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Original Article

Acupuncture at ST36 improves survival and ameliorates motor neuron defects by the activation of autophagy

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Abstract: Background: Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disorder with a rapidly deteriorating progression, and there is no evidence-based clinically therapy until now. Here, an acupuncture treatment is reported. This widely-accepted complementary and alternative medicine (CAM) has a great potential to benefit ALS patients. Materials & Methods: The 60-day-old hSOD1G93A mice were randomized into the 3 groups with 15 mice per group, Wild-type littermate control group (WT littermate, n=15), Model group (M group, n=15) no acupuncture treatment and Acupuncture treatment group (AT+M group, n=15). A pretreatment of acupuncture was applied at an interval of 2 days until the end of study. For acupuncture stimulation, stainless steel needle is vertically inserted into the ST36 in different groups. Morphological studies and autophagy-related proteins are used to evaluate the therapeutic outcomes of acupuncture. Results: Acupuncture at ST36 significantly delayed the onset, improved the motor function, and prolonged the survival time in the hSOD1^{G93A} transgenic mice. Morphological study demonstrated that acupuncture at ST36 can ameliorate the structure of spinal cord anterior horn motor neurons and rescue motor neurons from cell death. To determinate the underlying molecular mechanism of acupuncture in treatment of ALS, the expression level of hSOD1, Beclin1 and LC3-II was examined. The protein aggregate of hSOD1 is inhibited and the expression level of Beclin1, and LC3-II increases in the lumbar spinal cord. Conclusion: Therefore, acupuncture at ST36 appears to eliminate the hSOD1 aggregate in motor neurons via activating the autophagy process. This study suggests that the acupuncture treatment at ST36 might exert more than a CAM option in ALS, and provides an alternative therapy for ALS patients.

Keywords: Acupuncture, autophagy, amyotrophic lateral sclerosis (ALS), adjuvant treatment

Introduction

Amyotrophic lateral sclerosis (ALS) is an age-related neurodegenerative disorder and is one of the most common motor neuron disorders. The progressive degeneration of motor neurons is typically characterized by an early cortical dysfunction, proceeding with a lower motor neuron dysfunction and degeneration, which is divided into sporadic and familial ALS [1, 2]. For the last several decades, constant efforts have been made in understanding of ALS pathophysiology and diagnosis. However, ALS is still a fatal disease with a rapid deteriorating progression, and 90% of patients die within 5 years.

Accumulating evidence indicates that the mislocation of proteins and presence of cytoplasmic aggregates are typically pathological hallmarks of ALS, which would disrupt cellular function and contribute to cytotoxicity. The first evidence of aggregates was described in spinal cords of familial ALS patients carrying a mutation in the superoxide dismutase-1 (SOD1) gene [3]. With the advent of whole-exome sequencing, disease-causing protein mutations have been identified from patient tissues [4], such as TDP-43, FUS, OPTN, and UBQLN2, etc. [5-7]. In addition to those mutant protein aggregates, another putative hallmark of ALS is the presence of cytoplasmic glutamate aggregates in affected neurons. A cortical hyperexcitability is an early pathophysiological feature of ALS and releases glutamate from presynaptic neurons to activate postsynaptic specific ionotropic and metabotropic glutamate receptors [8]. In patients with ALS, glutamate-mediated excitotoxicity has been proven by evidence of a significant accumulation of glutamate in the synaptic cleft [9]. However, the persistence of

these aggregates in diseased neurons suggests defects in protein homeostasis, and impairments in protein degradation and clearance [10]. In eukaryotic cells, degradation of protein aggregates relies mainly on two systems: the ubiquitin-proteasome system (UPS) and autophagy, and autophagy is preferentially used for degrading long-lived proteins and entire organelles compared with the effects of UPS on short-lived, soluble proteins [11]. Indeed, emerging evidence from genetic and cellular studies of the etiology and pathogenesis of ALS has suggested that proper function in autophagy may contribute to degradation of ALS associated protein aggregates, and delay the development of ALS [12]. On the other hand, defects in autophagy have been implicated in the formation of pathological protein aggregates [13]. Therefore, autophagy induction has been a promising therapeutic strategy, and autophagy-modulating drugs have been developed recently. However, to take rapamycin for an example, the effect on the neuroprotection was controversial on certain ASL models, which suggests that rapamycin might have off-target effects [14]. More efforts are required for developing more specific and targeted agents without leading to cytotoxicity.

In Traditional Chinese Medicine (TCM), motor neuron disorders associated with muscle wasting are regarded as Atrophy Syndrome. Acupuncture has been used for such syndrome for hundreds of years in Asian countries, and it is gaining popularity as a complementary and alternative medicine (CAM) in the motor neuron disorders after stroke [15]. A Meta-analysis on the spasticity after stroke showed that acupuncture significantly decreased wrist, knee, and elbow spasticity [16]. In recent years, many acupuncturists have been trying to cure ALS with different modalities of acupuncture, and several case reports demonstrated that acupuncture offered symptomatic relief and dramatically improved quality of life. Moreover, pharmacological or electrical acupuncture at ST36 significantly enhanced motor function and decreased motor neuron death via engagement of endogenous immune modulatory system in the CNS in hSOD1^{G93A} transgenic mice [17-19]. Although no conclusion can be drawn whether acupuncture can cure ALS by several case reports, acupuncture is indeed a promising treatment as a CAM in ALS. Therefore, the purpose of this study was to investigate the underlying mechanisms of acupuncture and provides the evidence for further clinical trials.

In this study, acupuncture at ST36 point was found to significantly improve movement function, postpone the onset time of ALS, and prolong the survival time in the hSOD1^{G93A} transgenic mice. In addition, morphological studies demonstrated that the loss of anterior horn motor neurons in lumbar cord was ameliorated. More importantly, ST36 acupuncture could down-regulate the expression of hSOD1 and up-regulate the expression of LC3-II and Beclin1, the putative autophagy biomarkers. These findings suggest that ST36 acupuncture could promote clearance of protein aggregates by enhancing autophagy, which might offer a new perspective to explain the significant protection of motor neurons after acupuncture treatment.

Materials and methods

Human-SOD1 G93A transgenic (hSOD1^{G93A}) mice

The hSOD1^{G93A} transgenic mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Mice were identified by ear clipping, and the mouse tail-tip was cut for extraction of genomic DNA. Genotyping PCRs were performed on genomic DNA and amplified with human SOD1 primers (forward 5'-CCATCAGCCCTAATCCATCTGA-3', reverse 5'-CGCGACTAACAATCAAAGTGA-3') and control interleukin-2 receptor (IL-2R) primers (forward 5'-CTAGGCCACAGAATTGAAAGATCT-3', reverse 5'-GTAGGTGGAAATTCTAGCATCATCC-3'). Following PCR and agarose gel electrophoresis (1.5% agarose gel), IL-2R PCR products were visualized at 324 bp and human SOD1, if present at 236 bp. According to the animal instruction, the 14-week-old transgenic mice were considered symptomatic. To evaluate the effect of ST36 acupuncture on the onset of ALS, the 60-day-old mice were used in this study. Body weight was measured at two weeks intervals until the end of study. All mice were allowed access to water and food *ad libitum* and were maintained in an animal facility of Heilongjiang University of Chinese Medicine. All animal procedures were strictly conducted in accordance with the international ethical guidelines and national institutes of health guide concerning the care and use of laboratory animals and were

approved by the animal care and cse committee of heilongjiang university of chinese medicine.

Acupuncture methods and grouping

The ST36 points are located 3 mm lateral and distal to the wrist joint and between the radius and ulna in the forelimb. For acupuncture stimulation, stainless steel needle (0.17 mm in diameter, 7 mm in length) was vertically inserted into the ST36, inserting depth of 4 to 5 mm, and applied for 20 min in anesthetized mouse using inhaled isoflurane. The 60-day-old hSOD-1^{G93A} mice were randomized into the following groups with 15 mice per group, a pretreatment of acupuncture was applied at an interval of 2 days until the end of study. The grouping information is listed as follows: 1) Wild-type littermate control group (WT littermate, n=15); 2) Model group (M group, n=15), no acupuncture treatment; 3) Acupuncture treatment group (AT+M group, n=15), twenty minutes acupuncture treatment at ST36.

The onset of ALS and survival time

Beginning at 10 weeks, all animals were weekly assessed on two consecutive days with a tail suspension test in randomized order. The mice were evaluated for signs of motor deficit with the following a modified 4 point scoring system: 0 point if hind limbs fully stretch and for more than 2 seconds (no sign of motor dysfunction); 1 point if hind limb tremors are evident when suspended by the tail; 2 points if gait abnormalities are observed in a 30 minute walk; 1 point for dragging of at least one hind limb; 0 points for inability to right itself within 30 seconds [20]. Onset was defined as the earliest time when the mice showed the symptom of hind limb tremors (score=1) for 2 consecutive days. The survival time among groups was compared by recording the lifetime.

The rotarod performance test

The standard rotarod test was modified to assess the motor performance, muscular strength, and balance ability. A rotarod machine with automatic timers and falling sensors were used. An adaptive training began 5 days before the formal study, 6 mice in each group were trained to acquire the ability to maintain itself on a rod that turns at a constant speed of 15 r.p.m. For the formal rotarod test, the time for which an animal could remain on the rotating cylinder (3.5 cm) of a rotarod apparatus was measured. Each animal was given three tries at the intervals of 10 minutes and the longest latency to fall was recorded, whereas 180 seconds was chosen as the arbitrary cut-off time.

Morphology and immunohistochemistry

In total, 4 mice in each group were anesthetized using inhaled isoflurane, and blood was collected from retro-orbital veins, followed with a perfusion with 4% paraformaldehyde. The spinal cord tissues were removed and fixed in 4% paraformaldehyde for 3 days at 4°C until embedding. Briefly, the lumbosacral enlargement of spinal cord was embedded in paraffin, and the prepared tissues were cut into transverse sections (5 μm thick) and mounted on glass slides. Before staining, sections were deparaffinized in xylene and rehydrated in a graded series of ethanol followed by dH_2O.

Slices were incubated in hematoxylin followed by incubation in eosin and then mounted with Histomount medium for counting the number of anterior horn motor neurons. Nissl's staining was used to evaluate the morphological changes in this study. The deparaffinized slices were oven-dried, stained with 0.1% cresyl violet, dehydrated through a graded ethanol series (70%, 80%, 90%, and 100% × 2), placed in xylene, and covered with a coverslip after the addition of Histomount media. Three stained sections were counted per hSOD1G93A mouse. The number of anterior horn motor neurons cells were counted using Image J.

Immunofluorescence staining was processed as described in the instruction manual. The following primary antibodies, mouse monoclonal antibody to hSOD1 (1:1000), Rabbit polyclonal antibodies to Beclin1 (1:200) and LC3-II (1:200), were used for immunofluorescence staining. The sections were incubated with specific primary antibodies overnight at 4°C followed by the HRP-linked antibodies to rabbit IgG for 1 hour at room temperature. All pictures were photographed by the Carl Zeiss microscope at 400 × magnification.

Ultrastructure analysis

As mentioned above, the lumbosacral enlargement of spinal cord (each group, n=3) was collected and fixed in cold 2, 5-glutaraldehyde in

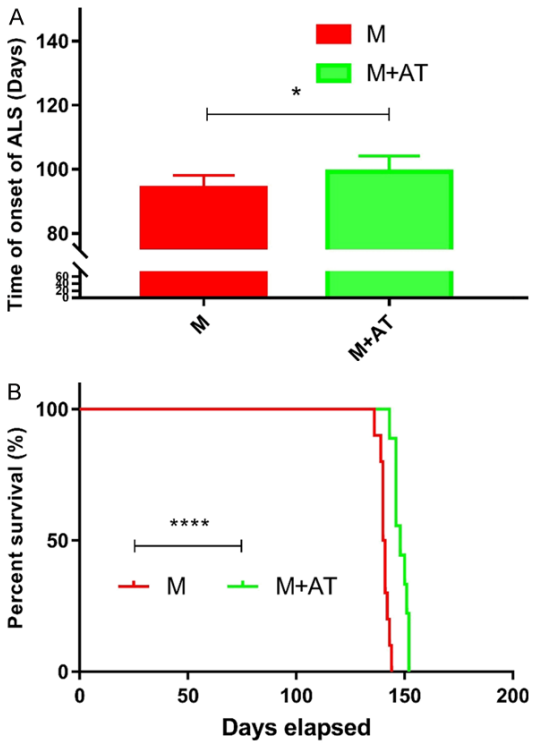

Figure 1. Beneficial effects of acupuncture at ST36 on disease onset and lifespan in the ALS mice. A. On the basis of the tail suspension test, acupuncture can significantly prolong disease onset in the ALS mice. B. Kaplan-Meier survival curves indicated survival in the WT-littermates or acupuncture-treated ALS mice. The data are presented as the mean ± SEM. *p<0.05; ****p<0.0001.

0.1 M/L cacodylate buffer (pH 7.3), postfixed in 1% OsO4, dehydrated through graded acetones and embedded in epoxy resin. Thick sections (about 1 μm) of lumbar spinal cord were stained with toluidine blue and observed by light microscopy in order to select fields. Ultrathin sections were mounted on copper grids, stained with uranyl acetate and lead citrate, and examined under an electron microscope (4200 ×).

Western blot analysis

The tissue of lumbar spinal cord was collected (each group, n=3). Tissues were sonicated in lysis buffer containing phosphatase and protease inhibitors, and the protein content of each sample was quantified following the conditions suggested by the manufacturer. SDS-PAGE was performed on Gradient NuPAGE 10% Bis-Tris gels. After samples had been transferred onto nitrocellulose membranes, the membranes were stained with Red Alert Western blot stain to ensure equal loading of lanes. According to the immunofluorescence staining, Western blot analysis was performed to quantify expression of proteins of interest. The hSOD1 (1:1000), Beclin1 (1:200), LC3-II (1:200), and β-actin (1:1000) were used. The membranes were incubated with specific primary antibodies overnight at 4°C followed by fluorescence-labeled secondary antibodies for 1 hour at room temperature. Subsequently, immunoreactive proteins were detected by using the Molecular Imager VersaDoc MP 5000 System (Bio-Rad) and analyzed using the Odyssey Infrared Imaging System.

Statistical analysis

All data are expressed as mean ± standard error of the mean (SEM). Mann-Whitney U-test for comparison of rotarod test results between acupuncture treated and untreated hSOD1G93A mice. Student's t-test was used to compare immunohistochemical data. One-way analysis of variance (ANOVA) was used to analyze the statistical differences among the groups. The *p*-values<0.05 were considered statistically significant. Statistical analyses and graphs were performed with SPSS 15.0 for Windows and GraphPad Prism 7.0 software.

Results

Acupuncture at ST36 delayed the ALS onset and lower the mortality rate

Acupuncture at ST36 started bilaterally and subcutaneously to treat the 60-day-old hSOD-1^{G93A} transgenic mice. To determine the effects of acupuncture on the onset of ALS, a tail suspension test based on the 4 point scoring system was used. The onset time of ALS in the M+AT group was delayed about 5 days compared to the ones in the M group (**Figure 1A**). Moreover, the life expectancy in M+AT group was significantly prolonged one weeks longer than those in the M group (**Figure 1B**). These results indicate that acupuncture was protective against the ALS progression in hSOD1^{G93A} mice.

Acupuncture at ST36 maintained the body weight and motor function

ALS manifests progressive atrophy and weakness of muscles, which will lead to the reduc-

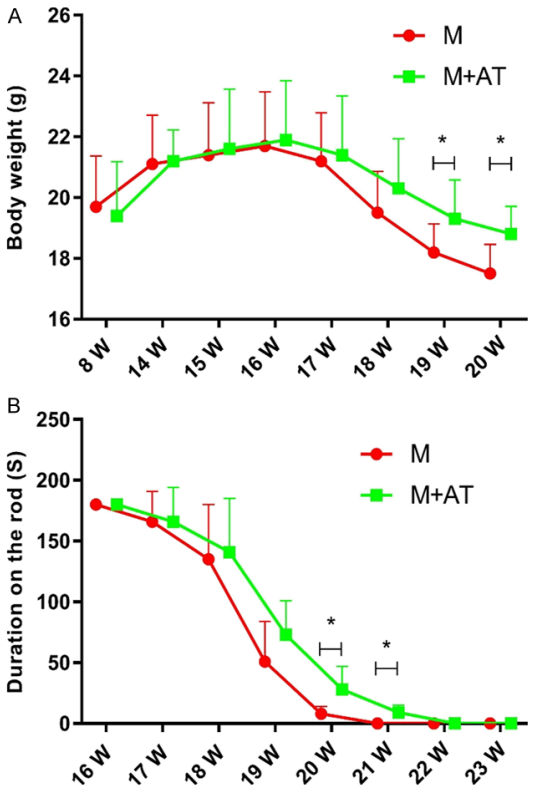

Figure 2. Acupuncture at ST36 prevented the loss of the bodyweight and maintained the motor function in ALS mice. A. Acupuncture treatment prevented the loss of bodyweight of end-stage in the ALS mice of M+AT group compared with those of M group. B. Acupuncture treatment ameliorated motor neuron defects. The data are presented as the mean ± SEM. *p<0.05.

tion of body weight. The body weight was measured at the interval of one week. Unfortunately, the body weight dropped dramatically after the onset of ALS, acupuncture could not prevent the disease progression. However, compared with the mice in the M group, acupuncture could significantly increase the end-stage body weight (**Figure 2A**). To confirm the effects of acupuncture on motor activity in symptomatic hSOD1^{G93A}, the rotarod behavioral test was performed from the onset of ALS to the spontaneous death at the interval of one week. Motor function of the mice in the M+AT group, such as the time of duration on the rotarod, significantly increased at the 17th and 18th week, compared to the ones in the M group (**Figure 2B**). These results illustrated that acupuncture could improve the motor activity and partially ameliorated the reduction of the end-stage body weight.

Acupuncture at ST36 protected the anterior horn motor neurons in number and structure

The degeneration of the anterior horn motor neurons is observed in ALS. To investigate whether improved motor activity induced by acupuncture at ST36 was dependent on the protective effect of motor neurons in the ALS animal model, the morphological studies in the spinal cord of symptomatic hSOD1^{G93A} mice were performed. As shown in **Figure 3**, the loss of anterior horn motor neurons was dramatically prevented in the lumbar spinal cord of acupuncture-treated mice as compared to the ones in the M group. Then, the number of Nissl's stained-motor neurons was quantitatively counted to prove that acupuncture treatment dramatically increased the number of neurons (**Figure 5A**). Furthermore, the cellular structure of neurons and nuclear staining became blurred and vacuoles interspersed in the H&E sections of M group, and acupuncture at ST36 could significantly improve the morphological structure in the mice of M+AT group. In addition, the results of the transmission electron microscope indicated that acupuncture at ST36 significantly mitigated the atrophy of motor neurons, relieved the edema of mitochondria and prevented the aggregation of heterochromatin. Moreover, those denatured neurons surrounded by the neuroglia cells formed the satellite phenomenon, which was significantly improved by the acupuncture treatment (**Figure 5B**).

Acupuncture at ST36 activated the autophagy pathways

Qualitative analysis of Beclin-1 and LC3-II expression in lumbar spinal cord was determined by immunofluorescent staining, and the protein levels of ANP and TNF-α were quantified by Western blotting analysis. The results indicated that Beclin-1 and LC3-II were lower in symptomatic hSOD1^{G93A} mice, suggesting that an obstruction of autophagy was associated with the progress of ALS. Acupuncture at ST36 significantly increased the protein production of Beclin-1 and LC3-II (**Figures 6**, **7**). To demonstrate whether the activation of autophagy was associated with the improvement of motor activity in the symptomatic hSOD1^{G93A} mice, the protein level of hSOD1 in the lumbar spinal cord was tested, and expression of hSOD1 was sig-

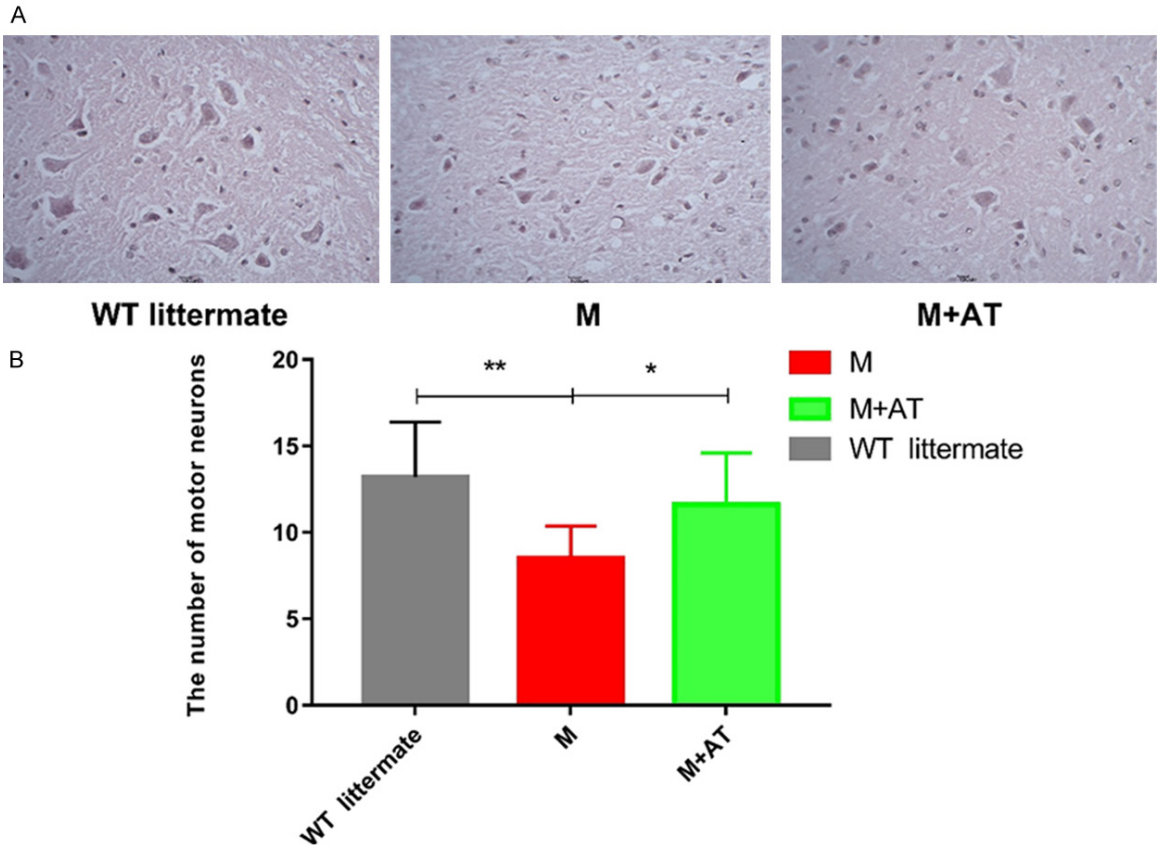

Figure 3. Acupuncture at ST36 prevented motor neuron death in ALS mice. A. H&E staining of the spinal cord from different groups of mice. B. Quantification of motor neurons in the anterior horn of lumbar spinal cord in different groups. The data are presented as the mean ± SEM. $^{*}p<0.05$; $^{**}p<0.01$.

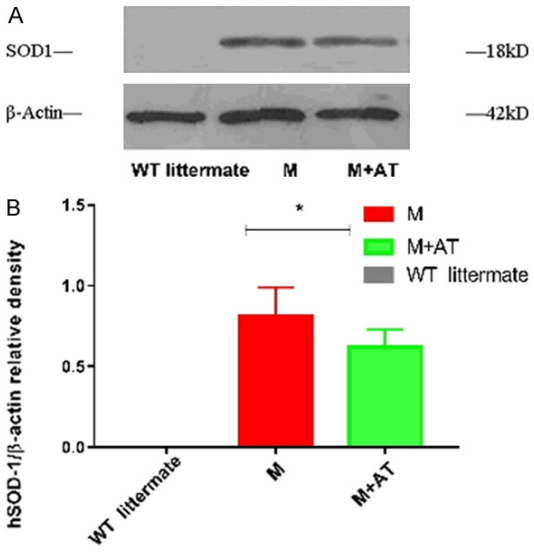

Figure 4. Acupuncture at ST36 reduced the expression of hSOD1 in ALS mice. A. Representative Western blots of hSOD1 in each group. B. Quantification of the expression of hSOD1 in the anterior horn of lumbar spinal cord in different groups. The data are presented as the mean ± SEM. $^{*}p<0.05$.

nificantly reduced by the acupuncture treatment (**Figure 4**).

Discussion

Although the pathogenesis and signaling pathways that induce ALS-related motor neuron disorders remain elusive, accumulation in the cell of misfolded or abnormal proteins attracts attention in ALS development. With the development of sequencing technology, more and more ALS-related aggregated mutant proteins have been identified [21]. On the other hand, autophagy, as one of major intracellular protein degradation pathways, is essential to recognize and remove those abnormal protein aggregates, while defects in autophagy have been proposed to contribute to ALS pathogenesis [22]. Accumulating evidence indicates that ALS might be an autophagy disease [23].

Generally, ALS is considered as an autophagy-related Neurodegenerative disease. The progressive degeneration of motor neurons is fol-

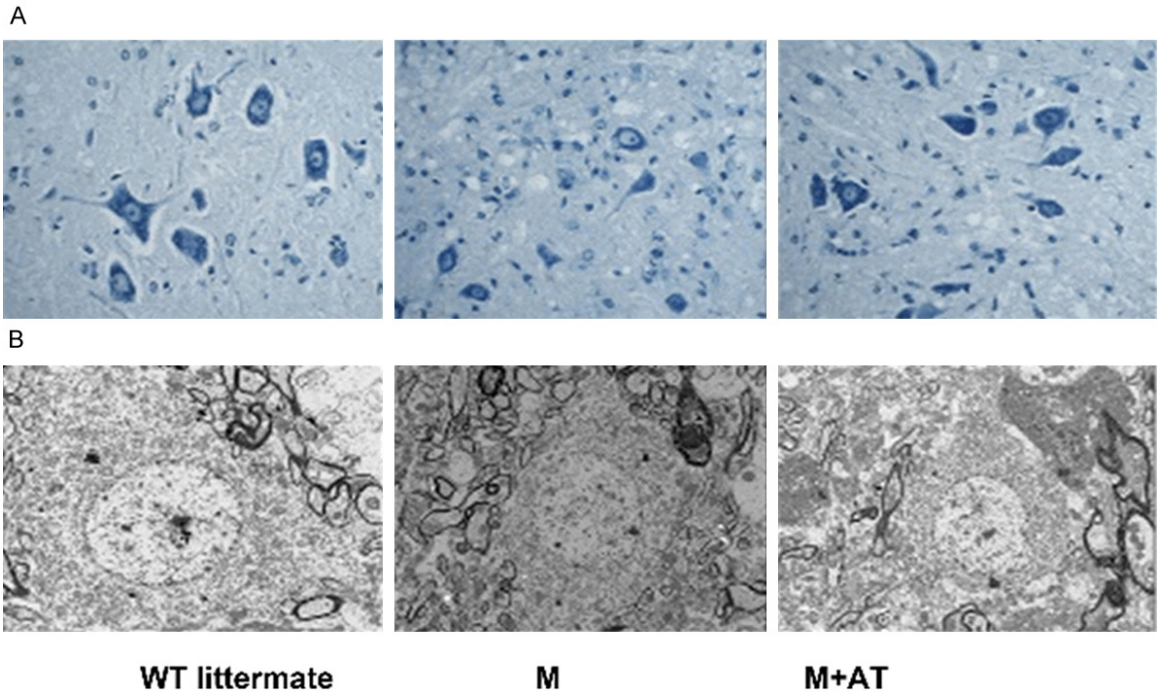

Figure 5. Acupuncture at ST36 protected the anterior horn motor neurons in number and structure. A. Representative images of Nissl's stained-motor neurons in the anterior horn of the spinal cord of SOD1G93A mice in different groups. B. Representative images of ultrastructure captured by electron microscopy.

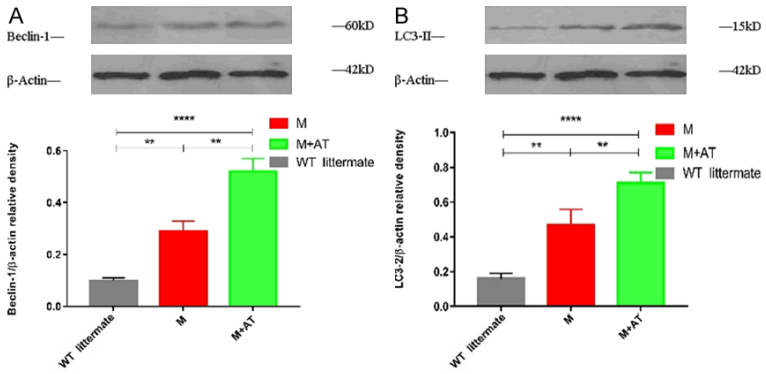

Figure 6. Acupuncture at ST36 increased the expression of Beclin-1 and LC3-II in ALS mice. A. Representative Western blots of Beclin-1 in each group, and quantification of the expression of Beclin-1 in the anterior horn of lumbar spinal cord in different groups. B. Representative Western blots of LC3-II in each group, and quantification of the expression of LC3-II in the anterior horn of lumbar spinal cord in different groups. The data are presented as the mean ± SEM. $^{**}p<0.01$; $^{****}p<0.0001$.

lowed by muscle atrophy, spasticity and quadriplegia, culminating in death within 3-5 years of disease onset due to respiratory failure [24]. Unfortunately, there is no an evidence-based clinically beneficial therapy until now, patients with ALS have to try at least one type of CAM conducted by a survey [25]. Acupuncture therapy has been widely used as a CAM especially in neurodegenerative diseases. A meta-analysis showed that acupuncture significantly decreased wrist, knee, and elbow spasticity in post-stroke patients [15]. Several clinical case reports claimed that acupuncture helps to control pains, relax muscles, and even to slow, stop or reverse progression [26]. The modern theory on the mechanisms of acupunctures relied on its significant anti-inflammatory effects by the sciatic and the vagus nerves modulating the production of catecholamine in the adrenal glands. [27, 28]. It is worthy of note that acupuncture could also alter inflammatory markers in ALS animal models [19, 29] and showed the beneficial effects of acupuncture. Recently, eight independent human genetic studies have uncovered a link between autophagy and neuroinflammation in ALS [30]. TANK-binding kinase 1 (TBK1) mutations not only resulted in impaired innate immunity but also distorted autophagy and contrib-

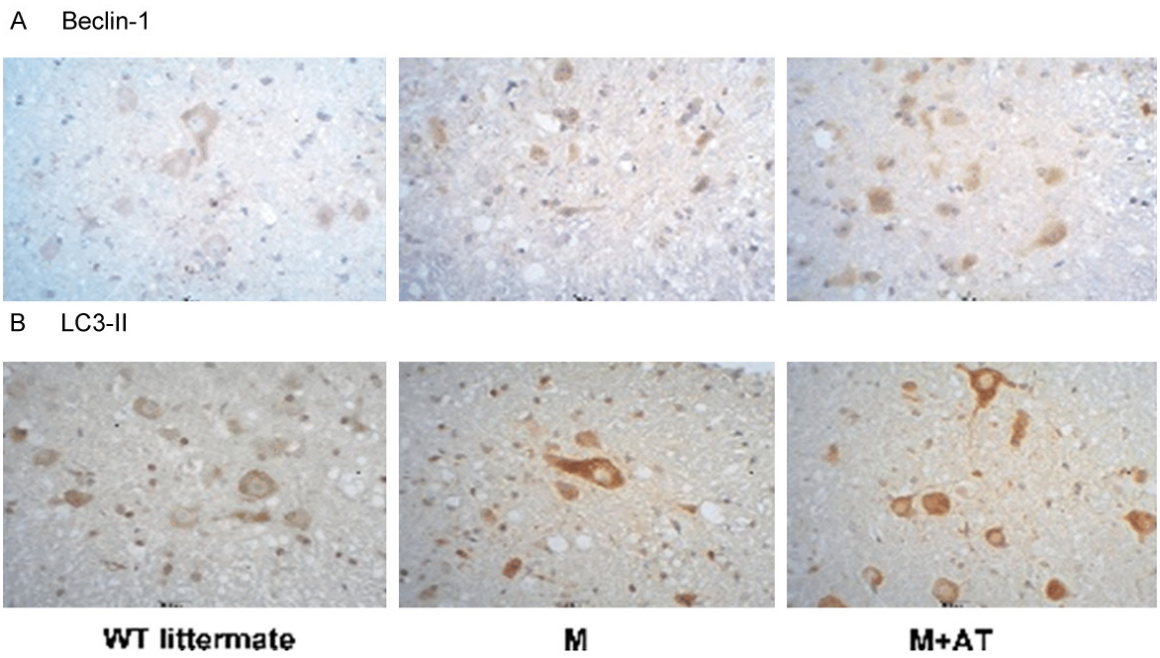

Figure 7. Acupuncture at ST36 increased the expression of Beclin-1 and LC3-II in ALS mice. A. Representative images of IHC staining of Beclin-1 in the anterior horn of the spinal cord of SOD1G93A mice in different groups. B. Representative images IHC staining of LC-II in the anterior horn of the spinal cord of SOD1G93A mice in different groups.

uted to the accumulation of protein aggregates. However, it has remained elusive whether acupuncture could target on the autophagy pathways to prohibit the abnormal protein aggregates.

This study sought to determine whether Acupuncture could prohibit the abnormal protein aggregates by targeting on the autophagy in a hSOD1^{G93A} transgenic ALS model. The results show that pre-treatment at ST36 point could prolong the onset and survival time as compared to age-matched, symptomatic hSOD-1^{G93A} mice by prevention of weight loss and improvement of motor function. The improvement of motor function was supposed to control pains, relax muscles, and help to maintain the balance and gesture on the rotarod. Regarding to the prevention of weight loss, it is reported that weight loss is nearly ubiquitous in ALS, the lower BMI index or rapid weight loss indicated the negative predictors of survival in ALS [31]. Given that the denervated muscular atrophy is the hallmark of ALS is because of the death of motor neurons [32], prevention of weight loss from acupuncture may have relied on its ability to rescue motor neurons from cell death biology by prohibiting the protein aggregates in motor neurons, and we examined the expression levels of hSOD1 in symptomatic mutant SOD1^{G93A} mice. The hSOD1 expression level of lumbar spinal cord was reduced by acupuncture at ST36.

To determine whether the effect of acupuncture at ST36 could be dependent on the activation of autophagy, we examined the expression of LC3-II and Beclin1 in lumbar spinal cord. The production of both proteins was upregulated by acupuncture at ST36 in the analysis of Western blotting, which was also confirmed by the immunohistochemistry staining (IHC). It is well acknowledged that autophagy is initiated by the formation of phagophore that is the precursor to the autophagosome, and Beclin1 as a key factor to constitute an autophagy initiation complex and form the phagophore [33]. With the elongation of phagophore, it seals around cytoplasmic substrates to form an autophagosome and release the LC3-II, the most widely-used marker. This process is called autophagosome maturation [34]. These results suggested that acupuncture at ST36 could activate the autophagy via the autophagy initiation and autophagosome maturation.

However, the placebo effect could not be ruled out for the lack of the “Sham” acupuncture due to the limitation of acupuncture modalities. Even the symptomatic mutant SOD1^{G93A} mouse

is thought as one of the best ALS models for the medicine development and pathogenesis investigation, various ALS-related mutant genes have been found for the last decades [23]. Moreover, the autophagosome delivers protein aggregates to lysosome for degradation. It has been reported that defects in lysosomal fusion might contribute to motor neuron death, which went beyond the scope of our research. All these limitations might restrict the experimental reproducibility in other transgenic animal model.

Taken together, these data suggest that acupuncture at ST36 maintained motor function and prevented motor neuron from death in the spinal cord compared to that observed in symptomatic mutant $SOD1^{G93A}$ mice. Furthermore, acupuncture at ST36 enhanced autophagy activities to facilitate the degradation of hSOD1 aggregates. Even more experimental studies or clinical trials are necessary to confirm our findings that the acupuncture might exert more than a CAM option in ALS, we hope this study would help patients make more informed treatment decisions.

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Disclosure of conflict of interest

None.

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