The paper explained

PROBLEM:

ALS is the most common adult-onset motor neuron disease in which motor neurons innervating skeletal muscles selectively and progressively undergo degeneration from undetermined mechanism. The progressive nature of the disease leads the patients with ALS to death from failure of respiratory muscles within a few years of onset without effective therapy. Recently, with the progress of pathogenic mechanism of ALS, several potential target molecules for therapy have been demonstrated. However, because motor neurons are localized widely in the nuclei of cranial motor nerves and the spinal cord, global delivery of therapeutic agents to the motor neurons is required to accomplish therapeutic effects. Although delivery of the therapeutic agents through the vasculature enables widespread delivery, the blood–brain-barrier prevents entrance of molecules from blood to the brain and spinal cord. Therefore, safe delivery of therapeutic agents widely to motor neurons using appropriate vehicles is required for development of ALS therapy.

RESULTS:

Progressive death of the motor neurons in the brains and spinal cords cause ALS phenotype and expression of abnormal GluA2 (a subunit of the AMPA receptor that is involved in the neuronal excitation in the brain and spinal cord) with glutamine residue at the glutamine/arginine (Q/R) site (GluA2Q) is a disease-specific and potentially death-causing molecular abnormality occurring in the motor neurons of the patients with sporadic form of ALS that accounts for the majority of ALS patients. Motor neurons normally express Ca^{2+}-impermeable AMPA receptors containing GluA2 with arginine residue at the Q/R site (GluA2R) in the assembly, but express Ca^{2+}-permeable AMPA receptors when GluA2Q is expressed, which leads motor neurons to death. Given that this event would be closely relevant to ALS etiology, we attempt to develop a therapeutic strategy for ALS by broadly delivering cDNA of ADAR2, the enzyme that converges GluA2Q to GluA2R, to motor neurons in the aim to enhance the expression of normal GluA2R. To achieve widespread and selective expression of the *ADAR2* gene in motor neurons through a peripheral route avoiding off-target delivery, we used a viral vector AAV9 and the neuron-specific SYN1 promoter. A single intravenous injection of AAV9-ADAR2 in AR2 mice, which comprise a mechanistic mouse model of sporadic ALS, effectively prevented progressive motor dysfunction and death of motor neurons by enhancing ADAR2 activity. Notably, AAV9-ADAR2 normalized the abnormal expression profile of TDP-43, which is the ALS-specific pathological change, in the remaining motor neurons. Thus, our therapeutic strategy semi-permanently normalized disease phenotype, neuronal death and the disease-specific molecular marker in the ALS model mice.

IMPACT:

This is the first report on successful pre-clinical ALS therapy based on plausible pathogenic mechanism, achieving semi-permanent therapeutic effects on a mechanistic disease mouse model. Potency of the therapy on the model mice and safety as demonstrated by the clinical use in some other diseases provide delivery of the *ADAR2* gene using AAV9 with SYN1 promoter as potential therapy applicable to patients with ALS. Intravenous route of delivery using a relatively safe vehicle would facilitate clinical trials.

peroxidase-conjugated affinipure donkey anti-goat IgG (H + L) (Jackson ImmunoResearch, West Grove, PA) (1:2000) were used as secondary antibodies. Visualization was conducted using ECL plus Western blotting detection reagents (GE Healthcare Bioscience, Piscataway, NJ, USA). Specific bands were detected using a LAS 3000 system (Fujifilm, Tokyo).

RNA extraction and reverse transcription

Total RNA was isolated from the cells and spinal cords of mice using an RNeasy micro kit (Qiagen) and trizol (Invitrogen) and treated with DNaseI as recommended by the manufacturer. First-strand cDNA was synthesized from the total RNA using a Onestep RT-PCR kit (Qiagen), Ready-To-Go You-Prime First-Strand Beads (GE Healthcare Bioscience) and 50 ng of random primers (Invitrogen) as recommended by the manufacturer.

Analysis of the conversion of adenosine to inosine at the GluA2 Q/R site and CYFIP2 K/E site editing

The efficiency of the conversion of adenosine to inosine in the GluA2 mRNA, pre-mRNA and the CYFIP2 mRNA was calculated using a Bioanalyzer 2100 (Agilent Technologies) following the digestion of PCR products with restriction enzymes (Bhalla et al, 2004; Kawahara et al, 2003, 2004). The amplified GluA2 mRNA and pre-mRNA PCR products were digested with *Bbv*I (New England Biolabs, Ipswich, MA). The amplified CYFIP2 mRNA PCR products were digested with *Mse*I (New England Biolabs).

The PCR products from edited GluA2 pre-mRNA molecules contain one intrinsic *Bbv*I 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 and GluA2 mRNA should produce two bands (129 and 71 bp, pre-mRNA; 200 and 44 bp, mRNA), whereas digestion of bands originating from the unedited GluA2 pre-mRNA or mRNA molecules should produce three bands (91, 38 and 71 bp, pre-mRNA; 119, 44 and 81 bp, mRNA). The density of the 71- or 44-bp band, which results from digestion of both the edited and unedited pre-mRNA or mRNA, and the 129- or 200-bp band, which is solely the product of the edited pre-mRNA or mRNA, were quantified and the editing efficiency was calculated as the ratio of former to the latter for each sample (Nishimoto et al, 2008; Sawada et al, 2009). Similarly, *Mse*I digestion of the RT-PCR product generated from edited CYFIP2 yields two bands (117 and 209 bp), whereas that generated from unedited CYFIP2

mRNA yields three bands (117, 60 and 149 bp) (Nishimoto et al, 2008). The PCR primers used in these assays are provided in Supporting Information Table S1.

Real-time quantitative polymerase chain reaction

Quantitative PCR was performed using a LightCycler System (Roche Diagnostics, Indianapolis, IN). 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), each primer at 0.5 μM and the Universal Probe Library (Roche Diagnostics) at 0.1 μM. We determined the expression level of ADAR2 mRNA using different primer pairs for mouse ADAR2, human ADAR2 and total (both human and mouse) ADAR2 cDNA (Supporting Information Table S2). 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; Supporting Information Table S2) and the same PCR conditions (Sawada et al, 2009; Yamashita et al, 2012c).

Immunohistochemistry

Under deep anesthesia with isoflurane mice, were transcardially perfused with 3.5% paraformaldehyde and 0.5% glutaraldehyde in phosphate-buffered saline (PBS). The brains and spinal cords were removed and immersed in serially increasing concentrations of sucrose–PBS solutions (final sucrose concentration of 30%). The sucrose-immersed spinal cords were cut at a thickness of 10 μm with a cryostat (Model LEICA CM1850; Leica). The sections were immunostained with a standard avidin–biotin–immunoperoxidase complex method using VECTASTAIN ABC IgGs (Vector Co.) for the secondary antibodies. Rabbit anti-TDP-43 (ProteinTech Group, Inc., 1:100) and sheep anti-rat RED1 (ADAR2; Exalpha Biologicals, Inc., 1:100) were used for the primary antibodies. Colour was developed using the HRP-DAB System (Vector Co.).

Immunofluorescent staining of the sections was performed using rabbit anti-TDP-43 (ProteinTech Group, Inc., 1:200) and sheep anti-rat RED1 (ADAR2; Exalpha Biologicals, Inc., 1:200) as the primary antibodies. Sections were then incubated with Alexa Fluor 555 donkey anti-sheep IgG (Invitrogen, 1:200) and Alexa Fluor 488 chicken anti-rabbit IgG (Invitrogen, 1:200), respectively, as the secondary antibodies. The sections were examined under an LSM-510 confocal microscope (Zeiss) after nuclear staining with 0.5 μM TO-PRO-3 for 30 min.

Morphological observation

Mice were killed by overdose of isoflurane, and the brains and spinal cords were removed and then either quickly frozen on dry ice (right hemisphere and the first cervical to the second lumbar spinal cord segments) or fixed with 3.5% paraformaldehyde and 1% glutaraldehyde in PBS (left hemisphere and the rest of the spinal cord). Frozen samples were stored at −80°C until use. Paraformaldehyde-fixed samples were immersed in the same fixative overnight and then rinsed in PBS. Sections of the fixed fifth lumbar (L5) spinal cord segment were sequentially immunostained for Flag (Flag-hADAR2) and TDP-43 using the immunofluorescence system. The fluorescent images were analysed using a fluorescence microscope (BIOREVO BZ-9000; Keyence Corp, Osaka, Japan). Large AHCs with diameters larger than 20 μm were separately counted for each mouse. TDP-43-positive AHCs (in the ventral grey matter ventral to the line running though the ventral edge of the central canal) were counted in four L5 sections for each mouse. The ventral roots of L5 were then postfixed in 1% phosphate-buffered osmium tetroxide. The signal intensity of TDP-43 was examined using Image J software. TDP-43-positive AHCs were counted when the signal intensity was more than threefold higher than the background. After three washes with phosphate buffer, each sample was dehydrated in a graded series of ethanol and embedded in Epon (Wako). Thin sections (1 μm) of the L5 ventral root were stained with 0.1% toluidine blue, digitized using a BIOREVO BZ-9000 (Keyence), and axons were counted manually by a researcher who was blind to the virus injection condition.

Tyramide signal amplification (TSA)

The perfusion-fixed, sucrose-immersed spinal cords were cut to a thickness of 12 μm using a cryostat (Model LEICA CM1850; Leica). The sections were incubated with 3.0% H_2O_2 in PBS and TNB blocking buffer [0.1 M Tris–HCl, pH 7.5, 0.15M NaCl and 0.5% blocking Reagent (PerkinElmer)]. Sections were serially incubated with goat anti-choline acetyltransferase (ChAT) (Millipore; 1:2000 in TNB blocjing buffer) at 4° C overnight, incubated with HRP-conjugated donkey anti-goat IgG (Abcam, 1:2000) for another 1 h at room temperature and finally incubated with tetramethylrhodamine plus amplification tyramide reagent (1:250 in amplification solution) for 20 min at room temperature. After washing, the sections were subjected to the next round of immunohistochemistry after blocking with 3.0% H_2O_2 in PBS. The sections were incubated with the rabbit anti-Flag antibody (Cell Signaling Tech; 1:200) in Can Get Signal buffer A (Toyobo) at 4°C overnight and then serially with HRP-conjugated chicken anti-rabbit IgG (Abcam, 1:2000) in Can Get Signal buffer A (Toyobo) for another 1 h at room temperature and with fluorescein plus amplification tyramide reagent (1:250 in amplification solution) for 20 min at room temperature. The sections were examined under a BIOREVO BZ-9000 microscope (Keyence Corp, Osaka, Japan) after nuclear staining with 0.5 μM TO-PRO-3 for 60 min. Bars represent 50 or 20 μm.

Statistical analysis

Average data are presented as means and s.e.m. Statistical analyses were conducted using JMP 9 software (SAS Institute, Inc.). For statistical comparisons of two groups, we used unpaired, two-tailed Student's *t* tests or Mann–Whitney *U* tests. Differences were considered significant when $p < 0.05$.

Author contributions

SK supervised the entire project. TY, KS, SM and SK conceived and designed the experiments. TY, HLC, SM and SK wrote the main text, and TY and HLC made the figures. TY, HLC, STe, KS and SM conducted the experiments and analysed the data. KS and SM generated the AAV9 constructs and virus for the studies. All of the co-authors (TY, HLC, STe, STs, KS, SM and SK) discussed the results and commented on the manuscript.

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Supporting Information is available at EMBO Molecular Medicine online.

The authors declare that they have no conflict of interest.

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