RETRACTED ARTICLE: Overexpression of IncRNA ANRIL aggravated hydrogen peroxide-disposed injury in PC-12 cells via inhibiting miR-499a/PDCD4 axis-mediated PI3K/Akt/mTOR/p70S6K pathway

Zhiliang Guo, Lanlan Li, Yu Gao, Xiaoyun Zhang and Min Cheng

ABSTRACT
Spinal cord injury (SCI) is a grievous neurolgy-related disorder that causes many devastating symptoms. This research planned to dig the function and latent mechanisms of long noncoding RNA (lncRNA) ANRIL on hydrogen peroxide (H2O2)-disposed injury in PC-12 cells. The PC-12 cells were disposed with H2O2 for 24 h to construct the SCI model. H2O2-disposed PC-12 cells was assessed by detecting cell viability, migration, invasion, apoptosis and autophagy. The level of ANRIL in H2O2-disposed PC-12 cells was analysed, afterwards, the impacts of ANRIL silencing on H2O2-disposed PC-12 cell injury was determined. The regulatory association between ANRIL and miR-499a, between miR-499a and PDCD4, as well as PDCD4 and PI3K/Akt/mTOR/p70S6K signals were investigated. H2O2 produced PC-12 cell injury and promoted the level of ANRIL. Silencing of ANRIL inhibited H2O2-disposed PC-12 cell injury through promoting cell viability, migration, invasion and inhibiting apoptosis and autophagy. Moreover, miR-499a was upregulated after silencing of ANRIL, and inhibition of miR-499a reversed the effects of silencing of ANRIL on H2O2-disposed PC-12 cell injury. Also, PDCD4 was a target of miR-499a. Furthermore, ANRIL silencing alleviated the H2O2-disposed injury in PC-12 cells possible by activating PI3K/Akt/mTOR/p70S6K signals, which was mediated by miR-499a/PDCD4 axis. Our results indicate that high level of ANRIL may sharpen the degree of SCI via targeting miR-499a/PDCD4 axis to regulate the briskness of PI3K/Akt/mTOR/p70S6K signals.

Introduction
Spinal cord injury (SCI) is a fatal neurological disorder that impacts significant financial burden on health care systems [1,2]. It can lead to severe motor, sensory and autonomic dysfunction [3]. Moreover, traumatic SCI can cause several devastating symptoms, including chronic pain and paralysis [4,5]. Despite great efforts made to improve the functional outcome, current effective treatment for SCI is limited [6]. Therefore, elucidating the key molecular mechanisms mediating SCI will facilitate to improve the functional outcomes of patients with this injury.

Long noncoding RNAs (lncRNAs) are a class of transcripts longer than 200 nucleotides and lack of protein-coding capacity [7]. Increasing evidence has pointed out that lncRNAs exhibit extensive functions in biological processes, such as gene expression regulation [8] and epigenetic control [9]. Moreover, lncRNA has gained more attention because their aberrant expression is implicated in several neurological disorders [10–12]. In recent years, several lncRNAs are pointed out to be crucial in the pathophysiology of SCI, such as lncSCIR1 [13], lncRNA H19 [14] and XIST [15]. However, there is limited report about the biological roles of lncRNAs in SCI. Antisense noncoding RNA in the INK4 locus (ANRIL) is a newly discovered IncRNA existing in the chromosome 9p21 region [16]. It is reported that ANRIL is involved in the development of multiple disease, such as cancers [17,18], coronary artery disease [19] and diabetic retinopathy [20]. However, the roles and possible mechanism of ANRIL in SCI has not been clarified.

Oxidative stress-mediated cellular injury is a major cause of neurodegenerative diseases, and PC-12 cells have been applied for model construction in analysing the molecular mechanism of SCI in vitro [21–23]. Moreover, SCI is reported as a fearful neurology-related disease and the latent therapeutic strategies for this injury have been grabbed in PC-12 cells [24]. Therefore, we chose hydrogen peroxide (H2O2) to produce injury in PC-12 cells, to imitate the process of SCI. H2O2-disposed injury in PC-12 cells was assessed by detecting cell viability, migration, invasion, apoptosis and autophagy. The abnormal level of ANRIL in H2O2-disposed PC-12 cells was analysed, then the impacts of ANRIL silencing on H2O2-disposed PC-12 cell injury was determined. To further...
elucidate the downstream mechanism of ANRIL on H2O2-disposed PC-12 cell injury, the regulatory associations between ANRIL and miR-499a, between miR-499a and PDCD4, as well as PDCD4 and PI3K/Akt/mTOR/p70S6K signals were investigated. Our findings will inform the future direction of treatments for patients with SCI.

Materials and methods

Cell culture, disposes and transfection

The PC-12 cells, obtained from ATCC (Manassas, VA) were seeded onto flasks at a density of 1×10⁴ cells/mL in DMEM medium mixed with 10% (v/v) FBS, 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco, Carlsbad, CA). They were maintained at a 37°C, 5% CO₂ humidified incubator.

For cell disposes, PC-12 cells were seeded in 96-well plates (Sangon Biotech, Shanghai, China) at a final density of 5×10³ cells/well for 24 h. To construct an oxidative stress model to stimulate SCI, PC-12 cells were then cultured in fresh medium containing 200 μM of H₂O₂ for the other 24 h. Cells in control group were disposed with the same fresh medium without H₂O₂ [25].

For cell transfection, short-hairpin RNAs against ANRIL (sh-ANRIL#1 (GGUCAUCUGCUAUCAUU) or sh-ANRIL#2 (GCCCAUUAUGCUGGGUA)) and small interfering RNAs against PDCD4 (si-PDCD4) were inserted into the U6/GFP/Neo plasmid (Sangon Biotech, Shanghai, China) to silence the lev-

els of ANRIL and PDCD4, respectively. MiR-499a mimics, miR-499a inhibitor and their respective NC were obtained from Sangon Biotech (Shanghai, China). PC-12 cells were then transfected with sh-ANRIL#1, sh-ANRIL#2, si-PDCD4, miR-499a mimics, miR-499a inhibitor and/or their respective negative control (NC) using lipofectamine 3000 reagent (Sangon Biotech, Shanghai, China). According to transfection efficiency, cells were harvested after 72 h of incubation. The silenced sequence for ANRIL#2 is sense 5'-TGCTTCTA TCCGCAATCAGG-3' and antisense 3'-GGGCCCTAGTTGCA CATACC-5', the silenced sequence for PDCD4 is sense 5'- GAAAGCGUAAGGAUAGGU-3' and antisense 3'- CATACC-5'.

Cell proliferation assay

PC-12 cells at a final density of 1×10⁵ cells/well were incubated in triplicate in 60-mm dishes. After plenty of time incubation, PC-12 cells were washed by PBS buffer. The live cell numbers were counted by trypan blue exclusion.

Migration and invasion test

We chose a modified two-chamber migration to evaluate cell migration assay. In brief, PC-12 cells were suspended in 200 μL of serum-free fresh medium. After that, cells were then plated on the upper chamber of 24-well Transwell with an aperture of 8 μm (BD Biosciences, San Jose, CA). The under chamber was padded with 600 μL of complete medium. After incubation at 37°C, traversed cells on the under chamber were fixed with methanol, stained with crys-
tal violet and then counted microscopically. The invaded behaviour of PC-12 cells was detected using a similar proto-
col as cell migration except that the Transwell chamber was pre-coated with 20 μg Matrigel (Takara Biotech, Kusatsu, Japan). The data are expressed as the average number of cells attached to the under chamber from five randomly chosen fields.

Apoptosis test

We chose flow cytometry to assess cell apoptosis. In brief, cells were fixed in 70% ethanol after they were washed by PBS. The fixed cells were then stained with Annexin V-FITC/PI apoptosis detection kit (Beijing Biosea Biotechnology, Beijing, China). After incubation for 1 h at room temperature darkly, we chose FACs can (Beckman Coulter, Fullerton, CA) for observing the apoptotic cells, and then chose FlowJo soft-
careware for calculation.

Luciferase reporter test

We amplified the coding sequence of PDCD4 containing the predicted binding site of miR-499a, then we inserted it into a pmirGLO Dual-luciferase miRNA Target Expression Vector (Promega, Madison, WI) to establish the vectors of PDCD4-wild-type (PDCD4-wt). The PDCD4-mutated-type (PDCD4-mut) reporter vector carrying the mutated binding site of miR-

499a in the PDCD4 was also constructed as NC. These reporter vectors and miR-499a mimics were also transfect-
ed into HEK 293T cells, and the luciferase activities of them were analysed by Dual-Luciferase Reporter Test System (Promega, Madison, WI).

Quantitative PCR

We isolated the total RNA from PC-12 cells by Trizol reagent (TaKaRa Biotech, Kusatsu, Japan). The One Step SYBR® PrimeScript®PLUS RT-RNA PCR Kit (TaKaRa Biotech, Kusatsu, Japan) and RNA PCR Kit (AMV) Ver.3.0 (TaKaRa Biotech, Kusatsu, Japan) were respectively used for the real-time qPCR analysis for detection of the expression lev-
els of ANRIL and PDCD4. After that, Taqman MicroRNA Reverse Transcription Kit was chosen for determining the levels of miR-499a in PC-12 cells (TaKaRa Biotech, Kusatsu, Japan). Fold changes of gene expression levels were then evaluated by $2^{- ΔΔCt}$ method as previous described [26]. GAPDH was chosen as internal control for normalizing the levels of them.

Western blot assay

We isolated the total protein from cells by RIPA lysis buffer (Sangon Biotech, Shanghai, China). Purity and concentration of the isolate protein were determined using the BCA™ Protein Assay Kit (Pierce, Appleton, WI). Bio-Rad Bis-Tris Gel system was applied for separating the protein samples after
we isolated the total protein from cells. The polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA) were thereby cultured with primary antibodies at 4°C overnight. Afterwards, the membranes were mixed with secondary antibody symboled by horseradish peroxidase for 1 h at room temperature. After washing three times, we chose Bio-Rad ChemiDoc™ XRS system to assess the membranes taking along blots and antibodies, after that, 200 μL Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA) was chosen for mixed with the membranes. We then chose Image Lab™ Software (Bio-Rad, Shanghai, China) to evaluate the protein signals under membranes. Notably, primary antibodies used in this study were totally obtained from Abcam (Cambridge, UK), and prepared in 5% blocking buffer at a dilution of 1:1000 before use.

**Statistical analysis**

We carried out all experiments independently with three times repeats. The obtained data are displayed as the mean ± standard deviation (SD). For comparison of difference between groups, the p values were calculated using a one-way ANOVA in GraphPad 6.0 statistical software (GraphPad, San Diego, CA). Statistically significance was presented when p < .05.

**Results**

**H$_2$O$_2$ induces injury in PC-12 cells**

The impacts of H$_2$O$_2$ on PC-12 cell injury were first investigated. Relative to control, H$_2$O$_2$ treatment markedly decreased PC-12 cell viability (p < .05, Figure 1(A)), migration (p < .01, Figure 1(B)) and invasion (p < .01, Figure 1(C)) in PC-12 cells. Moreover, H$_2$O$_2$ treatment resulted in distinct increase on the apoptotic cells (p < .01, Figure 1(D)). Consistent changes in the expressions of apoptotic proteins were also observed that the expression levels of Bax/Bcl-2, cleaved/pro-caspase-3 and cleaved/pro-caspase-9 were markedly increased after H$_2$O$_2$ exposes relative to control (Figure 1(D)). Furthermore, H$_2$O$_2$ treatment resulted in remarkable increases in the expression levels of LC3-II/I and Beclin-1 and obvious decrease in P62 expression (p < .01, Figure 1(E)), indicating that H$_2$O$_2$ treatment increased P12 cell autophagy.

**Silencing of ANRIL alleviates H$_2$O$_2$-disposed injury in PC-12 cells**

Notably, H$_2$O$_2$ treatment markedly promoted ANRIL expression in PC-12 cells relative to control (p < .01, Figure 2(A)), indicating the potential association between ANRIL and SCI. To further investigate the function of ANRIL on H$_2$O$_2$-disposed injury in PC-12 cells, ANRIL level was silenced in PC-12 cells by transfection with sh-ANRIL, and the results showed that ANRIL was markedly depressed in PC-12 cells post transfection with sh-ANRIL#1 or sh-ANRIL#2 in comparison to transfection with shNC (p < .01, Figure 2(B)). Moreover, compared to H$_2$O$_2$+shNC group, silencing of ANRIL by transfecting sh-ANRIL#1 or sh-ANRIL#2 dramatically alleviated H$_2$O$_2$-disposed the decreases of in PC-12 cell viability (p < .05, Figure 2(C)), migration (p < .05, Figure 2(D)) and invasion (p < .05, Figure 2(E)). Furthermore, silencing of ANRIL by transfecting sh-ANRIL#1 or sh-ANRIL#2 significantly inhibited the H$_2$O$_2$-disposed apoptosis and autophagy in PC-12 cells by changinover the levels of apoptotic and autophagy-related proteins (p < .05, Figure 3(F,G)). These data indicated that silencing of ANRIL alleviated H$_2$O$_2$-disposed injury in PC-12 cells.

**miR-499a is negatively associated with ANRIL and inhibition of miR-499a changeovers the impacts of silencing of ANRIL on H$_2$O$_2$-disposed PC-12 cell injury**

It is reported that IncRNAs could sponge miRNA to regulate miRNA expression via serving as a competing endogenous RNA (ceRNA) [27,28]. We therefore grabbed the correlation ship of ANRIL vs. miR-499a. We found that suppression of ANRIL after transfecting sh-ANRIL#1 or sh-ANRIL#2 significantly promoted the expression of miR-499a compared to shNC transfection (p < .01, Figure 3(A)). Afterwards, miR-499a level was high or low in PC-12 cells after transfecting miR-499a mimic or inhibitor, respectively (p < .01, Figure 3(B)). Furthermore, we found that relative to H$_2$O$_2$+sh-ANRIL#2 inhibitor NC group, inhibition of miR-499a in PC-12 cells transfected with H$_2$O$_2$+sh-ANRIL#2 and miR-499a inhibitor markedly changeovers the impacts of silencing of ANRIL on H$_2$O$_2$-disposed cell injury through inhibiting cell viability (p < .05, Figure 3(C)), migration (p < .05, Figure 3(D)) and invasion (p < .05, Figure 3(E)), and inducing apoptosis (p < .05, Figure 3(F)) and autophagy (p < .05, Figure 3(G)). All of these data uncovered that silencing of ANRIL might ease H$_2$O$_2$-disposed injury in PC-12 cells by negative regulation of miR-499a.

**PDCD4 is targeted by miR-499a**

As we all know, miRNAs function important roles in disease development via regulating their target genes [29,30]. We further predicted the potential targets of miR-499a according to the target information of TargetScanHuman. As shown in Figure 4(A), PDCD4 was identified as one of the targets of miR-499a (Figure 4(A)). To verify this, luciferase report assay was further performed and the luciferase report test showed us that of luciferase activity of PDCD4-wt was dramatically inhibited by miR-499a mimic (p < .05, Figure 4(B)), confirming that miR-499a could bind to the 3’UTR of PDCD4. Furthermore, our results revealed that the levels of PDCD4 in miR-499a mimic group were markedly depressed compared to those in scramble group, while obviously enhanced in miR-499a inhibitor group relative to those in inhibitor NC group (p < .01, Figure 4(C,D)), pointing out that PDCD4 was a target of miR-499a.
Inhibition of miR-499a aggravates H₂O₂-disposed injury in PC-12 cells but is changeover after depression of PDCD4

To confirm whether miR-499a regulated H₂O₂-disposed injury in PC-12 cells via targeting PDCD4, we depressed the level of PDCD4 in PC-12 cells by transfecting si-PDCD4. As predicted, PDCD4 in si-PDCD4 group was markedly lower relative to siNC group (p < .001, Figure 5(A)), deducting that PDCD4 was successfully depressed in PC-12 cells. Moreover, inhibition of miR-499a in H₂O₂+miR-499a inhibitor group further aggravated H₂O₂-disposed PC-12 cell injury via inhibiting cell viability (p < .05, Figure 5(B)), migration (p < .05, Figure 5(C)) and invasion (p < .05, Figure 5(D)), and inducing apoptosis (p < .01, Figure 5(E)) and autophagy (p < .05, Figure 5(F)). In comparison to H₂O₂+miR-499a inhibitor + siNC group, knockdown of PDCD4 concurrently in H₂O₂+miR-499a inhibitor + si-PDCD4 group remarkably alleviated the effects of miR-499a inhibition on aggravated H₂O₂-disposed PC-12 cell injury (p < .05, Figure 5(B–F)), indicating that inhibition of miR-499a might aggravate H₂O₂-disposed injury possible by targeting PDCD4.

ANRIL silencing decreased H₂O₂-disposed injury in PC-12 cells possible by activating PI3K/Akt/mTOR/p70S6K signals

The activated Akt/mTOR/p70S6K signal has been shown to be crucial in accelerating the regenerative environment for
SCI and may function as a promising tactics for its treatment [31]. We further investigated the regulatory association between ANRIL and PI3K/Akt/mTOR/p70S6K signals (Figure 5(G)). H2O2 dispose markedly decreased the levels of p/t-PI3K, p/t-AKT, p/t-mTOR and p-p70S6K in PC-12 cells, which were remarkably alleviated after silencing of ANRIL (all p < .05). Moreover, inhibition of miR-499a and silencing of ANRIL concurrently markedly changeover the impacts of silencing of

Figure 2. Silencing of ANRIL alleviated H2O2-disposed injury in PC-12 cells. (A) H2O2 promoted ANRIL expression in PC-12 cells compared to control. (B) The ANRIL was silenced after transfection with sh-ANRIL#1 or sh-ANRIL#2 compared to shNC. (C–E) Cell viability, migration and invasion after transfection with sh-ANRIL#1 or sh-ANRIL#2 in presence of H2O2 treatment; (F) cell apoptosis and the expressions of apoptosis-related proteins in PC-12 cells after transfection with sh-ANRIL#1 or sh-ANRIL#2 in presence of H2O2 treatment; (G) the expression of autophagy-related proteins in PC-12 cells after transfection with sh-ANRIL#1 or sh-ANRIL#2 in presence of H2O2 treatment. All experiments were repeated three times. Data are expressed as mean ± SD. *p < .05, **p < .01 and ***p < .001.
Figure 3. miR-499a expression was negatively regulated by ANRIL, and inhibition of miR-499a reversed the effects of silencing of ANRIL on H2O2-disposed PC-12 cell injury. (A) The miR-499a expression was increased after transfection with sh-ANRIL#1 or sh-ANRIL#1 compared to shNC. (B) The miR-499a expression was overexpressed and suppressed after transfection with miR-499a mimic and miR-499a inhibitor, respectively. (C–E) Cell viability, migration and invasion after cotransfection with sh-ANRIL#2 and miR-499a inhibitor in presence of H2O2 treatment; (F) cell apoptosis and the expressions of apoptosis-related proteins in PC-12 cells after cotransfection with sh-ANRIL#2 and miR-499a inhibitor in presence of H2O2 treatment; (G) the expression of autophagy-related proteins in PC-12 cells after cotransfection with sh-ANRIL#2 and miR-499a inhibitor in presence of H2O2 treatment. All experiments were repeated three times. Data are expressed as mean ± SD. *p < .05, **p < .01 and ***p < .001.
ANRIL alone on the levels of these PI3K/Akt/mTOR/p70S6K signal-related proteins in H2O2-disposed PC-12 cells, which were significantly reversed after inhibition of miR-499a, silencing of ANRIL and knockdown of PDCD4 simultaneously. These data deduced that ANRIL silencing might decrease H2O2-disposed injury in PC-12 cells possible by activating PI3K/Akt/mTOR/p70S6K signals, which was mediated by miR-499a/PDCD4 axis (Figure 5H).

Discussion

SCI is a fatal damage that brings about severe symptoms, and the prevention and treatment of this injury has aroused more attention [32]. In very recent studies, IncRNA SNHG5 has been shown to promote astrocytes and microglia viability in SCI [33]; and IncRNA ZNF667-AS1 impacts potential function in promoting recovery of SCI [34]. Moreover, IncRNA SNGH16 is shown to reduce H2O2-disposed cell injury in PC-12 cells, providing a new reference for remedying SCI [35]. These data suggest that IncRNAs may be pivotal factors in SCI.

There are plenty of cancer related researches rounding the pivotal roles of ANRIL; nevertheless, the impacts of ANRIL on SCI remain unfully discovered. In our study, we found that H2O2 disposed PC-12 cell injury and promoted ANRIL level. Silencing of ANRIL inhibited H2O2-disposed PC-12 cell injury through promoting cell viability, migration, invasion and inhibiting apoptosis and autophagy. Also, miR-499a was upregulated after silencing of ANRIL, and inhibition of miR-499a reversed the effects of silencing of ANRIL on H2O2-disposed PC-12 cell injury. Also, PDCD4 was a target of miR-499a. Furthermore, ANRIL silencing alleviated the H2O2-disposed injury in PC-12 cells possible by activating PI3K/Akt/mTOR/p70S6K signals, which was mediated by miR-499a/PDCD4 axis. ANRIL may provide a promising proposal for spinal cord repair.

Extensive studies have disclosed that IncRNAs are participating in the processes of plenty of diseases on the identity of can function as ceRNAs via sponging miRNAs [27,28], as well as in SCI by targeting miRNAs [36]. Moreover, miRNAs are involved in the biology and process of nerve development and injury repair [37,38]. As for this research, ANRIL showed a negative association with miR-499a, and PDCD4 was a target of miR-499a polymorphism was associated with the progression of several cancers, such as oral squamous cell carcinoma [39], breast cancer [40] and hepatocellular carcinoma [41]. The genetic polymorphism of miR-499 A>G (rs3746444) is also associated with the risk of ischemic stroke in a Chinese people [42]. Besides, PDCD4 was pointed out that its abnormal level was correlated with cell death of myocardium and brain tissue post injuries [43]. PDCD4 was targeted by miRNA-21, and this pattern can
protect spinal cords against ischemia-reperfusion injury [43]. Moreover, tetramethylpyrazine could enhance functional recovery after contusion SCI by regulating PDCD4 expression [44]. We indicated that inhibition of miR-499a changes the impacts of silencing of ANRIL on H2O2-disposed PC-12 cell injury, and the impacts of inhibition of miR-499a on aggravated H2O2-disposed PC-12 injury were reversed post knockdown of PDCD4. Although the role of miR-499a and PDCD4 in SCI has not been fully clarified, we speculate that ANRIL may sponge miR-499a to regulate PDCD4 expression, thus playing a significant role in SCI.

The PI3K/Akt signal is a major determinant in regulating multiple cellular processes, including SCI [45,46]. It is also reported that the activated PI3K/Akt signal is pivotal in improving recovery from SCI [24]. Also, the activated Akt/mTOR/p70S6K signals can help to improve motor function...
and the regenerative environment for SCI [31]. Furthermore, PI3K/Akt signal is discovered as a pivotal mediator participating in the transcriptional regulation of some lncRNAs, including ANRIL [47]. In current research, our data showed that ANRIL silencing alleviated the H_{2}O_{2}-disposed injury in PC-12 cells possibly by activating PI3K/Akt/mTOR/p70S6K signals, which was mediated by miR-499a/PDCD4 axis. We thus speculate that PI3K/Akt/mTOR/p70S6K signals are a latent pivotal downstream mechanism to mediate the role of ANRIL/miR-499a/PDCD4 axis in SCI.

In sum, our results indicate that high level of ANRIL may enhance SCI via targeting miR-499a/PDCD4 axis to regulate the briskness of PI3K/Akt/mTOR/p70S6K signals. Our study will lay a new sight in better understanding of the mechanism of SCI and provide a novel perspective for the treatment of this disorder.

Disclosure statement
No potential conflict of interest was reported by the authors.

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