Regular Article

LncRNA Snhg5 Attenuates Status Epilepticus Induced Inflammation through Regulating NF-κB Signaling Pathway

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Status epilepticus (SE) induced inflammation plays an important role in the pathogenesis of SE. Long non-coding RNA small nucleolar RNA host gene 5 (lncRNA Snhg5) has been reported in various inflammatory diseases. However, the mechanism of Snhg5 regulated inflammation in SE remains unclear. Therefore, this study aimed to clarify the role and mechanism of Snhg5 in SE-induced inflammation in vitro and vivo. In vitro, lipopolysaccharide (LPS)-induced inflammation in microglia was used to mimic the inflammation after SE. In vivo, SE model was induced by lithium chloride and pilocarpine. The level of Snhg5, p65, p-p65, p-inhibitor of kappaB (IkBα, IkBα and inflammatory factors (tumor necrosis factor (TNF)-α, interleukin (IL)-1β) were measured via quantitative real-time PCR or Western blot. The Nissl stain and immunohistochemical stain were performed to observe hippocampal damage and microglia proliferation. The results showed Snhg5 was up-regulated in the rat and microglia. Knockdown of Snhg5 inhibited LPS-induced inflammation and relative expression of p-65/p65, p-IκB/IκBα. Moreover, down-regulation of Snhg5 attenuated SE-induced inflammation and reduced the number of microglia in hippocampus. These findings indicated that Snhg5 modulates the inflammation via nuclear factor-kappaB (NF-κB) signaling pathway in SE rats.

Key words status epilepticus; nuclear factor-kappaB (NF-κB) signaling pathway; small nucleolar RNA host gene 5; inflammation; microglia

INTRODUCTION

Status epilepticus (SE) is a common neurological emergency with high morbidity and mortality. It is characterized by continual seizure activity that can vary widely in the intensity of convulsions.\(^1,\(^2\)\) Sustained seizures can cause extensive brain damage and even death.\(^3\) Various neurological diseases, which include brain trauma, central nervous system infection, brain tumors, stroke, and irregular use of anti-epileptic drug (AED) can cause SE.\(^4,\(^5\)\) Over the past decade, many clinical and animal studies have focused on understanding the pathophysiology, treatment, and long-term complications of SE. A large number of studies have confirmed that SE causes a rapid and intense inflammatory cascade in the brain, which activated microglia and released of inflammatory factors.\(^6,\(^7\)\) Inflammatory response after SE participates in the brain damage, especially in the hippocampus is vulnerable to damage.\(^5,\(^9\)\) A large number of studies have confirmed that the nuclear factor-kappaB (NF-κB) signaling pathway, as the core junction of multiple inflammatory signaling pathway, plays a vital role in SE-induced inflammation.\(^10,\(^11\)\) Currently studies have shown that inhibited the NF-κB signaling pathway can reduce the inflammatory response in the brain of SE rats and attenuate corresponding damage.\(^12,\(^13\)\) However, the exogenous inhibitors of NF-κB signaling pathway were limited clinical application for their toxicity and non-specificity.\(^14,\(^15\)\) Therefore, seeking specific molecules that endogenously regulated NF-κB signaling pathway may help solve this problem.

Long non-coding RNA (lncRNA) is a non-coding RNA with length of more than 200 nucleotides.\(^16\) It is involved in many important regulatory processes in the cell, mainly from epigenetics, transcription and post-transcription three different levels to achieve gene regulation.\(^17\) Many studies have shown that the expression of lncRNA small nucleolar RNA host gene 5 (Snhg5) was decreased in various inflammatory diseases such as ankylosing spondylitis, osteoarthritis and sepsis-related myocarditis.\(^18–21\) However, there is no findings reveal the role of lncRNA Snhg5 acting on inflammatory response in SE. Therefore, in this study, we aimed to confirm whether lncRNA Snhg5 regulated the SE-induced inflammation through NF-κB signaling pathway in vitro and vivo.

In this study, we hypothesized that lncRNA might involve in regulating the inflammation by NF-κB signaling pathway in status epilepticus. Considering this, a status epilepticus rat model and lipopolysaccharide (LPS)-induced microglia cell were separately constructed to explore the mechanism of lncRNA Snhg5. According to these investigations, we hope to provide some new insights and theory basic information for understanding and intervention of status epilepticus.

MATERIALS AND METHODS

Cell Culture Rat microglia was obtained from ScienCell (U.S.A.) and were cultured in Dulbecco’s modified Eagle’s medium (DMEM), a high glucose medium with 10% fetal bovine serum (FBS). The rat microglia were supported in a humid mixed atmosphere of 5% CO₂ and 95% air at 37°C. When the cell coverage in the culture dish reached 65%, it was treated with LPS (10 µg/mL). RNA and protein were extracted 48h after treatment with the reagent.
**Cell Transfection** To knockdown Snhg5, small interfering RNA (siRNA)-Snhg5 (si-Snhg5) were designed and synthesized by GenePharma (Shanghai, China). A scramble siRNA was used as negative control (si-NC). The related sequences were as follows: sense 5’-GCUUGGCUUCAUUCU-3’; antisense 5’-AAAGAUCAGAUAAGAACG-3’; si-NC: sense 5’-UUCCCGAAGCGUACGGC-3’; antisense 5’-ACGUGA CAGGUU-3’. Rat microglia were transfected with siRNAs using LipofectamineTM 2000 (Invitrogen) according to the manufacturer’s instructions. Forty-eight hours after transfection, cells were harvested for real-time (RT)-PCR analysis to examine the knockdown efficiency.

**Stereotactic Intrahippocampal Injection** Male Sprague-Dawley (SD) rats were randomly divided into three groups: control group, SH NC group and snhg5 group; Adeno associated virus 9 (AAV9) was injected into hippocampus of empty vector group and knockdown group. Aav9-sh-nc and aav9-sh-snhg5 viruses were separately packaged and thawed on ice. Rats were anesthetized by intraperitoneal injection of 10% chloral hydrate. Cut off the hair on the top of the rat’s head, drill the hole, carefully place the micro syringe, and was slowly pulled out, the wound was disinfected and sutured, and tumor necrosis factor (TNF)-α antibody) overnight at 4°C. The membrane was washed with TBST and promote with horseradish peroxidase (HRP)-conjugated Affinipure goat anti-rabbit immunoglobulin G (IgG) (H + L) for 1h at room temperature. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was the internal control for quantitative analysis of the relative express-sion levels of the proteins.

**RT-PCR** Rat hippocampus tissue or microglia were treated with Trizol; the tissues were separated thoroughly with a homogenizer, and the mRNA was extracted using chloroform and isomyl alcohol. The RNA sediment was dissolved in diethylpyrocarbonate (DEPC) water (TaKaRa, Shanghai, China), quantified using a spectrophotometer, and reverse transcribed into cDNA by using Prime Script reagent kits (TaKaRa). PCR was performed using SYBR Green PCR kits (TaKaRa) by a Step One Real-Time PCR System. Relative RNA Expression was normalized to GAPDH and calculated. The primers used in the study are listed as follows: Snhg5 (5’-GCTGCAAATTCCACCAAAGAGAAG-3’) and (5’-GCTGCUUUCATTCTATA-3’) GAPDH (5’-TTCGCCATGATGCAT-3’) and (5’-TAGGAGTCTCTCTATAC-3’), IL-β (5’-ATCTCAACAGCAGCATCTGCACAG-3’) and (5’-CACACTAGCAGGTCTACCC-3’), CD11b (5’-GCACTGATGCTAGCACT-3’) and (5’-CCCCGTCCATTTGAGATG-3’).

**Nissl Stain** Sacrificed the rats in each group according to the Chinese Medical Sciences Animal Experiment Guide. After the rats recovered for a week, we removed the rat brain tissues on ice. The fresh rat brain tissue was fixed in 4% formalin solution and dehydrate. Sliced into 5μm thick sections and put into Cresyl violet stain, place the dyeing tank in incubator (56°C) for 1h, and heat with alcohol lamp (10min). Put into Nissl Differentiation for 1 to 3 min and observed under a microscope until the background was nearly colorless. Dehydrated sections rapidly with Anhydrous ethanol and sealed with a neutral gum. Observe pyramidal neurons in hippocampus under microscope within a fixed area (400×) of the CA1 and CA3, as previous studies described. The images were processed by ImageJ software (U.S.A.), which calculated the number of neurons per 0.1 m² in CA1 and CA3 regions of stained hippocampus. The average number of positive cells was calculated: 3 rats in each group and one slice in each region.

**Immunohistochemistry** After the rats recovered for a week, we extracted the brain tissue on ice. Follow the immunohistochemical staining kit instructions. The fresh brain tissue sections (5μm) were heated at 60°C. Then dewaxed and permeabлизed the sections. Washing 3 times with PBS, goat serum was added for 20min to block non-specific antigen binding. Rabbit anti-Iba1 primary antibody was added and incubated overnight at 4°C and the sections were incubated with Goat Anti-Rabbit IgG (H + L) for 60min. After hematoxylin staining, rinse with 0.1% HCl-ethanol, then dehydrate in graded ethanol and fix in resin. Magnify 400 times with a microscope, select CA1 and CA3 region of hippocampus.
to observe the number of microglia, as previous studies described. Photos are processed by ImageJ software (U.S.A.), which count the number of Iba1-stained microglia in per 0.1 m² of CA1 and CA3 region of hippocampus. The average value was calculated for the number of positive cells, 3 rats each group, one slice per rat.

**Statistical Analysis** All data were analyzed using GraphPad Prism 8. The results are expressed as mean ± standard deviation (S.D.). Comparisons between groups were performed using one-way ANOVA and t-test. Pearson correlation analysis was used for two-variable correlation analysis. The p < 0.05 was considered to be statistically significant. All experiments were repeated at least three times.

**RESULTS**

**Knockdown of lncRNA Snhg5 Attenuated LPS-Induced Inflammation through Regulating the NF-κB Signaling Pathway in Microglia** *In vitro* microglia culture is powerful tools to study specific molecular pathways involved in inflammation. Thus, we used LPS-induced inflammation in microglia partly mimic the SE-induced neuroinflammation to explore the effect of Snhg5 on inflammation. As shown in Fig. 1a, it was demonstrated that Snhg5 expression were greatly increased in the LPS dose of 10 µg/mL. We thus chose a concentration of 10 µg/mL treated the microglia. And in the Fig. 1b, the Snhg5 expression was the most markedly increased at 48 h after LPS-treated. On the basis of this result, we extracted the protein and RNA 48 h after LPS-stimulation. In Fig. 1c, all three siRNAs could knock down the snhg5 in varying degrees, among which the si-1 was the most obvious. The Snhg5 were successfully knocked down in LPS-induced microglia (differential fold >1.5) in Fig. 1d. As seen in Fig. 1e, the M1 microglia markers (CD86 and inducible nitric oxide synthase (iNOS)) were decrease in LPS + si-Snhg5 group compared to LPS + si-NC group (#p < 0.05).
By contrast, the M2 microglia markers (CD206 and Arg1) increased in LPS + si-Snhg5 group in Fig. 1f (\#p < 0.05 vs. LPS + si-NC). Moreover, the LPS treated increased the protein level of inflammatory factors TNF-\(\alpha\) and IL-1\(\beta\) (*p < 0.05 vs. control). And the LPS + si-Snhg5 group compared with the LPS + si-NC group, the relative expression of TNF-\(\alpha\) and IL-1\(\beta\) were decreased (\#p < 0.05) in Fig. 1g. The expression of p-65 and p-I\(\kappa\)B\(\alpha\) were increased in LPS-treated group in Fig. 1h-i. And p-65 and p-I\(\kappa\)B\(\alpha\) were decreased in LPS + si-Snhg5 group compared to LPS + si-NC group (\#p < 0.05). The results in Fig. 1 suggested that knockdown of Snhg5 inhibited the M1/M2 polarization of microglia and attenuated LPS-induced inflammation by regulating NF-\(\kappa\)B signaling pathway.

**High Expression of Snhg5 Was Consistent with the Inflammation in SE Rats**

As shown in Fig. 2a that the expression of Snhg5 increased gradually 6h after SE compared to the normal group (*p < 0.05), and reached the highest level on 1w after SE (*p < 0.05 vs. Normal). The expression of NF-\(\kappa\)B signaling pathway related proteins (p-p65, p-p65, p-I\(\kappa\)B\(\alpha\), I\(\kappa\)B\(\alpha\), IL-1\(\beta\) and TNF-\(\alpha\)) were shown in Fig. 2b. From Fig. 2c the statistical analysis revealed the relative expression of p-p65 (p-65/p65) was increased at 1w after SE (*p < 0.05 vs. Normal). The result of Fig. 2e showed that the relative expression of p-I\(\kappa\)B\(\alpha\) (p-I\(\kappa\)B\(\alpha\)/I\(\kappa\)B\(\alpha\)) was increased mostly at 24h after SE (*p < 0.05 vs. Normal). Figures 2d and f showed that the expression of inflammatory factors (IL-1\(\beta\) and TNF-\(\alpha\)) were both increased obviously at 48h after SE in hippocampus tissues (*p < 0.05 vs. Normal). A positively correlation was
found between Snhg5 expression and p-p65/p65 in Fig. 2g 
(r = 0.6080, p < 0.05), which indicated Snhg5 expression is 
related to NF-κB signaling pathway. The results from Fig. 
2h suggested Snhg5 expression was also positively correlated 
with inflammatory factors TNF-α (r = 0.5014, p < 0.05). These 
results demonstrated that high expression of Snhg5 in SE rats 
may be related to SE-induced inflammation.

**Knockdown of Snhg5 Alleviated Hippocampal Neuron Injury in SE Rats** To further verify the results in vitro, we injected adeno-associated virus 9 (AAV9) into the hippocampus of rats to explore the effect of lncRNA Snhg5 on inflammation in vivo. As shown in Figs. 3a and b, compared with the normal group the number neurons in CA1 area of SE group were decreased (*p < 0.05). And the number of neurons in CA3 area was significantly reduced (*p < 0.05 vs. normal). Furthermore, in SE group the arrangement of neurons in CA3 was sparser than the normal group. And in Snhg5 knockdown group (SE + sh-snhg5) the number of hippocampal neurons were increased compared to the SE + sh-NC group (*p < 0.05). In addition, compared with SE + sh-NC group, the number of CA1 cells in SE group decreased slightly. These results indicated that the hippocampal neurons of SE group were damaged and Knockdown of lncRNA Snhg5 decreased the damage of CA3 hippocampal neurons.

**Knockdown of Snhg5 Inhibited the Proliferation of Hippocampal Microglia in SE Rats** Iba1 is a specific marker of microglia, thus in present study we used Iba1 to label microglia and investigated the effect of lncRNA Snhg5 on microglia proliferation. As shown in Figs. 4a and b, compared with the normal group, the number of microglia in CA1 and CA3 area of hippocampus were increased in SE group (*p < 0.05). In the knockdown group (SE + sh-snhg5), the number of microglia in both CA1 and CA3 were significantly decreased (*p < 0.05 vs. SE + sh-NC). The results showed that the proliferation of microglia in hippocampus of rats was not obvious one week after SE. Unexpectedly, the number of microglia in SE + sh-NC group were increased significantly both in CA1 and CA3 area of hippocampus (*p < 0.05 vs. SE). These results showed that the proliferation of microglia in hippocampus of SE rats was obvious. The increased number of microglia in hippocampus induced by exogenous AAV injection was significantly higher than SE. Knockdown of lncRNA Snhg5 could inhibit the proliferation of microglia in hippocampus of rats.

**Knockdown of Snhg5 Reduced SE-Induced Inflammation through Regulating the NF-κB Signaling Pathway in Rats** As shown in Fig. 5a, the expression of Snhg5 was successfully knocked down in SE + sh-snhg5 group (*p < 0.05 vs. SE + sh-NC) by AAV9. And in Figs. 5b and c, the expression of p-p65 and p-IκBα in SE group were significantly higher than those in control group (*p < 0.05). Compared with SE + sh-NC group, the expression of p-p65 and p-IκBα in SE + sh-Snhg5 group were decreased (p < 0.05). These results suggest that down-regulation of lncRNA Snhg5 could inhibited the Phosphorylation of p65 and IκBα in hippocampus of SE rats, which regulated the NF-κB signaling pathway. In
addition, compared with the normal group, the expression of
TNF-α and IL-1β in SE group was significantly higher than
that in the normal group (*p < 0.05). The protein expression
of inflammatory factors (TNF-α and IL-1β) were decreased in
SE + sh-Snhg5 group (#p < 0.05 vs. SE + sh-NC). And these
results were consistent with the results of cell verification in
vitro. These results suggested that lncRNA Snhg5 knockdown
inhibited inflammation by regulating NF-κB signaling path-
way in SE rats.

DISCUSSION

In the past few decades, a strong relationship between in-
flammation and SE has been reported both in animal models
and patients with SE.25,26 Increasing evidence has noted that
the inflammation during SE plays a decisive role in persist-
ent seizure and the long-term sequelae.13,27 And the activ-
eted microglia in the brain were considered to be the main
cells involved in the inflammatory response during SE.28,29
Therefore, in the current study we used lithium chloride and
pilocarpine induced SE rat model and LPS-treated microglia
to study the inflammation in vivo and vitro. The evidence
of inflammation was detected in the hippocampus of SE rats,
which consistent with those of other studies.30,31 And there
existed apparently inflammation in LPS-treated microglia to
partly mimic the inflammation during SE in vitro. Based on
these findings, early anti-inflammatory treatment of SE may
represent a promising strategy to improve the brain damage
cauised by seizure, as previous research supported.32,33 How-
ever, several anti-inflammatory drugs were failed to termi-
nate the recurrent seizure for non-specificity. Therefore, it is ne-
necessary to find drugs that specifically regulate inflammation in
SE.

There were several inflammatory signaling pathway ac-
tivated following prolonged seizure, which mainly include
NF-κB signaling pathway, mammalian target of rapamycin
(mTOR) signaling pathway and mitogen-activated protein
kinase (MAPK) signaling pathway.34,35 Many studies have
confirmed that the NF-κB signaling pathway plays a central
role in inflammation during SE.36,37 Hence, we detected the
NF-κB signaling pathway related protein family (p65, p-p65
and p-IκBα) both in SE rats and LPS-treated microglia. We
found that the level of p-p65/p65, p-IκBα/IκBα, IL-1β and
TNF-α were increased in SE rats and LPS-treated microglia.
Our findings further support the idea of the inflammation dur-
ing SE was mediated through the NF-κB signaling pathway
as prior studies reported.34,38 In addition, results in vitro con-
firm that LPS stimulated can activate the NF-κB signaling
pathway, release the p-p65 enter the nucleus and combine with
proinflammatory genes to initiate transcription of target genes,
as previous findings.39,40

LncRNA as an efficiently endogenous molecule can regulate
the inflammation through NF-κB signaling pathway in various
diseased.41 The latest research found that lncRNA Carlr
induce the inflammation in macrophages through inhibiting
the NF-κB signaling pathway in celiac patients.42 Similarly,
another study showed that lncRNA NKILA promoted the
inflammation in breast cancer through the NF-κB signaling
pathway.43,44 Additionally, the expression of lncRNA Snhg5
has been reported to change in many inflammatory diseases.45
Our study found that the expression of Snhg5 was increased
in SE rats and LPS-treated microglia. Expectedly, the level of
Snhg5 was up-regulated in SE rats and positively correlated
with the inflammatory factor TNF-α. Subsequently, Snhg5
knockdown was performed both in vitro and vivo. Snhg5
knockdown caused a reduction in the levels of p-p65 and
p-IκBα. Correspondingly, the level of inflammatory factors
(TNF-α and IL-1β) were reduced in LPS-treated microglia
and rat hippocampus. Further, Snhg5 knockdown was sufficient
to inhibit the LPS-induced inflammation, which can be explained
by the M1 microglia decreased and M2 microglia increased.
These findings indicated Snhg5 may regulate the inflammation
through NF-κB signaling pathway. Therefore, as an endo-
genous molecule with pro-inflammatory effect, lncRNA Snhg5
may become a new treatment for inflammation in SE.

There are, however, some deficiencies in the present study.
We did not observe a marked change of the microglia num-
ber in 24–48h of LPS-treated. We considered this based on
the following reasons. In vivo, the Iba1 labeled hippocampus
slices were taken after one week of SE, which was different
from the time in vitro. Besides, the chemotaxis of microglia
to sites of tissue damage is one of the reasons for the obvious
changes of microglia in SE rat hippocampus.46 Furthermore,
the precise mechanisms between Snhg5 and NF-κB signal-
ing pathway still unclear. Such considerations warrant further
studies. Therefore, in the future experiments, we should set to
verify and refine these problems.

CONCLUSION

In conclusion, our study revealed that lncRNA Snhg5 modulated inflammation in SE rats via regulating NF-κB signaling pathway. Our findings indicated that anti-inflammatory effect of Snhg5 might provide a promising treatment for SE.

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Author Contributions MW and YC conceived and designed the study. YX contributed to data analysis. MW performed the experiments and drafted the manuscript. YC and YS reviewed and edited the manuscript. All authors read and approved the final manuscript.

Conflict of Interest The authors declare no conflict of interest.

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