TDP-43DW1 (5′-TATTACGCGTCTACATTCCCCAGCCAGAAGACTTAG-3′). After gel purification, the PCR products were digested with *MluI* and cloned into a pCI mammalian expression vector (Promega, Madison, WI) that had been digested with the same restriction enzyme. For the construction of the TDP-43 fragments, each fragment was amplified using PCR with primers specific for residues 89–414 (TDP 89–414; forward, 5′-AAAAACGCGTATGGCTTCATCAGCAGTGAAAGTG-3′ and TDP-43DW1), or residues 220–414 (TDP 220–414; forward, 5′-AAAAACGCGTATGGTCTTCATCCCCAAGCCATTC-3′ and TDP-43DW1). The resulting products were digested with *MluI* and cloned into pCI (Promega). Mutant TDP-43 cDNAs carrying the A315T and M337V mutations were prepared using site-directed mutagenesis and cloned into the pCI vector. We sequenced the plasmids to confirm that there were no unwanted mutations.

The small interfering RNA (siRNA) used included human TDP-43 siRNA1 sense; r(GUAACAUGGUAACACUUAA)dTdT anti-sense; r(UUAAGUGUUACCAUGUUAC)dTdG human TDP-43 siRNA2 sense; r(CAAUAGCAAUAGACAGUUA)dTdT anti-sense; r(UAACUGUCUAUUGCUAUUG)dTdG and mouse TDP-43 siRNA1 sense; r(CGAUGAACCCAUUGAAAUA)dTdT anti-sense; r(UAUUUCAAUGGGUUCAUCG)dTdT mouse TDP-43 siRNA2 sense; r(GUAACAUGGUAACAUUAAA)dTdT anti-sense; r(UUUAAUGUUACCAUGUUAC)dTdG (Qiagen HP GenomeWide).

2.2. Cell culture and transfection

TetHeLaG2 cells (Sawada et al., 2009) were cultured in MEM-α medium (WAKO, Tokyo, Japan) supplemented with 10% Tet-System-Approved fetal bovine serum (TAKARA), 100 U/ml penicillin, 100 μg/ml streptomycin (Invitrogen), and with or without 0.75 μg/ml puromycin (TAKARA) in 5% CO_2 at 37 °C. Mouse neuroblastoma Neuro2a cells were cultured in high-glucose DMEM (WAKO, Tokyo, Japan) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin in 5% CO_2 at 37 °C. TetHeLa cells stably expressed the transfected GluA2 mini gene with constant editing efficiency of the pre-mRNA (Sawada et al., 2009) and Neuro2a cells expressed the intrinsic GluA2 gene with stable editing efficiency of the transcripts (Yamashita et al., 2012). The cells were grown to 70% confluence in 6-well culture plates and transfected with the expression constructs using Lipofectamine 2000 (Invitrogen) or Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions.

2.3. RNA extraction and reverse transcription

Total RNA was isolated from the cells using an illustra RNAspin RNA Isolation Kit (GE Healthcare Bioscience) and treated with DNaseI, as recommended by the manufacturer. First-strand cDNA was synthesized from the total RNA using Ready-To-Go You-Prime First-Strand Beads (GE Healthcare Bioscience) and 50 ng of random primers (Invitrogen) in a final volume of 33 μl, as recommended by the manufacturer. GluA2 was amplified as previously described (Sawada et al., 2009).

2.4. Analysis of the conversion of adenosine to inosine at the Q/R editing site

The efficiency of the conversion of adenosine to inosine in the GluA2 pre-mRNA was calculated following the digestion of PCR products with restriction enzymes using a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA) (Kawahara et al., 2003, 2004; Bhalla et al., 2004). The amplified GluA2 pre-mRNA PCR products were digested using *BbvI* (New England Biolabs, Ipswich, MA).

The PCR products from edited GluA2 pre-mRNA molecules contain one intrinsic *BbvI* recognition site, whereas the products originating from the unedited GluA2 contain an additional recognition site. Therefore, digestion of the PCR products from the edited GluA2 pre-mRNA should produce two bands (129 and 71 bp), whereas digestion of bands originating from the unedited GluA2 pre-mRNA molecules should produce three bands (91, 38 and 71 bp). The densities of the 71-bp band, which results from digestion of both the edited and unedited pre-mRNA, and the 129-bp band, which is only the product of the edited pre-mRNA, were quantified, and the editing efficiency was calculated as the former to the latter for each sample (Nishimoto et al., 2008; Sawada et al., 2009).

2.5. Real-time quantitative polymerase chain reaction

Quantitative PCR was performed using a LightCycler System (Roche Diagnostics, Indianapolis, IN). A set of standards and cDNA samples were 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 of each primer, and 0.1 μM of Universal Probe Library (Roche Diagnostics). The reaction was initially incubated at 95 °C for 10 min, and amplification of the templates was performed with a denaturing step at 95 °C for 10 s and a primer annealing step at 60 °C for 30 s. As an internal control, the expression of human β-actin was also measured in each sample using a LightCycler Primer/Probe Set (Roche Diagnostics) and the same PCR conditions (Sawada et al., 2009).

The PCR primers and probes were designed based on the cDNA sequence of GluA2 pre-mRNA, ADAR2 and β-actin, which were obtained from GenBank (GluA2, Accession Nos. NM000826, BC028736; GluA2 gene, Accession Nos. NC000004.11, NC000069.5; ADAR2, Accession Nos. NM015833, NM015834, NM130895, U76421, U76422; and β-actin, Accession Nos. NM001101, NM007393).

2.6. Western blot analysis

Neuro2a and TetHeLaG2m cells were homogenized by sonication in 20 volumes of extraction buffer (50 mM HEPES, pH 7.5, 1 mM EDTA, 100 mM NaCl, 10 mM DTT and 0.1% CHAPS). The homogenate was centrifuged at 1000 × *g* for 10 min at 4 °C. The supernatant was boiled with 4× SDS gel loading buffer, and subjected to SDS–PAGE. After electrophoresis, proteins were transferred to an Immobilon-P transfer membrane (Millipore, Bedford, MA) and immunoblotting for TDP-43 and β-actin was conducted. For the first antibodies, rabbit anti-human TDP-43 antibody (10782-1-AP, ProteinTech Group Inc., designated as TDP-43) (1:2500) and rabbit anti-β-actin antibody (IMGENEX Corp.) (1:2000) were used, and for the secondary antibodies, peroxidase-conjugated goat anti-rabbit IgG (Cell Signalling Technology, Inc., Danvers) (1:2000) were used. Visualization was carried out using ECL plus Western blotting detection reagents (GE Healthcare Bioscience, Piscataway, NJ, USA). Specific bands were detected with a LAS 3000 system (Fujifilm, Tokyo).

2.7. Statistical analysis

The data are presented as the mean ± SEM. Statistical analyses were carried out using the JMP 8 software (SAS Institute). For the statistical comparison of two groups, an unpaired, two-tailed Student's *t*-test was used. Differences were considered significant at $P < 0.05$.

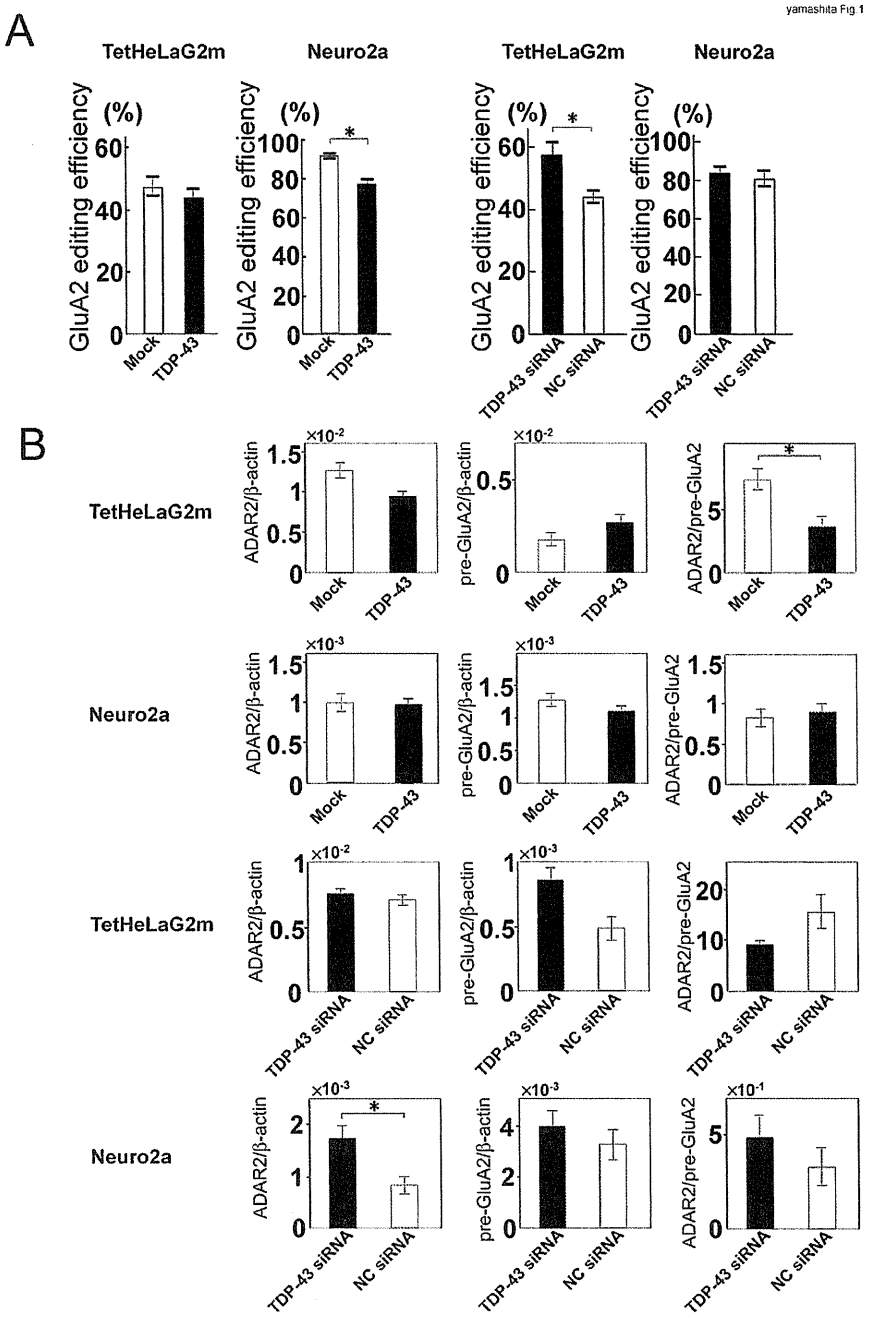

Fig. 1. The effects of altered TDP-43 expression on ADAR2 activity. The editing efficiency at the GluA2 pre-mRNA Q/R site was decreased after overexpression of human TDP-43 in Neuro2a cells but not in TetHeLaG2m cells. In contrast, the editing efficiency was increased in TetHeLaG2m cells, but not in Neuro2a cells, after TDP-43 knockdown. However, these changes were not correlated with the relative abundance of ADAR2 (the ratio of ADAR2 mRNA to GluA2 pre-mRNA), a determinant of ADAR2 activity. The data are presented as the mean ± SEM ($n = 3–5$, Student's t-test, $^*P < 0.05$).

3. Results

3.1. The effect of TDP-43 expression on ADAR2 activity

To test whether changes in TDP-43 expression could alter ADAR2 activity at the GluA2 Q/R site, we analyzed the editing efficiency and mRNA expression level of ADAR2 in TetHeLaG2m and Neuro2a cells that had been transfected with either the human TDP-43 expression constructs or an siRNA against TDP-43 (Supplementary Fig. S1). The effects of TDP-43 overexpression and knockdown of ADAR2 activity were inconsistent between the two cell lines: the overexpression of human TDP-43 decreased GluA2 pre-mRNA Q/R site editing (editing efficiency) in Neuro2a cells but not in TetHeLaG2m cells, whereas the knockdown of TDP-43 using siRNA increased the editing efficiency in TetHeLaG2m cells but not in Neuro2a cells (Fig. 1A). Previous studies have determined that ADAR2 activity is based on the expression level of ADAR2 relative to that of GluA2 mRNA or pre-mRNA in the human brain (Kawahara et al., 2003). Therefore, we examined the abundance of ADAR2 mRNA relative to GluA2 pre-mRNA using quantitative RT-PCR. Changes in the relative abundance of ADAR2 mRNA were observed in TetHeLaG2m cells overexpressing TDP-43 but not in

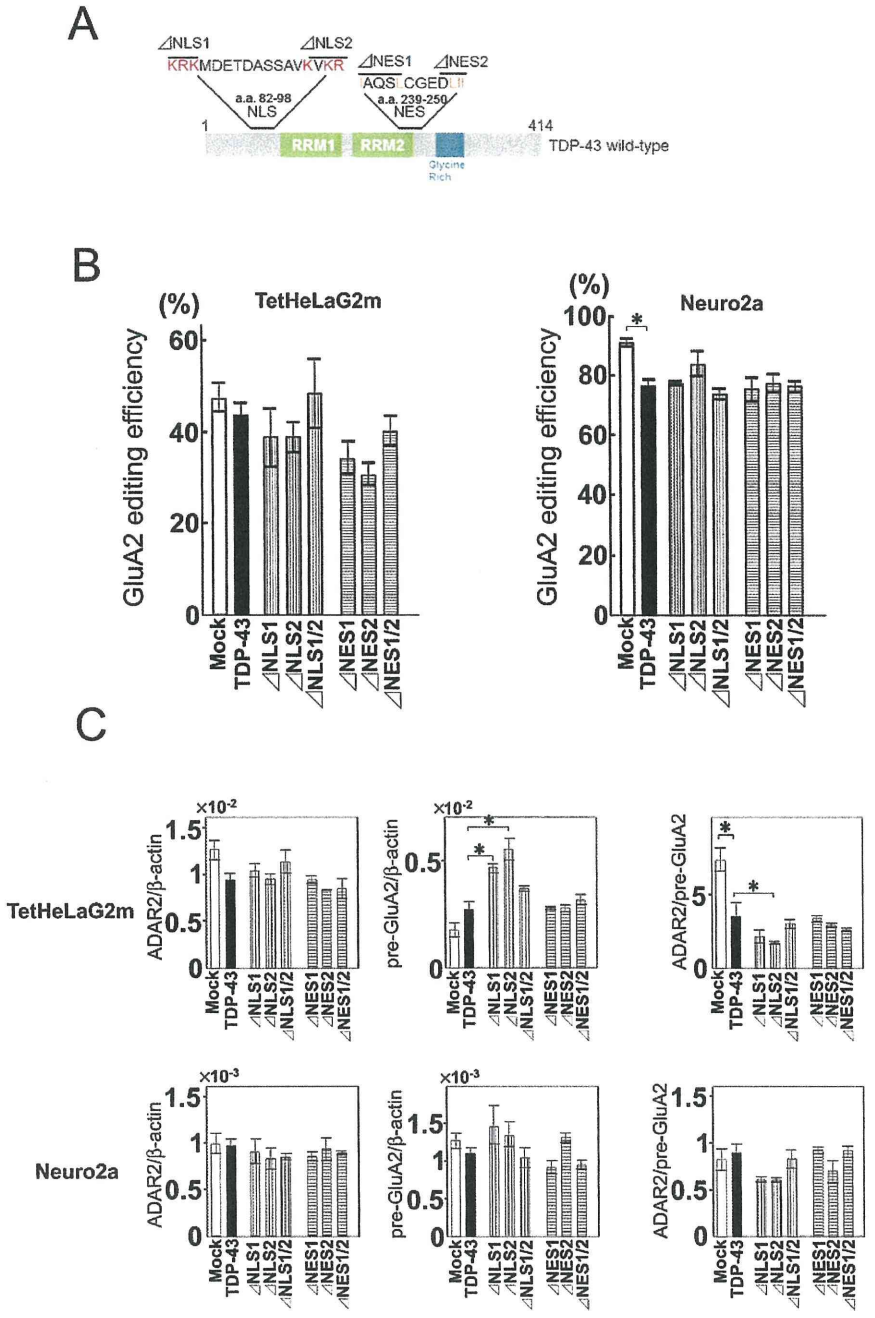

Fig. 2. The effects of the expression of TDP-43 lacking an NLS or NES on ADAR2 activity. (A) A schematic diagram of TDP-43 highlighting the location of the NLSs and NESs. Both sets of basic amino acids (red) in the NLS were mutated to generate three defective mutants (ΔNLS1, ΔNLS2, and ΔNLS1/2), and both sets of hydrophobic amino acids (orange) in the NES were altered to generate the ΔNES1, ΔNES2 and ΔNES1/2 mutants. (B) The editing efficiency in TetHeLaG2m and Neuro2a cells following expression of TDP-43 lacking an NLS (ΔNLS1, ΔNLS2, and ΔNLS1/2) or NES (ΔNES1, ΔNES2, and ΔNES1/2) is shown. None of the ΔNLS or ΔNES mutants changed the efficiency of GluA2 Q/R site editing. The data are presented as the means ± SEM (n = 3–5, Student's t-test, *P < 0.05). (C) Although the transfection of DNLS2 reduced the relative abundance of ADAR2 in TetHeLaG2m cells due to an increase in the GluA2 pre-mRNA level, there was no increase in the relative abundance of ADAR2 in Neuro2a cells transfected with DNLS2. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

the siRNA-treated TetHeLaG2m cells or the Neuro2a cells overexpressing TDP-43. Furthermore, the changes in the editing efficiency of GluA2 by TDP-43 overexpression in Neuro2a or knockdown in TetHeLaG2m cells were not associated with the changes in the expression levels of the ADAR2 mRNA, GluA2 pre-mRNA, or ADAR2 relative to the GluA2 pre-mRNA (Fig. 1A and B). The inconsistent results in the TetHeLaG2m and Neuro2a cells suggest that alterations in the expression level of TDP-43 do not play a significant role in the regulation of ADAR2 activity in these cultured cells.

3.2. The effect of removing the nuclear localization signal or the nuclear export signal of TDP-43 on ADAR2 activity

We investigated the effects of the expression of TDP-43 lacking either one or both of the nuclear localization signals (NLS1, ΔNLS2, or ΔNLS1/2) or TDP-43 lacking either one or both of the nuclear export signals (ΔNES1, ΔNES2, or ΔNES1/2) (Winton et al., 2008) because both TDP-43 and ADAR2 are nuclear proteins (Fig. 2A). None of the truncated forms of TDP-43 consistently changed the

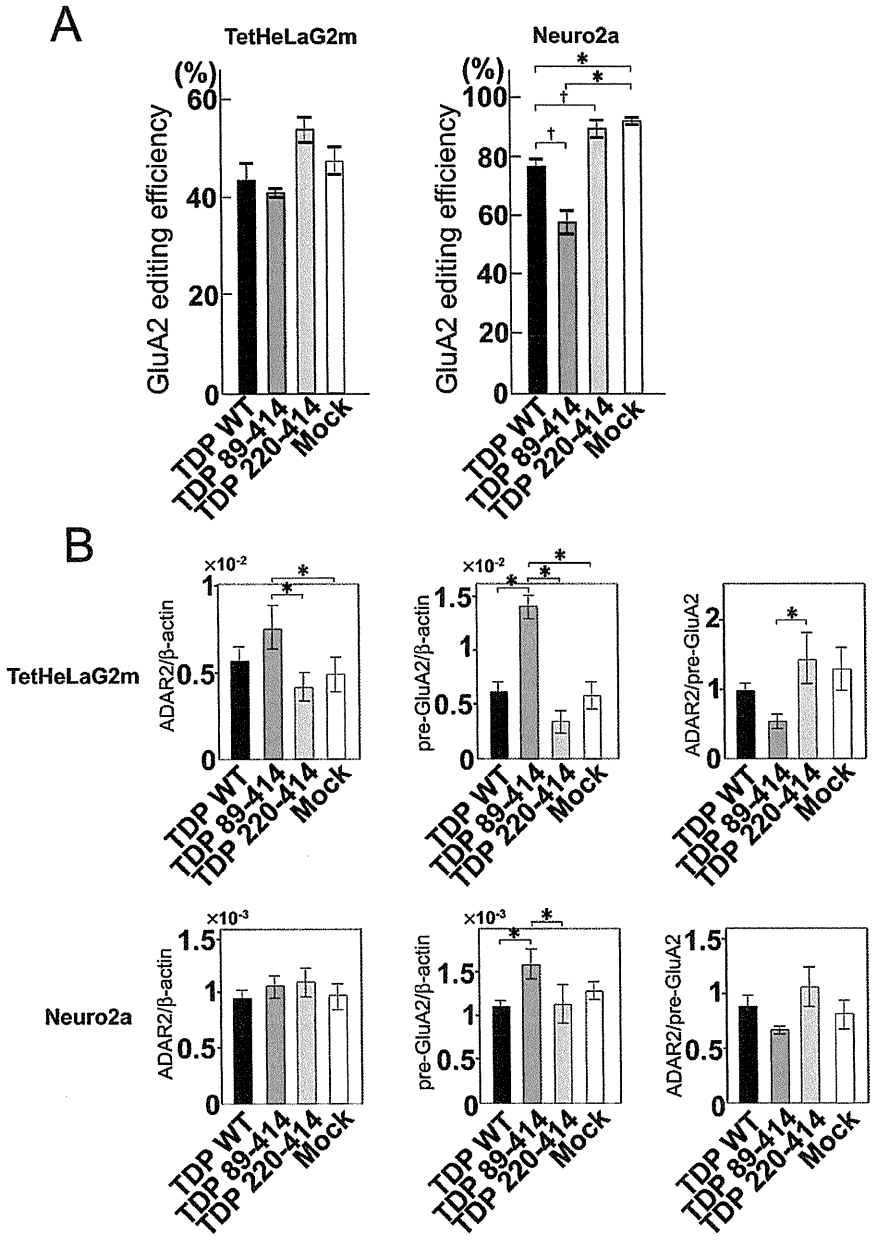

Fig. 3. The effects of caspase-3-cleaved TDP-43 fragments on ADAR2 activity. The editing efficiency in TetHeLaG2m and Neuro2a cells transfected with constructs expressing C-terminal fragments (CTFs) of TDP-43 (TDP 89–414 and TDP 220–414) is shown. GluA2 Q/R site editing is decreased in Neuro2a cells expressing TDP 89–414 but increased in Neuro2a cells expressing TDP 220–414. However, there is no change in the mRNA expression of ADAR2. The changes in editing efficiency may be the result of changes in the expression of the GluA2 pre-mRNA in Neuro2a cells expressing TDP 89–414. The data are presented as the means ± SEM ($n = 3$–5, Student's t-test, $^*P < 0.05$).

editing efficiency of ADAR2 (Fig. 2B). Because the expression level of ADAR2 mRNA is significantly reduced in ALS motor neurons (Hideyama et al., 2012), the abundance of the ADAR2 mRNA relative to the GluA2 pre-mRNA or to β-actin would have been decreased if nuclear TDP-43 had a regulatory role in the expression of ADAR2.

Although the transfection of ΔNLS2 reduced the relative abundance of ADAR2 in TetHeLaG2m cells due to an increase in the GluA2 pre-mRNA level (Fig. 2C), there was no increase in the relative abundance of ADAR2 in Neuro2a cells transfected with ΔNLS2 (Fig. 2B and C).

3.3. *The effect of caspase-3-cleaved C-terminal fragments of TDP-43 on ADAR2 activity*

Caspase-3 is thought to cleave TDP-43 into aggregation-prone C-terminal fragments (CTFs) that play a critical role in TDP-43 pathology by initiating the formation of cytoplasmic inclusions (Nonaka et al., 2009; Zhang et al., 2009). Therefore, we examined the effects of multiple TDP-43 CTFs on ADAR2 activity. A CTF consisting of amino acid residues 89–414 of TDP-43 (TDP 89–414) decreased the editing efficiency of ADAR2 in Neuro2a cells but not

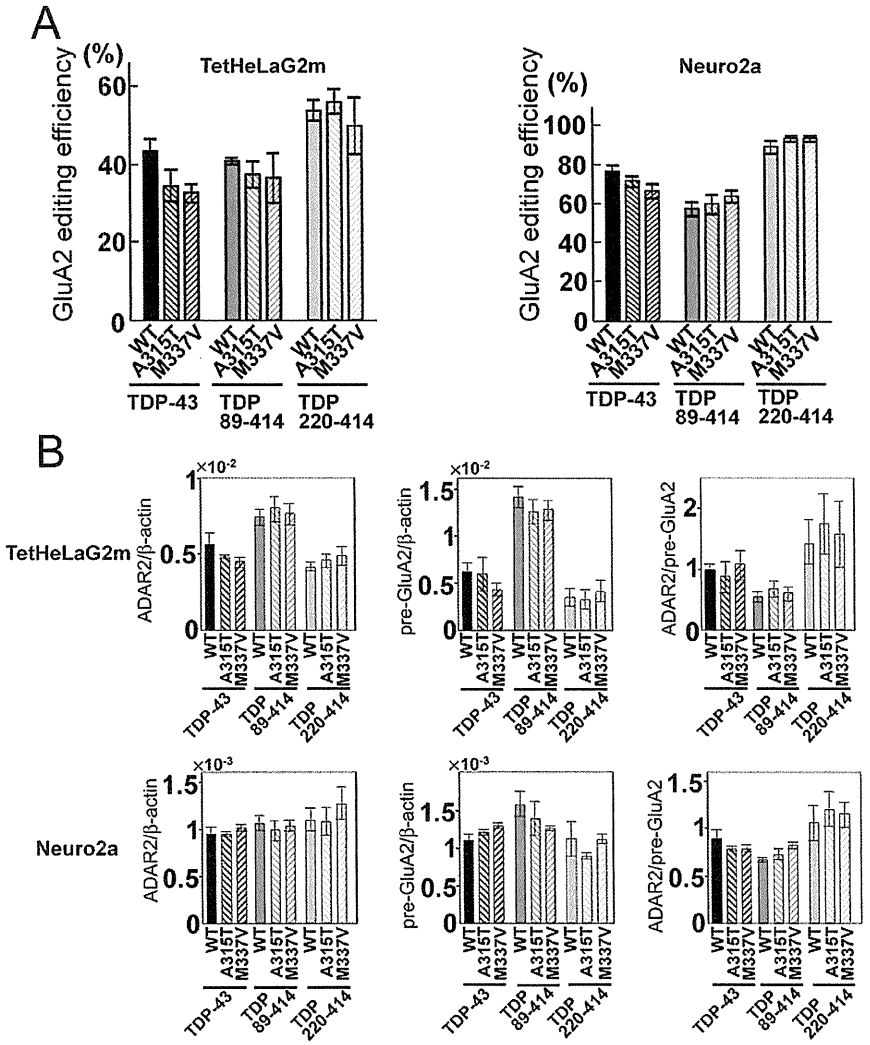

Fig. 4. The effects of mutated TDP-43 on ADAR2 activity. The editing efficiency in TetHeLaG2m and Neuro2a cells transfected with the constructs expressing full-length ALS-linked TDP-43 mutants or CTFs derived from these mutants is shown. The TDP-43 mutants did not affect GluA2 Q/R site editing. The data are presented as the means ± SEM (n = 3–5, Student's t-test, $^*P < 0.05$).

in TetHeLaG2m cells compared with the full-length human TDP-43; however, the CTF consisting of amino acid residues 220–414 of TDP-43 (TDP 220–414) increased the editing efficiency compared with full-length human TDP-43 in Neuro2a cells but not in TetHeLaG2m cells (Fig. 3A). The expression of ADAR2 mRNA relative to GluA2 pre-mRNA did not change in Neuro2a cells (Fig. 3B) but was increased in TetHeLaG2m cells without an associated increase in the editing efficiency (Fig. 3A and B).

3.4. The effect of ALS-linked TDP-43 mutations on ADAR2 activity

We tested the effects of ALS-linked TDP-43 mutations on ADAR2 activity by expressing full-length mutated TDP-43 (A315T and M337V) and CTFs (TDP 89–414 and TDP 220–414) derived from these mutants. Compared with cells transfected with wild-type TDP-43, neither the full-length TDP-43 mutants nor the CTFs from the TDP-43 mutants affected the editing efficiency (Fig. 4A) or the expression of ADAR2 mRNA relative to GluA2 pre-mRNA in TetHeLaG2m or Neuro2a cells (Fig. 4B).

4. Discussion

We aimed to determine if the abnormal processing of TDP-43 is upstream of inefficient GluA2 RNA editing and the molecular link between these two abnormalities in the motor neurons of patients with sporadic ALS (Kawahara et al., 2004; Aizawa et al., 2010). However, overexpression or knockdown of TDP-43, expression of TDP-43 lacking an NLS or NES, expression of CTFs from TDP-43 and expression of a full-length ALS-linked TDP-43 mutants or CTFs from the TDP-43 mutants failed to induce consistent changes in ADAR2 activity at the GluA2 Q/R site in two cultured cell lines. This result suggests that the abnormal processing of TDP-43 is likely not the cause of inefficient GluA2 RNA editing in ALS motor neurons.

A recent microarray study demonstrated that ADAR2 mRNA is a target RNA for TDP-43 (Sephton et al., 2011) and ADAR2 gene expression is reduced in the brain of TDP-43 knockdown mice (Polymenidou et al., 2011). These studies suggest a regulatory role of TDP-43 in the expression of ADAR2, which may induce downregulation of ADAR2 in ALS motor neurons that lack nuclear TDP-43. However, the extent of downregulation of ADAR2 in the TDP-43 knockdown animal brain (reduction by 22% compared to

wild-type mice) (Polymenidou et al., 2011) was much smaller than that in ALS motor neurons (approximately 70% compared to control motor neurons) (Hideyama et al., 2012). Normal motor neurons express only edited GluA2 (100% of GluA2 Q/R site-editing) unless the expression level of ADAR is kept at above half normal level in mouse motor neurons (Hideyama and Kwak, 2011; Hideyama et al., 2012). Therefore, only a modest decrease in ADAR2 expression does not explain the extensive expression of unedited GluA2 and the loss of ADAR2 immunoreactivity in motor neurons with TDP-43 pathology in patients with sporadic ALS (Aizawa et al., 2010).

TDP-43 pathology is not only observed in the motor neurons of ALS patients; it is also frequently observed in the cortical neurons of patients with various neurological diseases, including frontotemporal lobar degeneration with TDP-43-positive inclusions (FTLD-TDP), Alzheimer's disease (Tremblay et al., 2011; Wilson et al., 2011) and traumatic brain injury (McKee et al., 2010). In contrast, inefficient GluA2 RNA editing at the Q/R site occurs in the motor neurons of patients with sporadic ALS (Kawahara et al., 2004) and has not been observed in post-mortem brain tissue taken from patients with other neurological diseases, including Alzheimer's disease and FTLD (Suzuki et al., 2003). Furthermore, conditional ADAR2 knockout mice (AR2) exhibited a slow death of motor neurons resulting from a failure to edit the GluA2 Q/R site (Hideyama et al., 2010), suggesting a similar cell death mechanism may be occurring in ALS patients. Notably, both TDP-43 pathology and inefficient editing of the GluA2 Q/R site occur concomitantly in many of the motor neurons of patients with sporadic ALS, but neither of these two abnormalities were observed in the motor neurons of SOD1-associated ALS patients or SOD1 transgenic animals (Kawahara et al., 2006; Mackenzie et al., 2007; Tan et al., 2007), indicating that different pathways may underlie the pathogenesis of familial ALS (Kwak and Weiss, 2006) and that the link between these two abnormalities is specific to sporadic ALS. Our future work will test the hypothesis that the downregulation of ADAR2 can induce TDP-43-associated pathology.

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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.2012.02.015.

References

Aizawa, H., Sawada, J., Hideyama, T., Yamashita, T., Katayama, T., Hasebe, N., Kimura, T., Yahara, O., Kwak, S., 2010. TDP-43 pathology in sporadic ALS occurs in motor neurons lacking the RNA editing enzyme ADAR2. Acta Neuropathol. 120, 75–84.

Arai, T., Hasegawa, M., Akiyama, H., Ikeda, K., Nonaka, T., Mori, H., Mann, D., Tsuchiya, K., Yoshida, M., Hashizume, Y., Oda, T., 2006. TDP-43 is a component of ubiquitin-positive tau-negative inclusions in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. Biochem. Biophys. Res. Commun. 351, 602–611.

Bhalla, T., Rosenthal, J.J., Holmgren, M., Reenan, R., 2004. Control of human potassium channel inactivation by editing of a small mRNA hairpin. Nat. Struct. Mol. Biol. 11, 950–956.

Chen-Plotkin, A.S., Lee, V.M., Trojanowski, J.Q., 2010. TAR DNA-binding protein 43 in neurodegenerative disease. Nat. Rev. Neurol. 6, 211–220.

Hasegawa, M., Arai, T., Nonaka, T., Kametani, F., Yoshida, M., Hashizume, Y., Beach, T.G., Buratti, E., Baralle, F., Morita, M., Nakano, I., Oda, T., Tsuchiya, K., Akiyama, H., 2008. Phosphorylated TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. Ann. Neurol. 64, 60–70.

Hideyama, T., Kwak, S., 2011. When does ALS start? ADAR2-GluA2 hypothesis for the etiology of sporadic ALS. Front. Mol. Neurosci. 4, 33.

Hideyama, T., Yamashita, T., Aizawa, H., Tsuji, S., Kakita, A., Takahashi, H., Kwak, S., 2012. Profound downregulation of the RNA editing enzyme ADAR2 in ALS spinal motor neurons. Neurobiol. Dis. 45, 1121–1128.

Hideyama, T., Yamashita, T., Suzuki, T., Tsuji, S., Higuchi, M., Seeburg, P.H., Takahashi, R., Misawa, H., Kwak, S., 2010. Induced loss of ADAR2 engenders slow death of motor neurons from Q/R site-unedited GluR2. J. Neurosci. 30, 11917–11925.

Higuchi, M., Maas, S., Single, F.N., Hartner, J., Rozov, A., Burnashev, N., Feldmeyer, D., Sprengel, R., Seeburg, P.H., 2000. Point mutation in an AMPA receptor gene rescues lethality in mice deficient in the RNA-editing enzyme ADAR2. Nature 406, 78–81.

Kawahara, Y., Ito, K., Sun, H., Aizawa, H., Kanazawa, I., Kwak, S., 2004. Glutamate receptors: RNA editing and death of motor neurons. Nature 427, 801.

Kawahara, Y., Ito, K., Sun, H., Kanazawa, I., Kwak, S., 2003. Low editing efficiency of GluR2 mRNA is associated with a low relative abundance of ADAR2 mRNA in white matter of normal human brain. Eur. J. Neurosci. 18, 23–33.

Kawahara, Y., Sun, H., Ito, K., Hideyama, T., Aoki, M., Sobue, G., Tsuji, S., Kwak, S., 2006. Underediting of GluR2 mRNA, a neuronal death inducing molecular change in sporadic ALS, does not occur in motor neurons in ALS1 or SBMA. Neurosci. Res. 54, 11–14.

Kwak, S., Kawahara, Y., 2005. Deficient RNA editing of GluR2 and neuronal death in amyotropic lateral sclerosis. J. Mol. Med. 83, 110–120.

Kwak, S., Weiss, J.H., 2006. Calcium-permeable AMPA channels in neurodegenerative disease and ischemia. Curr. Opin. Neurobiol. 16, 281–287.

Lagier-Tourenne, C., Polymenidou, M., Cleveland, D.W., 2010. TDP-43 and FUS/TLS: emerging roles in RNA processing and neurodegeneration. Hum. Mol. Genet. 19, R46–R64.

Mackenzie, I.R., Bigio, E.H., Ince, P.G., Geser, F., Neumann, M., Cairns, N.J., Kwong, L.K., Forman, M.S., Ravits, J., Stewart, H., Eisen, A., McClusky, L., Kretzschmar, H.A., Monoranu, C.M., Highley, J.R., Kirby, J., Siddique, T., Shaw, P.J., Lee, V.M., Trojanowski, J.Q., 2007. Pathological TDP-43 distinguishes sporadic amyotrophic lateral sclerosis from amyotrophic lateral sclerosis with SOD1 mutations. Ann. Neurol. 61, 427–434.

McKee, A.C., Gavett, B.E., Stern, R.A., Nowinski, C.J., Cantu, R.C., Kowall, N.W., Perl, D.P., Hedley-Whyte, E.T., Price, B., Sullivan, C., Morin, P., Lee, H.S., Kubilus, C.A., Daneshvar, D.H., Wulff, M., Budson, A.E., 2010. TDP-43 proteinopathy and motor neuron disease in chronic traumatic encephalopathy. J. Neuropathol. Exp. Neurol. 69, 918–929.

Melcher, T., Maas, S., Herb, A., Sprengel, R., Seeburg, P.H., Higuchi, M., 1996. A mammalian RNA editing enzyme. Nature 379, 460–464.

Neumann, M., Sampathu, D.M., Kwong, L.K., Truax, A.C., Micsenyi, M.C., Chou, T.T., Bruce, J., Schuck, T., Grossman, M., Clark, C.M., McCluskey, L.F., Miller, B.L., Masliah, E., Mackenzie, I.R., Feldman, H., Feiden, W., Kretzschmar, H.A., Trojanowski, J.Q., Lee, V.M., 2006. Ubiquitinated TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. Science 314, 130–133.

Nishimoto, Y., Yamashita, T., Hideyama, T., Tsuji, S., Suzuki, N., Kwak, S., 2008. Determination of editors at the novel A-to-I editing positions. Neurosci. Res. 61, 201–206.

Nonaka, T., Kametani, F., Arai, T., Akiyama, H., Hasegawa, M., 2009. Truncation and pathogenic mutations facilitate the formation of intracellular aggregates of TDP-43. Hum. Mol. Genet. 18, 3353–3364.

Polymenidou, M., Lagier-Tourenne, C., Hutt, K.R., Huelga, S.C., Moran, J., Liang, T.Y., Ling, S.C., Sun, E., Wancewicz, E., Mazur, C., Kordasiewicz, H., Sedaghat, Y., Donohue, J.P., Shiue, L., Bennett, C.F., Yeo, G.W., Cleveland, D.W., 2011. Long pre-mRNA depletion and RNA missplicing contribute to neuronal vulnerability from loss of TDP-43. Nat. Neurosci. 14, 459–468.

Sawada, J., Yamashita, T., Aizawa, H., Aburakawa, Y., Hasebe, N., Kwak, S., 2009. Effects of antidepressants on GluR2 Q/R site-RNA editing in modified HeLa cell line. Neurosci. Res. 64, 251–258.

Seeburg, P.H., 2002. A-to-I editing: new and old sites, functions and speculations. Neuron 35, 17–20.

Sephton, C.F., Cenik, C., Kucukural, A., Dammer, E.B., Cenik, B., Han, Y., Dewey, C.M., Roth, F.P., Herz, J., Peng, J., Moore, M.J., Yu, G., 2011. Identification of neuronal RNA targets of TDP-43-containing ribonucleoprotein complexes. J. Biol. Chem. 286, 1204–1215.

Suzuki, T., Tsuzuki, K., Kameyama, K., Kwak, S., 2003. Recent advances in the study of AMPA receptors. Nihon yakurigaku zasshi Folia pharmacologica Japonica 122, 515–526.

Tan, C.F., Eguchi, H., Tagawa, A., Onodera, O., Iwasaki, T., Tsujino, A., Nishizawa, M., Kakita, A., Takahashi, H., 2007. TDP-43 immunoreactivity in neuronal inclusions in familial amyotrophic lateral sclerosis with or without SOD1 gene mutation. Acta Neuropathol. 113, 535–542.

Tremblay, C., St-Amour, I., Schneider, J., Bennett, D.A., Calon, F., 2011. Accumulation of transactive response DNA binding protein 43 in mild cognitive impairment and Alzheimer disease. J. Neuropathol. Exp. Neurol. 70, 788–798.

Wilson, A.C., Dugger, B.N., Dickson, D.W., Wang, D.S., 2011. TDP-43 in aging and Alzheimer's disease – a review. Int. J. Clin. Exp. Pathol. 4, 147–155.

Winton, M.J., Igaz, L.M., Wong, M.M., Kwong, L.K., Trojanowski, J.Q., Lee, V.M., 2008. Disturbance of nuclear and cytoplasmic TAR DNA-binding protein (TDP-43) induces disease-like redistribution, sequestration, and aggregate formation. J. Biol. Chem. 283, 13302–13309.

Yamashita, T., Tadami, C., Nishimoto, Y., Hideyama, T., Kimura, D., Suzuki, T., Kwak, S., 2012. RNA editing of the Q/R site of GluA2 in different cultured cell lines that constitutively express different levels of RNA editing enzyme ADAR2. Neurosci. Res., doi:10.1016/j.neures.2012.02.002.

Zhang, Y.J., Xu, Y.F., Cook, C., Gendron, T.F., Roettges, P., Link, C.D., Lin, W.L., Tong, J., Castanedes-Casey, M., Ash, P., Gass, J., Rangachari, V., Buratti, E., Baralle, F., Golde, T.E., Dickson, D.W., Petrucelli, L., 2009. Aberrant cleavage of TDP-43 enhances aggregation and cellular toxicity. Proc. Natl. Acad. Sci. U.S.A. 106, 7607–7612.

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RNA editing of the Q/R site of GluA2 in different cultured cell lines that constitutively express different levels of RNA editing enzyme ADAR2

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ABSTRACT

Adenosine deaminase acting on RNA 2 (ADAR2) catalyzes RNA editing at the glutamine/arginine (Q/R) site of GluA2, and an ADAR2 deficiency may play a role in the death of motor neurons in ALS patients. The expression level of ADAR2 mRNA is a determinant of the editing activity at the GluA2 Q/R site in human brain but not in cultured cells. Therefore, we investigated the extent of Q/R site-editing in the GluA2 mRNA and pre-mRNA as well as the ADAR2 mRNA and GluA2 mRNA and pre-mRNA levels in various cultured cell lines. The extent of the GluA2 mRNA editing was 100% except in SH-SY5Y cells, which have a much lower level of ADAR2 than the other cell lines examined. The ADAR2 activity at the GluA2 pre-mRNA Q/R site correlated with the ADAR2 mRNA level relative to the GluA2 pre-mRNA. SH-SY5Y cells expressed higher level of the GluA2 mRNA in the cytoplasm compared with other cell lines. These results suggest that the ADAR2 expression level reflects editing activity at the GluA2 Q/R site and that although the edited GluA2 pre-mRNA is readily spliced, the unedited GluA2 pre-mRNA is also spliced and transported to the cytoplasm when ADAR2 expression is low.

1. Introduction

Adenosine deaminase acting on RNA 2 (ADAR2) belongs to a family of mammalian RNA-editing enzymes (ADAR1–3) that have a catalytic domain responsible for the conversion of adenosine to inosine (A-to-I conversion) and two or three double-stranded RNA-binding domains (Kim et al., 1994; O'Connell et al., 1995, 1997; Melcher et al., 1996a,b; Chen et al., 2000). ADAR2 is expressed ubiquitously in mammalian organs and localizes predominantly in the nucleus (Desterro et al., 2003; Sansam et al., 2003; Aizawa et al., 2010). A-to-I conversions occur most abundantly in transcripts that are expressed predominantly in the central nervous system (CNS) of humans and other mammals, and ADAR2 plays a key role in regulating neuronal function by converting A into I in several pre-mRNAs (Kwak et al., 2008; Nishimoto et al., 2008; Riedmann et al., 2008; Sakurai et al., 2010), including the glutamine/arginine (Q/R) site in GluA2, a subunit of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptor (Brusa et al., 1995; Higuchi et al., 2000). In both mammalian and human CNSs, RNA editing at the GluA2 Q/R site occurs in neurons with virtually 100% efficiency throughout life beginning at the embryonic stage (Burnashev et al., 1992; Paschen and Djuricic, 1995; Carlson et al., 2000; Kawahara et al., 2003, 2004a,b). This conversion regulates the biologically indispensable properties of AMPA receptors, including permeability to Ca^{2+} and receptor trafficking (Hume et al., 1991; Burnashev et al., 1992; Lomeli et al., 1994). AMPA receptors containing an unedited GluA2 are permeable to Ca^{2+}, and ADAR2-null mice exhibit fatal epilepsy (Higuchi et al., 2000). In conditional ADAR2 knockout mice, motor neurons lacking ADAR2 slowly die as a result of the lack of GluA2 Q/R site-editing (Hideyama et al., 2010). Notably, motor neurons in patients with sporadic amyotrophic lateral sclerosis (ALS) express abundant unedited GluA2 mRNA (Takuma et al., 1999; Kawahara et al., 2004a; Kwak and Kawahara, 2005; Kwak et al., 2010), indicating that abnormal regulation of ADAR2 may play a role in the pathogenesis of sporadic ALS. Therefore, elucidation of the regulatory mechanisms underlying the ADAR2-mediated RNA editing of the GluA2 Q/R site is necessary.

In contrast to the neurons in the mammalian CNS, ADAR2-mediated GluA2 Q/R site-editing occurs with less than 100% efficiency in cultured cells (Rueter et al., 1995; Maas et al., 2001). Splicing and RNA editing are both post-transcriptional events that

Abbreviations: ADAR, adenosine deaminase acting on RNA; dsRNA, double-stranded RNA; ALS, amyotrophic lateral sclerosis; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate; GAPDH, glyceraldehyde-3 phosphate dehydrogenase.

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modify pre-mRNA. The double strand RNA structure required for ADAR2-mediated GluA2 Q/R site-editing is formed by an exon sequence encoding the Q/R site and the exon complementary sequence located in the downstream intron of GluA2 pre-mRNA. It is common that sites of selective editing are in the proximity of splice sites and that the editing event is dependent on intron sequence. In these cases, the complex of both editing and splicing might corroborate proper transcription (Bass, 2002; Nishikura, 2004). Several lines of evidence showed that the editing and splicing of the GluA2 pre-mRNA influence each other and the A-to-I conversion at the Q/R site has a positive effect on splicing (Gan et al., 2006; Ryman et al., 2007; Schoft et al., 2007).

In this study, we investigated the extent of Q/R site-editing of the GluA2 mRNA and pre-mRNA and the expression levels of the ADAR2 mRNA, GluA2 pre-mRNA and mRNA in several cultured cell lines. We found that the abundance of the ADAR2 mRNA relative to the GluA2 pre-mRNA was correlated with the extent of RNA editing at the Q/R site of the GluA2 pre-mRNA. We also found that although the edited GluA2 pre-mRNA was more readily spliced than unedited pre-mRNA, the unedited GluA2 mRNA was expressed when the ADAR2 level was low and that both the edited and unedited free GluA2 mRNA were transported to the cytoplasm.

2. Materials and methods

2.1. Cell culture

Tet-on-HeLa cells (TAKARA, Tokyo, Japan) were cultured in MEM-α medium (WAKO, Tokyo, Japan) supplemented with 10% Tet System Approved fetal bovine serum (TAKARA), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA, USA) with incubation condition at 37 °C with 5% CO_2. TetHeLaG2m cells (Nishimoto et al., 2008; Sawada et al., 2009) were cultured in the same condition except adding 0.75 μg/ml puromycin (TAKARA) in the medium. Rat INS1-D cells were cultured in RPMI-1640 (Invitrogen) with 11.1 mmol/l D-glucose (WAKO) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mmol/l HEPES (WAKO), 2 mmol/l L-glutamine (WAKO), 1 mmol/l sodium pyruvate (WAKO), and 50 μmol/l mercaptoethanol with incubation condition at 37 °C with 5% CO_2. Human neuroblastoma SH-SY5Y cells and human glioma U87MG and T98G cells were cultured in DMEM HAM F-12 (Invitrogen) and DMEM high glucose (WAKO), respectively, supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin in a 5% CO_2 at 37 °C. The culture medium was changed once after 24 h and then every 2 days. The cells were grown in 6-well plates at the density of 3.5×10^4 cell/cm^2 and were cultured for 24 h and 48 h, and then were harvested. In some experiments, we harvested cells after 72 h in culture (for Fig. 3).

2.2. Protein extraction and Western blot analysis

Nuclear and cytoplasmic fractions were separated with PARIS Protein and RNA Isolation System (Life Technologies) according to the manufacturer's instructions. The cultured cells (approximately 10^7 cells) were washed with cold PBS. The harvested cells were resuspended in 300 μl of ice-cold cell fractionation buffer of PARIS protein and RNA isolation system (Life Technologies), gently agitated, then incubated on ice for 10 min. The incubated cells were centrifuged at 500 × *g* at 4 °C for 5 min. The resultant supernatant was used as the cytoplasmic fraction and the pellet as the nuclear fraction. Aliquots of the nuclear and cytoplasmic fractions were boiled with 300 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, USA) and immunoblotting for histon protein (MAB052 (CHEMICON, Temecula, CA, USA) (1:1000), glyceraldehyde-3 phosphate dehydrogenase (GAPDH) (MAB374 (CHEMICON) (1:600) and ADAR2 (E-20, Santa Cruz Biotechnology, Santa Cruz, CA, USA) (1:5000) was conducted. For secondary antibodies, goat anti-mouse IgG (H+L) (Jackson ImmunoResearch, West Grove, PA, USA) (1: 5000), mouse and goat anti-mouse IgG (H+L) (Jackson ImmunoResearch, West Grove, PA, USA) (1:5000) and donkey anti-goat IgG HRP (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (1:5000) were used for respective primary antibodies. Visualization was carried out using ECL plus Western blotting detection reagents (GE Healthcare Bioscience, Piscataway, NJ, USA). Specific bands were detected with a LAS 3000 system (Fujifilm, Tokyo).

2.3. RNA extraction and reverse transcription

RNA was extracted from their cells in each well using an RNA-spin mini (GE Healthcare Bioscience) kit or PARIS Protein and RNA Isolation System (Life Technologies) according to the manufacturer's instructions. First-strand cDNA was synthesized from the total RNA extracted from nuclear and cytoplasmic fractions after treatment with *DNaseI* (Invitrogen) using Ready-To-Go You-Prime First-Strand Beads (GE Healthcare Bioscience) and 50 ng random primer (Invitrogen) in a final volume of 33 μl according to the manufacturer's instructions. Polymerase chain reaction (PCR) was performed under the condition as indicated in Table 1. The PCR amplification began with a 2 min denaturation step at 95 °C, followed by 30 cycles of denaturation at 95 °C for 10 s, annealing at 66 °C for 30 s and extension at 68 °C for 40 s. Nested PCR reaction was initiated at 95 °C for 2 min and amplification of templates was performed by 30 cycles of denaturation at 95 °C for 10 s annealing and extension at 68 °C for 30 s.

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

Extents of RNA editing at the Q/R site in GluA2 mRNA and pre-mRNA were expressed as the proportion (%) of edited transcripts in total transcripts calculated by quantitative analyses of the restriction digests of PCR products with a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA) (Kawahara et al., 2003, 2004b; Bhalla et al., 2004). The PCR products for GluA2 mRNA and GluA2 pre-mRNA were cleaved by *BbvI* (New England Biolabs, Ipswich, MA, USA).

Digestion of The PCR products with *BbvI* yields two bands at 129 and 71 bp when they were originated from edited GluA2 mRNA, whereas three bands at 91, 38 and 71 bp when they were originated from unedited GluA2 mRNA. The 71-bp band was originated from both edited and unedited GluA2 mRNA, while the 129-bp band was originated from only edited mRNA, hence the extent of editing was calculated as the molarity of the former to that of the latter for each sample.

2.5. Preparation of standard cDNA for quantitative polymerase chain reaction

Using the primers shown in Table 1, 2 μl of cDNA extracted from HeLa cells (human control) was subjected to PCR with 1 μl of advantage 2 polymerase mix (BD Biosciences Clontech, Palo Alto, CA, USA). After gel purification, PCR products were subcloned using the TOPO TA cloning kit (Invitrogen), and clones containing inserts were sequenced with an ABI PRISM 3100 sequencer (Applied Biosystems, Foster City, CA, USA). The concentration of each standard plasmid was measured spectrophotometrically at 260 nm (Nano Drop™ ND-1000; Nano Drop Technologies, Wilmington,

Table 1
Sequences of primers and TaqMan probes used for quantitative polymerase chain reaction.

		Oligonucleotide sequence	Universal probe library
Human GluA2 mRNA	hG2F2	5′-TTCCTGGTCAGCAGATTTAGCC-3′	
	hG2R2	5′-TTCCCTTTGGACTTCCGCAC-3′	
	hG2F4	5′-TCTGGTTTTCCTTGGGTGCC-3′	
	hG2R9	5′-AGATCCTCAGCACTTTCG-3′	
Human GluA2 pre-mRNA	hG2F2	5′-TTCCTGGTCAGCAGATTTAGCC-3′	
	hG2IR1	5′-GCAACATTCAAAGAACATTGTTC-3′	
	hG2F4	5′-TCTGGTTTTCCTTGGGTGCC-3′	
	hG2IR2	5′-CCGAAGCTAAGAGGATGTCCTTC-3′	
Rat GluA2 mRNA	hG2F2	5′-TTCCTGGTCAGCAGATTTAGCC-3′	
	rG2R1	5′-TTTCCTTTGGATTTCCGGACTC-3′	
	rG2F2	5′-TCTGGTTTTCCTTGGGTGCC-3′	
	rG2R2	5′-AGATCCTCAGCACTTTCG-3′	
Rat GluA2 pre-mRNA	hG2F2	5′-TTCCTGGTCAGCAGATTTAGCC-3′	
	rG2IR1	5′-TCAACATTCCAGGAACATTGTTC-3′	
	rG2F2	5′-TCTGGTTTTCCTTGGGTGCC-3′	
	rG2IR2	5′-ATGCCGATGGTAAAAATTTGCCCTC-3′	

		Oligonucleotide sequence	Universal probe library
Human GluA2 mRNA	hLC63F	5′-ATGCGATATTTCGCCAAGA-3′	probe: #63 (Roche cat. no. 04688627001)
	hLC63R	5′-CAGTCAGGAAGGCAGCTAAGTT-3′	
Human GluA2 pre-mRNA	hLC73F	5′-GATGGTGTCTCCCATCGAAA-3′	probe: #73 (Roche cat. no. 04688961001)
	hLC73R	5′-TCCATAAGCAATTTCTGTTTGCT-3′	
Human ADAR2	hLC42F	5′-GTGTAAGCACGCGTTGTACTG-3′	probe: #42 (Roche cat. no. 04688015001)
	hLC42R	5′-CGTAGTAAGTGGGAGGGAACC-3′	
Human β-actin	hLC64F	5′-GCAGAGGCGTACAGGGATAG-3′	probe: #64 (Roche cat. no. 04688635001)
	hLC64R	5′-GCAACCGCGAGAAGATGA-3′	
Rat GluA2 mRNA	rLC63F	5′-ATGCGATATTTCGCCAAGA-3′	probe: #63 (Roche cat. no. 04688627001)
	rLC63R	5′-CAGTCAGGAAGGCAGCTAAGTT-3′	
Rat GluA2 pre-mRNA	rLC73F	5′-TCAAAAGTGCATAGCTTTCTAAGTG-3′	probe: #73 (Roche cat. no. 04688961001)
	rLC73R	5′-CCAACTGGCTATCAGAAATAAGTG-3′	
Rat ADAR2	rLC42F	5′-TCCCGCCTGTGTAAGCAC-3′	probe: #42 (Roche cat. no. 04688015001)
	rLC42R	5′-TGGGCTTGGTGATCTTGG-3′	
Rat β-actin	rLC64F	5′-CCAACCGTGAAAAGATGACC-3′	probe: #64 (Roche cat. no. 04688635001)
	rLC64R	5′-ACCAGAGGCATACAGGGACA-3′	

DE). We prepared standard solutions by serial dilutions of the sample ranging from 10^{-11} to 10^{-10}, 10^{-9}, 10^{-8} and 10^{-6} μg/μl.

2.6. Real-time quantitative polymerase chain reaction

Quantitative PCR was performed using a LightCycler System (Roche Diagnostics, Indianapolis, IN, USA). A set of standard cDNA samples and test cDNA samples were amplified in a reaction mixture (20 μl in total volume) composed of 10 μl of 2× LightCycler 480 Probes Master Roche (Roche Diagnostics), 0.5 μM each primer, 0.1 μM Universal probes (Roche Diagnostics). The PCR reaction was initiated at 95 °C for 10 min and amplification of templates was performed by running denaturation at 95 °C for 10 s, primer annealing at 60 °C for 30 s. As an internal control, the expression of human β-actin was also measured with each sample using LightCycler-Primer/Probes Set (Roche Diagnostics) under the same PCR condition (Sawada et al., 2009).

The PCR primers and probes were designed from the cDNA sequences of GluA2, GluA2 mini-gene (preGluA2), ADAR2 and β-actin (as an internal control), which were obtained from GenBank (GluA2 (accession no. NM000826, BC028736), ADAR2 (accession no. NM015833, NM015834, U76421, U76422), β-actin (accession no. NM001101)).

2.7. Statistical analysis

The data are presented as the mean ± SEM. Statistical analyses were carried out using the JMP 8 software (SAS Institute). For the statistical comparison of two groups Mann–Whitney *U*-test was used. Correlation analysis was performed by Spearman rank correlation test. Differences were considered significant at $P < 0.05$.

3. Results

3.1. The extent of RNA editing at the GluA2 Q/R site in several cell lines

Nuclear and cytoplasmic fractions were isolated from Tet-on HeLa cells using the PARIS Protein and RNA Isolation System (Life Technologies). As detected using western blot, the ADAR2 protein was only detected in the nuclear fraction (Fig. S1).

Among the eight different cultured cell lines investigated, six cell lines (Tet-on HeLa, TetHeLaG2m, INS1-D, NSC-34, Neuro2a and SH-SY5Y) exhibited consistent extent of GluA2 Q/R site-editing over a given culture time, whereas two glioma cell lines demonstrated variable extent of RNA editing independent of culture time (Table 2 and Table S1). The extents of GluA2 mRNA editing were below 100% in SH-SY5Y cells and the glioma cell lines, but virtually 100% in the other five cell lines. By contrast, the extents of the GluA2 pre-mRNA editing were low, ranging from 4.6% to approximately 30% even in the cell lines expressing only edited GluA2 mRNAs (Fig. 1 and Table S1). Because of the unique profile of SH-SY5Y cells, which consistently expressed a large proportion of unedited GluA2 mRNAs,

Table 2
The extent of editing at the GluA2 Q/R (%) site in several cell lines.

U87MG	50.7 ± 4.2	$n=32$
T98G	77.5 ± 8.1	$n=16$
SH-SY5Y	53.5 ± 2.0	$n=18$
INS-1D	100 ± 0.0	$n=24$
Tet-on HeLa	100 ± 0.0	$n=10$
TetHeLaG2m	96.3 ± 2.1	$n=20$

Each value represents the mean ± SEM.

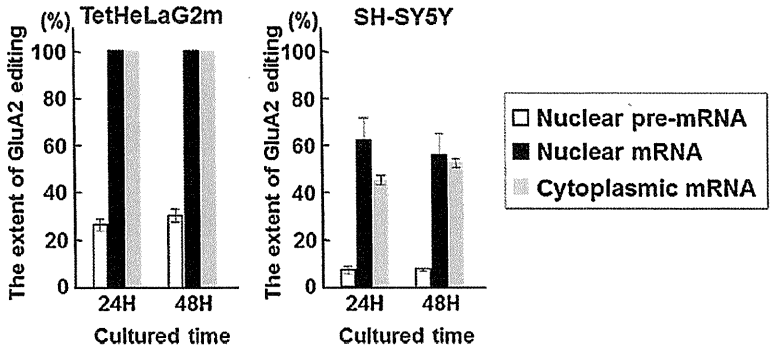

Fig. 1. Q/R site-editing of the GluA2 mRNA and pre-mRNA in the nuclear and cytoplasmic fractions. The proportion of edited GluA2 mRNAs and pre-mRNAs in TetHeLaG2m and SH-SY5Y cells. The white, black and gray columns represent the proportion of nuclear pre-mRNA, nuclear mRNA and cytoplasmic mRNA transcripts that were edited, respectively. The data are presented as the mean ± SEM ($n = 6$). The mean values for the GluA2 pre-mRNA are significantly lower than those for the GluA2 mRNA in the nucleus or in the cytoplasm (Mann–Whitney U-test, $^{**}P < 0.001$).

we compared the extents of RNA editing and expression levels of transcripts in SH-SY5Y cells and TetHeLaG2m cells.

3.2. The extent of the GluA2 mRNA and pre-mRNA editing in nuclear and cytoplasmic fractions

Next, we investigated the extent of the GluA2 mRNA and pre-mRNA editing in nuclear and cytoplasmic fractions isolated from the TetHeLaG2m cells and SH-SY5Y cells after 24 h or 48 h of culture. The extent of the GluA2 mRNA editing in TetHeLaG2m cells was 100 ± 0% in both the nuclear and cytoplasmic fractions irrespective of the time in culture (Fig. 1). However, the extent of RNA editing in SH-SY5Y cells decreased slightly from 61.9 ± 9.7% at 24 h to 55.8 ± 8.9% at 48 h in the nuclear fraction and increased from 45.3 ± 2.0% at 24 h to 52.6 ± 2.1% at 48 h in the cytoplasmic fraction (Fig. 1). The extents of the GluA2 pre-mRNA editing in both

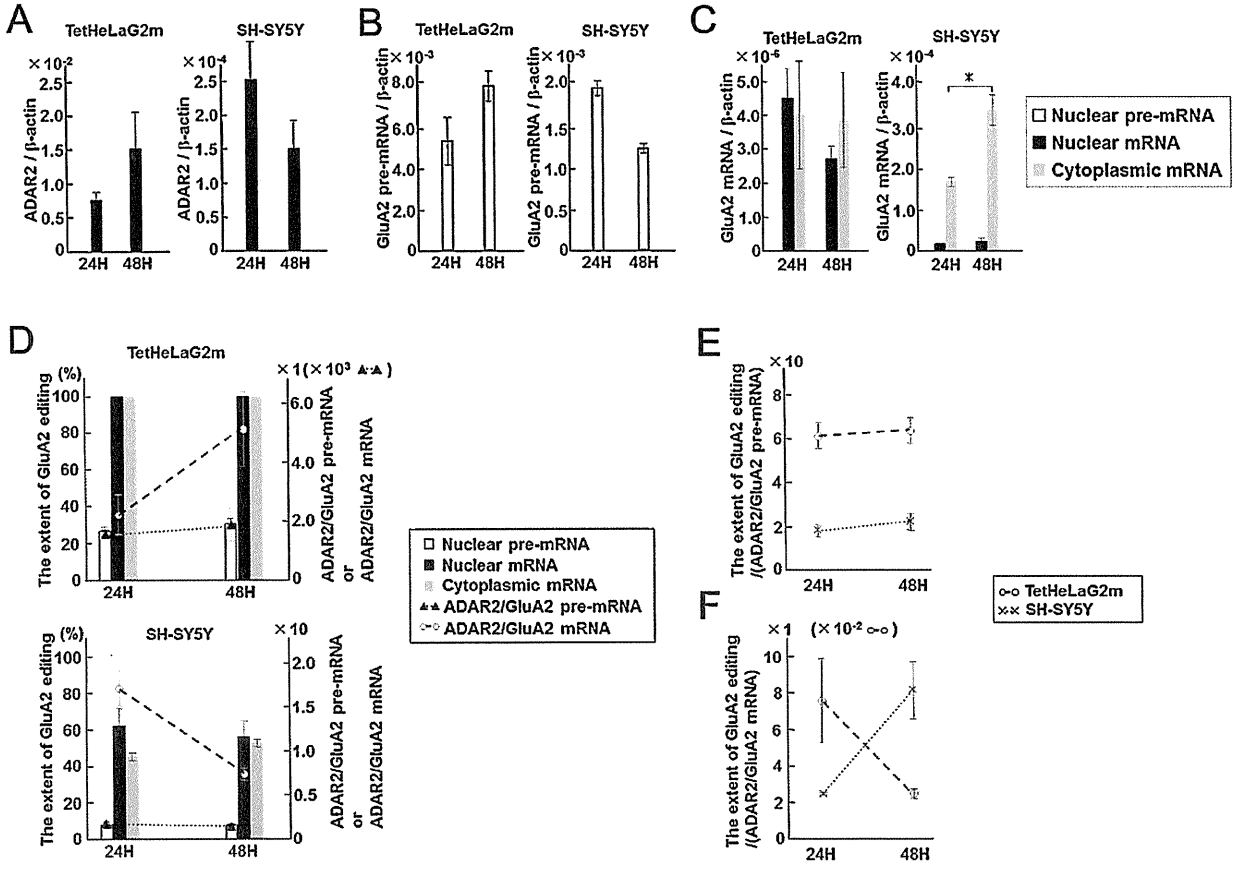

Fig. 2. Time-dependent changes in RNA editing and expression of the ADAR2 mRNA, GluA2 mRNA and pre-mRNA. The expression levels of the ADAR2 mRNA (A), GluA2 pre-mRNA (B) and GluA2 mRNA (C) in TetHeLaG2m and SH-SY5Y cells in culture for 24 h or 48 h; all values were normalized to β-actin expression. Compared with TetHeLaG2m cells, SH-SY5Y cells expressed significantly less ADAR2 mRNA and GluA2 pre-mRNA, but more GluA2 mRNA. The data are presented as the mean ± SEM ($n = 6$). The cytoplasmic GluA2 mRNA level in SH-SY5Y cells after 48 h is significantly higher than that after 24 h (Mann–Whitney U-test, $^{*}P < 0.006$). (D) The extent of GluA2 Q/R site-editing correlates more closely with the ADAR2 mRNA level relative to the GluA2 pre-mRNA level than with the ADAR2 mRNA level relative to the GluA2 mRNA level. (E and F) The extent of the GluA2 pre-mRNA editing correlates with the abundance of the ADAR2 mRNA relative to the GluA2 pre-mRNA (E), whereas the extent at the GluA2 mRNA Q/R site-editing does not correlate with the abundance of the ADAR2 mRNA relative to the GluA2 mRNA (F).

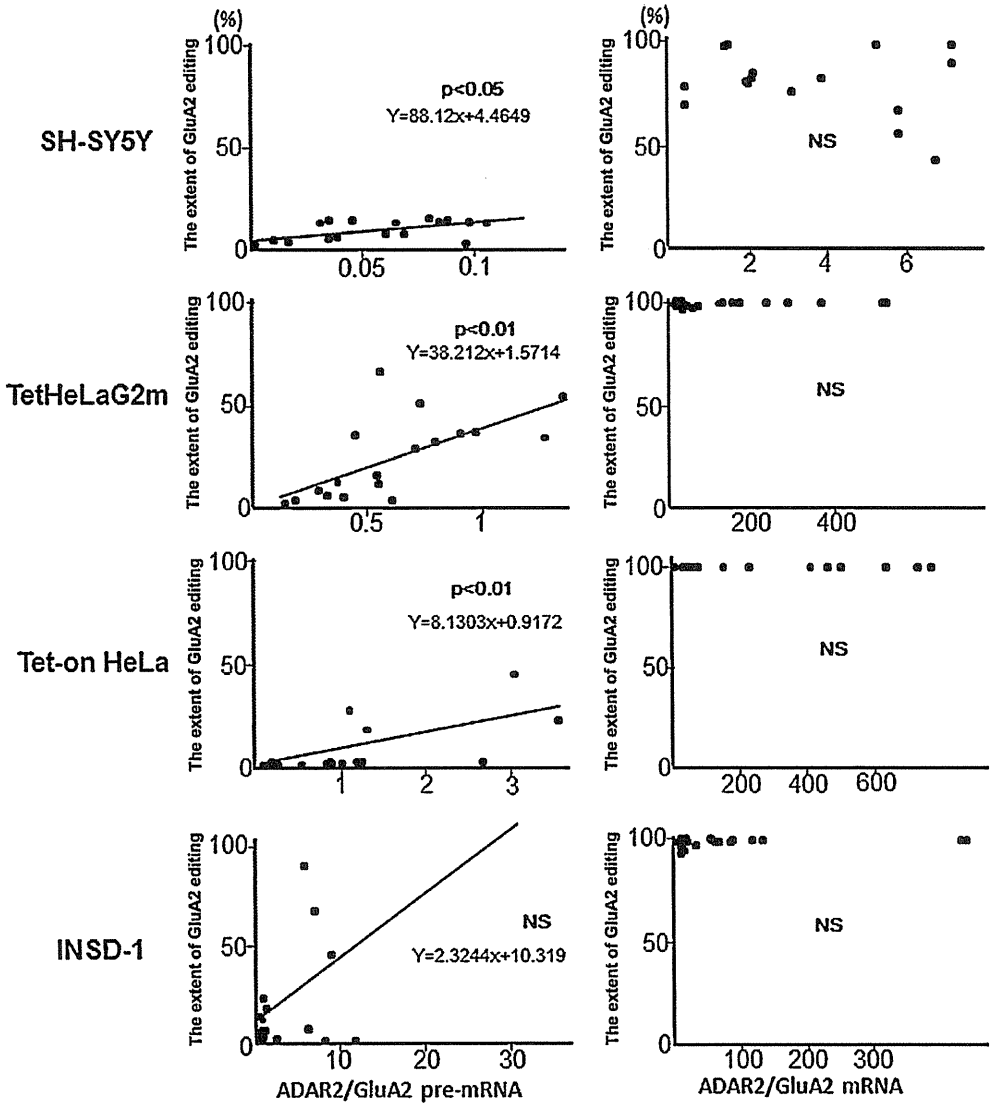

Fig. 3. The correlation between the extent of editing and the relative abundance of the ADAR2 mRNA in different cell lines. The extent of the GluA2 pre-mRNA editing significantly correlates with the ADAR2 mRNA level relative to the GluA2 pre-mRNA level in SH-SY5Y ($n = 18$, $r_s = 0.586$; $P < 0.05$), TetHeLaG2m ($n = 18$, $r_s = 0.661$; $P < 0.01$) and Tet-on HeLa cells ($n = 18$, $r_s = 0.666$; $P < 0.01$) but not in INSD-1 cells ($n = 15$, $r_s = 0.333$; NS) (left column). There was no correlation between the extent of the GluA2 mRNA editing and the ADAR2 mRNA level relative to the GluA2 mRNA level (right column). Each symbol represents the value for a culture dish at a given culture time. Correlation analysis was performed by Spearman rank correlation test.

TetHeLaG2m cells and SH-SY5Y cells were significantly lower than those of the GluA2 mRNA and did not change after 24 h or 48 h of culture ($P < 0.001$).

3.3. The expression levels of the ADAR2 mRNA, GluA2 mRNA and GluA2 pre-mRNA

Following normalization to β-actin expression, the expression levels of the transcripts differed among the cell lines (Fig. 2A–C and Table S1). SH-SY5Y cells expressed a one order of magnitude lower level of ADAR2 mRNA ($P < 0.005$) and a modestly lower level of GluA2 pre-mRNA ($P < 0.01$) but a one order of magnitude higher level of GluA2 mRNA than TetHeLaG2m cells (Fig. 2A–C).

The expression level of the ADAR2 mRNA was increased in TetHeLaG2m cells, but decreased in SH-SY5Y cells, after 48 h of culture when compared with the expression after 24 h of culture (Fig. 2A). The expression level of the GluA2 pre-mRNA changed in parallel with the ADAR2 expression level in both cell lines (Fig. 2B). The nuclear and cytoplasmic expression level of the GluA2 mRNA in TetHeLaG2m cells did not change with time, whereas the cytoplasmic expression level in SH-SY5Y cells after 48 h of culture was significantly greater than the expression after 24 h of culture ($P < 0.006$) (Fig. 2C).

3.4. Correlation between the extent of the GluA2 pre-mRNA editing and the abundance of the ADAR2 mRNA relative to the GluA2 pre-mRNA

We next compared the extent of Q/R site-editing with the abundance of the ADAR2 mRNA relative to the GluA2 mRNA and pre-mRNA. In SH-SY5Y cells, the expression level of the ADAR2 mRNA relative to the GluA2 pre-mRNA or the GluA2 mRNA was three orders of magnitude and two orders of magnitude lower than the levels in TetHeLaG2m cells, respectively (Fig. 2D). In both SH-SY5Y cells and TetHeLaG2m cells, the time-dependent change in the abundance of the ADAR2 mRNA correlated with that of the

GluA2 pre-mRNA but not with that of the GluA2 mRNA (Fig. 2D). The extent of Q/R site-editing correlated with the abundance of the ADAR2 mRNA relative to the GluA2 pre-mRNA at different culture time points (Fig. 2E), but not with that relative to the GluA2 mRNA (Fig. 2F).

There was a slight time-dependent change in the extent of Q/R site-editing and the abundance of the ADAR2 mRNA relative to the GluA2 pre-mRNA correlated with the extent of the GluA2 pre-mRNA editing in SH-SY5Y ($n=18$, $r_s=0.586$; $P<0.05$), TetHeLaG2m ($n=18$, $r_s=0.661$; $P<0.01$) and Tet-on HeLa ($n=18$, $r_s=0.666$; $P<0.01$) cells (Fig. 3), although the relative abundance of the ADAR2 mRNA markedly differed among the different cell lines examined (Fig. 2 and Table S1). There was no correlation between the relative abundance of ADAR2 mRNA and the extent of the GluA2 mRNA editing in all of the cell lines examined (Fig. 3).

4. Discussion

In this study, the extent of the Q/R site in the GluA2 mRNA editing was virtually 100% in five cell lines but approximately 50% in SH-SY5Y cells. Interestingly, the expression level of the ADAR2 mRNA in SH-SY5Y cells was more than one order of magnitude lower than the expression level seen in the other five cell lines. The extent of the GluA2 pre-mRNA editing was significantly lower than that of the GluA2 mRNA in all of the cell lines examined (Fig. 1 and Table S1), but the extent of editing was correlated with the abundance of the ADAR2 mRNA relative to the GluA2 pre-mRNA in the same cell line at different culture times. However, the abundance of the ADAR2 mRNA relative to the GluA2 pre-mRNA differed markedly among the cell lines examined (Fig. 2 and Table S1).

The low ADAR2 activity in SH-SY5Y cells may be important because of the expression of the unedited GluA2 mRNA in both the nucleus and the cytoplasm. The proportion of edited GluA2 pre-mRNA was correlated with the abundance of ADAR2 relative to the GluA2 pre-mRNA in SH-SY5Y cells, similar to what was seen in TetHeLaG2m (Fig. 2), Tet-on HeLa and INSI-D cells (data not shown). In addition, the expression level of the GluA2 pre-mRNA in SH-SY5Y cells did not markedly differ from that in TetHeLaG2m cells. These results suggest that ADAR2 edits the GluA2 pre-mRNA faster than splicing occurs and that the unedited GluA2 pre-mRNA can also undergo splicing in conditions with low ADAR2 levels, such as in SH-SY5Y cells (Fig. 2A). Notably, the cytoplasmic GluA2 mRNA level was markedly higher in SH-SY5Y cells compared with TetHeLaG2m cells (Fig. 2C), and approximately half of the GluA2 mRNA molecules in SH-SY5Y cells were edited despite the low proportion (less than 10%) of edited GluA2 pre-mRNAs (Fig. 2B). The high proportion of edited GluA2 mRNAs compared with GluA2 pre-mRNAs suggests that in SH-SY5Y cells, the edited GluA2 pre-mRNA is preferentially spliced compared with the unedited GluA2 pre-mRNA, consistent with previous reports detailing the inhibition of GluA2 pre-mRNA splicing at the exon harboring the Q/R site in the absence of editing (Higuchi et al., 2000) and that edited GluA2 pre-mRNAs are more readily spliced than unedited GluA2 pre-mRNAs (Ryman et al., 2007; Schoft et al., 2007). These results also suggest that free GluA2 mRNA molecules (i.e., unassociated with ADAR2) are readily transported to the cytoplasm irrespective of editing in SH-SY5Y cells because only edited GluA2 mRNA was associated with ADAR2 in human cerebellar nuclei (Nishimoto et al., 2008). Thus, Q/R site-editing may be a preferential determinant of the splicing of the GluA2 pre-mRNA but not a determinant of the extranuclear transport of the GluA2 mRNA.

A reduced mRNA level of ADAR2 relative to GluA2 mRNA is associated with the expression of unedited GluA2 mRNAs in the human brain (Kawahara et al., 2003, 2004b). In the human and mouse CNS, virtually all GluA2 mRNAs and the majority of the GluA2 pre-mRNAs are edited at the Q/R site (Higuchi et al., 2000; Kawahara et al., 2003, 2004a; Hideyama et al., 2010), in contrast to some cell lines in which unedited GluA2 pre-mRNAs are expressed abundantly. It is likely that the abundance of the ADAR2 mRNA relative to the GluA2 pre-mRNA correlates better than relative abundance to the GluA2 mRNA in the mammalian CNS, as in cultured cells. However, the high proportion of edited GluA2 pre-mRNAs also makes the abundance of ADAR2 relative to the GluA2 mRNA a suitable marker of ADAR2 activity at the GluA2 Q/R site in the human and mammalian CNS (Kawahara et al., 2003). The relative abundance of the ADAR2 mRNA determines the extent of the GluA2 pre-mRNA editing in the same cells, but the marked difference among the cell lines suggests that cell type-specific cofactors or regulators play roles as well (Peng et al., 2006; Marcucci et al., 2011). The present results also suggest that increased expression of the ADAR2 mRNA may activate GluA2 Q/R site-editing in human motor neurons and, therefore, may be a therapeutic strategy for the treatment of sporadic ALS, in which unedited GluA2 is abundantly expressed (Kwak et al., 2010).

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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.2012.02.002.

References

Aizawa, H., Sawada, J., Hideyama, T., Yamashita, T., Katayama, T., Hasebe, N., Kimura, T., Yahara, O., Kwak, S., 2010. TDP-43 pathology in sporadic ALS occurs in motor neurons lacking the RNA editing enzyme ADAR2. Acta Neuropathol. 120, 75–84.

Bass, B.L., 2002. RNA editing by adenosine deaminases that act on RNA. Annu. Rev. Biochem. 71, 817–846.

Bhalla, T., Rosenthal, J.J., Holmgren, M., Reenan, R., 2004. Control of human potassium channel inactivation by editing of a small mRNA hairpin. Nat. Struct. Mol. Biol. 11, 950–956.

Brusa, R., Zimmermann, F., Koh, D.S., Feldmeyer, D., Gass, P., Seeburg, P.H., Sprengel, R., 1995. Early-onset epilepsy and postnatal lethality associated with an editing-deficient GluR-B allele in mice. Science 270, 1677–1680.

Burnashev, N., Monyer, H., Seeburg, P.H., Sakmann, B., 1992. Divalent ion permeability of AMPA receptor channels is dominated by the edited form of a single subunit. Neuron 8, 189–198.

Carlson, N.G., Howard, J., Gahring, L.C., Rogers, S.W., 2000. RNA editing (Q/R site) and flop/flip splicing of AMPA receptor transcripts in young and old brains. Neurobiol. Aging 21, 599–606.

Chen, C.X., Cho, D.S., Wang, Q., Lai, F., Carter, K.C., Nishikura, K., 2000. A third member of the RNA-specific adenosine deaminase gene family, ADAR3, contains both single- and double-stranded RNA binding domains. RNA 6, 755–767.

Desterro, J.M., Keegan, L.P., Lafarga, M., Berciano, M.T., O'Connell, M., Carmo-Fonseca, M., 2003. Dynamic association of RNA-editing enzymes with the nucleolus. J. Cell Sci. 116, 1805–1818.

Gan, Z., Zhao, L., Yang, L., Huang, P., Zhao, F., Li, W., Liu, Y., 2006. RNA editing by ADAR2 is metabolically regulated in pancreatic islets and beta-cells. J. Biol. Chem. 281, 33386–33394.

Hideyama, T., Yamashita, T., Suzuki, T., Tsuji, S., Higuchi, M., Seeburg, P.H., Takahashi, R., Misawa, H., Kwak, S., 2010. Induced loss of ADAR2 engenders slow death of motor neurons from Q/R site-unedited GluR2. J. Neurosci. 30, 11917–11925.

Higuchi, M., Maas, S., Single, F.N., Hartner, J., Rozov, A., Burnashev, N., Feldmeyer, D., Sprengel, R., Seeburg, P.H., 2000. Point mutation in an AMPA receptor gene rescues lethality in mice deficient in the RNA-editing enzyme ADAR2. Nature 406, 78–81.

Hume, R.I., Dingledine, R., Heinemann, S.F., 1991. Identification of a site in glutamate receptor subunits that controls calcium permeability. Science 253, 1028–1031.
Kawahara, Y., Ito, K., Sun, H., Aizawa, H., Kanazawa, I., Kwak, S., 2004a. Glutamate receptors: RNA editing and death of motor neurons. Nature 427, 801.
Kawahara, Y., Ito, K., Sun, H., Ito, M., Kanazawa, I., Kwak, S., 2004b. Regulation of glutamate receptor RNA editing and ADAR mRNA expression in developing human normal and Down's syndrome brains. Brain Res. Dev. Brain Res. 148, 151–155.
Kawahara, Y., Ito, K., Sun, H., Kanazawa, I., Kwak, S., 2003. Low editing efficiency of GluR2 mRNA is associated with a low relative abundance of ADAR2 mRNA in white matter of normal human brain. Eur. J. Neurosci. 18, 23–33.
Kim, U., Garner, T.L., Sanford, T., Speicher, D., Murray, J.M., Nishikura, K., 1994. Purification and characterization of double-stranded RNA adenosine deaminase from bovine nuclear extracts. J. Biol. Chem. 269, 13480–13489.
Kwak, S., Hideyama, T., Yamashita, T., Aizawa, H., 2010. AMPA receptor-mediated neuronal death in sporadic ALS. Neuropathology 30, 182–188.
Kwak, S., Kawahara, Y., 2005. Deficient RNA editing of GluR2 and neuronal death in amyotropic lateral sclerosis. J. Mol. Med. 83, 110–120.
Kwak, S., Nishimoto, Y., Yamashita, T., 2008. Newly identified ADAR-mediated A-to-I editing positions as a tool for ALS research. RNA Biol. 5, 193–197.
Lomeli, H., Mosbacher, J., Melcher, T., Hoger, T., Geiger, J.R., Kuner, T., Monyer, H., Higuchi, M., Bach, A., Seeburg, P.H., 1994. Control of kinetic properties of AMPA receptor channels by nuclear RNA editing. Science 266, 1709–1713.
Maas, S., Patt, S., Schrey, M., Rich, A., 2001. Underediting of glutamate receptor GluR-B mRNA in malignant gliomas. Proc. Natl. Acad. Sci. U.S.A. 98, 14687–14692.
Marcucci, R., Brindle, J., Paro, S., Casadio, A., Hempel, S., Morrice, N., Bisso, A., Keegan, L.P., Del Sal, G., O'Connell, M.A., 2011. Pin1 and WWP2 regulate GluR2 Q/R site RNA editing by ADAR2 with opposing effects. EMBO J. 30, 4211–4222.
Melcher, T., Maas, S., Herb, A., Sprengel, R., Higuchi, M., Seeburg, P.H., 1996a. RED2, a brain-specific member of the RNA-specific adenosine deaminase family. J. Biol. Chem. 271, 31795–31798.
Melcher, T., Maas, S., Herb, A., Sprengel, R., Seeburg, P.H., Higuchi, M., 1996b. A mammalian RNA editing enzyme. Nature 379, 460–464.
Nishikura, K., 2004. Editing the message from A to I. Nat. Biotechnol. 22, 962–963.
Nishimoto, Y., Yamashita, T., Hideyama, T., Tsuji, S., Suzuki, N., Kwak, S., 2008. Determination of editors at the novel A-to-I editing positions. Neurosci. Res. 61, 201–206.
O'Connell, M.A., Gerber, A., Keller, W., 1997. Purification of human double-stranded RNA-specific editase 1 (hRED1) involved in editing of brain glutamate receptor B pre-mRNA. J. Biol. Chem. 272, 473–478.
O'Connell, M.A., Krause, S., Higuchi, M., Hsuan, J.J., Totty, N.F., Jenny, A., Keller, W., 1995. Cloning of cDNAs encoding mammalian double-stranded RNA-specific adenosine deaminase. Mol. Cell. Biol. 15, 1389–1397.
Paschen, W., Djuricic, B., 1995. Regional differences in the extent of RNA editing of the glutamate receptor subunits GluR2 and GluR6 in rat brain. J. Neurosci. Methods 56, 21–29.
Peng, P.L., Zhong, X., Tu, W., Soundarapandian, M.M., Molner, P., Zhu, D., Lau, L., Liu, S., Liu, F., Lu, Y., 2006. ADAR2-dependent RNA editing of AMPA receptor subunit GluR2 determines vulnerability of neurons in forebrain ischemia. Neuron 49, 719–733.
Riedmann, E.M., Schopoff, S., Hartner, J.C., Jantsch, M.F., 2008. Specificity of ADAR-mediated RNA editing in newly identified targets. RNA 14, 1110–1118.
Rueter, S.M., Burns, C.M., Coode, S.A., Mookherjee, P., Emeson, R.B., 1995. Glutamate receptor RNA editing in vitro by enzymatic conversion of adenosine to inosine. Science 267, 1491–1494.
Ryman, K., Fong, N., Bratt, E., Bentley, D.L., Ohman, M., 2007. The C-terminal domain of RNA Pol II helps ensure that editing precedes splicing of the GluR-B transcript. RNA 13, 1071–1078.
Sakurai, M., Yano, T., Kawabata, H., Ueda, H., Suzuki, T., 2010. Inosine cyanoethylation identifies A-to-I RNA editing sites in the human transcriptome. Nat. Chem. Biol. 6, 733–740.
Sansam, C.L., Wells, K.S., Emeson, R.B., 2003. Modulation of RNA editing by functional nucleolar sequestration of ADAR2. Proc. Natl. Acad. Sci. U.S.A. 100, 14018–14023.
Sawada, J., Yamashita, T., Aizawa, H., Aburakawa, Y., Hasebe, N., Kwak, S., 2009. Effects of antidepressants on GluR2 Q/R site-RNA editing in modified HeLa cell line. Neurosci. Res. 64, 251–258.
Schoft, V.K., Schopoff, S., Jantsch, M.F., 2007. Regulation of glutamate receptor B pre-mRNA splicing by RNA editing. Nucleic Acids Res. 35, 3723–3732.
Takuma, H., Kwak, S., Yoshizawa, T., Kanazawa, I., 1999. Reduction of GluR2 RNA editing, a molecular change that increases calcium influx through AMPA receptors, selective in the spinal ventral gray of patients with amyotrophic lateral sclerosis. Ann. Neurol. 46, 806–815.

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Profound downregulation of the RNA editing enzyme ADAR2 in ALS spinal motor neurons

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ABSTRACT

Amyotrophic lateral sclerosis (ALS) is the most common adult-onset fatal motor neuron disease. In spinal motor neurons of patients with sporadic ALS, normal RNA editing of GluA2, a subunit of the L-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor, is inefficient. Adenosine deaminase acting on RNA 2 (ADAR2) specifically mediates RNA editing at the glutamine/arginine (Q/R) site of GluA2 and motor neurons expressing Q/R site-unedited GluA2 undergo slow death in conditional ADAR2 knockout mice. Therefore, investigation into whether inefficient ADAR2-mediated GluA2 Q/R site-editing occurs universally in motor neurons of patients with ALS would provide insight into the pathogenesis of ALS. We analyzed the extents of GluA2 Q/R site-editing in an individual laser-captured motor neuron of 29 ALS patients compared with those of normal and disease control subjects. In addition, we analyzed the enzymatic activity of three members of the ADAR family (ADAR1, ADAR2 and ADAR3) in ALS motor neurons expressing unedited GluA2 mRNA and those expressing only edited GluA2 mRNA. Q/R site-unedited GluA2 mRNA was expressed in a significant proportion of motor neurons from all of the ALS cases examined. Conversely, motor neurons of the normal and disease control subjects expressed only edited GluA2 mRNA. ADAR2, but not ADAR1 or ADAR3, was significantly downregulated in all the motor neurons of ALS patients, more extensively in those expressing Q/R site-unedited GluA2 mRNA than those expressing only Q/R site-edited GluA2 mRNA. These results indicate that ADAR2 downregulation is a profound pathological change relevant to death of motor neurons in ALS.

Introduction

Amyotrophic lateral sclerosis (ALS) is the most common adult-onset fatal motor neuron disease with unknown etiology. In spinal motor neurons of patients with ALS, normal RNA editing of GluA2, a subunit of the L-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor, is inefficient (Kawahara et al., 2004a; Kwak and Kawahara, 2005; Takuma et al., 1999). This is in marked contrast to the fact that all GluA2 mRNA expressed in the spinal motor neurons was edited in control subjects (Kawahara et al., 2004a; Kwak and Kawahara, 2005; Takuma et al., 1999), in patients with motor neuron diseases other than sporadic ALS (Kawahara et al., 2006; Kwak and Kawahara, 2005) and in dying neurons in neurodegenerative diseases, including degenerating Purkinje cells of patients with spinocerebellar degeneration (Akbarian et al., 1995; Kawahara et al., 2004a; Suzuki et al., 2003).

Conversion of glutamine (Q) to arginine (R) at the Q/R site of GluA2 affects multiple AMPA receptor properties, including Ca^{2+} permeability of receptor-coupled ion channels, receptor trafficking, and assembly of receptor subunits (Burnashev et al., 1992; Greger et al., 2003; Greger et al., 2002; Sommer et al., 1991). In the GluA2 pre-mRNA, the adenosine coding for the Q/R site is converted to inosine (A-to-I conversion) by adenosine deaminase acting on RNA 2 (ADAR2) (Higuchi et al., 1993; Melcher et al., 1996), and inosine is read as guanosine during translation, thereby converting the genomic

Abbreviations: ALS, amyotrophic lateral sclerosis; ADAR2, adenosine deaminase acting on RNA 2; AH, anterior horn; AMPA, L-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; BLCAP, bladder cancer associated protein; CYFIP2, cytoplasmic fragile X mental retardation protein interacting protein 2; GluA2, AMPA receptor subunit 2; MSA, multiple system atrophy; PBP, progressive bulbar palsy; PH, posterior horn; Q/R, glutamine/arginine; RT-PCR, reverse transcription polymerase chain reaction.

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Table 1
Profile of the cases with sporadic ALS.

Individual	Age at onset (year)	Clinical type of ALS	Duration of illness	Initial symptom
A1	77	Classic	2 y	U/E
A2	60	Classic	2 y 3 mo	U/E
A3	39	Classic	13 y	U/E, L/E
A4	77	Classic	1 y 7 mo	L/E
A5	69	Classic	2 y	U/E
A6	31	Classic	5 y	U/E
A7	57	Classic	1 y 9 mo	U/E
A8	72	Classic	5 y 3 mo	U/E
A9	67	Classic	2 y	U/E
A10	54	Classic	3 y	U/E
A11	58	Classic	3 y	U/E
A12	42	Classic	6 mo	U/E, L/E
A13	64	Classic	7 mo	U/E
A14	70	Classic	1 y 3 mo	U/E, L/E
A15	70	Classic	4 y	U/E
A16	71	Classic	2 y 9 mo	U/E
A17	80	Classic	2 y	U/E
A18	56	Classic	5y4 mo	U/E
A19	22	Baso	8 mo	U/E
A20	39	PBP	1 y 4 mo	Bulbar
A21	77	PBP	2 y	Bulbar
A22	78	PBP	1 y 4 mo	Bulbar
A23	85	PBP	1 y 8 mo	Bulbar
A24	43	PBP	1 y 5 mo	Bulbar, U/E
A25	70	PBP	4 y	Bulbar
A26	58	ALS-D	10 mo	U/E, L/E
A27	76	ALS-D	2 y	U/E
A28	67	PBP	4 y 5 mo	Bulbar
A29	75	PBP	2 y 6 mo	Bulbar

Classic: limb-onset classical ALS, PBP: progressive bulbar palsy, ALS-D: ALS with dementia, Baso: ALS with basophilic inclusion body, y: year, mo: month, Bulbar: dysarthria and/or dysphagia, U/E: weakness and/or amyotrophy in upper extremities, L/E: weakness and/or amyotrophy in lower extremities.

Q codon (CAG) to an R codon (CGG) at the Q/R site of GluA2. Because A-to-I conversion at the GluA2 Q/R site occurs in all GluA2 expressed in neurons, only Q/R site-edited GluA2 is expressed in virtually all neurons in the mammalian brain (Seeburg, 2002). Failure of Q-to-R conversion at this site produces Ca^{2+}-permeable AMPA receptors, resulting in increased excitation of neurons, which has been shown to culminate in fatal status epilepticus in mice (Brusa et al., 1995; Feldmeyer et al., 1999; Higuchi et al., 2000). Moreover, ADAR2-deficient motor neurons undergo slow death due to failure of GluA2 Q/R site-editing with expression of unedited GluA2 in conditional ADAR2 knockout (ADAR2$^{flox/flox}$VAChT-Cre.Fast or AR2) mice (Hideyama et al., 2010). Because A-to-I nucleotide conversion in the pre-mRNA for the GluA2 Q/R site is critical to survival of motor neurons, we investigated whether the expression of unedited GluA2 with a reduction of ADAR2 occurs in motor neurons of patients with sporadic ALS irrespective of the phenotype.

Materials and methods

All studies were carried out in accordance with the Declaration of Helsinki, and the Ethics Committee of the University of Tokyo approved the experimental procedures used.

Study population and spinal cord and brain tissue samples

Frozen spinal cords from pathologically proven patients with ALS (n = 29; age 23–87 years) were used in this study (Table 1). Spinal cords from patients with multiple system atrophy (MSA) (n = 5; age 65–78 years) and from control subjects (n = 12; age 27–82) were used as disease and normal controls, respectively (Table 2). The ALS group included classical limb-onset ALS, progressive bulbar palsy (PBP), ALS with long clinical course, ALS with basophilic inclusion body, and ALS with dementia. All patients were clinically examined by neurologists prior to death and none had relatives with ALS. Previously reported cases (Kawahara et al., 2004a) were included in this study, but we obtained additional samples from these cases. Spinal cord tissues were rapidly frozen on dry ice immediately after removal at autopsy and were kept at −80 °C until use. Written informed consent was obtained from all of the subjects prior to death or from their relatives.

Samples of the anterior horn (AH), posterior horn (PH) and white matter (SpW) of the spinal cord were obtained by dissecting frozen axial spinal cord sections under a binocular microscope within a freezing chamber. In addition, single motor neurons were dissected with a laser microdissector (Leica AS LMD, Leica Microsystems) as previously described (Kawahara et al., 2004a; Kawahara et al., 2003b; Takuma et al., 1999). Total RNA was isolated from dissected tissues, and first-strand cDNA was synthesized and treated with DNAase I (Invitrogen) as previously described (Kawahara et al., 2003a; Kawahara et al., 2003b).

Analysis for editing efficiency at A–I sites

Editing efficiencies at the Q/R sites in GluA2 mRNA were calculated by quantitative analyses of the digests of RT-PCR products with *BbvI* as previously described (Kawahara et al., 2004a; Kawahara et al., 2003a; Kawahara et al., 2003b; Takuma et al., 1999). In brief, 2 μl of cDNA was subjected to first PCR in duplicate in a reaction mixture of 50 μl containing 200 mM each primer, 1 mM dNTP Mix (Eppendorf AG), 5 μl of 10× PCR buffer and 1 μl of Advantage 2 Polymerase mix (BD Biosciences Clontech). The PCR amplification began with a 1-min denaturation step at 95 °C, followed by 40 cycles of denaturation at 95 °C for 10 s, annealing at 58 °C for 30 s and extension at 68 °C for 40 s. Nested PCR was conducted on 2 μl of the first PCR product under the same conditions with the exception of the annealing temperature (56 °C). Primer pairs used for each PCR were listed in Table S1. After gel purification using the Zymoclean Gel DNA Recovery Kit according to the manufacturer's protocol (Zymo Research), an aliquot (0.5 mg) was incubated with *BbvI* (New England BioLabs) at 37 °C for 12 h. The PCR products originating from Q/R site-edited GluA2 mRNA had one intrinsic restriction enzyme recognition site, whereas those originating from unedited mRNA had an additional recognition site. Thus, restriction digestion of the PCR products originating from edited GluA2 mRNA should produce different numbers of fragments (two bands at 116- and 66-bp) from those originating from unedited GluA2 mRNA (three bands at 35-, 81- and 66-bp). As the 66-bp band would originate from both edited and unedited mRNA, but the 116-bp band would originate from only edited mRNA, we

Table 2
Profile of the cases with sporadic ALS, control, and disease control (MSA).

Cases	Sporadic ALS				Control	MSA
	Classic	Baso	PBP	ALS-D		
	n = 18	n = 1	n = 8	n = 2	n = 12	n = 5
Age at onset (year) mean ± SD (range)	61.9 ± 13.7 (31–80)	22	66.8 ± 16.8 (41–87)	67.0 (58–76)	52.4 ± 18.0 (27–82)	71.6 ± 5.5 (65–78)
Duration of illness (month) mean ± SD (range)	38.2 ± 34.2 (6–156)*1	8	31.8 ± 12.5 (16–53)	17 (10–24)		

*1; with long duration (1 case): 156-mo, Classic: classical limb-onset ALS; PBP: progressive bulbar palsy; ALS-D: ALS with dementia; Baso: ALS with basophilic inclusion body; Control: neurologically free control subjects; MSA: multiple system atrophy. Please refer to Table 1 for a more detailed profile of the cases with sporadic ALS.

quantified the molarity of the 116- and 66-bp bands using the 2100 Bioanalyzer (Agilent Technologies) and calculated the editing efficiency as the ratio of the former to the latter for each sample (Supplementary Table 1).

With similar methods, we calculated the editing efficiencies at the Q/R sites in GluR6 mRNA and in GluA2 pre-mRNA, the R/G site in GluA2 mRNA, and the K/E sites in cytoplasmic fragile X mental retardation protein interacting protein 2 (CYFIP2) mRNA and pre-mRNA (Supplementary Table 1) (Kawahara et al., 2004a; Kawahara et al., 2003a; Kawahara et al., 2003b; Kwak et al., 2008; Nishimoto et al., 2008; Paschen et al., 1994; Takuma et al., 1999). Following restriction enzymes were used for restriction digestion of the respective A-to-I sites; *BbvI* for the Q/R sites, *NlaIV* (New England BioLabs) for the R/G sites, and *MseI* (New England BioLabs) for the K/E site. Primer pairs used for each PCR and sizes of restriction digests of PCR products were indicated in Supplementary Table 1.

Quantitative PCR

The expression levels of ADAR1, ADAR2, ADAR3, GluA2 and β-actin mRNAs were measured using the LightCycler system (Roche Diagnostics, Indianapolis, IN) (Kawahara et al., 2004a; Kawahara et al., 2003a; Takuma et al., 1999). To prepare an internal standard for quantitative PCR, gene-specific PCR products of approximately 1 kb in length were amplified from human cerebellar cDNA with the same primer pairs as previously reported (Supplementary Table 2) (Kawahara et al., 2004a; Kawahara et al., 2003a; Takuma et al., 1999). Each cDNA sample was amplified in a reaction mixture (20 ml total volume) composed of 10 μl of 2× LightCycler 480 Probes Master Roche (Roche Diagnostics), 0.5 mM of each primer set and 0.1 mM probes (Universal Probe Library Set, Human, Roche Diagnostics) (Supplementary Table 3).

DNA sequence

Editing efficiency at the Y/C site in mRNA of bladder cancer associated protein (BLCAP) was evaluated by sequencing the PCR products with a 3100 Genetic Analyzer sequencer (Applied Biosystems, Foster City, CA) (Supplementary Table 1).

Statistical analysis

Differences and correlations between two groups were evaluated using Mann–Whitney *U*-test with SPSS software (version 15; SPSS Inc., Chicago, IL) and Pearson product-moment correlation coefficient with GraphPad Prism version 5 (GraphPad Software, Inc., La Jolla, CA), respectively. Moreover, differences between three groups were evaluated using repeated ANOVA with GraphPad Prism version 5. Differences were considered statistically significant with $p<0.05$ and highly significant with $p<0.01$.

Results

Inefficient GluA2 editing in motor neurons is a universal molecular abnormality in patients with sporadic ALS

We analyzed the extent of GluA2 Q/R site-editing in spinal cord and motor neuron lysates from 29 cases of pathologically proven sporadic ALS of various phenotypes (Table 1). Efficiency of RNA editing at the GluA2 Q/R site in the anterior horn (AH) was nearly 100% in the control subjects, whereas efficiency was significantly lower in all of the ALS cases examined (Fig. 1A). To test whether unedited GluA2 mRNA was universally expressed in motor neurons in ALS cases, we examined the extent of GluA2 Q/R site-editing in lysates of motor neurons. Reverse transcription polymerase chain reaction (RT-PCR) products for GluA2 mRNA were not always yielded from lysates of a single motor neuron and approximately one third of the lysates yielded amplicons for GluA2 mRNA. When we could not effectively amplify RT-PCR products from any of the lysates of a single motor neuron in a case, we used lysates of 30 motor neurons dissected from the same case. We dissected more than 10,000 motor neurons, including 500 motor neurons of control subjects, 600 motor neurons of patients with MSA and 1270 motor neurons of patients with ALS. These lysates except those of 100 motor neurons from two ALS case (A28, A29) yielded GluA2 RT-PCR amplicons. The number of motor neurons examined and the mean editing efficiency in each case were shown in Supplementary Table 1. We found that at least one motor neuron in each of the 27 ALS cases expressed detectable amounts of unedited GluA2 mRNA. The proportion of unedited GluA2 varied among motor neurons in each ALS case, ranging from 0% to 100%. Conversely, all of the motor neurons from control subjects and patients with MSA expressed only edited GluA2 mRNA (Fig. 1B and Supplementary Table 1).

ADAR2 is downregulated in all the motor neurons including apparently normal motor neurons that express only edited GluA2

In the AH, the expression level of ADAR2 mRNA relative to GluA2 mRNA in ALS cases was decreased to less than one third of that in control cases. In contrast, expression levels in the PH and the SpW of the spinal cord of ALS cases were not different from those of the control cases (Fig. 2A). After examining whether unedited GluA2 mRNA was contained in each single motor neuron lysate, we pooled cDNA from 100 single motor neuron lysates of four ALS cases that contained only edited GluA2 mRNA (ALS^{edit}). We pooled together cDNA from 47 motor neurons that expressed unedited GluA2 mRNA into a sample called ALS^{uned} (Fig. 3). In addition, we obtained lysates of 100 or 30 motor neurons from control subjects and MSA cases. We analyzed the expression level of ADAR2 mRNA in these lysates relative to GluA2 mRNA (Fig. 2B) and β-actin mRNA (Fig. 2C). Both analyses demonstrated that the motor neurons of ALS patients expressed significantly lower amounts of ADAR2 mRNA than those of the control subjects or patients with MSA. Notably, the expression level of ADAR2 mRNA was much lower for ALS^{uned} than for ALS^{edit} (Figs. 2B and C).

Next, we examined the efficiencies of A-to-I conversion at ADAR2-mediated conversion positions. A-to-I conversion at the Q/R site of GluA2 pre-mRNA and the K/E site in CYFIP2 are specifically mediated by ADAR2 (Kwak et al., 2008; Nishimoto et al., 2008; Riedmann et al., 2008), and A-to-I conversion at the Q/R site of GluR6 is partially mediated by ADAR2 (Higuchi et al., 2000; Paschen et al., 1994). The editing efficiencies at these sites in lysates were lower in ALS cases than in control cases, although the difference in the efficiency of GluR6 Q/R site-editing between ALS and control cases did not reach statistical significance (Figs. 2D–F). Furthermore, efficiencies of editing at these sites were significantly correlated with the efficiency of GluA2 Q/R site-editing (Supplementary Figs. 1A and B). These results indicate that ADAR2 activity is significantly decreased in ALS motor neurons.

ADAR1 and ADAR3, other members of the ADAR family, play no role in ALS pathogenesis

There are three members of the ADAR family, including ADAR1, ADAR2 and ADAR3. ADAR1 catalyzes A-to-I editing in vivo with different but overlapping site-selectivity from ADAR2 (Levanon et al., 2005; Nishimoto et al., 2008; Wang et al., 2004), whereas editing activity has not been demonstrated for ADAR3 that predominantly localizes in brains (Chen et al., 2000). The expression levels of ADAR1 mRNA in lysates of ALS motor neurons (both ALS^{edit} and ALS^{uned}) were similar to or even higher than those in lysates of control motor neurons (Fig. 4A). In addition, efficiencies of ADAR1-

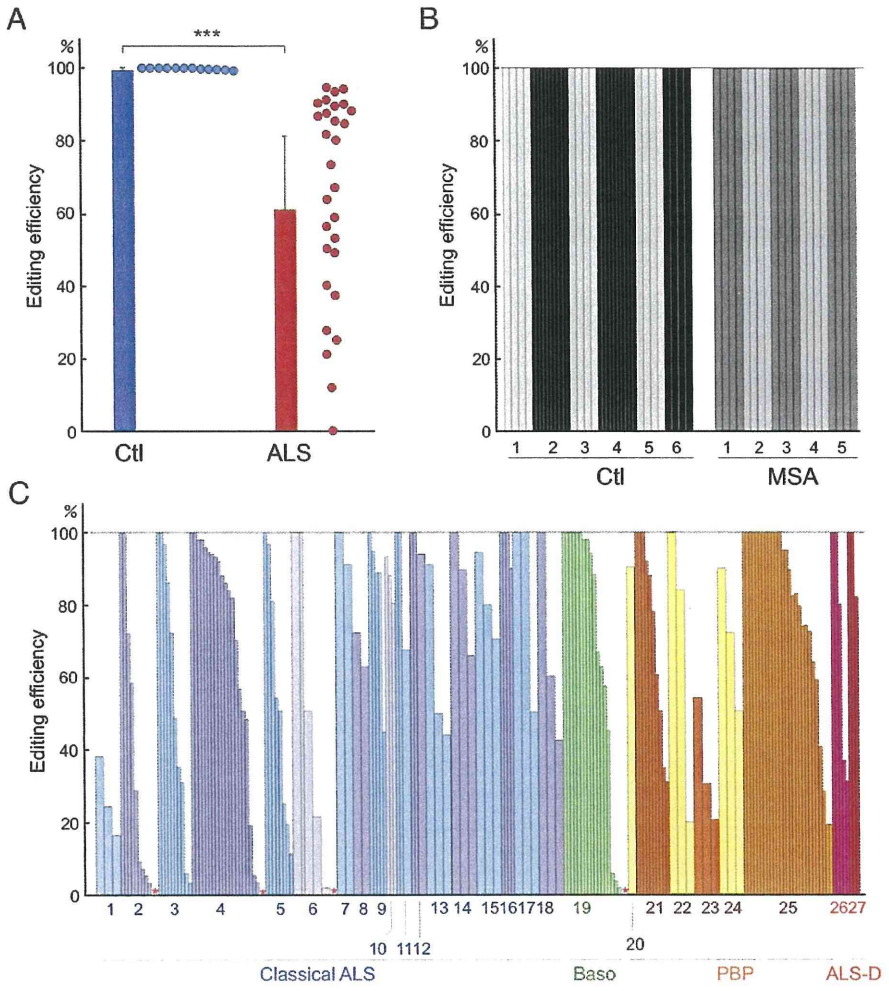

Fig. 1. Reduction of GluA2 Q/R site-editing in ALS motor neurons. (A) Editing efficiencies at the GluA2 Q/R site in anterior horn (AH) lysates of spinal cord from sporadic ALS patients (ALS) and control subjects (Ctl). Editing efficiencies for the ALS group (ALS, n = 29) was significantly lower than that of the control group (Ctl, n = 12) (61.0% ± 22.7% vs. 99.4% ± 0.7%; ***p < 0.001). Each circle represents the extent of GluA2 Q/R site-editing in each ALS case (red) or control case (blue), illustrating that values representing editing efficiency were less than 100% in all ALS cases, whereas those in the control subjects were approximately 100%. Columns and bars represent the mean ± SEM. (B and C) Extents of GluA2 Q/R site-editing in motor neuron lysates. Each narrow bar represents the editing efficiency in the lysate of one motor neuron, and each wide bar represents the editing efficiency in the lysate of 30 motor neurons. The results from the lysates of an individual case are shown in the same color. Editing efficiency was 100% in all motor neurons from all control subjects and patients with multiple system atrophy (MSA). Note that the extent of editing was less than 100% in at least one motor neuron of all 27 ALS cases examined, regardless of the clinical phenotype. Red asterisks indicate lysates showing 0% editing efficiency. Classical ALS: limb-onset ALS (blue); Baso: ALS with basophilic inclusion body (green); PBP: progressive bulbar palsy (orange); ALS-D: ALS with dementia (red).

mediated A-to-I conversion at the R/G site of GluA2 mRNA (Wang et al., 2004) and the Y/C site of bladder cancer associated protein (BLCAP) mRNA (Kwak et al., 2008; Nishimoto et al., 2008) in the ALS AH lysates did not differ from those in the control AH lysates (Figs. 4B and C and Supplementary Fig. 1C). Finally, ADAR3 mRNA expression level in the lysate of the ALS AH did not differ from that of the control AH (Supplementary Fig. 1D).

Discussion

The consistent results obtained from a large number of single motor neurons derived from a considerable number of ALS cases clearly demonstrate that inefficient GluA2 Q/R site-editing is a disease-specific molecular abnormality observed among sporadic ALS patients with different clinical manifestations, including classical limb-onset ALS, progressive bulbar palsy and ALS with dementia. GluA2 Q/R site-editing is fully preserved throughout life from the embryonic stage in mammalian brains (Burnashev et al., 1992; Nutt and Kamboj, 1994), including human brains (Kawahara et al., 2004b). A-to-I conversion at the GluA2 Q/R site is specifically mediated by ADAR2 (Higuchi et al., 2000), and motor neurons deficient in ADAR2 undergo slow death specifically due to a failure to edit this site, as observed in conditional ADAR2 knockout (AR2) mice (Hideyama et al., 2010). Expression of unedited GluA2 is also toxic in cultured cells (Mahajan and Ziff, 2007). Therefore, inefficient GluA2 Q/R site-editing is likely a common molecular abnormality and one cause of motor neuron death in ALS. Notably, the present study demonstrated that Q/R site-unedited GluA2 mRNA was expressed in motor neurons of two patients with ALS with dementia as occurred in patients with other clinical phenotypes of sporadic ALS. Based on the recent observation that ALS is not infrequently associated with the pathology in fronto-temporal lobar degeneration with ubiquitin-immunoreactive inclusion bodies (FTLD-U) and that degenerating neurons in both patients with ALS and those with FTLD-U exhibit TDP-43 pathology, it is believed that there is a common pathogenic mechanism between ALS and FTLD-U (Cairns et al., 2007). The present result is in agreement with this concept and lends support to the hypothesis that inefficient GluA2 Q/R site-

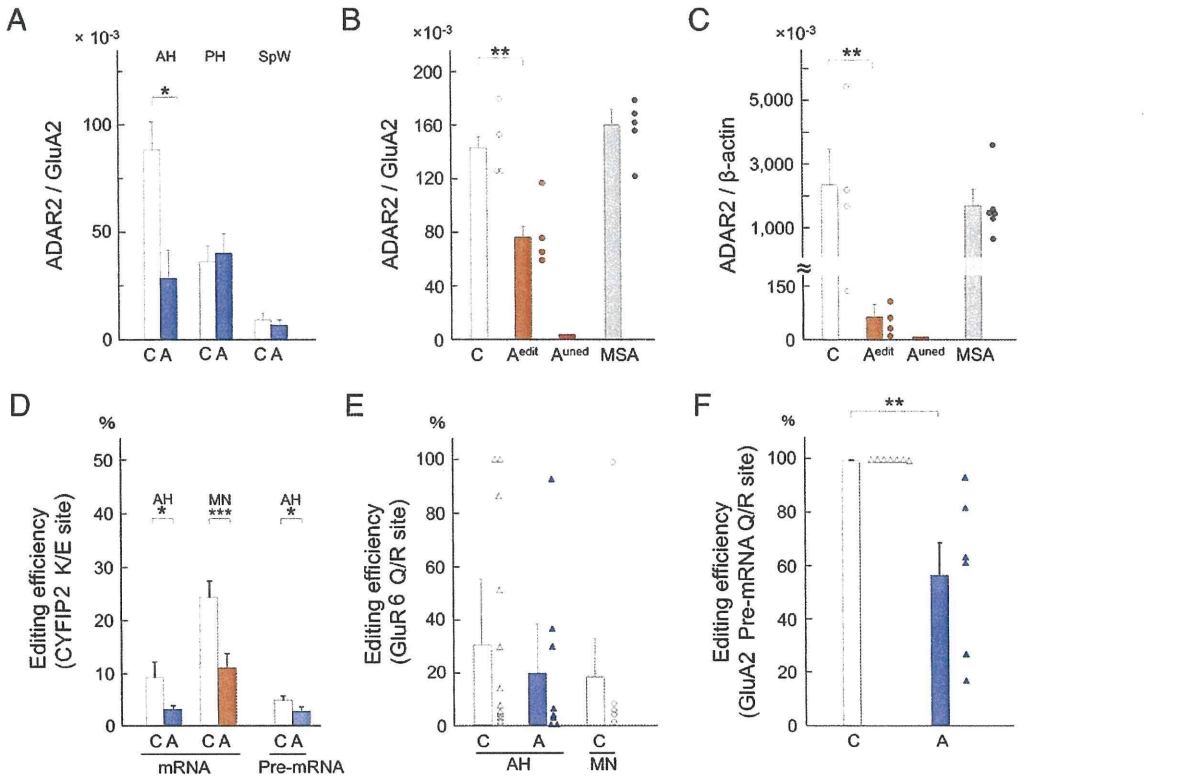

Fig. 2. Downregulation of ADAR2 mRNA and editing efficiency of ADAR2 substrates in ALS motor neurons. (A) The relative abundance of ADAR2 mRNA in the GluA2 mRNA base in the spinal cord anterior horn (AH) was significantly less in the ALS group (A) than in the control group (C) (*$p<0.05$). In contrast, relative abundance in the posterior horn (PH) or white matter (SpW) of the spinal cord did not differ between the two groups. (B and C) Relative abundance of ADAR2 mRNA in lysates of motor neurons in the GluA2 mRNA base (B) and β-actin mRNA base (C). The expression level of ADAR2 mRNA in ALS^{edit} (A^{edit}; $n=4$) was significantly lower than in the lysates of motor neurons of control subjects (C; $n=4$; **$p<0.02$), and that in ALS^{uned} (A^{uned}) was much lower than in A^{edit}. On the contrary, ADAR2 mRNA level in the MSA group did not differ from the C group. (D) Editing efficiency at the K/E site of CYFIP2 mRNA in lysates of AH (*$p<0.03$) and motor neurons (MN) (***$p<0.005$) and editing efficiency of CYFIP2 pre-mRNA in the AH (*$p<0.02$) were significantly lower in the ALS group (A) than in the control group (C). (E) Editing efficiency at the Q/R site of GluR6 mRNA was lower in the ALS AH than in the control AH, although this difference was not statistically significant. Editing efficiency at the GluR6 Q/R site was not significantly different between AH and the lysate of 100 MN from control spinal cord. (F) Editing efficiency at the Q/R site of GluA2 pre-mRNA in the AH lysates from the ALS group was lower than that of the lysates of the control group (**$p<0.01$). Columns and bars represent mean ± SEM. Each triangle represents a value for the AH lysate, and each circle represents a value for the MN lysate from a single case. C: Lysate of 100 motor neurons from control subjects ($n=4$). A^{edit}: lysate of 100 motor neurons expressing only Q/R site-edited GluA2 mRNA from ALS patients. A^{uned}: lysate of 47 motor neurons expressing Q/R site-unedited GluA2 mRNA from ALS patients. MSA: lysate of 30 motor neurons from the patients with multiple system atrophy ($n=6$).

editing is a common death-causing molecular abnormality in sporadic ALS.

Of the three members of the ADAR family, only ADAR2 was downregulated in ALS motor neurons. Although ADAR1 catalyzes A-to-I conversions that are critical during embryogenesis (XuFeng et al., 2009) and ADAR3 is predominantly localized in the central nervous system (Chen et al., 2000), neither ADAR1 nor ADAR3 participates in the inefficient GluA2 Q/R site-editing in ALS motor neurons. In addition, ADAR2 was downregulated in all ALS motor neurons, even in those motor neurons expressing only edited GluA2 (ALS^{edit}) and more extensively in those motor neurons expressing unedited GluA2 (ALS^{uned}), indicating profound downregulation of ADAR2 associated with sporadic ALS. Deficient ADAR2 induces death of motor neurons via failure to edit the GluA2 Q/R site, and expression of edited GluA2 by genetic engineering of the endogenous GluA2 gene rescues motor neurons lacking ADAR2 from death in AR2 mice (Hideyama et al., 2010). Investigations on the conditional ADAR2 knockout mice in which one ADAR2 gene allele was ablated in motor neurons (HeteroAR2; ADAR2$^{flox/+}$/VAChT-Cre.Fast) demonstrated that the motor neurons with reduced ADAR2 expressed unedited GluA2 mRNA, and although the unedited GluA2 accounted for no more than 30% of all GluA2 mRNA, these motor neurons underwent slow death (Hideyama and Kwak, 2011). Therefore, expression of unedited GluA2, even in a small proportion, is not favorable for the survival of motor neurons in mice and expression of ADAR2 sufficient to edit all the GluA2 mRNA rather than the expression level of ADAR2 per se is the critical factor for motor neuron survival. The critical role of GluA2 Q/R site-editing in neuronal death indicates that ALS motor neurons can survive, provided they express only edited GluA2, despite reduced ADAR2 activity. However, the expression level of ADAR2 likely decreases further with progression of the disease, and once the expression level of ADAR2 decreases below a threshold that is necessary to edit the Q/R site of all GluA2, motor neurons enter into a death cascade (see Fig. 5). This interpretation is consistent with the finding that the proportion of unedited GluA2 among all the GluA2 expressed in single neurons varied widely from 0% to 100% in a single ALS cases (Fig. 1C) (Kawahara et al., 2004a) and with the pathological finding that both healthy-appearing and shrunken motor neurons are observed in the ventral horn of the same ALS patients (Weller et al., 1997), as well. Therefore, it is likely that progressive downregulation of ADAR2 is closely relevant to the pathogenesis of ALS, in which the failure of A-to-I conversion at the GluA2 Q/R site is critical. We previously demonstrated that the threshold level of ADAR2 mRNA expression that enables editing of the Q/R sites of all GluA2 mRNA is approximately 20×10^{-3} relative to GluA2 mRNA in human brains (Kawahara et al., 2003a). We obtained consistent results in the present study; the expression level of ADAR2 mRNA in ALS^{edit} was well above this threshold

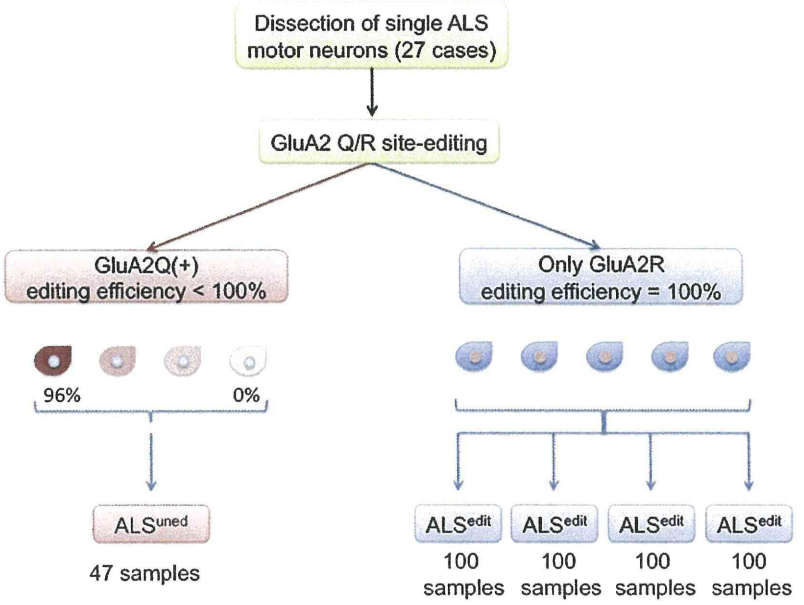

Fig. 3. Collection of ALS single motor neurons. We dissected single motor neurons from 27 sporadic ALS cases using a laser microdissector (see Table 1). After examining the efficiency of GluA2 Q/R site-editing in each lysate of a single motor neuron, we separately collected the remaining cDNA of the lysate that contained only Q/R site-edited GluA2 (ALS^{edit}) and lysate that contained unedited GluA2 mRNA (ALS^{uned}). Each ALS^{edit} contained cDNA from 100 motor neurons that expressed only Q/R site-edited GluA2 (n = 4), and one ALS^{uned} contained cDNA from 47 motor neurons that expressed unedited GluA2. ALS^{edit} and ALS^{uned} were used for analysis of the expression levels of ADAR1 mRNA and ADAR2 mRNA in ALS motor neurons shown in Figs. 2B and C.

($78.5\times10^{-3}\pm26.1\times10^{-3}$; range $60.0–117\times10^{-3}$), whereas the expression level in ALS^{uned} was far below this threshold (3.48×10^{-3}) (Figs. 2B and C).

In the present study, we showed that the expression level of ADAR2 mRNA is a factor regulating the ADAR2 activity in human spinal motor neurons, and that reduced expression level of ADAR2 transcripts was the cause of reduced ADAR2 activity in ALS motor neurons. We previously demonstrated that all the motor neurons express ADAR2 immunoreactivity in the spinal cord of normal human subjects, whereas approximately half of motor neurons lack ADAR2 immunoreactivity in patients with sporadic ALS (Aizawa et al., 2010). The present results provided us with knowledge that the lack of ADAR2 protein in ALS motor neurons resulted from reduced expression level of ADAR2 transcripts but not from accelerated degradation of ADAR2 protein, as shown in ischemic rat brain (Mahajan et al., 2011). Molecular mechanism regulating the level of ADAR2 mRNA expression and ADAR2 activity has been poorly elucidated. At least 48 different RNA variants are expressed in human brain, among which only three variants accounting for less than 10% of total amount of the transcripts encode active form of ADAR2 (Kawahara et al., 2005). Aside from the regulation by the expression level of ADAR2 mRNA, ADAR2 activity was positively regulated by the presence of inositol hexaphosphate in the catalytic domain (Macbeth et al., 2005) and negatively regulated by excessive expression of ADAR1 in glioma cells (Cenci et al., 2008) and cleavage of ADAR2 protein by Ca^{2+}-dependent protease (Mahajan et al., 2011).

Because ADAR2 is an RNA regulatory protein, the present findings support the emerging hypothesis that defects in RNA processing play a role in neurodegenerative diseases, including ALS (Lagier-Tourenne and Cleveland, 2009). For example, the mislocalization of TDP-43 and FUS/TLS has been reported in the motor neurons of ALS patients, including sporadic ALS. We previously found that ADAR2-deficient motor neurons exhibit TDP-43 pathology, whereas ADAR2-expressing motor neurons exhibit normal TDP-43 subcellular localization in sporadic ALS patients (Aizawa et al., 2010), indicating that there is a molecular link between these two RNA processing proteins. Whether TDP-43

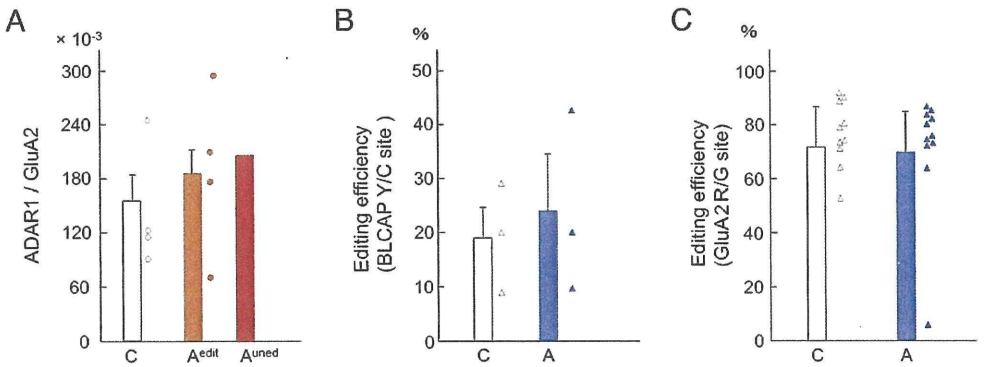

Fig. 4. Normal ADAR1 and ADAR3 in ALS motor neurons. (A) The expression levels of ADAR1 mRNA in ALS motor neurons, both ALS^{edit} (A^{edit}) and ALS^{uned} (A^{uned}) were in the same level as in control motor neurons (C). (B) Extents of RNA editing at the Y/C site of bladder cancer associated protein (BLCAP) mRNA in ALS AH did not differ from those in control AH. (C) There was no significant difference in editing efficiency at the R/G site of GluA2 mRNA in AH between ALS and control cases. Columns and bars represent mean ± SEM. Each triangle represents a value for the AH lysate, and each circle represents a value for the MN lysate from a single case.