Inositol Hexakisphosphate Kinase 2 is a Presymptomatic Biomarker for Amyotrophic Lateral Sclerosis

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Objective: Inositol hexakisphosphate kinase 2 ($InsP_6K2$), an enzyme that converts inositol hexakisphosphate ($InsP_6$) to diphosphoinositol pentakisphosphate ($InsP_7$), induces cell death. $InsP_6K2$ is abundant in the central nervous system, especially anterior horn cells of spinal cord. To identify the role of $InsP_6K2$ in amyotrophic lateral sclerosis (ALS), we investigated the expression levels of $InsP_6K2$ in transgenic mice expressing mutant superoxide dismutase-1 (SOD1) (mSOD1 Tg mice).
Methods: The specimens of spinal cords were obtained from mSOD1 Tg mice and age-matched wild-type mice. We investigated the expression of $InsP_6K2$ at the gene and protein levels of the spinal cord in mSOD1 Tg and wild-type mice.
Results: The gene expression levels of $InsP_6K2$ in mSOD1 Tg mice was significantly higher than that in wild-type mice before ALS symptoms developed. In immunohistochemistry and western blotting results showed that $InsP_6K2$ translocated from the nucleus to the cytoplasm in mSOD1 Tg mice.
Conclusion: These findings suggest that $InsP_6K2$ activates in mSOD1 Tg mice before the onset of ALS. Therefore, $InsP_6K2$ might be a presymptomatic biomarker for ALS.

Key words: amyotrophic lateral sclerosis (ALS), biomarker, inositol phosphate, superoxide dismutase (SOD), cell death, inositol hexakisphosphate kinase 2 ($InsP_6K2$)

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder impairing both upper and lower motor neurons. Its main symptoms are limb weakness, bulbar palsy, and respiratory failure. The cause of ALS is unclear, but aging, environmental factors, and genetic factors are considered to be involved. More than 90% of ALS cases are sporadic, whereas the remaining 10% of cases are familial. The most common mutation in familial ALS is a mutation in the human superoxide dismutase-1 (mSOD1) gene, and G93A mSOD1 Tg mice have been used to elucidate the mechanisms of ALS neuropathogenesis [1, 2]. Riluzole is the only therapeutic agent for this condition; however, even with riluzole treatment, ALS is usually progressive and fatal [3, 4].

Inositol phosphates act as important signaling molecules in cells. Inositol 1, 4, 5-trisphosphate ($InsP_3$) is a well-known inositol phosphate that acts as a second messenger for calcium release in cells. $InsP_3$ serves as a precursor of highly phosphorylated inositol polyphosphates such as inositol tetrakisphosphate ($InsP_4$), inositol pentakisphosphate ($InsP_5$), and inositol hexakisphosphate ($InsP_6$). There are also higher inositol phosphates, such as disphosphoinositol pentakisphosphate ($InsP_7$) and bis-(disphospho)-inositol tetrakisphosphate ($InsP_8$) [5–7]. $InsP_7$ can compete for pleckstrin homology (PH) domain binding with PtdIns(3, 4, 5)P_3 and serve as a phosphate donor to other proteins [8]. In mammals, the formation of $InsP_7$ is mediated by a family of inositol hexakisphosphate kinases ($InsP_6Ks$), which constitute three subtypes: $InsP_6K1$, $InsP_6K2$, and $InsP_6K3$. Notably, $InsP_6K2$ is usually localized exclusively in the nucleus, whereas the other two enzymes are found in both the cytoplasm as well as the nucleus [9–12]. When $InsP_6K2$ is activated, it is translocated to the cytoplasm [13, 14].

We had previously reported that $InsP_6K2$ induces cell death by converting $InsP_6$ to $InsP_7$ [14, 15]. $InsP_6K2$ is abundant in the central nervous system, especially in the anterior horn cells of the spinal cord. To elucidate the role of $InsP_6K2$ in ALS, we investigated the expression level of $InsP_6K2$, employing G93A mSOD1 Tg mice.

MATERIALS AND METHODS

The investigation conforms with The Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publication No. 85–23, revised 1996).

Mice

We used G93A mSOD1 Tg mice at postnatal week 6 (n = 6), week 12 (before the onset of motor neuron disorders, n = 6), week 17 (presenting motor neuron

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disorders, n = 7), and after week 17 (terminal stage of motor neuron disorders, n = 7) and compared them with wild-type littermates in the C57BL6/J background (n = 31) of the same age. Specimens of the spinal cord were obtained from these mice.

Immunostaining against InsP$_6$K2

Anesthesia was induced in the mice with 4% isoflurane/66% N_2O/30% O_2 and maintained with 1.5% isoflurane. The mice were perfused from the apex of the heart with phosphate-buffered saline (PBS) and perfusion-fixed with 4% paraformaldehyde (PFA) in PBS. Spinal cords were taken from the mice and immersion-fixed overnight at 4°C in 4% PFA with rocking and subsequently cryoprotected in 10% (2 h), 15% (2 h), 20% (2 h), and 25% (overnight) sucrose in PBS at 4°C. The slices were then embedded in OCT compound (Miles Scientific, DE, USA) and quickly frozen in isopentane. Coronal frozen sections (10 μm) were prepared on a cryostat and stored at −80°C until use. The frozen sections were thawed, washed thrice in PBS, permeabilized with 0.1% Triton X-100/PBS at room temperature for 5 min, and then blocked in 5% skim milk/3% bovine serum albumin (BSA)/PBS for 60 min. InsP$_6$K2 was detected with the use of anti-InsP$_6$K2 antibody that we prepared ourselves. The slides were incubated with primary antibodies (1 : 200) at 4°C overnight and with the secondary antibodies at room temperature for 2 h, and immunoreactivity was visualized by the ABC method. The immunohistochemical images were taken by OLYMPUS BX51 with the use of DP Controller software.

Gene expression of InsP$_6$Ks

Fresh spinal cord tissues were conserved in RNALater (Life Technologies, CA, USA) at −20°C and homogenized. Total RNA was extracted from these spinal cord tissues with Trizol RNA extraction reagent. Extracted total RNA was treated with DNase I and purified with RNeasy Mini Kit (Qiagen, Venlo, Netherlands). cDNA was synthesized using a SuperScriptIII cDNA synthesis kit (Invitrogen, CA, USA). The Step One Plus Real-Time PCR System (Applied Biosystems, CA, USA) was used with each custom-designed, gene-specific primer/probe set to amplify and quantify each mouse InsP6K. The reactions contained each of the forward and reverse primers [mouse InsP$_6$K1 (Mm 01229851), mouse InsP$_6$K2 (Mm 01232055, Mm 01232056), or mouse InsP$_6$K3 (Mm 00557208)] for the relevant TaqMan Gene Expression Assays (Applied Biosystems, CA, USA). Polymerase chain reaction (PCR) amplification and real-time detection were performed with TaqMan Fast Advanced Master Mix (Applied Biosystems, CA, USA), activation at 95°C for 20 s, followed by 40 cycles of denaturation at 95°C for 3 s and annealing/extension 30 s at 60°C for 30 s. The data were analyzed with Applied Biosystems Step One Software V2.1. Mouse β-actin (4352341E−1112017) was used as an endogenous control for normalization of the input target RNA. Relative quantification of the real-time PCR data was performed by the comparative threshold (Ct) method ([16]). InsP$_6$K1, InsP$_6$K2, and InsP$_6$K3 mRNA expression in wild-type mice at postnatal week 6 was set as 1.0 for calibration.

Protein expression

The samples were homogenized and fractionated to the cytoplasm and the nucleus with a ProteoExtract Subcellular Proteome Extraction Kit (Merck KGaA, Darmstadt, Germany). These samples were separated by electrophoresis through 4%−12% gradient gels. After electrophoretic transfer to a polyvinylidene fluoride membrane (Immobilon-P; Millipore, MA, USA), the membranes were blocked with 4% BSA in PBS. The membranes were washed and incubated with the primary antibodies at 4°C overnight. After incubation with the primary antibodies, the membranes were washed with PBS-T (0.1% Tween 20) and incubated with secondary antibodies (Vector Laboratories, CA, USA) for 2 h at room temperature. The membranes were then examined with an enhanced chemiluminescence western blotting system (Amersham Pharmacia, NJ, USA). In this study, we compared the density ratio of InsP$_6$K2 protein expression in the cytoplasm and the nucleus among these mice.

Statistical analysis

Welch's t-test was used for statistical analyses with SPSS Statistics (IBM, NY, USA). A P value < 0.05 was considered to indicate a statistically significant difference. Data are expressed as means ± SD.

RESULTS

InsP$_6$K2 translocated from the nucleus to the cytoplasm in mSOD1 Tg mice

The anterior horn cells of the spinal cord in a wild-type mouse were strongly stained with anti-InsP$_6$K2 antibody. InsP$_6$K2 was mainly stained in the nucleus in wild-type mice, whereas it was stained in the cytoplasm in the mSOD1 Tg mice in each period (Fig. 1). The ratios of the band density of the cytoplasmic/nuclear fractions in mSOD1 Tg mice were higher than those in wild-type mice at each developmental stage, especially in postnatal weeks 12 and 17, when they were significantly higher (Fig. 2).

InsP$_6$K2 in mSOD1 Tg mice is expressed before the onset of clinical symptoms of ALS in the spinal cord compared with wild-type mice

The gene expression levels of InsP$_6$K1 in mSOD1 Tg mice at each week were not significantly different from those in wild-type mice (Fig. 3). InsP$_6$K3 was very faintly expressed compared with InsP$_6$K1 and InsP$_6$K2 (data not shown). The gene expression levels of InsP$_6$K2 in mSOD1 Tg mice at postnatal week 12 were 2.6 times higher than those in wild-type mice at postnatal week 12. However, the gene expression levels of InsP$_6$K2 in mSOD1 Tg mice at postnatal week 17 were lower than those in wild-type mice at postnatal week 17 (Fig. 4).

DISCUSSION

ALS is a neurodegenerative disorder that is progressive, intractable, and fatal. ALS is often difficult to diagnose, especially in its early stages. The standard clinical criteria for the diagnosis of ALS are the revised El-Escorial criteria, also known as the Airlie House

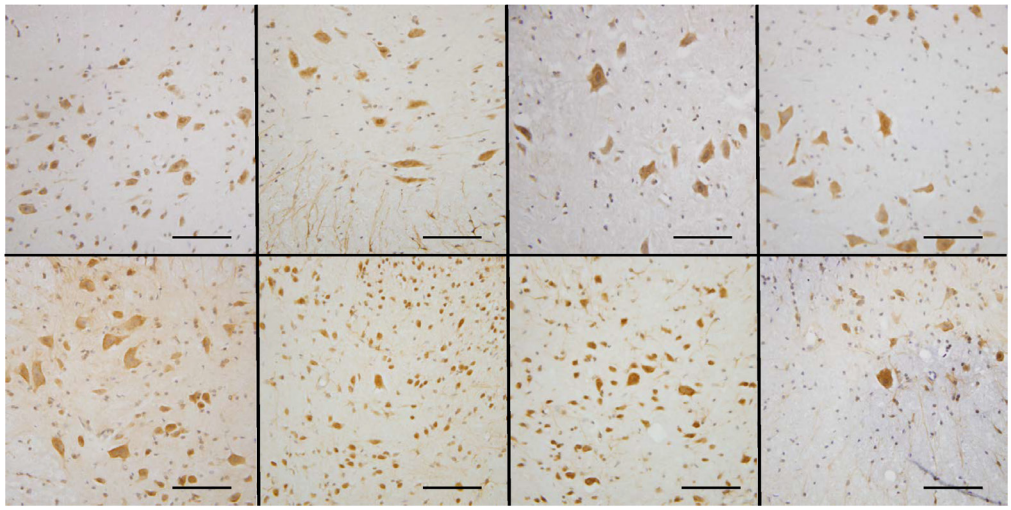

Fig. 1 Immunohistochemistry of inositol hexakisphosphate kinase 2 (InsP$_6$K2) in the anterior horn cells of mouse spinal cord at each developmental stage (Fig. 1A: reduced images of high-power field, Fig. 1B: high-power field). InsP$_6$K2 was stained mainly in the nucleus in wild-type mice, whereas it was stained in the cytoplasm in mSOD1 Tg mice at each developmental stage. The immunohistochemical images were taken at 400 × magnification.

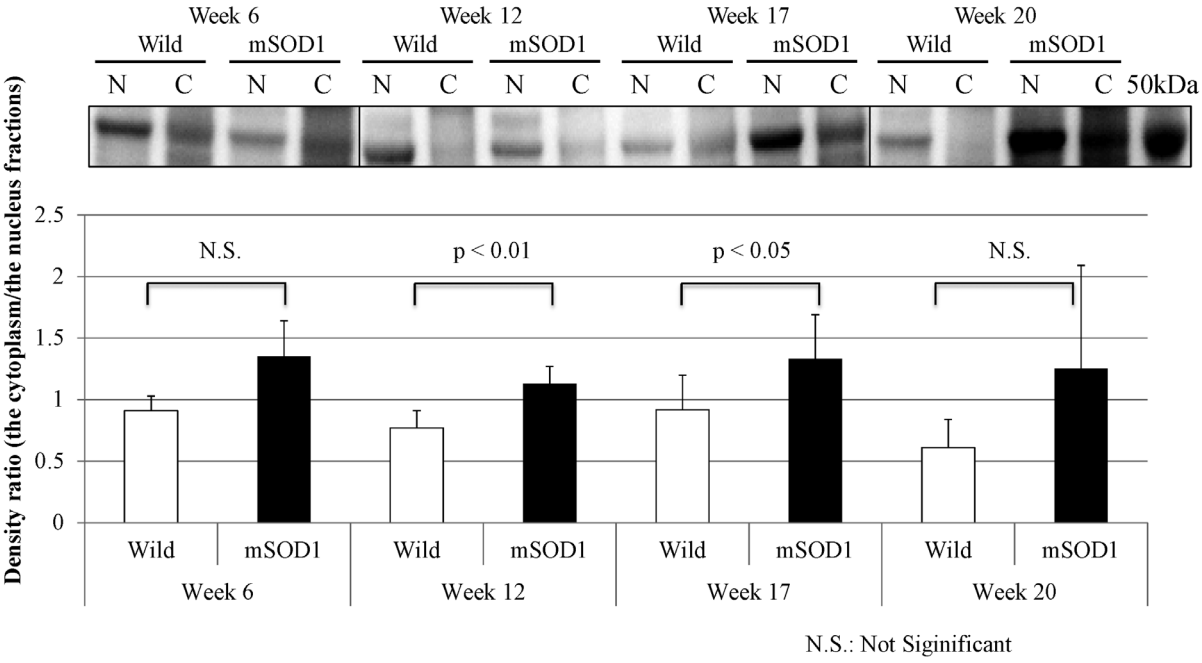

Fig. 2 Immunoblotting against anti-inositol hexakisphosphate kinase 2 (InsP$_6$K2) antibody on cell fractions of mouse spinal cord at each developmental stage. The ratios of the band density of the cytoplasmic/nuclear fractions in transgenic mice expressing mutant superoxide dismutase-1 (SOD1) (mSOD1 Tg mice) mice were higher than those in wild-type mice. These findings show that InsP$_6$K2 is activated in the mSOD1 Tg mouse. The immunoblot figure was spliced from three sections. C, cytoplasm; N, nucleus.

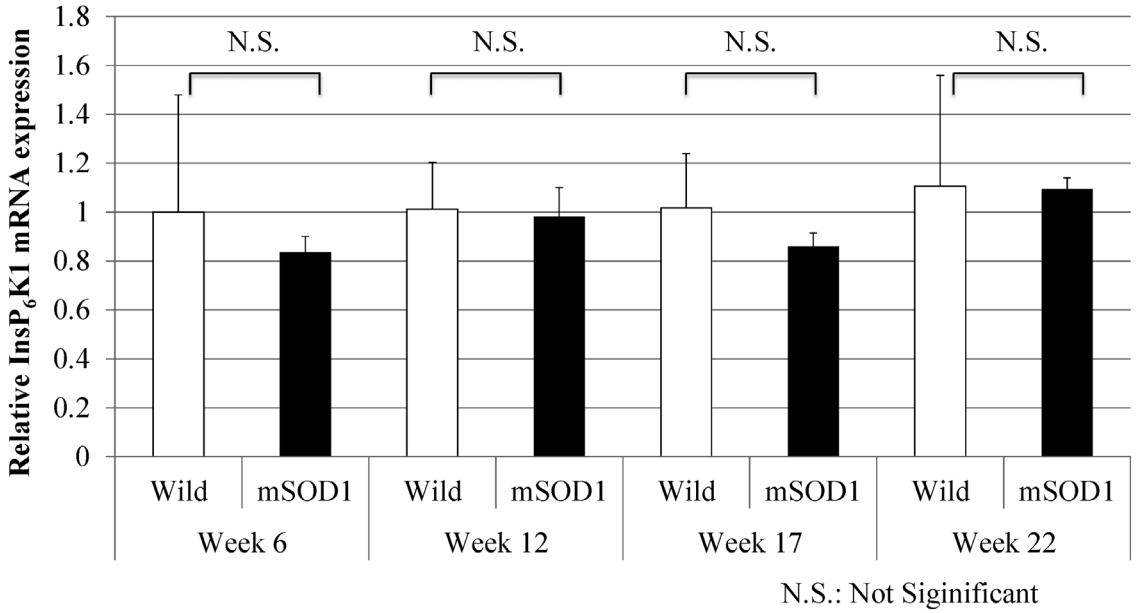

Fig. 3 Quantitative PCR of inositol hexakisphosphate kinase 1 (InsP$_6$K1) mRNA expression in spinal cord of wild-type mice and transgenic mice expressing mutant superoxide dismutase-1 (SOD1) (mSOD1 Tg mice) at each development stage. The gene expression levels of InsP$_6$K1 in mSOD1 Tg mice at each week were not significantly different from those of wild-type mice.

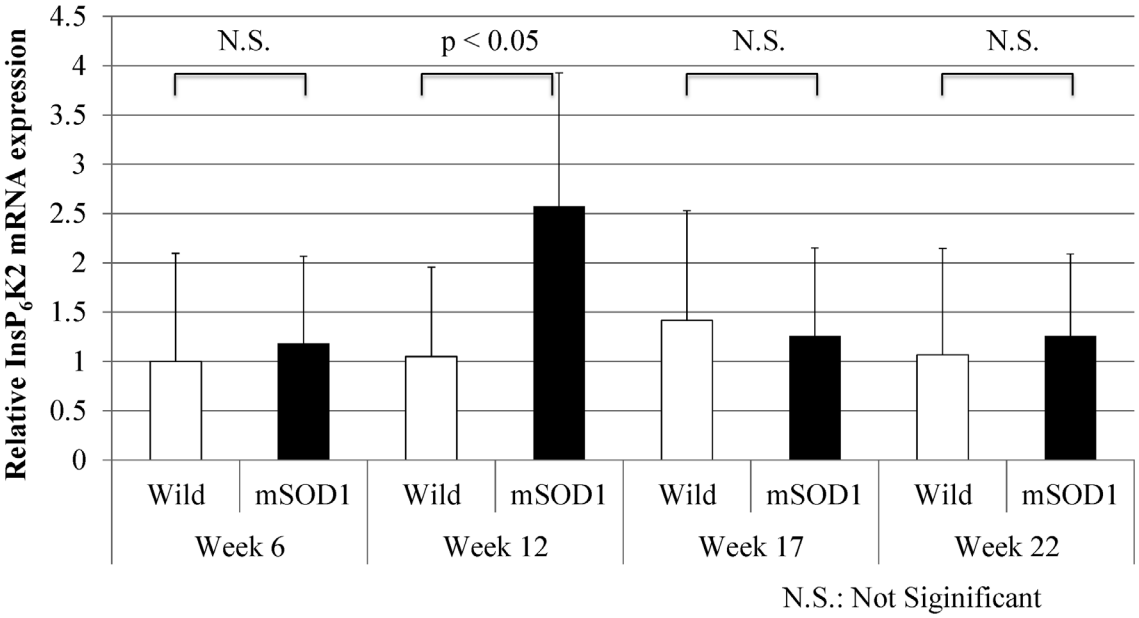

Fig. 4 Quantitative PCR of inositol hexakisphosphate kinase 2 (InsP$_6$K2) mRNA expression in spinal cord of wild-type mice and transgenic mice expressing mutant superoxide dismutase-1 (SOD1) (mSOD1 Tg mice) at each developmental stage. The gene expression levels of InsP$_6$K2 in mSOD1 Tg mice at postnatal week 12 were 2.6 times higher than those of wild-type mice at postnatal week 12. However, the gene expression levels of InsP$_6$K2 in mSOD1 Tg mice at postnatal week 17 were lower than those of wild-type mice at postnatal week 17.

criteria. The revised El-Escorial criteria were designed to be of limited sensitivity for ALS, although their specificity was higher, particularly in the early stages of ALS [17–19]. Consequently, significant diagnostic delays are inevitable, leading to a delay in the establishment of neuroprotective therapies and recruitment into therapeutic trials, perhaps beyond the therapeutic window period. The neurophysiologically based Awaji criteria were developed for use in conjunction with the clinical criteria, in an attempt to minimize diagnostic delays [20]. With the use of the revised El-Escorial and Awaji criteria, ALS is diagnosed by clinical upper and lower motor neuron signs and electromyographic findings. A more useful diagnostic marker is required for ALS, such as a peripheral blood test, a cerebrospinal fluid (CSF) test, or an imaging study.

TAR DNA-binding protein 43 (TDP-43) has been identified as a major component of ubiquitin-positive inclusions in the brain and spinal cord of patients with frontotemporal lobar degeneration with ubiquitinated inclusions or sporadic ALS. Mutations in the corresponding gene have also been identified [21, 22]. TDP-43 levels in CSF are elevated in ALS patients compared with those in patients with other neurodegenerative or inflammatory diseases [23]. Thus, the sensitivity of CSF TDP-43 measurement for diagnosing ALS is relatively low.

Various biomarkers for ALS have been previously reported. Among the biochemical markers, phosphorylated NF-H [24] and the ratio of CD14 to S100 beta [25] in CSF are elevated in ALS patients, whereas cystatin C [26], CCL4, CXCL10 [27], angiotensin II [28] follistatin, interleukin-1 alpha, and kallikrein-5 [29] are decreased. On the other hand, among the biophysiological markers, a high rate of abnormalities of central motor conduction on transcranial magnetic stimulation was found in patients with ALS [30]. For bioimaging of ALS, voxel-based morphometry [31] and diffusion tensor imaging [32, 33] have been reported. However, to date, we have found no suitable specific biomarkers for ALS.

InsP$_6$Ks and inositol phosphates also play important roles in export of mRNA from the nucleus to the cytoplasm in Saccharomyces cerevisiae [34]. Moreover, InsP$_6$K2 is mainly localized to the nucleus when the cell is in a normal state. Upon activation, InsP$_6$K2 is translocated to the cytoplasm [14]. InsP$_6$K2 activation generates InsP$_7$ from InsP$_6$. We previously demonstrated that InsP$_7$ competes with PtdIns(3, 4, 5)P$_3$ for binding to several mammalian PH-domain-containing proteins, including Akt, PIKE, and Tiam [8]. Because InsP$_7$ can compete with Akt for binding, a high concentration of InsP$_7$ would occupy more of the PH domain of Akt, thereby inhibiting Akt phosphorylation. Consequently, the activation of mTOR, which is located downstream in the Akt/PIP3 pathway, would be suppressed [35]. InsP$_6$K2 is a proapoptotic protein regulated by HSP90 [36], which binds and inactivates it, as well as by casein kinase 2, which phosphorylates InsP$_6$K2 to enhance its proteasomal degradation. Apoptotic stimuli stabilize, activate, and facilitate the nuclear translocation of InsP$_6$K2, where it binds p53 and augments its apoptotic actions [37]. We reported that cell death was augmented in the presence of cytoplasmic TDP-43 aggregations and activated InsP$_6$K2. In the presence of both TDP-43 aggregation and activated InsP$_6$K2 in the cytoplasm of cells, the expression levels of HSP90 and casein kinase 2 and the activity of Akt decreased [38].

As mentioned above, InsP$_6$K2 is a part of the apoptotic pathway. In this study, InsP$_6$K2 might have been activated in mSOD1 Tg mice. Notably, the expression of InsP$_6$K2 and the translocation of InsP$_6$K2 protein to the cytoplasm were increased at postnatal week 12 before the onset of motor neuron disorders in mSOD1 Tg mice. The gene expression levels of InsP$_6$K2 in mSOD1 Tg mice at postnatal week 12 were significantly higher than those of wild-type mice at postnatal week 12. However, the gene expression levels of InsP$_6$K2 in mSOD1 Tg mice at postnatal week 17 were lower than those in wild-type mice at postnatal week 17. It is considered that anterior horn cells in the spinal cord of mSOD1 mice were degenerated and reduced in its stage [39–41]. These results that InsP$_6$K2 might be activated in mSOD1 Tg mice before the onset of motor neuron disorders. Moreover, InsP$_6$K2 can serve as a diagnostic biomarker for an early stage of ALS.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interests.

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CONTRIBUTORS

YM, EN, and ST planned the experiments. SH provided the animals and information on the animals. YM, EN, TS, and HO performed the experiments. YM and EN interpreted the data and wrote the manuscript. All authors reviewed the results and approved the final version of the manuscript.

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