

## Original Article

# Study on the role of PI3K $\delta$ /AKT in regulating glucocorticoid resistance in sudden sensorineural hearing loss

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**Abstract:** Objective: To investigate the role of PI3K $\delta$ /AKT in regulating glucocorticoid resistance in Sudden Sensorineural Hearing Loss (SSNHL). Methods: From March 2017 to February 2018, 27 patients with SSNHL were enrolled in this study and divided into the efficacy group and inefficacy group according to the conditions of glucocorticoid resistance. Peripheral blood mononuclear cells (PBMCs) were collected from both groups. HDAC2 mRNA and protein expression levels were detected by qPCR and Western blot. p-AKT/AKT protein expression was detected by Western blot. HDAC2 and p-AKT/AKT expression in PBMCs were compared between both groups. The H<sub>2</sub>O<sub>2</sub>-induced oxidative stress model of human histiocytic lymphoma cells (U937) was established. U937 cells were divided into the H<sub>2</sub>O<sub>2</sub> group, H<sub>2</sub>O<sub>2</sub>+Lenti-EGFP group, and H<sub>2</sub>O<sub>2</sub>+lenti-PI3K $\delta$  siRNA group according to the cell transfection. U937 cells were divided into the H<sub>2</sub>O<sub>2</sub> group and H<sub>2</sub>O<sub>2</sub>+IC-87114 group according to whether cells were treated with PI3K $\delta$  specific inhibitor. HDAC2 and p-AKT/AKT expression were compared among the above groups. Results: After treatment, HDAC2 mRNA and protein expression of PBMCs in the efficacy group were significantly higher than those in the inefficacy group (all P < 0.05) while p-AKT/AKT protein expression in PBMCs from the efficacy group was obviously lower than that in the inefficacy group (P < 0.001). Compared with the H<sub>2</sub>O<sub>2</sub> group and H<sub>2</sub>O<sub>2</sub>+Lenti-EGFP group, the HDAC2 mRNA and protein expression in the H<sub>2</sub>O<sub>2</sub>+lenti-PI3K $\delta$  siRNA group were markedly higher and p-AKT/AKT protein expression was obvious lower (all P < 0.05). In contrast to the H<sub>2</sub>O<sub>2</sub> group, HDAC2 mRNA and protein expression in the H<sub>2</sub>O<sub>2</sub>+IC-87114 group was markedly higher and the p-AKT/AKT protein expression was significantly lower (all P < 0.05). Conclusions: For glucocorticoid resistance in patients with SSNHL, HDAC2 expression was significantly decreased and p-AKT/AKT protein expression was obviously increased. The activation of the PI3K $\delta$ /AKT signaling pathway could lead to glucocorticoid resistance in SSNHL.

**Keywords:** Sudden sensorineural hearing loss, glucocorticoid resistance, PI3K $\delta$ /AKT signal pathway, histone deacetylase-2

## Introduction

Sudden sensorineural hearing loss (SSNHL) is defined as hearing impairment over 30 dB in three adjacent frequencies, and it is a common otorhinolaryngological disease [1, 2]. The incidence of SSNHL has become increasingly high. The etiology of SSNHL, which is not clear, may involve many factors such as virus infection, vascular deficiencies, autoimmunity and metabolic disorders [3]. Glucocorticoids (GC) are the main therapy option for SSNHL. However, a

large number of patients are insensitive and resistant to GC therapy [4]. Therefore, it is important to identify the mechanism of GC resistance so that it may help to improve the prognosis of patients with SSNHL.

It was reported that the expression of histone deacetylase-2 (HDAC2) could enhance the sensitivity of GC therapy by affecting the deacetylation of glucocorticoid receptors (GR) [5]. Reduced expression of HDAC2 is considered an important cause of GC resistance in SSNHL.

Many studies have reported that oxidative stress induced by reactive oxygen species may lead to SSNHL [6]. The activity of HDAC2 is decreased through oxidative stress-induced posttranscriptional modifications, such as nitrication of tyrosine residues, phosphorylation of serine residues causing GC resistance in patients with SSNHL [7]. Previous studies have reported that in the development of GC resistance in respiratory inflammation, phosphatidylinositol-3-kinase delta/protein kinase B (PI3K $\delta$ /AKT) is activated by oxidative stress and leads to reduced expression of HDAC2 [8]. In addition, To et al. reported that amionophylline had antioxidant effects and low-dose amionophylline could decrease the expression of PI3K $\delta$  and then increase the activation of HDAC2 in the development of chronic obstructive pulmonary disease [9]. It was reported that amionophylline could increase HDAC2 expression and improve the effect of GC in LPS-induced hearing loss in guinea pigs [4]. However, it is still unclear whether oxidative stress can inhibit HDAC2 expression and lead to GC resistance through PI3K $\delta$ /AKT signal pathway.

In this study, we observed the expression levels of HDAC2 and p-AKT/AKT in SSNHL patients with GC resistance. The H<sub>2</sub>O<sub>2</sub>-induced oxidative stress model of human histiocytic lymphoma cells (U937) was established to explore the role of PI3K $\delta$ /AKT in regulating HDAC2 expression in GC resistance. The results of this study may provide new ideas for clinical treatment of SSNHL patients with GC resistance.

### Materials and methods

#### Subjects

From March 2017 to February 2018, 27 SSNHL patients who were admitted to the Department of Otolaryngology in Nanjing Drum Tower Hospital were enrolled in this study. The inclusion criteria was as follows: pure tone audiometry (PTA) (0.25~8 kHz) was more than 60 dB and patients met the diagnostic criteria of SSNHL published in 2019 [10]; patients were more than 18-years old and newly diagnosed; related drugs such as glucocorticoid and amionophylline that affect experimental results were used in recent three months; patients with high compliance finished the prescribed course of treatment; and the clinical data of patients was complete. The exclusion criteria were as fol-

lows: patients with history of acoustic neuroma, family history of sensorineural deafness, use of ototoxic drugs, history of systemic infectious disease, history of autoimmune disease, and history of hematonosis disease, and patients who were loss to follow-up and dropped out of this study. All patients gave their informed consent and this study was approved by the hospital ethic committee.

SSNHL patients underwent conventional therapy based on glucocorticoid as follows: after admission, methylprednisolone (Pfizer Inc, USA) was given at 80 mg per day through an intravenous drip for 4 days. Then, methylprednisolone was reduced to 40 mg per day for 3 days. Finally, methylprednisolone was reduced to 20 mg per day for 3 days. Through an intravenous drip, patients also received Extract of Ginkgo biloba (Dr. Willmar Schwabe GmbH & Co. KG, German) 105 mg per day and Sodium Monosialotetrahexosylganglioside injection (Qilu Pharmaceutical Co. Ltd, China) 40 mg per day. The treatment time was 10 days. After that, patients were switched to oral administration of Ginkgo leaf tablets and Mecobalamin Tablets for two months. All patients were followed up for three months. Patients were divided into the efficacy group (N = 17) and inefficacy group (N = 10) according to whether the improvement degree of PTA was more than 15 dB [11].

#### Separation of PBMCs

PBMCs were isolated using the method of density gradient centrifugation. Fifteen mL of peripheral venous blood was drawn from patients in each group and equal amounts of PBS solutions was added. The above mixture was slowly added to the liquid surface of Lymphocytes Separation Medium and a distinct interphase was kept in this process. Then, it was centrifuged for 20 min at the speed of 2000 rpm/min. The contents in centrifuge tube were divided into four layers. PBMCs were located in the second layer. This layer was extracted using a micropipettor and kept in a new centrifuge tube. After centrifugation for 10 min at the speed of 1500 rpm/min, the supernatant was discarded. Then after washing with PBS solutions, PBMCs was used for further experiments.

**Table 1.** Primer sequence

Primers	Sequences
HDAC2-F	ATGGCG TACAGTCAAGGAGG
HDAC2-R	TGCGGATTCTATGAGGCTTCAA
$\beta$ -actin-F	GTCCACCGCAAATGCTTCTA
$\beta$ -actin-R	TGCTGTACCTTCACCGTTC

#### Cell culture and grouping

U-937 cells (Cell bank of Chinese academy of sciences, China) were cultured in RPMI1640 (Gibco, USA) containing 10% fetal bovine serum (FBS) (Gibco, USA) and 100  $\mu$ g/mL streptomycin and 100  $\mu$ g/mL penicillin. The cells were incubated in the conditions of 5% CO<sub>2</sub> and 37°C. The oxidative stress model of cells was established following treatment with 0.2 mmol/L of H<sub>2</sub>O<sub>2</sub> for 20 min. The transfection of cells with PI3K $\delta$  siRNA (Genechem, China) was done using lentivirus, they were divided into three groups: H<sub>2</sub>O<sub>2</sub> group, H<sub>2</sub>O<sub>2</sub>+Lenti-EGFP group, and H<sub>2</sub>O<sub>2</sub>+lenti-PI3K $\delta$  siRNA group. According to whether application of IC-87114 (PI3K $\delta$  specific inhibitor), the cells were divided into two groups: H<sub>2</sub>O<sub>2</sub> group and H<sub>2</sub>O<sub>2</sub>+IC-87114 group.

#### Real-time PCR assay

Total RNA in each group was extracted with Trizol Reagent (Invitrogen, USA) according to the operation instructions. TaqMan MicroRNA reverse transcription Kit (Promega, USA) was applied to synthesize the above RNA into cDNA.  $\beta$ -actin served as normalization. Real-time PCR was performed with SYBRH Premix Ex TaqTM (TaKaRa, Japan). The primer design is shown in **Table 1**. PCR amplification was performed by the following conditions: predenaturation 5 min in 95°C, denaturation 25 s in 95°C and annealing/extension 50 s at 60°C, a total of 35 cycles. The relative expression amount of HDAC2 mRNA was calculated according to the 2- $\Delta\Delta$ Ct method through ABI 7300 System software.

#### Western blot assay

Total protein in each group was extracted with RIPA protein lysis buffer following the operation instructions. BCA method was used to detect the concentration of protein. Proteins were separated using SDS-PAGE gel electrophoresis

and subsequently transferred onto a PVDF membrane. Then at room temperature the membrane was incubated with TBST containing 5% non-fat milk powder and blocked for 1 h. The primary antibodies; p-AKT (dilution 1:500, Santa Cruz, USA), AKT (dilution 1:800, Santa Cruz, USA) and HDAC2 (dilution 1:1000, Santa Cruz, USA) were incubated with the membranes overnight. Next, HRP-conjugated secondary antibody (dilution 1:1000, Santa Cruz, USA) was incubated with a PVDF membrane for 30 min. Finally, ECL solution was applied to evaluate the expression of the targeted proteins. Bio-Rad image software was applied to quantify the target band intensities.  $\beta$ -actin served as an internal control.

#### Statistical analysis

SPSS 22.0 software was used for the statistical analysis of data in this study. The measurement data are expressed as mean  $\pm$  standard deviation (SD). The comparison between both groups was performed by t test. The comparison between groups was performed by One-way ANOVA. Enumeration data was presented as cases or percentage and the comparison among groups was conducted through  $\chi^2$  test. P < 0.05 indicated significant statistical differences.

## Results

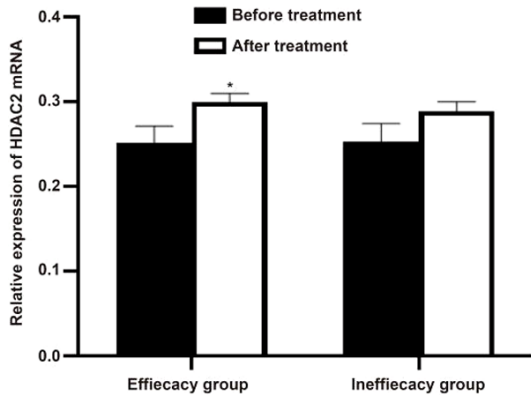
#### Comparison of HDAC2 mRNA in PBMCs between both groups

Before treatment, there was no significant difference in HDAC2 mRNA of PBMCs between the efficacy group and inefficacy group (0.252 $\pm$ 0.019 vs 0.253 $\pm$ 0.021). After treatment, HDAC2 mRNA of PBMCs in the efficacy group was significantly higher than that in the inefficacy group, and there was clear statistical differences (0.300 $\pm$ 0.010 vs 0.289 $\pm$ 0.011, t = 2.661, P = 0.013), as shown in **Figure 1**.

#### Comparison of HDAC2 protein expression in PBMCs between both groups

There was no remarkably difference for HDAC2 protein expression in PBMCs between the efficacy group and the inefficacy group before treatment (0.509 $\pm$ 0.011 vs 0.507 $\pm$ 0.018). HDAC2 protein in PBMCs after treatment in the efficacy group was remarkably higher than

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**Figure 1.** Comparison of HDAC2 mRNA in PBMCs between the efficacy group and inefficacy group. Compared with the inefficacy group after treatment, \* $P < 0.05$ .

that in the inefficacy group, and there was a remarkable statistical difference ( $0.607 \pm 0.018$  vs  $0.510 \pm 0.017$ ,  $t = 13.790$ ,  $P < 0.001$ ), as seen in **Figure 2**.

### Comparison of p-AKT/AKT expression in PBMCs between both groups

There was no obvious difference for p-AKT/AKT expression in PBMCs between the efficacy group and inefficacy group before treatment ( $0.203 \pm 0.048$  vs  $0.193 \pm 0.046$ ). p-AKT/AKT expression in PBMCs after treatment in the efficacy group was obviously lower than that in the inefficacy group, and there was a remarkable statistical difference ( $0.121 \pm 0.032$  vs  $0.188 \pm 0.053$ ,  $t = 4.118$ ,  $P < 0.001$ ), as seen in **Figure 3**.

### The effect of PI3K $\delta$ siRNA on HDAC2 expression in the H<sub>2</sub>O<sub>2</sub>-induced oxidative stress model of U937 cells

The efficacy of transfection with recombinant lentivirus in U937 cells was 70%, as shown in **Figure 4**. As shown in **Figure 5**, compared with those in the H<sub>2</sub>O<sub>2</sub> group ( $0.337 \pm 0.051$ ), and H<sub>2</sub>O<sub>2</sub>+Lenti-EGFP group ( $0.387 \pm 0.072$ ), HDAC2 mRNA expression in the H<sub>2</sub>O<sub>2</sub>+Lenti-PI3K $\delta$  siRNA group ( $0.837 \pm 0.090$ ) was significantly higher, and there were significant statistical differences (all  $P < 0.001$ ). There was no significant difference for HDAC2 mRNA expression between the H<sub>2</sub>O<sub>2</sub> group and the H<sub>2</sub>O<sub>2</sub>+Lenti-EGFP group. In contrast to those in the H<sub>2</sub>O<sub>2</sub> group ( $0.117 \pm 0.025$ ), and the H<sub>2</sub>O<sub>2</sub>+Lenti-EGFP group ( $0.127 \pm 0.036$ ), the HDAC2 protein

expression in the H<sub>2</sub>O<sub>2</sub>+Lenti-PI3K $\delta$  siRNA group ( $0.702 \pm 0.090$ ) was markedly higher, and there were significant statistical differences (all  $P < 0.05$ ). There was no significant difference for HDAC2 protein expression between the H<sub>2</sub>O<sub>2</sub> group and H<sub>2</sub>O<sub>2</sub>+Lenti-EGFP group.

### The effect of PI3K $\delta$ siRNA on p-AKT/AKT expression in the H<sub>2</sub>O<sub>2</sub>-induced oxidative stress model of U937 cells

As shown in **Figure 6**, p-AKT/AKT expression in the H<sub>2</sub>O<sub>2</sub>+Lenti-PI3K $\delta$  siRNA group ( $0.076 \pm 0.017$ ) was significantly lower than that in the H<sub>2</sub>O<sub>2</sub> group ( $0.796 \pm 0.037$ ) or the H<sub>2</sub>O<sub>2</sub>+Lenti-EGFP group ( $0.757 \pm 0.081$ ), and there were significant statistical differences (all  $P < 0.05$ ). There was no significant statistical difference for p-AKT/AKT expression between the H<sub>2</sub>O<sub>2</sub> group and H<sub>2</sub>O<sub>2</sub>+Lenti-EGFP group.

### The effect of PI3K $\delta$ inhibitor IC-87114 on HDAC2 expression in the H<sub>2</sub>O<sub>2</sub>-induced oxidative stress model of U937 cells

As shown in **Figure 7**, HDAC2 mRNA expression in the H<sub>2</sub>O<sub>2</sub>+IC-87114 group was significantly higher than that in the H<sub>2</sub>O<sub>2</sub> group, and there were significant statistical differences between both groups ( $0.537 \pm 0.023$  vs  $0.692 \pm 0.026$ ,  $t = 9.984$ ,  $P < 0.001$ ). HDAC2 protein expression in the H<sub>2</sub>O<sub>2</sub>+IC-87114 group was also obviously higher, compared with that in the H<sub>2</sub>O<sub>2</sub> group, and a significant difference could be found between both groups ( $0.317 \pm 0.052$  vs  $0.452 \pm 0.050$ ,  $t = 4.185$ ,  $P = 0.003$ ).

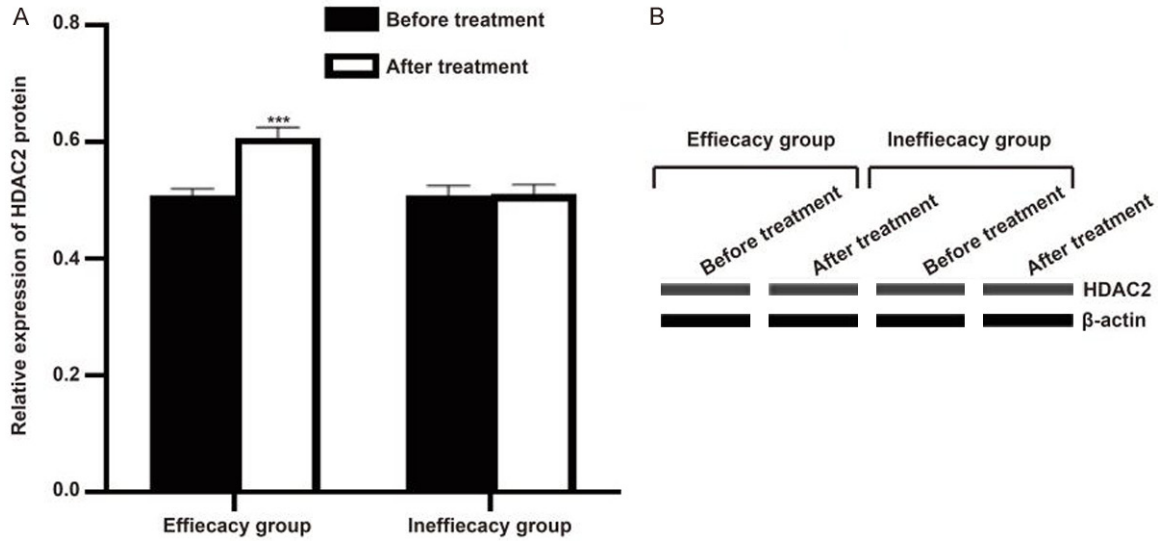
### The effect of IC-87114 on p-AKT/AKT expression in the H<sub>2</sub>O<sub>2</sub>-induced oxidative stress model of U937 cells

p-AKT/AKT expression in the H<sub>2</sub>O<sub>2</sub>+IC-87114 group was remarkably lower than that in the H<sub>2</sub>O<sub>2</sub> group, and a significant difference was found between both groups ( $0.632 \pm 0.041$  vs  $0.452 \pm 0.049$ ,  $t = 6.300$ ,  $P < 0.001$ ), as shown in **Figure 8**.

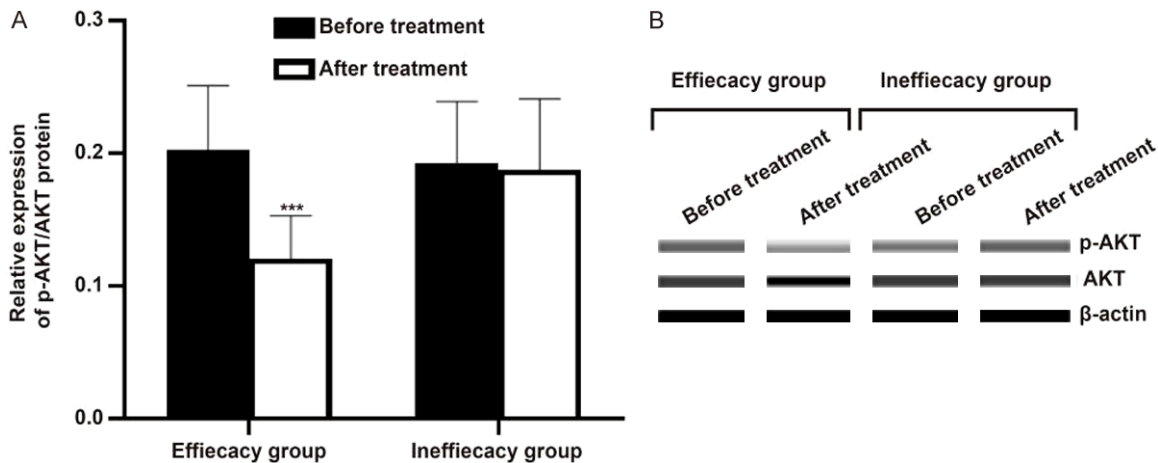
## Discussion

In this study, we explored the role of PI3K $\delta$ /AKT in glucocorticoid resistance using an H<sub>2</sub>O<sub>2</sub>-induced oxidative stress model of human histiocytic lymphoma cells and PBMCs from SSNHL patients. We demonstrated for the first

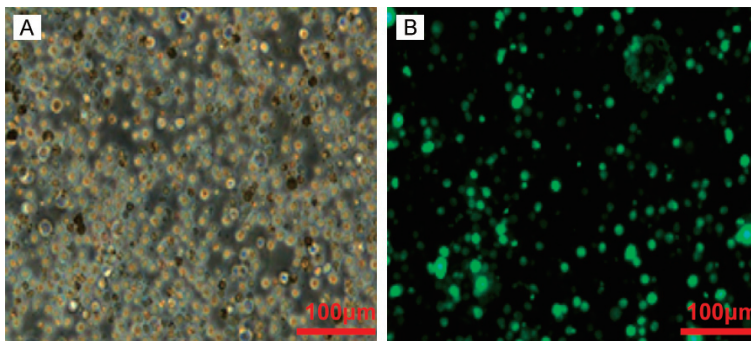
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**Figure 2.** Comparison of HDAC2 protein expression in PBMCs between the efficacy group and inefficacy group. A. Quantitative analysis of HDAC2 protein expression; B. Western blot of HDAC2 protein. Compared with the inefficacy group after treatment, \*\*\* $P < 0.001$ .



**Figure 3.** Comparison of p-AKT/AKT protein expression in PBMCs between the efficacy group and inefficacy group. A. Quantitative analysis of p-AKT/AKT protein expression; B. Western blot of p-AKT/AKT protein. Compared with the inefficacy group after treatment, \*\*\* $P < 0.001$ .

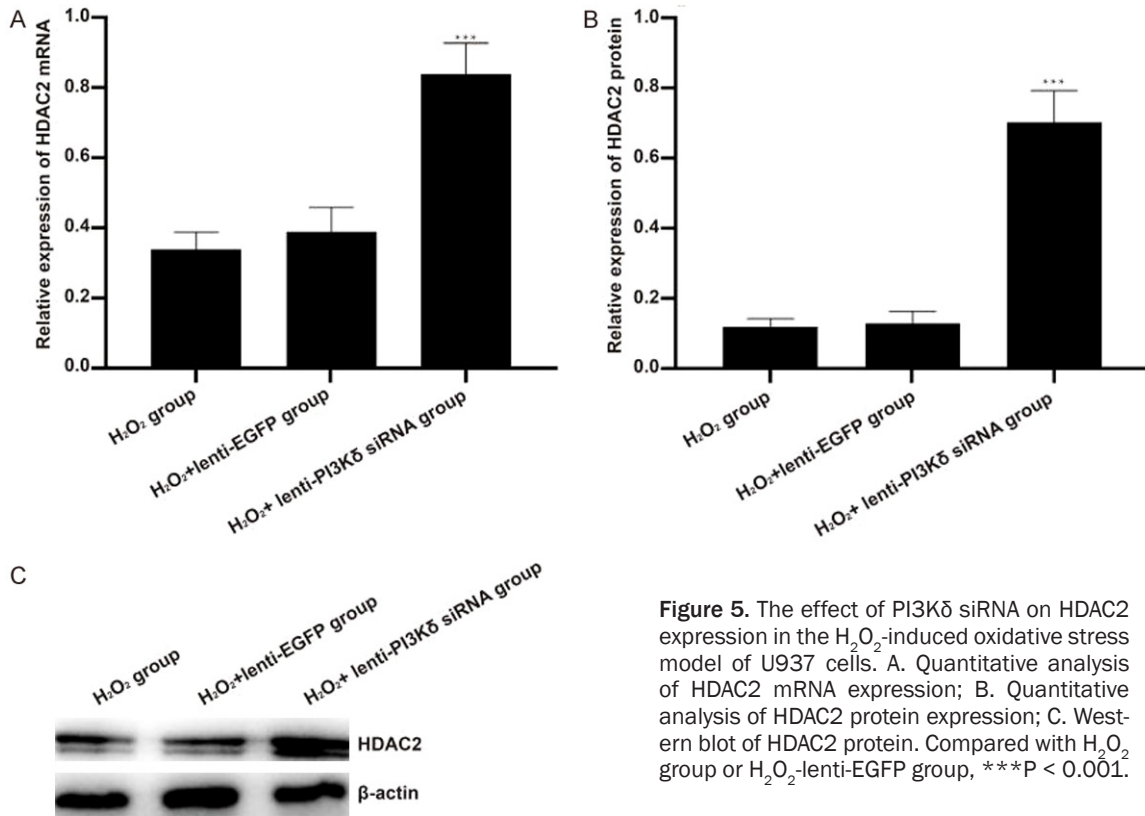


**Figure 4.** U937 cells were transfected with PI3K $\delta$  siRNA using recombinant lentivirus. A. Observation under white light. B. Observation under fluorescence light.

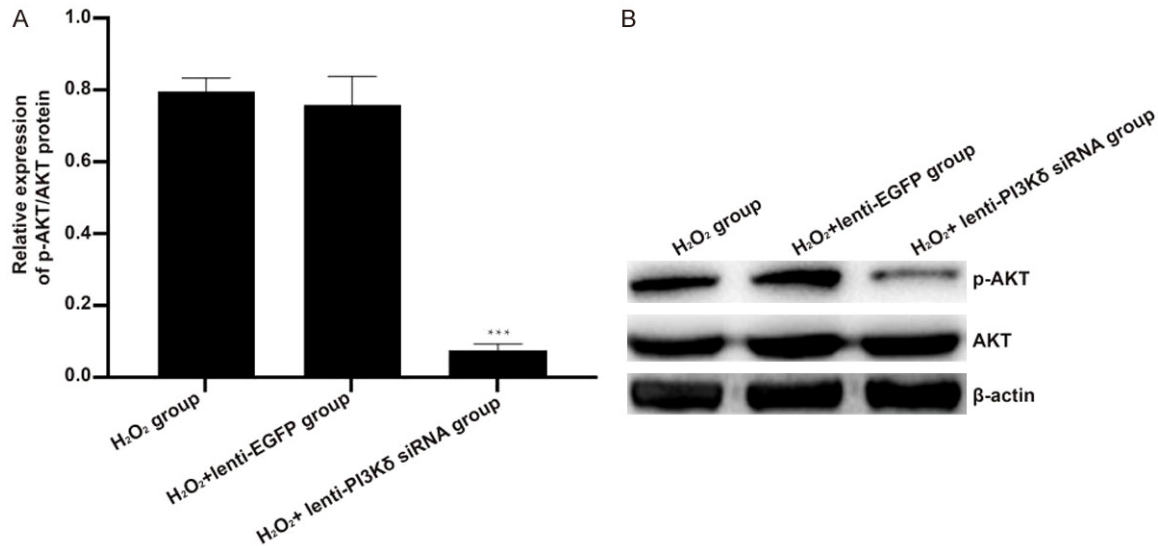
time that the PI3K $\delta$ /AKT signaling pathway plays an important role in glucocorticoid resistance induced by oxidative stress in SSNHL. Our results also indicated that inhibiting the activation of PI3K $\delta$ /AKT could reduce glucocorticoid resistance.

At present, glucocorticoids are widely clinically used for SSNHL, but a lot of patients show resistance to glucocorti-

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**Figure 5.** The effect of PI3K $\delta$  siRNA on HDAC2 expression in the H<sub>2</sub>O<sub>2</sub>-induced oxidative stress model of U937 cells. A. Quantitative analysis of HDAC2 mRNA expression; B. Quantitative analysis of HDAC2 protein expression; C. Western blot of HDAC2 protein. Compared with H<sub>2</sub>O<sub>2</sub> group or H<sub>2</sub>O<sub>2</sub>-lenti-EGFP group, \*\*\*P < 0.001.

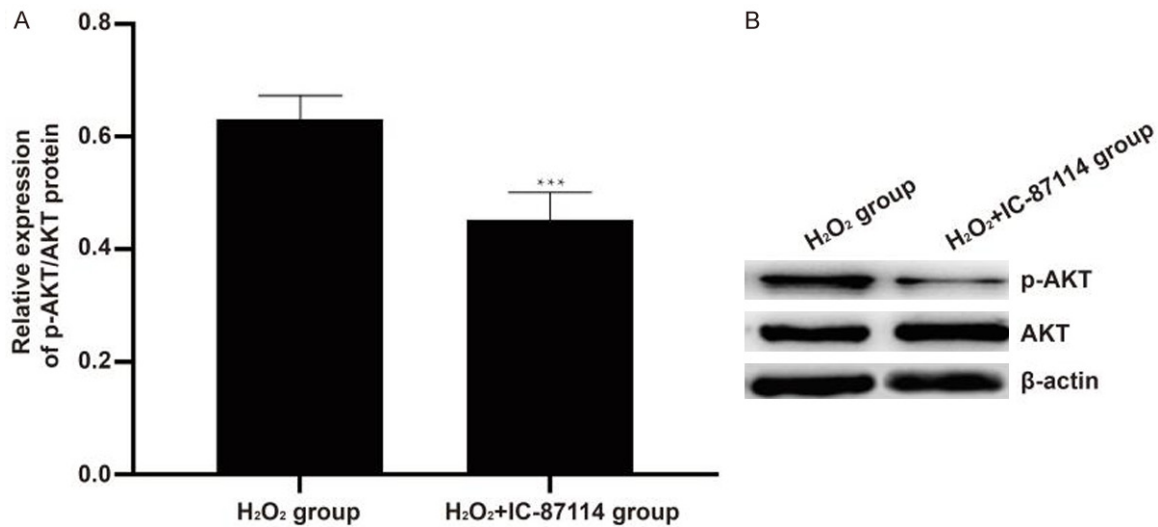
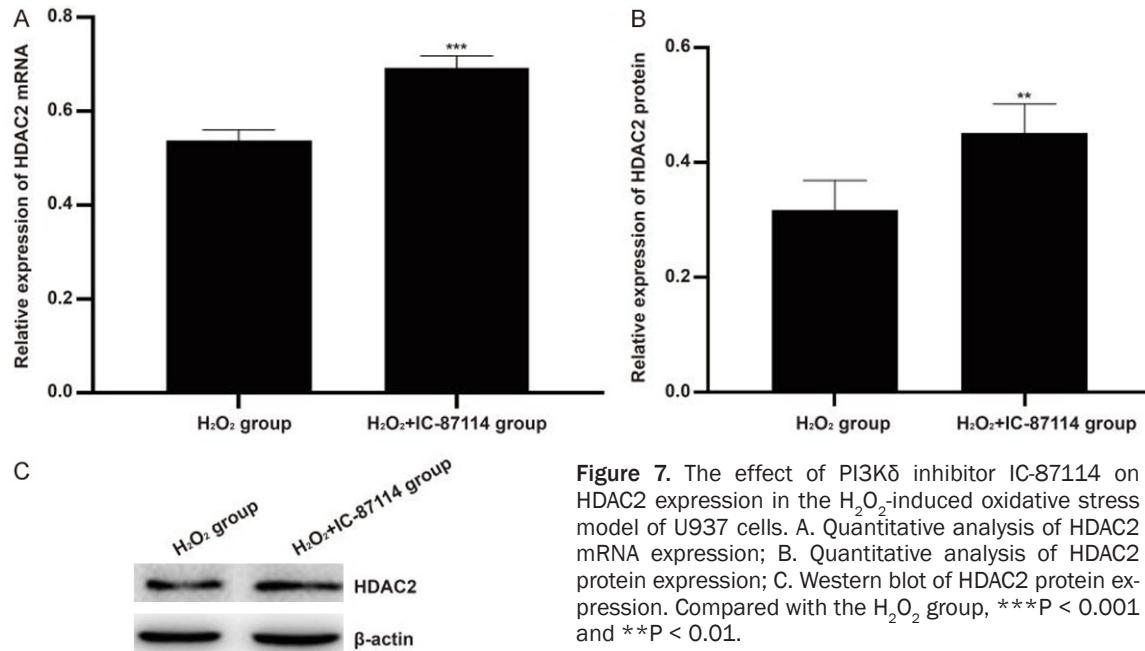


**Figure 6.** The effect of PI3K $\delta$  siRNA on p-AKT/AKT expression in the H<sub>2</sub>O<sub>2</sub>-induced oxidative stress model of U937 cells. A. Quantitative analysis of p-AKT/AKT protein expression; B. Western blot of p-AKT/AKT protein expression. Compared with the H<sub>2</sub>O<sub>2</sub> group or H<sub>2</sub>O<sub>2</sub>-lenti-EGFP group, \*\*\*P < 0.001.

coid treatment. According to the epidemiological survey, SSNHL patients with glucocorticoid resistance usually have a poor prognosis [12]. This is often contributed to adverse effects resulting from the long-term use of glucocorti-

coids, besides primary disease itself. Compared with SSNHL patients with glucocorticoids sensitivity, these patients have much higher rates of hospitalization [13]. Therefore, glucocorticoid resistance not only affects the physical

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and psychological health, but also exerts an economic burden for these SSNHL patients.

In recent years, the mechanism of glucocorticoid resistance in SSNHL has been a hot area of research. Many factors are involved in the molecular mechanism of glucocorticoid resistance [14]. Oxidative stress has been confirmed as one of the mechanisms in glucocorticoids resistance [15]. It has been found that HDAC2 expression was associated with glucocorticoid

resistance [16]. Kazuhiro, et al. reported that in patients with COPD, oxidative stress could induce glucocorticoid insensitivity by impaired HDAC2 expression and that overexpression of HDAC2 in glucocorticoid-insensitive alveolar macrophages was able to restore the sensitivity of glucocorticoid [17]. Adenuga, et al. [18] reported that abnormal oxidant burden was correlated with obviously reduced expression of HDAC2 and glucocorticoid resistance, and that HDAC2 deficiency could lead to glucocorti-

coid resistance in inhibiting the lung inflammatory response due to an oxidant/antioxidant imbalance. Another study reported that in pediatric B-cell acute lymphoblastic leukemia (B-ALL), PI3K inhibitor could sensitize B-ALL cells to dexamethasone by restoring translocation of the GC receptor and counteract stroma-induced dexamethasone-resistance, suggesting that PI3K inhibition may be a promising strategy for treating B-ALL patients by overcoming GC-resistance [19]. To et al. found that theophylline was a potent selective inhibitor of oxidant-activated PI3K- $\delta$ , which was up-regulated in peripheral lung tissue of patients with COPD, and knock-down of PI3K- $\delta$  failed to induce GC resistance with oxidative stress [9]. However, the roles of PI3K in regulating HDAC2 in glucocorticoid resistance for SSNHL patients are still unknown.

Increasing evidence shows that oxidative stress plays a crucial role in GC resistance in SSNHL. In this study, it was demonstrated that compared with the inefficacy group p-AKT/AKT expression in PBMCs from the efficacy group was significantly increased while HDAC2 expression was obviously decreased. Previous studies have reported that p-AKT/AKT is a direct target of PI3K and a surrogate marker for PI3K activation in different cell systems. These results indicated that an increased level of p-AKT/AKT expression may be associated with low expression of HDAC2, which may lead to GC resistance in SSNHL. This was in accordance with the results reported by Yuan et al. [20]. In addition, the H<sub>2</sub>O<sub>2</sub>-induced oxidative stress model of human histiocytic lymphoma cells was applied in this study. The results showed that the reduction of HDAC2 activity was reversed using Lenti-PI3K $\delta$  siRNA and IC87114, a selective inhibitor. PI3K $\delta$  is one of the isoforms of the catalytic subunit in Class I-PI3K. It was reported that PI3K $\delta$  can regulate neutrophil activation, trafficking, and directional movement [21]. It was found that PI3K $\delta$  can inhibit neutrophil accumulation with theophylline [22]. Other studies have shown that the PI3K $\delta$  isoform is involved in GC insensitivity in the smoking murine model [23]. Marwick, et al. [24] reported that PI3K $\delta$  knockout resulted in protection against GC resistance and reduced HDAC2 activity after exposure to oxidative stress and this agrees with the results shown here.

In summary, in SSNHL patients with glucocorticoid resistance, p-AKT/AKT expression was obviously increased and HDAC2 expression was markedly decreased. In the H<sub>2</sub>O<sub>2</sub>-induced oxidative stress model of human histiocytic lymphoma cells, high expression of p-AKT/AKT and low expression HDAC2 were revealed, and inhibition of PI3K $\delta$ /AKT can manifest the reversal of the above results, indicating improvement of glucocorticoid sensibility. However, there are some limitations in this research. First, the H<sub>2</sub>O<sub>2</sub>-induced oxidative stress model of human histiocytic lymphoma cells does not completely represent the clinical pathogenesis of SSNHL. More studies can be conducted using cell models more suitable for SSNHL studies. Secondly, this is the first study to use a PI3K $\delta$ /AKT inhibitor for the treatment of an inner ear disease, the functions of PI3K $\delta$ /AKT are very complex, and there are many unclear aspects that remain to be investigated. The role of PI3K $\delta$ /AKT in an animal model of SSNHL may also be explored in future experiments.

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### Disclosure of conflict of interest

None.

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