involved in the A-to-I conversion of various pre-mRNAs and specifically catalyzes GluR2 Q/R site-editing. AMPA receptors containing GluR2 which is unedited at the Q/R site have significantly higher Ca^{2+} permeability than those containing edited GluR2. This factor plays a crucial role in neuron survival [5, 31]. Neurons in the mammalian brain only express Q/R site-edited GluR2 mRNA, and mice unable to edit this site die from status epilepticus early in life [14]. GluR2 Q/R site-editing occurs with 100% efficiency in normal human motor neurons, but is characterized by high variability (from 0 to 100%) among individual motor neurons in individual cases of ALS [16]. Therefore, ADAR2-positive

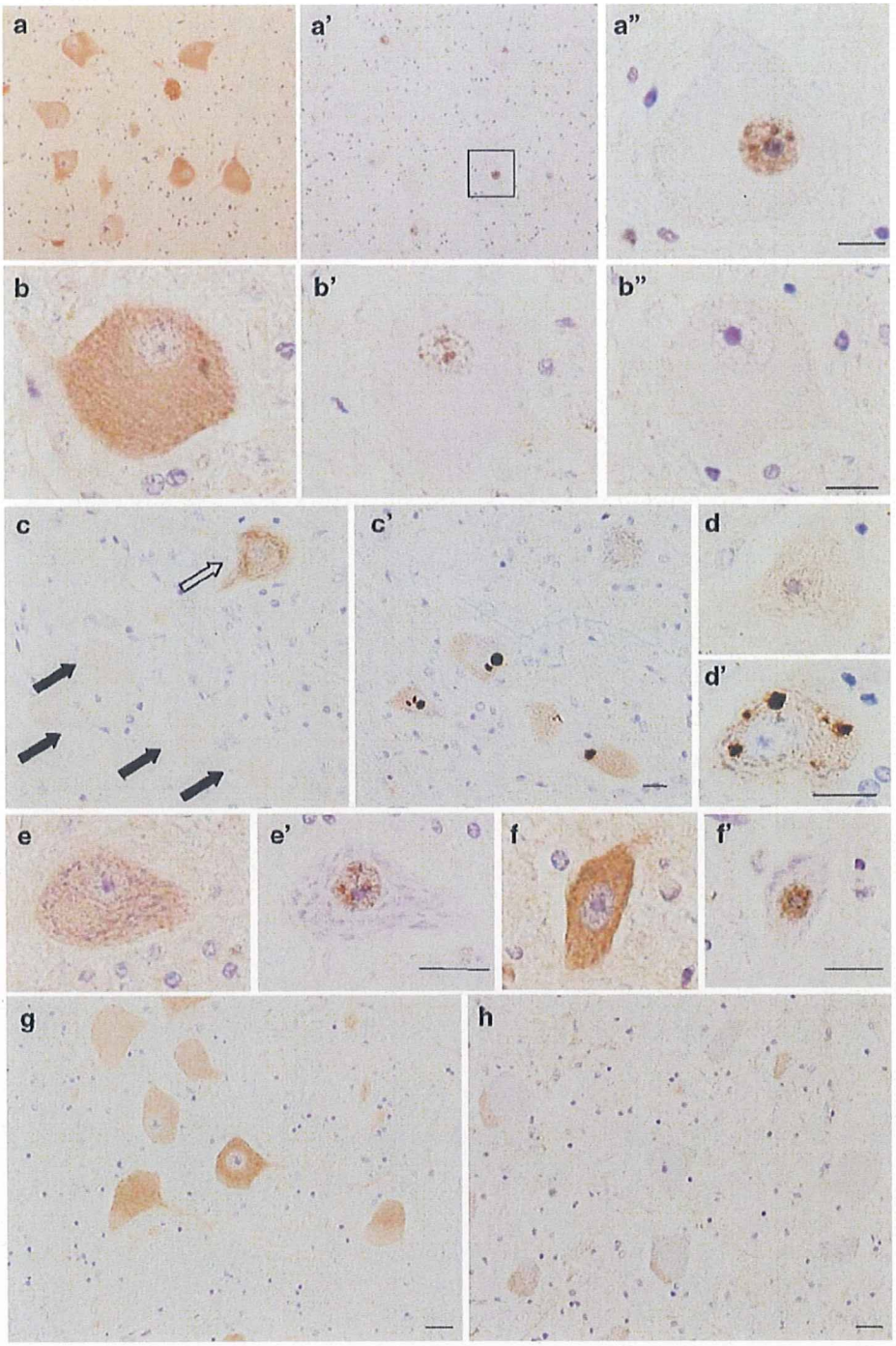

Fig. 4 Expression of ADAR2, phosphorylation-dependent TDP-43 and phosphorylation-independent TDP-43 in spinal motor neurons from adjacent sections. A low-magnification view of the lumbar spinal anterior horn in a control subject (case 8) shows that all the motor neurons are immunoreactive for ADAR2 (**a**). These neurons show phosphorylation-independent TDP-43 (piTDP-43) immunoreactivity in the nucleus in an adjacent section (**a′**, **a″**: a high-magnification view of the open square in **a′**). A lumbar spinal motor neuron from a control subject (case 12) shows diffuse ADAR2 immunoreactivity in the cytoplasm (**b**). The adjacent section shows piTDP-43 immunoreactivity in the nucleus (**b′**), but does not show phosphorylation-dependent TDP-43 (pTDP-43) immunoreactivity (**b″**). All ADAR2-negative neurons (*closed arrows* in *c*; case 6) display pTDP-43-positive inclusions in the cytoplasm (**c′**), whereas an ADAR2-positive neuron from a patient with ALS (*open arrow* in **c**) has no pTDP-43 immunoreactivity (**c′**). An ADAR2-negative neuron (**d**; case 6) has multiple pTDP-43-positive inclusions in the cytoplasm (**d′**). ADAR2-positive neurons (**e** case 6, **f** case 3) show phosphorylation-independent TDP-43 immunoreactivity in the nucleus (**e′**, **f′**). ADAR2 immunoreactivity in the cytoplasm (**g**) disappeared when recombinant ADAR2-preabsorbed anti-ADAR2 antibody was used as the primary antibody (**h**) (case 12). *Bar* indicates 20 μm

motor neurons likely represent normal neurons expressing Q/R site-edited GluR2, whereas ADAR2-negative motor neurons represent those expressing Q/R site-unedited GluR2 [16, 20]. The present results indicating the presence of both ADAR2-positive and -negative motor neurons are consistent with high variability in the efficiency of GluR2 Q/R site-editing (from 0 to 100%) among individual motor neurons in ALS patients [16]. These findings strengthen the hypothesis that reduced ADAR2 activity is closely associated with the pathogenesis of sporadic ALS [20].

The presence or absence of ADAR2 immunoreactivity in the cytoplasm of motor neurons was conversely related to pTDP-43 immunoreactivity in the cytoplasm. Abnormally processed TDP-43 was initially identified as a

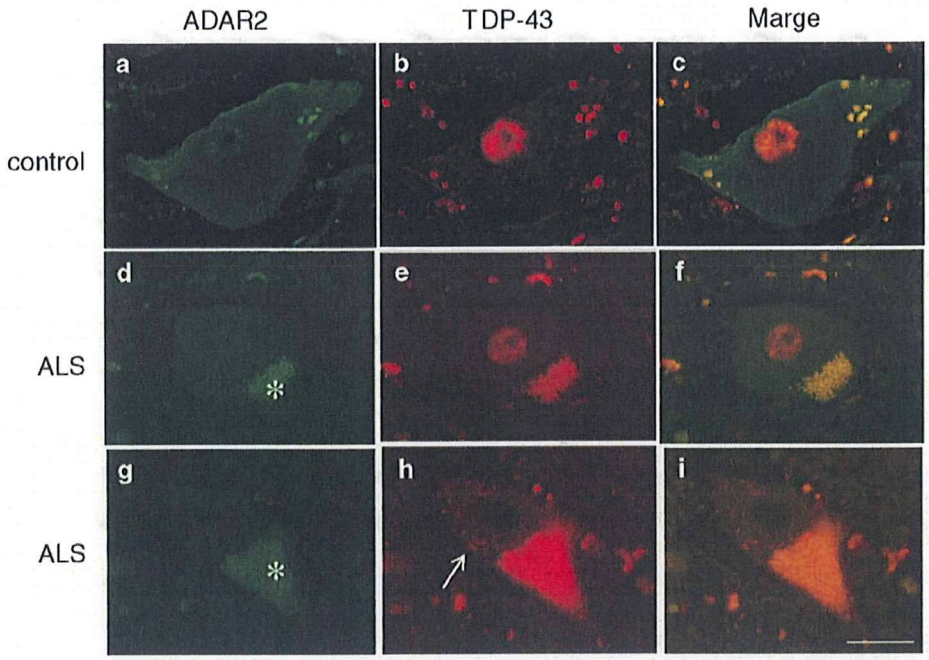

Fig. 5 Double-labeled immunofluorescence study using anti-ADAR2 and anti-piTDP-43 antibodies. **a–c** Control motor neuron was immunopositive for ADAR2 in the cytoplasm and nucleus and piTDP-43 in the nucleus. **d–f** An ALS motor neuron immunoreactive to ADARA2 in the cytoplasm showed immunoreactivity to piTDP-43 in the nucleus. **g–i** An ALS motor neuron lacking immunoreactivity to ADAR2 showed piTDP-43 positive cytoplasmic inclusions (*arrow*) and loss of piTDP-43 immunoreactivity in the nucleus. Asterisks indicate lipofuscin autofluorescence. *Bar* indicates 20 μm

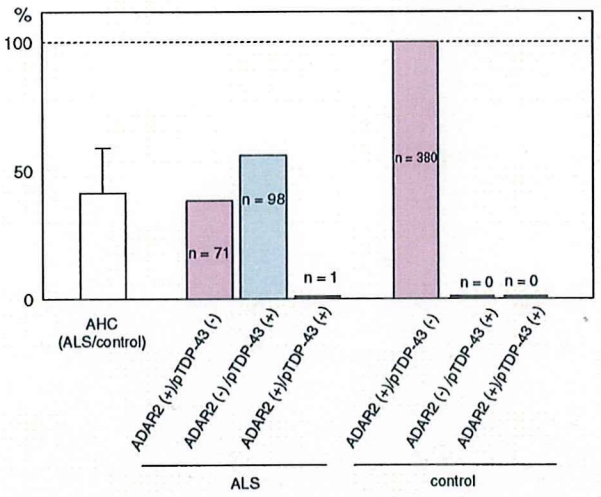

Fig. 6 Motor neurons with different immunoreactivities in ALS and control cases. The number of anterior horn cells (motor neurons) (AHCs) in 7 sporadic ALS cases was reduced to 39 ± 21% (mean ± SD) of the number in control cases ($p < 0.0001$, Mann–Whitney *U* test). In ALS cases, 42% of total AHCs were ADAR2-positive and pTDP-43-negative (*pink bar*); 58% were ADAR2-negative and pTDP-43-positive (*blue bar*). Only one AHC out of 170 (0.2%) was positive for both ADAR2 and pTDP-43, and none were negative for both ADAR2 and pTDP-43. All lumbar spinal motor neurons ($n = 380$) from 6 control cases were ADAR2-positive and pTDP-43-negative (*pink bar*). *AHC* anterior horn cell (motor neuron)

protein component of ubiquitin-positive and tau-negative inclusions in the brains of patients with FTD and ALS [3, 25]. Subsequently, abnormal TDP-43-positive inclusions were found in various proportions in neurons from patients with other neurodegenerative disorders, such as Parkinson's disease dementia and dementia with Lewy bodies [24], Parkinsonism-dementia complex and ALS in Guam [8, 11], corticobasal degeneration [36] and Alzheimer's disease [2, 13, 36]. These results imply that aberrant processing of TDP-43 may be involved in a common pathway of the neurodegenerative process and that the accumulation of pTDP-43 in the cytoplasm of motor neurons is not a disease-specific event in ALS [2, 13, 24, 36].

This study demonstrates that all ADAR2-negative motor neurons showed pTDP-43-positive inclusions in the cytoplasm in cases of sporadic ALS, suggesting a molecular association between reduced ADAR2 activity and the formation of pTDP-43-positive inclusions in ALS motor neurons. Both TDP-43 and ADAR2 are nuclear proteins, playing roles in the regulation of RNA processing; TDP-43 regulates RNA splicing [4, 28] and ADAR2 catalyzes RNA editing. However, there is no report regarding the functional link between the two molecules. We found that pTDP-43-positive inclusions showed no ADAR2 immunoreactivity, indicating that the trapping of ADAR2 protein in the inclusions due to direct protein–protein interaction is unlikely. Reduced ADAR2 activity increases Ca^{2+} permeable AMPA receptors by failure to edit the Q/R site of GluR2 [5, 6, 14], but it is not known whether an increase of the Ca^{2+} overload influences the TDP-43 processing. Thus, it is not clear from the present immunohistochemical study whether the reduced ADAR2 expression is a cause or a consequence of TDP-43 pathology. Interestingly, neither pTDP-43-positive inclusions [23, 35] nor a reduction of GluR2 Q/R-site-editing [19] was associated with

SOD1-related familial ALS or SBMA, an X-linked hereditary lower motor neuron disease associated with expanded CAG repeats in the androgen receptor gene. Consistent with the absence of pTDP-43-positive inclusions in the spinal motor neurons of *SOD1*-associated familial ALS, present study demonstrated that all the motor neurons examined were ADAR2-positive in $SOD1^{G93A}$ transgenic mouse spinal cords. Elucidation of the molecular mechanism underlying the co-occurrence of reduced ADAR2 activity and abnormal TDP-43 pathology in the same motor neurons may provide a clue to the neurodegenerative process of sporadic ALS.

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Journal of Pharmacological Sciences

Forum Minireview

Novel Etiological and Therapeutic Strategies for Neurodiseases: RNA Editing Enzyme Abnormality in Sporadic Amyotrophic Lateral Sclerosis

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Abstract. The motor neurons of patients with sporadic amyotrophic lateral sclerosis (ALS) express abundant Q/R site–unedited GluR2 mRNA, whereas those of patients with other motor neuron diseases including familial ALS associated with mutated SOD1 (ALS1) and those of normal subjects express only Q/R site–edited GluR2 mRNA. Because adenosine deaminase acting on RNA type 2 (ADAR2) specifically catalyzes GluR2 Q/R site–editing, it is likely that ADAR2 activity is not sufficient to edit this site completely in motor neurons of patients with sporadic ALS. Because these molecular abnormalities occur in disease- and motor neuron–specific fashion and induce fatal epilepsy in mice, we have hypothesized that GluR2 Q/R site–underediting due to ADAR2 underactivity is a cause of neuronal death in sporadic ALS. We found that cytoplasmic fragile X mental retardation protein interacting protein 2 (CYFIP2) mRNA had an ADAR2-mediated editing position using RNA interference knockdown. Our review will include a discussion of new ADAR2 substrates that may be useful for research on sporadic ALS.

Keywords: RNA editing, amyotrophic lateral sclerosis (ALS), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptor, GluR2 Q/R, cytoplasmic fragile X nuclear retardation protein interacting protein 2 (CYFIP2), neurodisease

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease characterized by progressive paralysis with muscle wasting due to selective loss of upper and lower motor neurons. More than 90% of cases of ALS are sporadic, while the remaining cases of ALS have more than one other affected family member (familial ALS). Gene mutations causative of familial ALS, including those of the superoxide dismutase 1 (SOD1) gene, have not been detected in the majority of sporadic cases of ALS (1), indicating that the pathogenesis of sporadic ALS differs from that of familial ALS. Several hypotheses concerning the pathogenesis of sporadic ALS have been suggested, including those related to excitotoxicity (2), toxicity (3), autoimmunity (4), infection (5), and oxidative stress (6). Among these, considerable evidence supports the excitotoxic hypothesis (7 – 9).

The mechanism of initiation of motor neuronal death appears to involve excessive influx of Ca^{2+} through α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors (10). The determinants of this Ca^{2+} influx include the Ca^{2+} permeability of AMPA receptors, which is due to the presence of the GluR2 subunit and related to the reduction of GluR2 Q/R editing, and the density of receptors on the cell surface, which is regulated by many factors including regulatory proteins, direct

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phosphorylation, and RNA editing at the GluR2 Q/R site (Fig. 1) (11).

We have demonstrated that RNA editing of GluR2, a subunit of the AMPA receptor, at the Q/R site is decreased in motor neurons of a small number of sporadic ALS cases in disease-specific and neuronal class–selective fashion (12, 13). Functional AMPA receptors are tetrameric assemblies of GluR1, GluR2, GluR3, and GluR4, in various combinations produced in nonstochastic fashion. In mammals, all GluR2 mRNAs in neurons are completely edited and the majority of AMPA receptors have GluR2 in their composition, making AMPA receptors Ca^{2+}-impermeable. In the motor neurons in patients with ALS, due to underediting of GluR2 mRNA at the Q/R site, the proportion of Ca^{2+}-permeable AMPA receptors may be increased, resulting in neuronal death (14) . RNA editing at the GluR2 Q/R site is specifically catalyzed by ADAR2 (15). Reduction of this enzyme activity is probably the cause of the underediting observed in ALS motor neurons.

2. RNA editing and ADARs

RNA editing is a posttranscriptional modification of mRNA that alters the amino acids specified by the gene. The resulting change in amino acid residues alters the biological function of translated molecules; this is most clearly demonstrated in alterations of channel properties including those of the Ca^{2+} permeability of GluRs, a subunit of AMPA, and kainate receptors (16, 17). In human and rodent brains, the efficiency of editing at each editing site of GluRs is developmentally and regionally regulated (18 – 24), and abnormal RNA editing may result in animal or human diseases affecting the central nervous system. However, no consistent findings have been reported regarding alteration of these editing sites in the case of temporal lobe epilepsy (25), depression (26), and schizophrenia (27). In contrast, abnormal editing has been demonstrated to be associated with certain neurological diseases including amyotrophic lateral sclerosis (28, 29).

These alterations are catalyzed by the adenosine deaminases, which recognize a specific target sequence of nucleotides and convert an adenosine (A) to an inosine (I), which the ribosome translates as a guanosine (G).

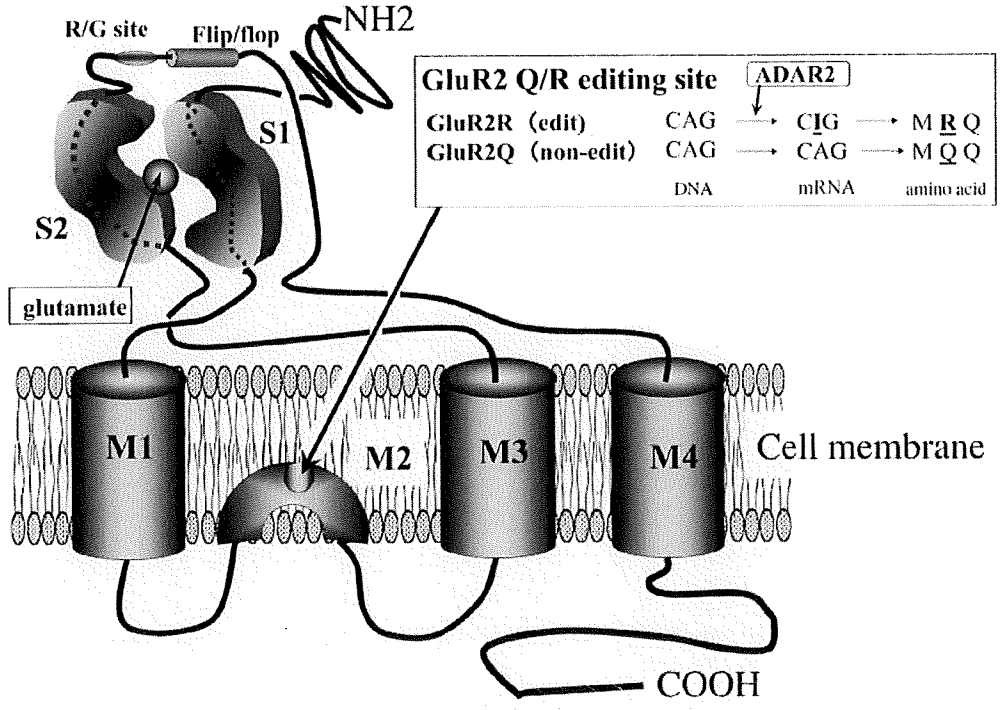

Fig. 1. Structure of the GluR subunit. GluR2 subunit has four membrane domains (M1 – M4). The Q/R site (Q/R) is located in the M2 domain and this editing site is the determinant of Ca^{2+} permeability of the AMPA receptor. The R/G site (R/G) and the flip/flop alternative splicing site are located between the M3 and M4 domains, and these sites are the determinants of channel desensitization.

This A-to-I RNA editing controls 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 humans. A lot of novel A-to-I editing sites have been identified in vitro in mRNAs abundantly expressed in mammalian organs by means of computational genomic techniques (30), although the enzyme catalyzing editing at these sites has yet to be determined.

ADAR2 predominantly catalyzes RNA editing at the Q/R site of GluR2 both in vivo and in vitro (15, 31, 32), whereas both ADAR1 and ADAR2 catalyze the Q/R sites of GluR5 and GluR6, which are subunits of kainate receptors (15, 32). ADAR3, a third member of the ADAR family, is exclusively expressed in the central nervous system but is catalytically inactive on both extended dsRNA and known pre-mRNA editing substrates (33, 34).

3. New substrates of ADAR2

Using immunoprecipitation and the RNA interference (RNAi) knockdown system in vitro, we investigated whether the recently reported A-to-I editing sites in CYFIP2, filamin A (FLNA), bladder cancer associated protein (BLCAP), and insulin-like growth factor binding protein 7 (IGFBP7) mRNAs (35) are the substrates of ADAR1 or ADAR2 in humans. We also examined whether these mRNAs form complexes with ADAR2 in humans, by means of ADAR2-immunoprecipitation of nuclear extracts of human cerebellum (36).

Using RNAi knockdown, we found that CYFIP2 mRNA had an ADAR2-mediated editing position and that BLCAP mRNA had an ADAR1-mediated editing position (Table 1) (36, 37). In addition, we found that ADAR2 formed complexes 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 the immunoprecipitation method. CYFIP2 mRNA was ubiquitously expressed in human tissues with variable extents of K/E site-editing (Fig. 2) (36, 37).

Table 1. Novel A-to-I positions

Editing site	CYFIP2 K/E	BLCAP Y/C	Reference
Normal mouse brain (%)	90	50	(38)
Mouse neuronal primary culture (%)	54.5	36.5	(38)
$ADAR1^{-/-}$ mouse neuronal primary culture (%)	50	8.5	(38)
$ADAR2^{-/-}$ mouse brain (%)	approx. 11	33.5	(38)
Human cerebellum (%)	84	approx. 30	(37)
Hela cell (%)	1.9	13	(37)
ADAR1 siRNA in Hela cell (%)	0.4 – 1.1	0	(37)
ADAR2 siRNA in Hela cell (%)	0	16 – 18	(37)

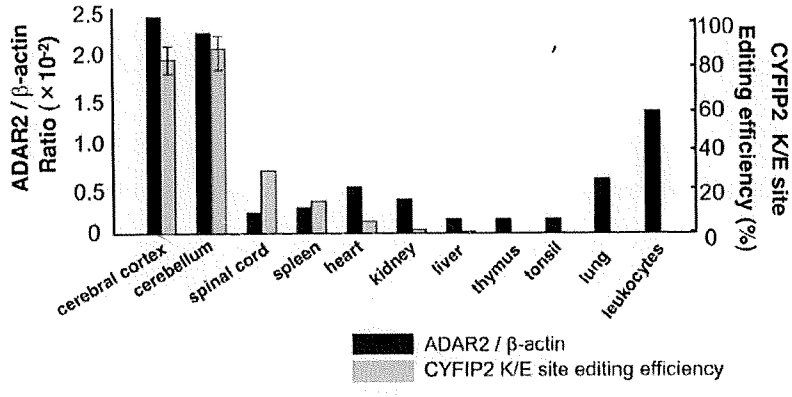

Fig. 2. Extent of CYFIP2-mRNA editing and level of expression of ADAR2 mRNA in human tissues. Tissues with high expression levels of ADAR2 mRNA in the β-actin mRNA base (black columns) tend to show higher extent of RNA editing at the CYFIP2 K/E site (gray columns) than those with low ADAR2 mRNA expression 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) and represents the mean for the rest of tissue samples (n < 3). Reproduced from Ref. 37.

4. Conclusion

CYFIP2 mRNA is particularly abundant in the central nervous system including motor neurons in the spinal cord, and the extent of site-editing in it ranges from 30% to 85% in the human central nervous system (37, 38). Because ADAR2 underactivity may be a cause of death of motor neurons in sporadic ALS, the CYFIP2 K/E site, a newly identified ADAR2-mediated editing position, may become a useful tool for ALS research. To investigate whether deficiency of ADAR2 activity induces slow neuronal death as seen in motor neurons of sporadic ALS patients, we have generated genetically modified mice in which the ADAR2 gene is conditionally knocked out in motor neurons using the Cre-loxP system.

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Symposium: Advances in amyotrophic lateral sclerosis research

AMPA receptor-mediated neuronal death in sporadic ALS

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α - amino - 3 - hydroxy - 5 - methyl - 4 - isoxazolepropionate (AMPA) receptor-mediated excitotoxicity has been proposed to play a role in death of motor neurons in amyotrophic lateral sclerosis (ALS). We demonstrated that RNA editing of GluR2 mRNA at the glutamine/arginine (Q/R) site was decreased in autopsy-obtained spinal motor neurons, but not in cerebellar Purkinje cells, of patients with sporadic ALS. This molecular change occurs in motor neurons of sporadic ALS cases with various phenotypes, but not in degenerating neurons of patients with other neurodegenerative diseases, including SOD1-associated familial ALS. Because GluR2 Q/R site-editing is specifically catalyzed by adenosine deaminase acting on RNA 2 (ADAR2), it is likely that regulatory mechanism of ADAR2 activity does not work well in the motor neurons of sporadic ALS. Indeed, ADAR2 expression level was significantly decreased in the spinal ventral gray matter of sporadic ALS as compared to normal control subjects. It is likely that ADAR2 underactivity selective in motor neurons induced deficient GluR2 Q/R site-editing, which results in the neuronal death of sporadic ALS. Thus, among multiple different molecular mechanisms underlying death of motor neurons, it is likely that an increase of the proportion of Q/R site-unedited GluR2-containing Ca^{2+}-permeable AMPA receptors initiates the death of motor neurons in sporadic ALS. To this end, normalization of ADAR2 activity in motor neurons may become a therapeutic strategy for sporadic ALS.

Key words: ADAR2, ALS, AMPA receptor, GluR2, RNA editing.

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THE AMPA RECEPTOR-MEDIATED NEURONAL DEATH HYPOTHESIS

Molecular mechanisms leading motor neurons to death have not been elucidated in ALS, even in SOD1-associated familial ALS (ALS1) on which a tremendous number of investigations have been conducted for more than a decade. Although different causative genes have been identified in several different familial ALS, the majority of sporadic ALS cases do not carry mutations in these causative genes, indicating that none of them play a causal role in sporadic cases.[1] Therefore, it is likely that there are multiple different death pathways in motor neurons. Among the hypotheses proposed to explain the etiology of sporadic ALS, excitotoxicity mediated by α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors, a subtype of ionotropic glutamate receptors, has attracted much attention due to the fact that motor neurons are particularly vulnerable to AMPA receptor-mediated neurotoxicity in cultured spinal cord neurons. Although mechanisms underlying excitotoxic neuronal injury are complex and incompletely understood, intracellular Ca^{2+} overload is an important trigger and an increased influx of Ca^{2+} through activated AMPA receptor-coupled channels appears to play a key role in slow death of motor neurons in culture.[2–4]

MOLECULAR MECHANISM UNDERLYING AMPA RECEPTOR-MEDIATED NEUROTOXICITY IN SPORADIC ALS

Functional AMPA receptors are tetrameric assemblies that are composed of four subunits, GluR1, GluR2, GluR3 and GluR4, in various combinations. The Ca^{2+} conductance of AMPA receptors differs markedly depending on whether the receptor has the GluR2 subunit in its subunit assembly. AMPA receptors that contain at least one GluR2 subunit

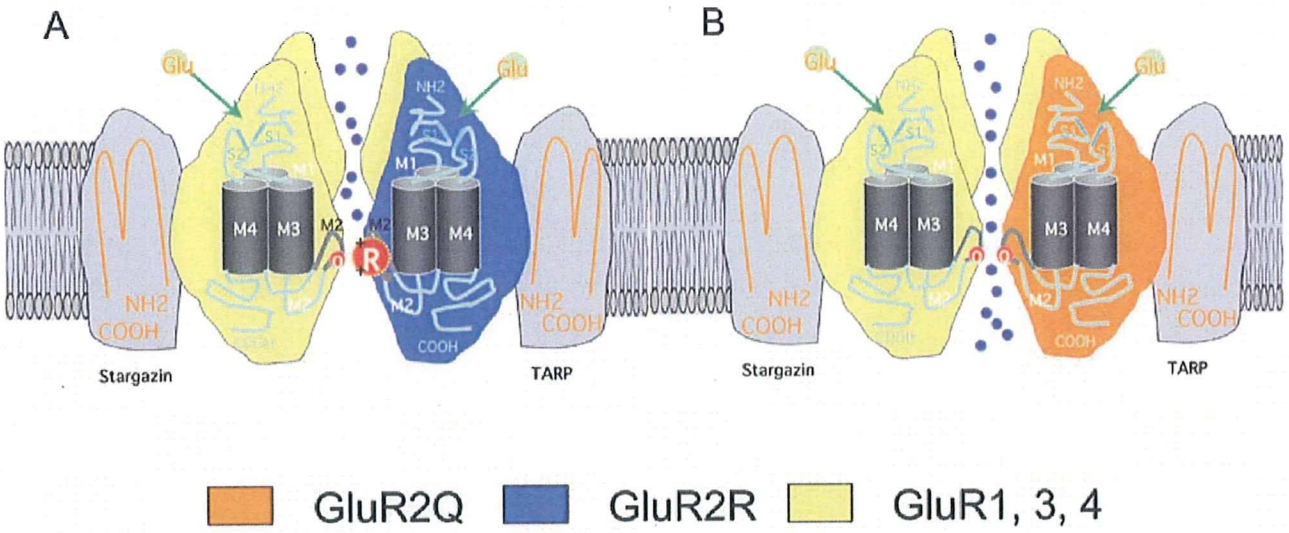

Fig. 1 α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptor and Ca^{2+}-permeability. A. AMPA receptor containing GluR2 subunit edited at the glutamine/arginine (Q/R) site (blue) in M2 (curved line in dark gray). Due to the positive charge of arginin (R) residue at the Q/R site, Ca^{2+} (blue circle) cannot pass through the channel pore. Majority of the AMPA receptors expressed on neurons is Q/R site-edited GluR2-containing AMPA receptors. B. Q/R site-unedited GluR2-containing (orange) or GluR2-lacking AMPA receptors. When none of the subunits have R at the Q/R position, AMPA receptors are Ca^{2+}-permeable. GluR1, 3, 4 subunits are in yellow.

have low Ca^{2+} conductance (Fig. 1A), whereas those lacking a GluR2 subunit are Ca^{2+} permeable.[5] These properties of GluR2 are generated by a single nucleotide conversion from adenosine (A) to inosine (I) by post-transcriptional RNA processing called RNA editing (A-to-I editing), during which inosine is recognized as guanosine during translation, glutamine (Q) codon (CAG) is substituted by arginine (R) codon (CIG; CGG) at the position called the Q/R site in the putative second membrane domain (M2) (Fig. 1B).[6] Analyses of adult rat, mouse and human brain RNA have demonstrated that almost all GluR2 mRNA *in vivo* has R at the Q/R site, whereas Q remains at this critical position in the GluR1, GluR3 and GluR4 subunits. The change in amino acid residue at the Q/R site of GluR2 results in marked alterations in channel properties of AMPA receptors, including Ca^{2+} permeability,[7-9] trafficking,[10] subunit assembly[11] and kinetic aspects of channel gating.[12] Furthermore, failure of GluR2 Q/R site-editing led mice to fatal status epilepticus.[13] Therefore, reductions in both GluR2 expression and GluR2 Q/R site-editing increase Ca^{2+} influx through AMPA receptor-coupled ion channels. We found that extents of GluR2 Q/R site-editing, but not of GluR2 mRNA expression were decreased in motor neurons of sporadic ALS. Because mice deficient for GluR2 Q/R site-editing died young from status epilepticus,[13] an increase of Q/R site-unedited AMPA receptors is likely a death-inducing molecular change in sporadic ALS.

Motor neurons undergo progressive degeneration in ALS, while other neuronal subsets undergo degeneration at a much later disease stage, if ever. We found that the reduction of GluR2 Q/R site-editing did not occur in the cerebellar Purkinje cells of patients with sporadic ALS.[14] Additionally, we found that dying cerebellar Purkinje cells of patients with spinocerebellar degeneration (i.e. dentatorubral pallidoluysian atrophy (DRPLA) and multiple system atrophy (MSA-c)) expressed only normally Q/R site-edited GluR2 mRNA.[14] Moreover, GluR2 Q/R site-editing has been reported to be preserved even in the brain areas severely affected in neurodegenerative diseases including the striatum of Huntington disease, the neocortex and hippocampus of Alzheimer and Pick diseases, and the cerebellum of spinocerebellar degeneration.[15-18] Therefore, the defect in GluR2 Q/R site-editing is not a non-specific phenomena occurring in dying neurons, but is a molecular abnormality relevant to the pathogenesis of sporadic ALS.

DEATH OF MOTOR NEURONS IN OTHER MOTOR NEURON DISEASES

SOD1-associated familial ALS (ALS1) is the most frequent familial ALS,[19] and mutated human SOD1 transgenic animals have been studied extensively as a disease model of ALS1, and sometimes of ALS in general.[20] GluR2 mRNA in the motor neurons of symptom-manifesting $SOD1^{G93A}$ and $SOD1^{H46R}$ transgenic rats was completely edited at the Q/R site.[21] Therefore, the death-inducing molecular mechanisms may be different between sporadic ALS and ALS1. Indeed, disease-causative mutations of the SOD1 gene were found in only a small percentage of

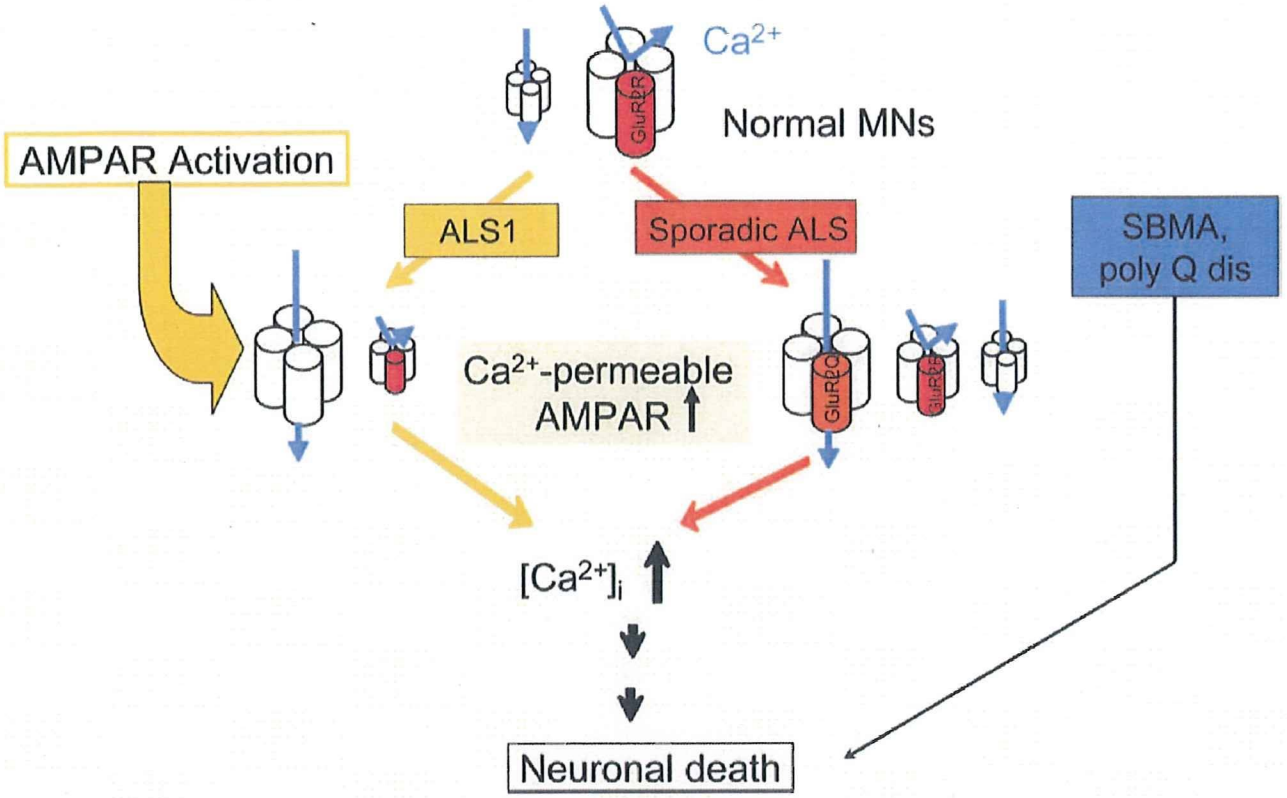

Fig. 2 α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptor-mediated and -unmediated pathways in death of motor neurons: Motor neurons normally express abundant Ca^{2+}-impermeable AMPA receptors and a small proportion of GluR2-lacking Ca^{2+}-permeable AMPA receptors, as well. Motor neurons in sporadic ALS patients express a significant proportion of glutamine/arginine (Q/R) site-unedited GluR2, resulting in an increased of Q/R site-unedited GluR2-containing Ca^{2+}-permeable AMPA receptors. By contrast, motor neurons in SOD1-associated familial ALS (ALS1) patients express greater amounts of GluR2-lacking AMPA receptors than those in control subject due to up-regulation of GluR3 and neuronal death occurs when AMPA receptors are activated. AMPA receptor-mediated neuronal death does not play a role in death of motor neurons in spinal and bulbar spinal atrophy (SBMA), a genomic glutamine codon (CAG)-repeat expansion disease.

patients with sporadic ALS.[27] Furthermore, abnormal accumulation of TDP-43 demonstrated in motor neurons of sporadic ALS[23,24] was not observed in motor neurons of SOD1-associated familial ALS.[25,26]

However, many recent studies support critical roles of Ca^{2+}-permeable AMPA receptors in motor neuron degeneration in ALS1. Transgenic animal studies have recently solidified the link between Ca^{2+}-permeable AMPA receptors and motor neuron loss in ALS1. Specifically, crossing $SOD1^{G93A}$ transgenic mouse models of ALS1 with either mice lacking GluR2 entirely[37] or mice expressing a modified GluR2 gene that encodes aspargine (N) at the Q/R site (GluR-B(N)), which is equivalent to Q/R site-unedited GluR2Q,[38] resulted in marked acceleration of the disease. Conversely, when mice with decreased numbers of Ca^{2+}-permeable AMPA receptors in their motor neurons (via targeted GluR2 overexpression) were crossed with the $SOD1^{G93A}$ transgenic mice, the disease was significantly delayed.[39] Because all the GluR2 mRNA was edited at the Q/R site in ALS1 model rat motor neurons,[21] these lines of evidence may indicate that an increase of Ca^{2+}-permeable AMPA receptors resulted from an increase of GluR2-lacking, but not of Q/R site-unedited GluR2-containing, AMPA receptors in ALS1. On the other hand, an increase in GluR3 mRNA has been reported in the motor neurons of $SOD1^{G93A}$ mice,[30] and the survival of these mice can be prolonged by the administration of GluR3 antisense protein nucleic acid.[31] Similarly, there was an up-regulation of total AMPA receptor subunit mRNAs due to a selective increase of GluR3 mRNA in motor neurons after the initiation of kainic acid infusion.[32]

Therefore, AMPA receptor-mediated neuronal death plays a pivotal role in both sporadic ALS and familial ALS1, but via different molecular mechanisms (Fig. 2). Investigation into the link between abnormal TAR DNA-binding protein of Mr 43 kDa (TDP-43) and GluR2 Q/R site under-editing may promote our understanding about the ALS pathogenesis.

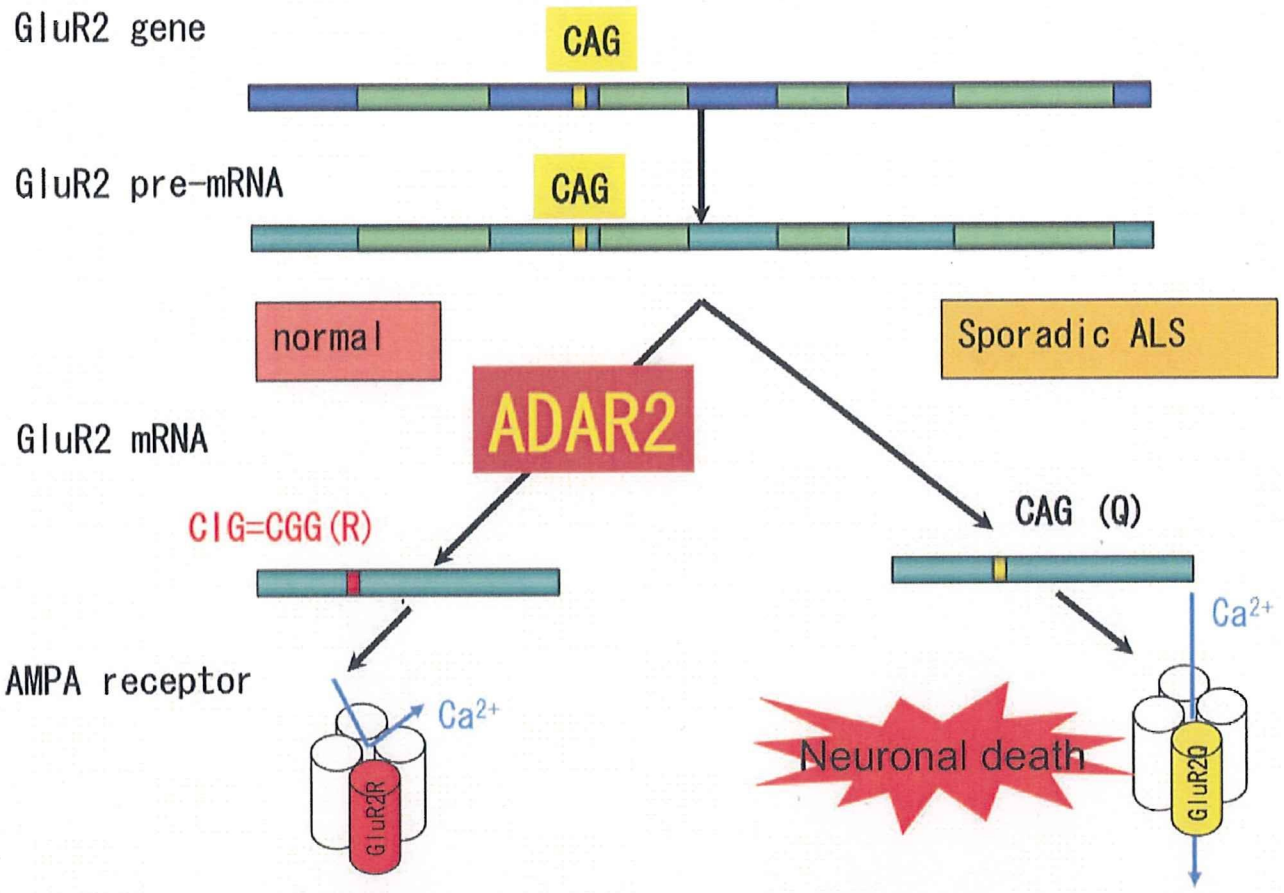

Fig. 3 RNA editing and neuronal death in sporadic ALS. The genomic glutamine codon (CAG) is converted to CIG post-transcriptionally due to A-to-I conversion by ADAR2, which is translated as CGG (arginine; R) in normal neurons. By contrast, due to ADAR2 underactivity, unedited mRNA is translated as GluR2 with glutamine (Q) at the Q/R site in motor neurons of sporadic ALS. α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors containing Q/R site-unedited GluR2 are Ca^{2+}-permeable and mediate neuronal death.

Another example of motor neuron disease is spinal and bulbar spinal atrophy (SBMA), in which the CAG-repeat expansion in the androgen receptor gene has been demonstrated[33] and pharmacological castration is therapeutically effective in animal models.[34,35] *N*-methyl-D-aspartate (NMDA) receptor-mediated neurotoxicity but not AMPA receptor-mediated neurotoxicity plays a role in the neuronal death in model mice of Huntington disease, another CAG-repeat expansion disease,[36] and we found that GluR2 Q/R site-editing was not affected in dying motor neurons in the autopsied SBMA spinal cord.[31]

Taken together, it is likely that there are multiple different death pathways in motor neurons, and motor neurons in sporadic ALS, ALS1 and SBMA die by different death cascades (Fig. 2).[37]

ADAR2 AND SPORADIC ALS

Enzymes responsible for the A-to-I conversion have been termed adenosine deaminases acting on RNA (ADARs), and three structurally related ADARs (ADAR1 to ADAR3) have been identified in mammals.[38] ADAR2 recognize the adenosine residue to be edited in the Q/R site of GluR2 through the structure of the duplex that is formed between the editing site and its editing site complementary sequence (ECS), which is located in the adjacent downstream intron of the precursor (pre-) mRNA[39] and catalyzes conversion of A-to-I at the Q/R site of GluR2 mRNA exon 11.

ADAR2 knockout mice died young from status epilepticus while expressing a markedly high proportion of Q/R site-unedited GluR2, but the ADAR2 knockout mice additionally expressed Q/R site-edited GluR2 without ADAR2 ($GluR\text{-}B^R$) and exhibited normal phenotype without developing seizure activity.[40] It is likely therefore that ADAR2-deficiency induces neuronal death via failure to edit the GluR2 Q/R site, which is demonstrated by our newly developed mouse line in which ADAR2 was conditionally targeted in the motor neurons using the Cre/LoxP system. The mutant mice exhibited

ALS-like phenotype via failure to edit the GluR2 Q/R site completely.[41]

One of the determinants of GluR2 Q/R site-RNA editing is the expression level of ADAR2 mRNA in human brain,[42] and the expression level of ADAR2 mRNA was significantly reduced in ventral gray matter of the autopsy-obtained spinal cord of sporadic ALS cases compared with that of control subjects.[43,44] Therefore, it is likely that a reduction in ADAR2 activity causes death of motor neurons via failure to edit efficiently the GluR2 Q/R site in sporadic ALS (Fig. 3).

These lines of evidence suggest that the drugs that up-regulate GluR2 Q/R site-editing may be potential therapeutic tools for sporadic ALS. We developed a modified HeLa cell line that stably expresses half-edited GluR2 pre-mRNA, and using this cell line, we searched for compounds that increase GluR2 Q/R site-editing. We found that several antidepressants have the potency at a concentration of μM orders.[45] Our results suggest that antidepressants have the potency to enhance GluR2 Q/R site-editing by either up-regulating the ADAR2 mRNA expression level or other unidentified mechanisms. It is important to investigate whether these antidepressants could enhance GluR2 Q/R site-editing *in vivo*. To this end, markers representing a wide range of ADAR2 activity may become useful tools for evaluation of the efficacy of therapy aiming at enhancement of ADAR2 activity. Messenger RNA of cytoplasmic fragile X mental retardation protein interacting protein 2 (CYFIP2) is ubiquitously expressed and is particularly abundant in the central nervous system, including motor neurons in the spinal cord. Extents of CYFIP2 K/E site-editing are in the range of 30% to 85% in human brain and spinal cord.[46] Therefore, the extent of CYFIP2 K/E site-editing may become an additional marker for ADAR2 activity in neuronal and other types of cells *in vivo*, as well as *in vitro*.[47]

Because it is apparent that the pathogenesis of sporadic ALS is different from familial ALS cases, including ALS1, research based on patient-derived death-inducing molecular abnormalities is absolutely necessary in sporadic ALS. Although little has been elucidated about the precise pathways of death cascade, evidence that ADAR2 underactivity is the specific molecular change seen in autopsied tissues of patients, will provide both new insight into the pathogenesis of sporadic ALS and also novel therapeutic strategies.

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