HnRNP G Reduces Neuron Death in Amyotrophic Lateral Sclerosis by Preventing Abnormal TDP-43 Accumulation

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Abstract

Heterologous ribonucleoprotein (hnRNP) G protein was found to significantly down-regulated in the spinal cord of amyotrophic lateral sclerosis (ALS) mouse model in our previous study, but the down-regulated effects of hnRNP G in ALS haven't been known up to now. Therefore, we further studied the possible effects of hnRNP G on spinal neuron death in TG(SOD1*G93A)1Gur (TG) mice. Eighteen TG mice and eighteen SOD1 wild-type (WT) mice were divided into 6 groups. The hnRNP G of the spinal cord was analyzed using immunofluorescent histochemistry and Western blot. hnRNP G-siRNA was transfected into PC12 cells and observed the alteration of proliferation rate and intracellular proteins using the CCK8 and Western blot. We reported that the distribution of hnRNP G positive cells in the posterior horn was more than that in the central canal and its surrounding gray matter more than that in the anterior horn. hnRNP G protein mainly expressed in neurons. hnRNP G expression in the cervical and thoracic segments of TG mice in the pre-onset group was significantly higher than that in the control group. hnRNP G expression in the thoracic and lumbar segments of TG mice in onset group was lower than that in control group. hnRNP G expression in the cervical and thoracic segments of TG mice in progressive group decreased, while that in the lumbar segment of TG mice in progression group significantly increased. After hnRNP G was silenced, the proliferation rate of PC12 cells was slower than that in the control group, SOD1 expression didn't significantly change, both TDP-43 and Bax expression significantly increased in PC12 cells after silenced. Our study revealed that the distribution of hnRNP G in the spinal cord of ALS mice showed the possible protective effect on the progression of ALS, its mechanism maybe prevent neuron death by reducing abnormal TDP-43 accumulation in the spinal cord of ALS-like mice.

Introduction

Amyotrophic lateral sclerosis (ALS) is the commonest motor neuron disease (MND), which is characterized by the damage of both upper and lower motor neurons, manifested as progressive weakness, muscular atrophy, and fascicular fibrillation of muscle as well as pyramidal tract signs. Most patients died of respiratory paralysis within 3–5 years [1]. Current clinical used drugs only could partly postpone the disease progression of ALS, their effect mechanisms include the elimination of oxygen free radicals and the protection of neurons. Up to now, there haven't been any reliable specific drugs curing ALS. Several studies have found that the pathogenesis of ALS is closely related to genetic factors, the toxicity of excitatory amino acids, the damage of oxidative stress, the damage of axonal transport, the defect of mitochondrial function, and the abnormal aggregation of protein so on. Their effects can exert synergistic effects on each other [2]. Recently found pathogenic mechanisms of ALS include RNA metabolic dysfunction, the defects of cytoplasmic transport, and the dynamics damage of RNA particles and other ribonucleoprotein body [1]. These pathogenic factors can lead to the disorder of gene transcription, splicing, translation, and other related processes, and further damage cells and /or tissues.

The pathological feature of ALS is the generation of cytoplasmic inclusion bodies, aggregating in damaged motor neurons and oligodendrocytes. Inclusion bodies not only exist in the spinal cord but also

exist in multiple brain regions, such as frontal cortex, temporal cortex, hippocampus and cerebellum [3, 4]. The aggregation of abnormal proteins and the generation of inclusion bodies have been considered as the pathological characteristics of several neurodegenerative diseases including ALS. Among them, the aggregation of abnormal proteins and the generation of inclusion bodies are the commonest in spinal motor neurons. Ubiquitin positive inclusion body is a sign of ALS pathology. In ALS, some proteins encoded by mutant genes, such as SOD1, TDP-43, FUS, OPTN, Ubqln2 and NEFH, are the important components of inclusion bodies [5–7].

In 1993, the first mutant SOD1 gene [8] was found in the patient with familial ALS (fALS). From then on, more than 50 potential pathogenic genes of ALS have been found up to now, among them, including c9orf72, TDP-43, FUS, heterologous ribonucleoprotein (hnRNP) A1, Sqtstm1, VCP, OPTN, Pfn1 [9]. These genes are roughly divided into the following categories: The genes affected the control of protein stability and expression quality, the genes affected the normal function and metabolism of RNA, and the genes interfered with the cytoskeleton dynamics of distal axons of motor neurons. The advances in studied technologies have prompted researchers to sequence a wide range of DNA in patients with sporadic ALS (sALS), and found that the genetic variation of ALS gene isn't uncommon. 1–3% of sALS patients are caused by the missense mutations in SOD1 gene [10], and 5% or more sALS are caused by the abnormal amplification of introns in c9orf72 gene [11].

In 2006, Lee et al. found that RNA binding protein (RBP) TDP-43 deposited in the brain and spinal cord of ALS patients, and aggregated in cytoplasm. TDP-43 mislocation is now widely recognized as the characteristic of partial sALS and fALS [12]. Subsequently, the mutations of other ALS related proteins including FUS and hnRNP A1 were identified. These proteins were mutated after bound with RNA, which made RNA's biological role be more concerned in the pathogenesis of ALS. Because the mutations of RBPs gene are found in neurodegenerative diseases, the dysfunction of RBPs is clearly evolving into the central theme of neurodegenerative diseases, and the dysfunction of RNA processing and the phagocytosis of aggregation protein become an important mechanism related to ALS [13]. The RBPs closely related to ALS include TDP-43, FUS, c9orf72, hnRNP A1, hnRNP A2/B1, Matr3, SETX, ELP3, atxn2, ANG, SMN1 and SMN2 [14, 15]. In addition, many other RBPs have been found to show changes in subcellular distribution in the neurons and/or glial cells of ALS patients, but the mutations caused ALS have not been known [16], which indicates that even if the gene of RBPs does not mutate, it can also cause the destruction of RNA stability in ALS, or affect the metabolism and stability of RNA during the occurrence and development of ALS. Only 4% of fALS patients show the gene mutation of TDP-43, about 97% of ALS patients had dis-localization and the inclusion body of TDP-43 protein. It suggests that even if there is no relevant mutation, the cytoplasmic and intranuclear inclusion bodies are common in ALS patients [17].

Both TDP-43 and FUS are involved in RNA related pathways, they play some roles at many steps of RNA regulation, including RNA transcription, splicing, transportation, translation and microRNA production [18]. Both TDP-43 and FUS proteins interact directly with polyphase ribonucleoprotein complexes, regulate RNA splicing and transport, both TDP-43 and FUS have similar biological roles in RNA related

pathways [19]. The cellular cytotoxicity of mutants and/or cytoplasmic dislocated TDP-43 and FUS might be caused by the following reasons: (1) the loss of normal nuclear function leads to the disorder of nuclear RNA processing; (2) obtains the additional cytoplasmic RNA binding activity; (3) the polymerization dependent toxicity [7, 20–22]. More and more evidence show that neurotoxicity generates through various cellular pathways, such as the RNA mismatch and the transcription reduction of c9orf72 gene, the disorder of transport between nuclear and cytoplasm, the stress of nuclear protein and DNA damage [23–27]. The break of DNA strand is the most serious type of DNA damage. If the broken DNA are not repaired properly, which usually leads to cell death. The hnRNP A1 is a member of the hnRNP family, involves in a variety of RNA metabolism [28]. Several studies find that the hnRNP A1 negatively regulates its mRNA expression by inhibiting the intron 10 splicing of hnRNP A1 pre-mRNA in cell models. This mechanism might be the self-regulation of hnRNP A1 expression, and the low-level hnRNP A1 overexpression can cause cytotoxicity by activating the pathway of mitochondrial apoptosis [29, 30]. It is suggested that the level of hnRNP A1 is strictly controlled within a certain range by the self-regulation of mRNA, so that no cytotoxicity of hnRNP A1 expression occurs at the physiological condition.

Among recently discovered RBPs, hnRNP G is the most prominent in the familial members of more than 20 RBPs proteins. TDP-43, FUS and hnRNP A1 are members of hnRNP protein family that regulate RNA metabolism at each stage of RNA life cycle [31–33]. hnRNPs help to control the maturation of newly formed hnRNA and pre-mRNA, stabilize mRNA and control its translation during cell transport. hnRNPs are key proteins in cellular nucleic acid metabolism [34]. Since neurons are the permanent cells, the steady state of mRNA needs to be strictly regulated, and the steady state of mRNA is very vulnerable to the dysfunction of RBPs including hnRNPs [35].

hnRNP G is the product of RNA binding motif protein and RNA binding motif protein X-linked (RBMX) gene, is the only glycosylated hnRNP that can directly bind to RNA. The RNA binding motif protein Y-linked gene is located on Y chromosome, is formed by the reverse transposition of RBMX [36–38]. hnRNP G can be used as a pre-splicing factor of mRNA, involves in the selection of specific splicing sites, is related to the post transcriptional new mRNA of RNA polymerase II, and regulates the selection of pre-mRNA alternative splicing sites [39]. Recent studies have shown that hnRNP G is an active regulator of the splicing mechanisms in neurodegenerative diseases. Firstly, hnRNP G promotes the expression of exon 7 of survival motor neuron. The homozygous deletion of hnRNP G gene leads to the common motor neuron disease called Spinal Muscular Atrophy [40]. Secondly, the microtubule associated protein like Tau is regulated by the interaction of cis- and trans- factors including hnRNP G. In human genes, the exon 10 of Tau is a binding domain of selective splicing, the wrong splicing of Tau gene results in the frontotemporal dementia or Parkinson's syndrome [41]. It is obvious that hnRNP G has various and complex functions. Our studied groups found that the hnRNP G protein in the spinal cord of TG(SOD1*G93A)1Gur (TG) mice was significantly reduced through the methods of proteomic analysis [27]. The purpose of this study is to further explore the possible roles and mechanisms of hnRNP G in the pathogenesis of ALS.

Materials And Methods

Cell Line and Animal

The PC12 cell line (Cell Storage Center of Wuhan University) used in the experiment derives from the pheochromocytoma of rats. The culture conditions of PC12 cells were as follows: The constant temperature of 37°C, 5% of carbon dioxide, and low sugar DMEM medium. TG mice and wild-type (WT) mice were provided by the Model Animal Research Institute of Nanjing University. All experimental animals included the rationale of testing animals were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of China, and protocols were reviewed and approved by the Animal Care and Use Ethics Committee (ACUEC Number 2019-11-02) of Jiangxi Provincial People's Hospital, First Affiliated Hospital of Nanchang Medical College, Affiliated People's Hospital of Nanchang University, China.

Extraction of Mouse DNA

Approximate 0.3cm mouse tails were taken into EP tube, added 75ul 50mM NaOH. The mixed solution was put in the water bath at 97°C for 30 min. Added 7.5ul 1M Tris HCl into a specimen, centrifuged by 4,000g for 1 min. The supernatant was then extracted DNA.

PCR Amplification

The primers (Shanghai biotechnology company) of PCR were as follows: The upstream primer of mouse-G93A*SOD1: 5'-cat cag cct act ccc tga-3', downstream primer: 5'-cgc gac taa caa tca atc aaa gtg a-3'. The upstream primer of mouse-IL-2: 5'-cta ggc aca gaa ttg aaa gat ct-3', downstream primer: 5'-gta ggt gga aaa ttc tag cat cat cc-3'. The PCR reaction condition of extracted mice DNA was as follows: 25ul reaction mix solution, 2ul primer (10pmol/ul), 2ul template DNA, 21ul ddH₂O, 50ul total volume. Pre-denaturation at 95°C for 5 min once, 35 reaction cycles of denaturation at 94°C for 30 sec, annealing 60°C for 1 min, and extend at 72°C for 1 min, final extension at 72°C for 2 min once, hold at 16°C. Detected PCR results by the electrophoresis of agarose gel. The G93A*SOD1 positive mice were used in experimental groups, the G93A*SOD1 negative mice (WT) were used in control groups.

Animal Experiment Group

Eighteen TG mice were randomly selected as experimental groups and 18 WT mice were randomly used as control groups. We designed randomization of animals to experimental groups. After the genotype identification of mice, TG and WT mice were randomly selected for the study. Firstly, we marked a code for each mouse. The samples of mice were then blindly stained and observed, the staining and the observing researcher didn't know the identity of the sample. Finally, we matched the results of each sample to its true identity and summarize for analysis.

Experimental groups and control groups were divided into three subgroups by using the random number labeled method. The mice bred to 60–70 days were used as pre-onset groups, that bred to 90–100 days were used as onset groups, those bred to 120–130 days were used as progression groups. Each

subgroup consisted of six mice. Each subgroup was randomly divided into immunofluorescence stained groups and Western blot groups. After the mice was anesthetized, the spinal cord were dissected at 4°C. If the mice died unexpectedly during the raise or the operation of specimen preparation was failed, the experimental mice can be supplemented randomly.

Treatment of Animal Experimental Materials

When mice grew to experimental asked days, the spinal cord was dissected. The procedures of spinal cord dissection were as follows: Mice were anesthetized by diethyl ether after intraperitoneally injected 0.1-0.2ml of 10% chloral hydrate based on the counting of body weight/each kilogram. Firstly, approximately 20 ml pre-cooled 0.9% sodium chloride injection was perfused. If specimens were used for the analysis of Western blot, the spinal cord was taken out immediately at 4°C. If specimens were used in immunofluorescence stain, then continued to slowly infuse approximate 20 ml 4% paraformaldehyde. The whole spinal cord was immersed in 4% paraformaldehyde at 4°C overnight. After paraformaldehyde fixation, spinal cord was conducted gradient dehydration in the alcohol of different concentrates, then immersed in sucrose solution and placed in a 4°C refrigerator overnight. The dehydrated spinal cord was divided into cervical, thoracic and lumbar segments, were embedded with OCT solution (Sakura Finetek Japan Co. Ltd).

Immunofluorescence Stain of Mouse Spinal Cord

The spinal cord of each mouse was cut into 12μl thick sections by the technique of constant temperature frozen section. Anti hnRNP G antibody and anti-NeuN antibody were used in experimental groups and control groups. The NeuN positive cells showed green fluorescence, the hnRNP G positive cells showed red fluorescence. Staining steps are as follows: added 0.3% Triton X-100 for 10 min, washed with 0.01M PBS for 3 times of 5 min. The tissue sections were sealed with 5% BSA for 1 h, then incubated by primary antibody in accordance with following concentrate: Rabbit anti hnRNP G antibody: 1:200 (ab190352, Abcam Co. Ltd), mouse anti NeuN antibody: 1:400 (ab104224, Abcam Co. Ltd). The tissue sections with primary antibodies were kept in a dark box overnight at 4°C. The next day, the slice was washed with 0.2% Tween-20 for 5 times of 5 min. Then slices were incubated by secondary antibody in dark RT for 2 h, after incubated by secondary antibody, slices were washed 5 times with 0.2% Tween-20 for 5 min each time. The concentrate of secondary antibodies was goat anti-mouse IgG (FITC) 1:200 (ab150113, Abcam Co. Ltd), donkey anti-rabbit IgG (TRITC) 1:250 (ab150075, Abcam Co. Ltd). Slices were washed 3 times with 0.01M PBS for 5 min each time, sealed it with the anti-fluorescence quenching sealing solution. Used the fluorescence microscope with a camera to observe and took pictures of all parts.

PC12 Cells Transfection of hnRNP G siRNA

To find the appropriate transfection concentration, the concentration gradient was screened in the pre-experiment. The transfection experiment was divided into the following five groups: blank control group adding normal culture medium, negative control group adding negative control transfection complex, hnRNP G siRNA 1 transfection group adding sequence 1 transfection complex of hnRNP G, hnRNP G siRNA 2 transfection group adding sequence 2 transfection complex of hnRNP G, hnRNP G siRNA 3

transfection group adding sequence 3 transfection complex of hnRNP G. The sequences of siRNAs (Shanghai GenePharma Co. Ltd) were shown in the following: The forward and reverse primers of hnRNP G siRNA1 sequence were GCU CUU UAU UGG UGG GCU UTT and AAG CCC ACC AAU AAA GAG CTT, that of siRNA2 were CCC GAG AGA UGA UGG AUA UTT and AUA UCC AUC AUC UCU CGG GTT, that of siRNA3 were GCG UGA CUA UUC GGA UCA UTT and AUG AUC CGA AUA GUC ACG CTT. The results of transfection gradient experiment were in Additional file 1: Table S1-2.

The $1-1.5 \times 10^5$ cells were added into each pore with a 1,000 μ l growth medium for 18–24 h before the transfection experiment. Lip2000 transfection reagent (YG0398, Shanghai Kemin Biotechnology Co. Ltd) was mixed gently in RT before being used. 95 μ l DMEM was added into sterile EP tube, 3 μ l hnRNP G siRNA (20 μ M) was added into the EP tube containing DMEM culture medium, and mixed well. Then, 2 μ l lip2000 transfection reagent was added into EP tube and mixed well at RT for 20 min. The processes of transfection were as following: Firstly, 900 μ l fresh DMEM medium was added into each plate of 12 wells. 100 μ l transfection mixtures were added into each pore, the final volume was 1,000 μ l. Shook pore plate gently and cultured cells in 5% CO₂ incubator at 37°C. Total RNA can be extracted after the culture of 24–96 h.

RNA Extraction from PC12 Cells Transfected with hnRNP G siRNA and Reverse Transcription PCR

A proper amount of Trizol was added into PC12 cells, placed the split cells were in a ribozyme free EP tube. Added 1/5 Trizol of chloroform. Centrifuged by 12,000g at 4°C for 20 min, put the supernatant into another EP tube with an equal volume of isopropanol. Centrifuged by 12,000g at 4°C for 15 min, and discarded the supernatant. A proper amount of anhydrous ethanol was added. Centrifuged by 12,000g at 4°C for 10 min, and discarded the supernatant. DEPC (Diethyl pyrocarbonate) water was added into the dissolve RNA pellet. The concentration of RNA samples was measured by a spectrophotometer. The extracted RNA was used for reverse transcription PCR (RT-PCR). The experimental steps were as follows: 2 μ l 5x primescript™ reverse transcription Master Mix, 2 μ l total RNA, and added RNase free ddH₂O to 10 μ l. The reverse transcription procedure was as follows: Reverse transcription reaction at 37°C for 15 min, the inactivation reaction of reverse recording enzyme at 85°C for 5 sec.

Detection of hnRNP G Expression in Each Group by Real-time Fluorescence Quantitative PCR

The steps of real-time fluorescence quantitative PCR (QRT PCR) were as follows: The primers of hnRNP G and internal reference β -actin were as follows: the upstream primer of hnRNP G was 5'-aaa ctt tgg acc aca cat atc c-3', the downstream primers was 5'-aag cca cgc tta cac ata cta-3', the upstream primer of β -actin was 5'-ccg tga aaa gat gcc cag at-3', the downstream primers was 5'-GGA CAG TGA GGC CAG GAT AGA-3', Prepared the QRT PCR reaction solution on ice according to the following components: 10 μ l TB Green premix Ex Taq II (2x), 0.8 μ l PCR forward primer (10uM), 0.8 μ l PCR reverse primer (10uM), 0.4 μ l ROX reference dye or dye II (50x), 2 μ l 1x reverse transcription reaction solution (cDNA solution from RT-PCR),

6μl sterile water. Used the stepone plus real-time PCR system to conduct PCR reaction, standard PCR amplification procedures were as follows: Stage 1: pre-denaturation of 95°C for 30 sec once, stage 2: PCR reaction of 95°C for 5 sec, 60°C for 30 sec and total repeated 40 times. After amplification, observed the amplification curve and fusion curve of qRT-PCR, obtained the effective CT value, and analyzed using Graphpad Prism software.

CCK8 Detection of Cell Growth Curve after Gene Silenced

After the gene was silenced, the growth curve of PC12 cells under the normal condition was detected by the CCK8 kit. The steps were as follows: added PC12 cell suspension of 100μl/each well into 96 wells plate, and placed it in an incubator for 24 h. The transfection reagent was added to the pored plate and placed in the incubator for further incubation for 24–48 h. The transfected PC12 cells were changed to the new culture medium and added 10μl CCK8 solution was into each pore. After the culture dish was placed in the incubator for 1 h, the absorbance of each pore at 450nm was measured by the enzyme analyzer. Data analysis was used by Graphpad Prism software.

Western Blot

The operation steps of spinal protein extraction and quantification were as follows: Mouse spinal cord was ground by a glass homogenizer, added a proper amount of tissue Ripa lysis solution containing 1mM PMSF. Lysis liquid was centrifuged by 13,000g at 4°C for 15 min, the supernatant was extracted proteins. The extracted proteins were quantified according to instructions of the BCA kit (Solarbio Co. Ltd, Beijing, China).

The protein extraction and quantification procedures of PC12 cells after hnRNP G silenced are as follows: Took 1ml of Ripa solution into the solution of PC12 cell, added 10μl PMSF solution, washed 3 times with PBS, added 100–150μl lysate solution into each cell of twelve pore plates, centrifuged by 10,000–14,000g at 4°C for 3–5 min, the supernatant was extracted proteins. The quantification of protein concentration was detected by the BCA kit.

Western blot was used for the semi-quantitative analysis of spinal proteins in each group. Protein samples (20–50μg) were conducted electrophoresis by SDS-PAGE gel, the electrophoresis of 100mA current and 80V voltage was performed for 30 min, then voltage was changed to 120V electrophoresis. Conducted the membrane transfer of protein at 200mA and 100V for 30–60 min. Sealed the PVDF membrane using 5% skimmed milk solution for 1–2 h at RT, took out PVDF membrane and washed with 1x TBST 3 times for 10 min each time. Put PVDF membrane into the solution of corresponding primary antibody and incubated at 4°C overnight, washed the membrane with 1x TBST 3 times for 10 min each time. Put PVDF membrane into a secondary antibody to incubate at RT for 1–2 h, and washed membrane 3 times for 10 min each time. Added mixed luminescent liquid for 30 sec-2 min, and put it in the dark box for exposure, used Image J software to analyze protein band density. The concentrations of primary antibody were as follows: α-Tubulin 1:1,000 (Cat.No.66031-1-Ig, Proteintech Co. Ltd), hnRNP G 1:1,000 (ab190352, Abcam Co. Ltd), SOD1 1:1,000 (Cat.No.67480-1-Ig, Proteintech Co. Ltd), Bax 1:1,000 (Cat.No.60267-1-Ig, Proteintech Co. Ltd), TDP-43 1:1,000 (Cat.No.66734-1-Ig, Proteintech Co. Ltd) and β-

Actin 1:2,000 (Cat.No.66009-1-Ig, Proteintech Co. Ltd). The horseradish peroxidase (HRP)-secondary antibody 1:1,500-2,500 (ab181658, Abcam Co. Ltd) was selected according to corresponding samples and primary antibodies.

To verify the specificity of primary antibodies, negative controls included fluorescent immunohistochemical staining and Western blot that was incubated in the absence of primary antibody or in a presence of nonimmune normal serum in the same dilution as the primary antibody, as well as antigen–antibody pre-absorption experiments with the native antigen at 4°C for 24 h, and other procedures are same in the same sample.

Statistical Analysis

The Image J software was used to count the hnRNP G positive cell number of immunofluorescence image and measure the gray value of Western blot image, and Photoshop was used to adjust the images. The total amount of hnRNP G positive cells in each distinct anatomic region of the spinal cord was counted at 200 magnifications in 10 sections, the hnRNP G positive cells sum of all 10 sections was divided by the section number, 5 mice per group were used, the averaged amount of hnRNP G positive cells was used for the quantitative analysis. All data were analyzed with the IBM SPSS Statistics 25, the normality and variance homogeneity was assessed, For unpaired samples, student's t-test was used if it obeys normal distribution and variance was homogeneous; Welch's t-test was used if the normal distribution was obeyed but variance was not uniform; Mann-Whitney U test was used if the normal distribution was not obeyed. $P < 0.05$ showed the statistical difference.

Results

Distribution of hnRNP G Positive Cells in Mouse Spinal Cord

It was found that the hnRNP G positive cells were widely distributed in the anterior horn, the central canal, the posterior horn and other parts of spinal cord, and there were significant differences in the distribution of hnRNP G positive cells in the different segments of spinal cord and in the different regions of the same segments. The distribution of hnRNP G positive cells in the different regions of same spinal cord in the cervical, thoracic and lumbar segments showed a pattern of posterior horn more than the central canal and its peripheral gray matter more than the anterior horn. The results showed that the number of hnRNP G positive cells distributed in the anterior horn of spinal cord at the pre-onset stage of TG group (Mice age of 60–70 days) was significantly higher than that of WT group ($P < 0.0001$, Fig. 1, the detailed data see Additional file 2: Table S1-3). The distribution of hnRNP G positive cells in the central canal and its surrounding gray matter of lumbar spinal cord in the TG mice of pre-onset group was also significantly higher than that of WT mice ($P < 0.0001$, Fig. 2, the detailed data see Additional file 3: Table S1-3). The distribution of hnRNP G positive cells in the posterior horn of thoracic spinal cord in the TG mice of the pre-onset group was significantly lower than that of WT mice ($P < 0.01$, Fig. 3, the detailed data see Additional file 4: Table S1-3). The number of hnRNP G positive cells in the spinal anterior horn of TG mice (90–100 days) in the onset group was significantly lower than that of WT mice ($P < 0.001$, Fig. 1, the

detailed data see Additional file 2: Table S1-3). The number of hnRNP G positive cells in the central canal and its surrounding gray matter of each spinal segment in the TG mice of onset group was significantly reduced compared with that in the WT mice ($P < 0.01$, Fig. 2, the detailed data see Additional file 3: Table S1-3). The number of hnRNP G positive cells in the posterior horn of spinal cervical and lumbar segments at the progressive group of TG mice (120–130 days) was significantly lower than that of WT mice ($P = 0.037$, Fig. 3, the detailed data see Additional file 4: Table S1-3). The number of hnRNP G positive cells in the spinal central canal and its surrounding gray matter of TG mice at the progressive group was also decreased compared with that of WT mice ($P < 0.01$, Fig. 2, the detailed data see Additional file 3: Table S1-3).

The Double Immunocytochemistry in Mouse Spinal Cord

In this study, both the hnRNP G antibody and the NeuN antibody of neuron biomarker were used to double label in the spinal different segments of TG mice in the onset group (Fig. 4). It was observed that there was the immunologic co-localization of hnRNP G positive cells and neurons in the different spinal segments at the onset stage. Moreover, the hnRNP G positive cells were mainly distributed in the central canal.

Western Blot of Mouse Spinal Cord

Western blot was used to analyze the expression of hnRNP G protein in the spinal cord at the different periods and stages of WT and TG mice (Fig. 5, the detailed data see Additional file 5: Table S1-3). The results showed that the hnRNP G protein of TG mice in the cervical segment was significantly higher than that of WT mice in the pre-onset group ($P < 0.01$), and the hnRNP G protein of TG mice was significantly lower than that of WT mice in the progression group ($P = 0.16$) (Fig. 5A, the detailed data see Additional file 5: Table S). The hnRNP G protein of TG mice in the thoracic segment was significantly higher than that of WT mice in the pre-onset group ($P = 0.01$), the hnRNP G protein of TG mice was significantly lower than that of WT mice in the onset group ($P = 0.03$), and the hnRNP G protein of TG mice was significantly higher than that of WT mice in the progression group ($P < 0.01$) (Fig. 5B, the detailed data see Additional file 5: Table S2). The protein of hnRNP G decreased significantly in the progression TG mice compared with WT mice ($P < 0.0001$) (Fig. 5C, the detailed data see Additional file 5: Table S3).

Detection of Silencing Efficiency of hnRNP G-siRNA Gene in PC12 Cells

The mRNA level of hnRNP G gene detected by the real-time fluorescent quantitative PCR was significantly lower than that of control groups including both blank and negative control groups (Fig. 6A, the detailed data see Additional file 6: Table S1). The quantitative analysis of hnRNP G protein in cells by Western blot showed that the content of hnRNP G protein in the hnRNP G gene silenced group was significantly lower than that in the control groups including both blank and negative control groups ($P < 0.001$) (Fig. 6B, C, the detailed data see Additional file 6: Table S2).

Effect of hnRNP G Gene Silenced on PC12 Cells

In this experiment, the CCK8 method was used to draw the growth curve of PC12 cells without any treatment (Fig. 7A), and it was observed that the cell survival rate of PC12 cells decreased after being interfered by the hnRNP G-siRNA ($P < 0.01$, Fig. 7B, the detailed data see Additional file 7: Table S1-3).

Expression of TDP-43/Bax/SOD1 in PC12 Cells after hnRNP G Gene Silenced

Western blot was used to detect the expression of TDP-43, SOD1 and Bax (Fig. 7C-F, the detailed data see Additional file 7D: Table S4-5, Additional file 7E: Table S6-7 and Additional file 7F: Table S8-9). It was found that the expression of SOD1 protein was not significantly changed in the PC12 cells after the interference of hnRNP G-siRNA, the expression of Bax protein involved in the apoptosis was significantly increased, and the expression of TDP-43 protein was also significantly increased ($P < 0.01$), which suggested that hnRNP G might be related to apoptosis and the abnormal accumulation of TDP-43 protein.

Discussion

Our study further studied the distribution and expression alteration of hnRNP G protein in the spinal cord of SOD1*G93A transgenic ALS model mice and the possible mechanism of hnRNP G in the pathogenesis of ALS on the basis of our previous study. The results showed that hnRNP G were widely distributed in the anterior horn, the central canal and it's around grey, the posterior horn and other regions of spinal cord, and there were significant differences in the distribution of hnRNP G in different segments of spinal cord and in the different regions of the same segment. The expression of hnRNP G protein in the spinal cord of TG mice significantly increased at the pre-onset stage, but the expression of hnRNP G protein in the spinal cord of TG mice decreased at the onset stage, and also decreased compared with the progression TG mice. It is suggested that the hnRNP G protein plays a certain effects in the pathogenesis of ALS model mice, which is that the decrease of hnRNP G protein expression may promote the development of ALS in mice. In order to further understand the effect of hnRNP G in neuronal damage or apoptosis, we conducted the hnRNP G-siRNA interference in the PC12 cells by silenced the partial hnRNP G gene. The experimental results showed that after reducing the expression of hnRNP G in PC12 cells, there was no significant change in the expression of SOD1, but the expression of TDP-43 and Bax significantly increased, and the cell survival rate significantly decreased. These results indicated that hnRNP G was very important for the normal expression of TDP-43 and Bax. Because the abnormal expression of TDP-43 can lead to the abnormal distribution and aggregation of hnRNP G, ultimately cause the damage and death of neurons and promote the occurrence and development of ALS [6, 12, 19, 22]. The hnRNP G protein might play a positive role in the occurrence and development of ALS by regulating the TDP-43 expression and controlling apoptosis, because the Bax protein accelerated the neuron apoptosis [42] significantly increased and the cell death significantly increase on the inhibition of

hnRNP G. Our result found that hnRNP G extensively distributed in neurons. Therefore, we suggested that hnRNP G was closely associated with the death of neurons, because the more cells damaged in ALS were neurons [1, 3].

Effect of hnRNP G on Neurons Damage and/or Apoptosis in ALS

It has been found that the expression of hnRNP G increased at the early stage of neuron damage, and gradually decreased to the normal level after reaching the peak value, and the immunofluorescence stain found that there was co-localization between hnRNP G and neuron and the activated caspase-3 [43]. The neuron apoptosis is an important part of neuron damage. Caspase-3 is an important regulator of neuronal apoptosis, which has been proved to play a key role in the pathological neuron death of nervous system. In the adult mice with the acute spinal cord injury, the expression of hnRNP G peaked on the first day after injury, and then returned to the basic level on the 14th day [44]. Moreover, the hnRNP G is also involved in the brain formation of zebrafish. If the hnRNP G gene is knocked down, zebrafish have characteristic brain morphological defects, such as the enlarged tectum and ventricles. In the zebrafish of knockdown hnRNP G gene, the abnormal development of nerve, muscle and spinal cord was also detected [45]. The role of hnRNP G in the brain development of zebrafish suggests that the human hnRNP G gene may be involved in the functional and behavioral defects caused nervous system diseases [46].

In this study, we found that the hnRNP G protein in the spinal cord of TG mice increased at the pre-onset stage, but significantly decreased at the onset stage, and increased at the progression stage relative to the onset stage, which was the same as the past reported results of increased expression and then decreased expression in neurons after the injury of spinal cord [44]. Our results showed that the expression of hnRNP G significantly increased at the pre-onset stage of TG mice due to the aggregation of SOD1 protein, which resulted in the neuron damage and/or apoptosis. As for the increase of hnRNP G in the spinal cord of progression TG mice, it might be due to the reason that hnRNP G, like other hnRNPs, could negatively regulate itself by closely binding with its own RNA in the nucleus [30].

The double labeling stain of immunofluorescence showed that biomarkers of neurons and astrocytes had doubly labeled with the hnRNP G immunoreactivity, while microglia had no double label with hnRNP G immunoreactivity. The expression of hnRNP G was mainly increased in neurons and astrocytes. In addition, the hnRNP G proliferating cellular nuclear antigen and the hnRNP G activated caspase-3 was detected in astrocytes and neurons respectively [44]. The expression of Bcl-2, Bax and the activated caspase-3 in mice with acute spinal cord injury is also changed, which will lead to secondary tissue injury, regeneration damage, and cell dysfunction [47, 48]. In our study, only doubly labeled hnRNP G and the neuron marker NeuN were found to have immune-co-localization. Therefore, the decrease of hnRNP G expression may be related to the death of spinal neurons in TG mice.

Effect of hnRNP G on TDP-43 in ALS

Although the role of TDP-43 in the RNA metabolism has been basically clear, there are still some problems to need be deeply comprehended, for example, does the change of RNA function play a role in diseases? Do hnRNPs related to ALS play a sole independent or synergistic role? Like many other hnRNPs, the functions of TDP-43 and hnRNP A1 may be the extremely dose sensitive, so they can be self-regulated by closely binding with their own RNA in the nucleus [30, 49]. When the cytoplasmic aggregates deplete TDP-43 in the nucleus, the level of TDP-43 is up-regulated due to the lack of self-regulation [50], which results in a vicious cycle of TDP-43 RNA expression increase and cytoplasmic aggregation. Deshaies et al. reported that the loss of TDP-43 function resulted in the hnRNP A1 protein increase in the cytoplasm, and the level of hnRNP A1 protein decreased in the ALS patient's neuronal nucleus [51]. The activation of RBPs compensation in the ALS patients with the TDP-43 gene mutation leads to the increase of splicing repressor expression in order to offset the loss of TDP-43 function. It was found that the overexpression of hnRNP u and hnRNP A1/A2 can inhibit the TDP-43-induced neuronal cell death in mice [52].

The expression of hnRNP G significantly increased at the pre-onset stage of TG mice, then decreased to the basic level at the progression stage of TG mice, which may be due to the fact that hnRNP G, like other hnRNP, can regulate itself negatively by closely binding with its own RNA in the nucleus, but this needs to be further proved. There was co-localization of hnRNP G positive cells and neurons in the different segments of mice spinal cord at the different stages, the decrease of hnRNP G expression might be related to the neuron death in the spinal cord of TG mice. After PC12 cells were interfered with by hnRNP G-siRNA, the proliferation activity of PC12 cells was lower than that of the control groups including both blank and negative control groups, and the expression of TDP-43 and Bax protein in PC12 cells significantly increased as well as the expression of hnRNP G significantly decreased, which might further lead to the abnormal aggregation of TDP-43 and the apoptosis of neurons in ALS.

In this study, we only observed the immune-co-localization of NeuN labeled neurons and hnRNP G, and did not doubly label other neural cells with the immunofluorescence stain. Relationships between hnRNP G protein and astrocytes, microglia, oligodendrocytes and undifferentiated neurons were not observed. If that, it would further clarify the target cells of hnRNP G protein. Moreover, this study did not up-regulate the expression of hnRNP G protein and did not observed the expression of related proteins in cells after the up-regulation, did not clarify the mechanism and effect of abnormal accumulation of TDP-43 caused by down-regulating hnRNP G in ALS.

Conclusions

Our results showed that the expression of TDP-43 and Bax were significantly increased after down-regulating the expression of hnRNP G in PC12 cells. It is suggested that the absence of hnRNP G might stimulate the increase of TDP-43 transcription, or destroy the automatic regulation of TDP-43 mRNA and slow down the degradation of TDP-43 protein. At the onset stage of TG mice, the content of hnRNP G protein significantly decreased, which might further aggravate the abnormal accumulation of TDP-43 and lead to the damage of neural cells.

Abbreviations

hnRNP G: Heterologous ribonucleoprotein G; ALS: Amyotrophic lateral sclerosis; PC 12: Pheochromocytoma 12 cells; CCK8: Cell counting kit 8; TDP43: TAR DNA-binding protein 43; Bax: BCL2-associated X protein; SOD1 Superoxide dismutase 1; FUS: Fused in sarcoma/translocated in liposarcoma; OPTN: Optineurin; Ubqln2: Ubiquilin 2; NEFH: Neurofilament, heavy polypeptide

Declarations

Acknowledgments

Not applicable

Ethics approval

Consent to participate

Animals were handled in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health [(NIH) publication no. 85-23, revised 1996]. All methods are reported in accordance with ARRIVE guidelines. All animal experiments and maintenance were approved by the Laboratory Animal Ethics Committee of Jiangxi Provincial People's Hospital, First Affiliated Hospital of Nanchang Medical College, Affiliated People's Hospital of Nanchang University (ACUC Number 2019-11-02).

Consent for publication

All authors have approved the manuscript.

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

All data generated or analyzed during this study are included in this article. All raw data used in this manuscript are available on reasonable request.

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Authors' contributions

FY, XW and RX designed the study and analyzed the data. FY, WC, YZ and SJ performed western blots, immunohistochemistry, cell culture experiment and wrote the manuscript. FY, WC, YZ and SJ performed the statistical analysis and animal dissection for isolating spinal cord. FY, WC and YZ generated the TG(SOD1*G93A)1Gur transgenic mice. Further, FY, WC and YZ did character isolation and validation of the transgenic mice model and wrote the manuscript. All authors read and approved the final manuscript.

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Figures

Figure 1

Figure 1

The distribution of hnRNP G in the anterior horn of spinal cord of mice at the different stages. a The representative images of hnRNP G positive cells distribution in the anterior horn of spinal different segments of mice at different stages. b The statistical analysis of hnRNP G positive cells number in the anterior horn of spinal different segments of mice at different stages. The distribution of hnRNP G positive cells in the spinal lumbar segment of TG mice at the pre-onset stage was significantly higher

canal and its surrounding gray matter of spinal different segments of mice at different stages. b The statistical analysis of hnRNP G positive cells number in the central canal and its surrounding gray matter of spinal different segments of mice at different stages. The distribution of hnRNP G positive cells in the whole spinal cord of TG mice at the onset stage was significantly less than that in the control group (**** $P < 0.0001$, $n = 5$ per group). The distribution of hnRNP G positive cells in the spinal lumbar segment of TG mice was significantly higher than that in the control group at the pre-onset stage (** $P < 0.01$, $n = 5$ per group), and significantly lower than that in the control group at the progressive stage (** $P < 0.01$, $n = 5$ per group). The arrow points to hnRNP G positive cells. There were 3 technical replicates performed.

Figure 3
a Posterior horn

The different segments of spinal cord, periods of WT mice and stages of TG(SOD1*G93A)Gur mice

Figure 3

The distribution of hnRNP G in the posterior horn of spinal cord of mice at the different stages. a The representative images of hnRNP G positive cells distribution in the posterior horn of spinal different segments of mice at different stages. b The statistical analysis of hnRNP G positive cells number in the posterior horn of spinal different segments of mice at different stages. The distribution of hnRNP G positive cells in the spinal cervical and lumbar segments of TG mice at the progressive stage was

significantly lower than that in the control group (*P<0.05, n=5 per group). The arrow points to hnRNP G positive cells. There were 3 technical replicates performed.

Figure 4

Figure 4

The double labeled immunofluorescent staining of hnRNP G and NeuN in the anterior horn, the central canal and its surrounding gray matter, and the posterior horn of different spinal cord segments of TG mice. The immunofluorescent double labeled representative images of hnRNP G and NeuN in the cervical

segment a, the central canal and its surrounding gray matter (b) and the posterior horn (c) of spinal cord in TG mice. The neurons were doubly labeled by NeuN and hnRNP G, showed immunocolocalization.

Figure 5

Figure 5

The semi-quantitative analysis of hnRNP G protein in different spinal cord segments of mice. The Western blot of hnRNP G protein in the different segments of spinal cord of mice at different stages. a The expression of hnRNP G protein in the cervical segment of spinal cord in TG mice was significantly

higher than that in the control group at the pre-onset stage (**P<0.01) and significantly lower at the progression stage (*P<0.05, n=3 per group). b The expression of hnRNP G protein in the thoracic segment of spinal cord in TG mice significantly increased at the pre-onset stage (*P<0.05), significantly decreased at the onset stage (*P<0.05), and significantly increased at the progression stage compared with the control group (***P<0.001, n=3 per group). c The expression of hnRNP G protein in the lumbar segment of spinal cord of TG mice was significantly lower than that of control group at both onset (**P<0.01) and progression stage (***P<0.001, n=3 per group).

Figure 6

Figure 6

a The QRT PCR detection of hnRNP G mRNA expression in PC12 cells after the interference of hnRNP g-siRNA compared with control group. b The image of Western blot. c The semi-quantitative analysis of hnRNP G protein after hnRNP G gene silenced in PC12 cells by Western blot. After the interference of hnRNP G-siRNA in PC12 cells, the results of QRT PCR showed that the silenced efficiency of three siRNA sequences was significant (***P<0.001). The expression of hnRNP G protein in the transfection group

was significantly lower than that in the blank and negative control groups (**P<0.001). There were at least 3 technical replicates performed.

Figure 7

Figure 7

The cell growth curve before and after hnRNP G gene silenced, the Western blot semi-quantitative analysis of TDP-43, Bax, SOD1 expression in PC12 cells after hnRNP G-siRNA interfered. a The normal growth curve of PC12 cells. b The growth curve of PC12 cells after the interference of hnRNP G-siRNA. It

showed that the survival rate of PC12 cells after the interference of hnRNP G-siRNA was lower than that of normal and negative control cells (** $P < 0.01$). c After the gene was silenced, the expression of related proteins in PC12 cells was detected by Western blot. d TDP43 protein significantly increased compared with control group (** $P < 0.01$), e Bax protein also significantly increased compared with control group (* $P < 0.05$). f SOD1 protein did not significantly change compared with control group. There were at least 3 technical replicates performed.

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