

Oxidative stress induces B lymphocyte DNA damage and apoptosis by upregulating p66shc

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Abstract. – **OBJECTIVE:** B lymphoma is a type of malignant tumor originating from the lymphatic hematopoietic system. The pathogenesis and treatment methods are not clear. The change of oxidative stress is closely related to the cell DNA damage and cell apoptosis, which may be served as a target for cancer treatment. This study aims to illustrate the role of oxidative stress in the regulation of B lymphocytoma.

PATIENTS AND METHODS: The tumor tissue was collected from patients with B lymphocytoma. The p66shc level was detected by Western blot. Hydrogen peroxide (H₂O₂) was assessed by the kit. The oxidative stress model of B lymphoma cell was established by H₂O₂ treatment. ROS inhibitor or RNAi was used to regulate ROS level. ROS level was determined by flow cytometry. 8-OHdG level (DNA damage product) was tested by the kit. Cell apoptosis was evaluated by annexin V-PI.

RESULTS: P66shc expression was significantly reduced, while H₂O₂ production was significantly decreased in tumor tissue of B lymphoma compared with adjacent normal control. H₂O₂ stimulation markedly elevated ROS level and p66shc expression ($p < 0.05$), accompanied by the aggravation of DNA damage and increase of apoptosis. ROS inhibitor or p66shc RNAi treatment significantly attenuated DNA damage and declined cell apoptosis ($p < 0.05$).

CONCLUSIONS: ROS production promotes p66shc expression, induced DNA damage, and facilitated cell apoptosis. Upregulation of p66shc in oxidative stress could be treated as a therapeutic target for B lymphoma.

Key Words:

p66shc, Oxidative stress, B lymphoma.

Its incidence may be related to genetic mutation, viruses and other pathogens infection, radiation, chemical drugs, and autoimmune diseases^{1,2}.

Evidence showed that the change of cell oxidative stress played an important role in maintaining chromosome stability and had a close relationship with cell DNA damage and apoptosis, which, therefore, can be treated as a treatment target for cancer. Current researches suggested that induction of oxidative stress may promote cell apoptosis to regulate tumor progress³. Adapter protein P66Shc, encoded by proto-oncogene Shc, is involved in the physiological process of metabolism, oxidative stress, and cell aging in mammals^{6,7}. It is a key protein associated with oxidative stress in the cell and can induce cell apoptosis through cytochrome C in the mitochondria for the production of ROS⁸. It was found that p66shc expression was changed in multiple tumor cells. Moreover, its dysregulation status was in diversity in different cell types. In human colorectal cancer cell line HCT8, the knock-down of p66shc inhibited cancer cell survival via PI3K/AKT/Mdm-2/p53 signaling pathway⁹. On the contrary, it was reported that p66shc-miR-21-Sod2 signaling pathway suppressed hepatic cancer¹⁰. However, the expression and mechanism of p66shc in B lymphoma have not been elucidated. In this experiment, we aimed to explore the expression of p66shc in human B lymphocytoma and the regulation effect of ROS-p66shc signaling pathway

Materials and Methods

Main Reagents

Total protein extraction kit was purchased from Keygen (Nanjing, Jiangsu, China). Radio-immunoprecipitation assay (RIPA) and bicinchoninic acid (BCA) protein quantification kit

Introduction

B lymphoma is a kind of malignant tumor originated from the lymphatic hematopoietic system.

were provided by Beyotime (Suzhou, Jiangsu, China). The p66shc antibody was bought from Proteintech (Wuhan, Hubei, China). Horseradish peroxidase (HRP-labeled goat anti-rabbit IgG (H+L) was got from ZSbio (Beijing, China). MTT reagent was obtained from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), and penicillin-streptomycin were provided by Gibco (Rockville, MD, USA). Si p66shc and si control were designed and synthesized by Genepharma (Shanghai, China).

Main Instruments

The clean bench was provided by Boxun (Shanghai, China). Gel imaging system UVP Multispectral Imaging System was purchased from Bio-Rad (Hercules, CA, USA). PS-9 semi-dry transfer electrophoresis was obtained from Jim-x Scientific (Dalian, Liaoning, China). BD FACSCalibur was bought from BD Biosciences (San Diego, CA, USA). CO₂ incubator and Thermo-354 microplate reader were got from Thermo Fisher (Waltham, MA, USA).

Cell Culture

Human B lymphoma cell line RAMOS was bought from Cell Bank, Institute of Chinese Academy of Sciences (Shanghai, China). The cells were maintained in DMEM medium containing 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. The cells were cultured under 37% and 5% CO₂. The cells were observed under the inverted microscope (Olympus, Tokyo, Tokyo, Japan). The cells in logarithmic phase were selected for the experiment.

Clinical Samples

Human B lymphoma tumor tissue and adjacent normal tissue were obtained from the lymphoma patients in our hospital. All of the 15 patients had signed informed consent. The sample was collected from the surgery and stored at 80 °C. All procedures were approved by the Animal Ethics Committee of the First Affiliated Hospital of Zhejiang University.

H₂O₂ Level Determination

Tissue H₂O₂ level detection kit was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China). A total of 100 mg tissue was homogenated in 1 ml ddH₂O. The supernatant was used to test H₂O₂ level according to the manual.

Cell Transfection and Drug Administration

The p66shc siRNA were obtained from the reference and synthesized by Genepharma (Shanghai, China)^{11,12}. si p66Shc sense: CCGCTTTGAAAGT-GTCAGTCA; anti-sense: TCAGCTACCACATG-GACAATC. The cell line was passaged one day before transfection. The cells were seeded in the 24-well plate at confluence 30-50%. 100 µl of 1.25 µl siRNA at 20 µM were solved in 100 µl Opti-MEM to prepare solution A, while 100 µl Lipofectamine 2000 or LipofectamineTM RNAi MAX was solved in Opti-MEM to prepare solution B. After 5 min mix, solution A and B were mixed for 5 min and added to the plate. The medium was changed back to DMEM containing 10% FBS after 4 h incubation. P66shc expression was detected to calculate the transfection efficiency.

After administration, cells were treated by NAC at 200 µM for 4 h and then added by H₂O₂ at 4 h for measuring. Next, ROS level, p66shc expression, DNA damage, and cell apoptosis, were detected.

ROS Measurement

CM-H₂DCFDA was hydrolyzed to DCFH in the cells. DCFH was oxidized to DCF with high fluorescence intensity by an oxidizing agent in the cells. The cells were digested to cell suspension after transfection. Next, the cells were incubated with CM-H₂DCFDA for 30 min and washed by DMEM for three times. At last, the solution was detected on BD FACSCalibur (San Jose, CA, USA).

MTT Assay

The cells in logarithmic phase were digested with 0.25% trypsin and seeded in 96-well plate. After transfected with p66shc siRNA at 50 nM for 6 h, the cells were further cultured in DMEM medium containing 10% FBS for 18 h. The cells were incubated in 10 µl MTT solution for 4 h and observed under the microscope. After the supernatant was removed, the cells were added with 150 µl dimethyl sulfoxide (DMSO) and tested at 490 nm after 12-15 h to calculate cell survival rate.

Cell survival rate (%) = (OD value in experimental group – OD value in blank)/(OD value in control – OD value in blank).

8-OHdG Level Detection

8-OHdG detection kit was purchased from Keygen (Nanjing, Jiangsu, China). A total of 100 mg tissue were homogenated in 1 ml ddH₂O. The supernatant was used to test 8-OHdG level according to the manual.

Western Blot

Western blot was performed according to the reference¹³. The cells were washed with phosphate-buffered saline (PBS) and lysed by phenyl-methylsulfonyl fluoride (PMSF) on ice for 5-10 min. Then, the cell debris were moved to a new Eppendorf (EP) tube and centrifuged at 4°C and 12000 g for 5 min. The supernatant was quantified by bicinchoninic acid (BCA) method and boiled for 5 min. Next, the sample was separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membrane at 300 mA for 1 h. Then, the membrane was incubated with p66shc antibody (1:1000) at 4°C overnight and incubated with secondary antibody (1:1000) at 37°C for 2 h. At last, the membrane was developed by chemiluminescence.

AV-PI Staining

The cells were seeded in 6-well plate and transfected with lentivirus; then, they were resuspended as single cell suspension and incubated with AV and PI on ice avoid of light for 10 min. At last, the cells were tested on BD FACSCalibur. Each group was repeated for three times.

Statistical Analysis

All data analyses were performed on SPSS 19.0 software (IBM Corp., IBM SPSS Statistics for Windows, Armonk, NY, USA). The measurement data were presented as mean $\pm$ standard deviation and received by normality test and homogeneity test of variance. The data in normal distribution were compared by *t*-test or one-way ANOVA with post-hoc test of LSD. $p < 0.05$ was considered statistical significance.

Results

H₂O₂ and p66shc Expressions in Human B Lymphocytoma Samples

In this study, we first tested H_2O_2 level in B lymphocytoma and adjacent normal tissue. As shown in Figure 1A, H_2O_2 content was significantly reduced in B lymphocytoma tissue compared with adjacent normal tissue ($p < 0.05$). p66shc is the main factor of mitochondria in producing ROS. Thus, we also detected p66shc expression via Western blot. As shown in Figure 1B, p66shc level significantly declined in B lymphocytoma tissue compared with para-carcinoma tissue.

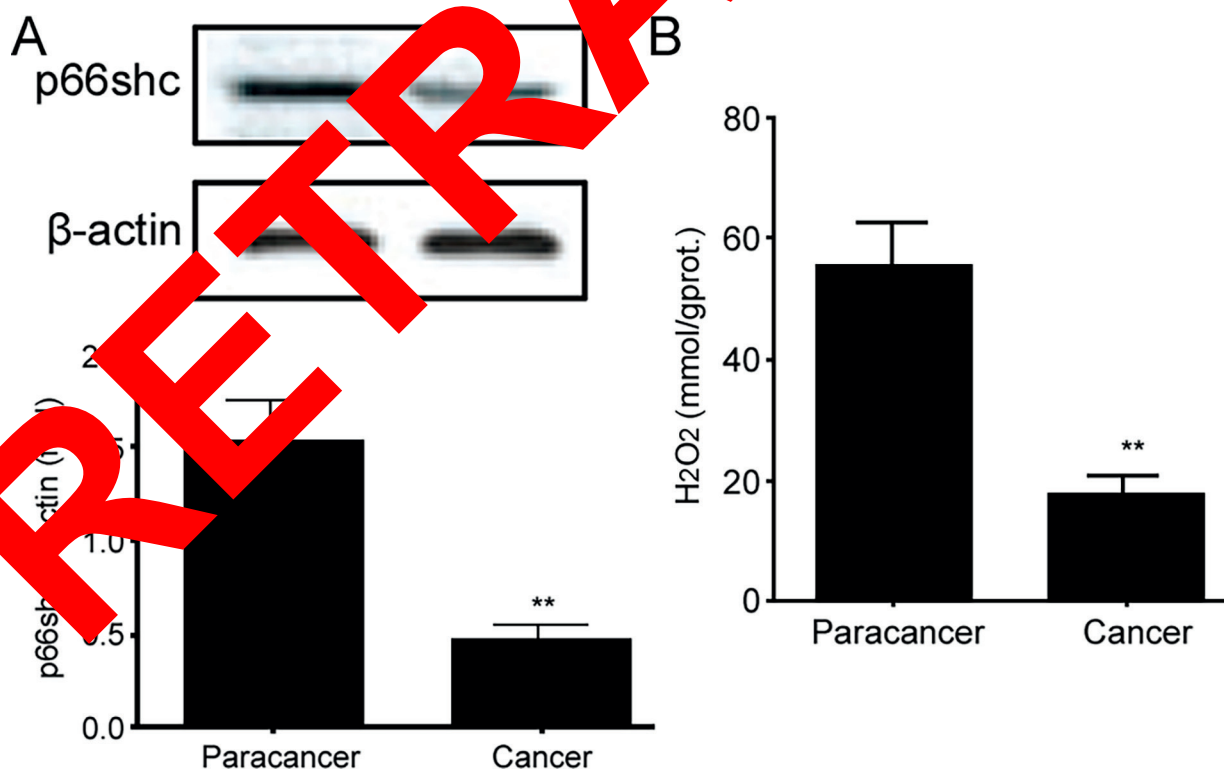


Figure 1. H_2O_2 and p66shc expressions in human B lymphocytoma samples. **A**, p66shc protein expression. **B**, H_2O_2 expression. ** $p < 0.05$, compared with paracarcinoma tissue.

Moreover, p66shc level was positively correlated with H_2O_2 in the tissue. We speculated that the reduction of p66shc led to the decrease of ROS production that promoted tumor progression.

The Impact of H_2O_2 on ROS Production, p66shc Expression, and Cell Survival Rate in B Lymphocytoma Cell Line

H_2O_2 -induced oxidative stress is a direct factor in leading to cell injury; thus, it is usually used to establish oxidative stress injury model. Therefore, we adopted different concentrations of H_2O_2 on B lymphoma cell line to observe its influence on the levels of ROS production, p66shc expression, and cell survival rate. As shown in Figure 2A, H_2O_2 markedly restrained cell survival in a dose-dependent manner. Furthermore, we selected two concentrations to explore the impact of H_2O_2 on p66shc expression. As shown in Figure 2B, H_2O_2 apparently upreg-

ulated p66shc expression as the dose gradually increased. The data on ROS level showed that it was significantly elevated following H_2O_2 treatment (Figure 2C).

The Influence of H_2O_2 on ROS, DNA Damage, and Cell Apoptosis in B Lymphocytoma Cell Line

To investigate the influence of H_2O_2 on DNA damage and cell apoptosis, we further tested 8-OHdG level and cell apoptosis changes. As shown in Figure 3A and B, H_2O_2 significantly enhanced cell oxidative damage and cell apoptosis levels compared with control.

The Effect of p66shc Knockdown on ROS, DNA Damage, and Cell Apoptosis in B Lymphocytoma Cell Line

To confirm whether p66shc could be treated as the target to promote DNA damage, we applied siR-

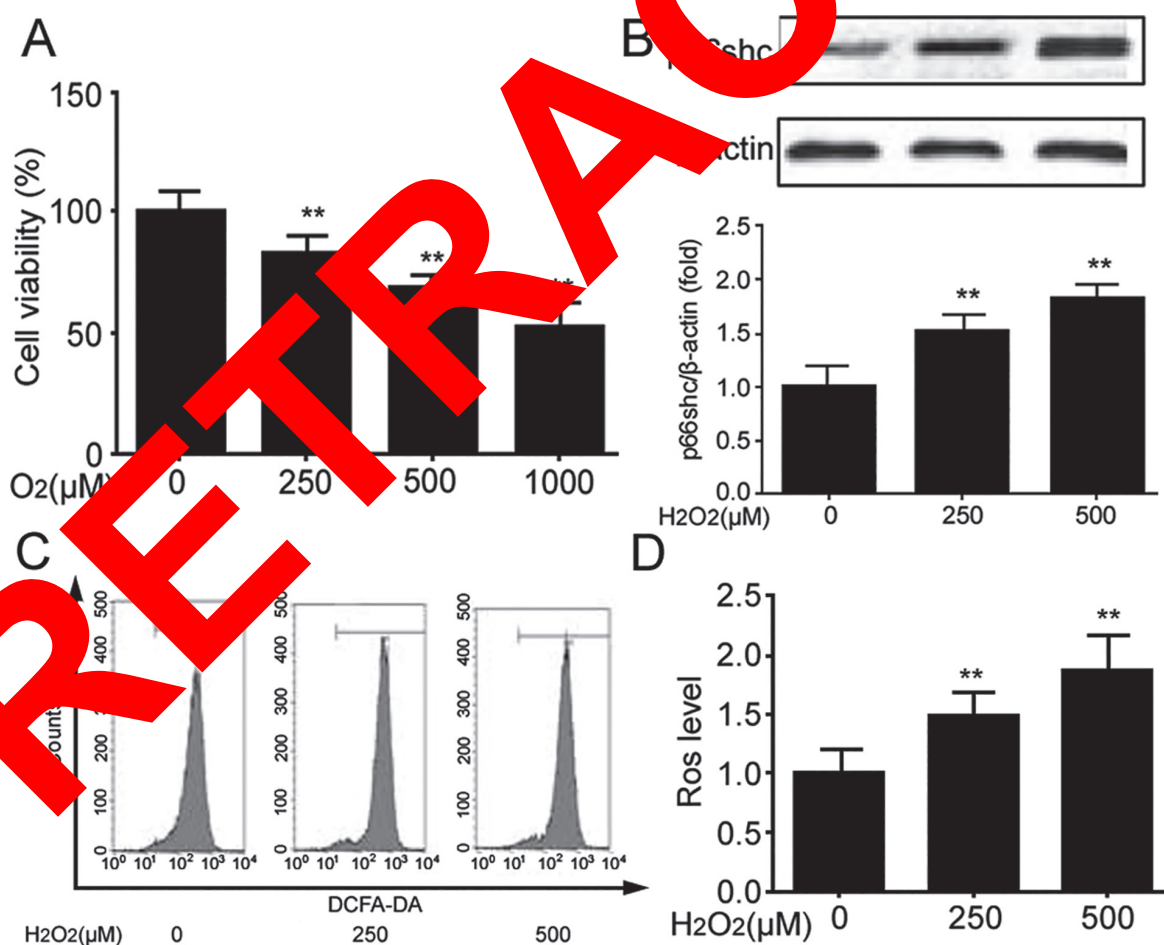


Figure 2. The impact of H_2O_2 on ROS production, p66shc expression, and cell survival rate in B lymphocytoma cell line. **A**, Cell survival rate; **B**, p66shc protein expression; **C**, ROS level. ** $p < 0.05$, compared with control.

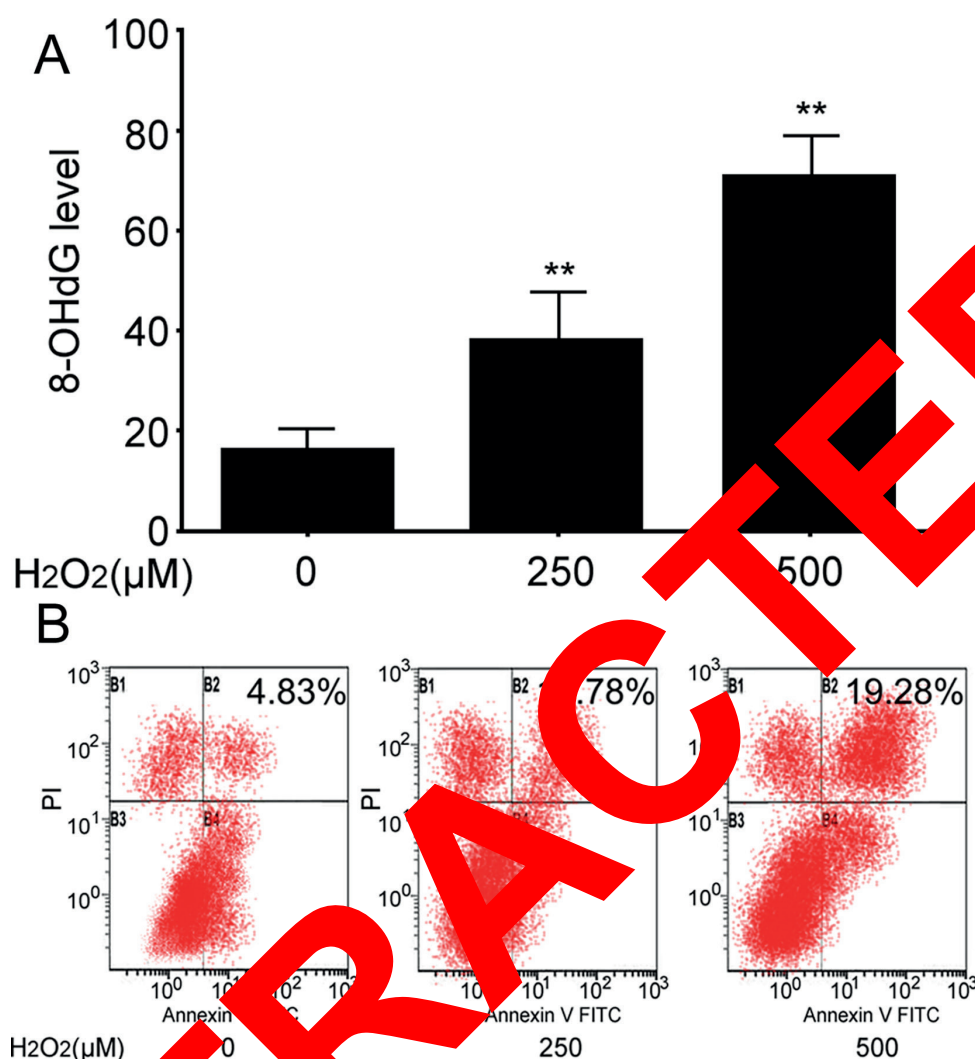


Figure 3. The influence of H₂O₂ on oxidative damage and cell apoptosis. **A**, 8-OHdG level; **B**, Cell apoptosis level. ** $p < 0.05$, compared with control.

NA to knock down p66shc level and explored ROS level, DNA damage and cell apoptosis in B lymphocytoma cell line. As shown in Figure 4A, p66shc expression was markedly reduced after transfection, indicating successful transfection with siRNA. As shown in Figure 4B, C, and D, ROS, DNA damage, cell apoptosis were apparently reduced after knock-down of p66shc. It revealed that H₂O₂ induced ROS up-regulation, DNA damage aggravation, and cell apoptosis enhancement, via p66shc overexpression.

The Impact of NAC on ROS, p66shc Expression, DNA Damage, and Cell Apoptosis in B Lymphocytoma Cell Line

Since the activation of p66shc can produce a large amount of ROS, we explored whether p66shc or ROS caused cell injury¹⁴. NAC, as the

precursor of the reductive glutathione, belongs to the oxygen free radical scavenger in the body, which can balance the ROS level without affecting the expression level of p66shc. After NAC treatment, we tested ROS, p66shc expression, DNA damage, and cell apoptosis in B lymphocytoma cell line. As shown in Figure 5A, no statistical difference of ROS level was found in cells intervened by H₂O₂ compared with that in normal control after NAC treatment. However, p66shc upregulation still existed in cells after treated by H₂O₂ and NAC, while it was reduced compared with H₂O₂ model group, indicating that the regulation of NAC on ROS level was independent of p66shc expression (Figure 5B). We further detected DNA damage and cell apoptosis. As shown in Figure 5C and D, 8-OHdG level

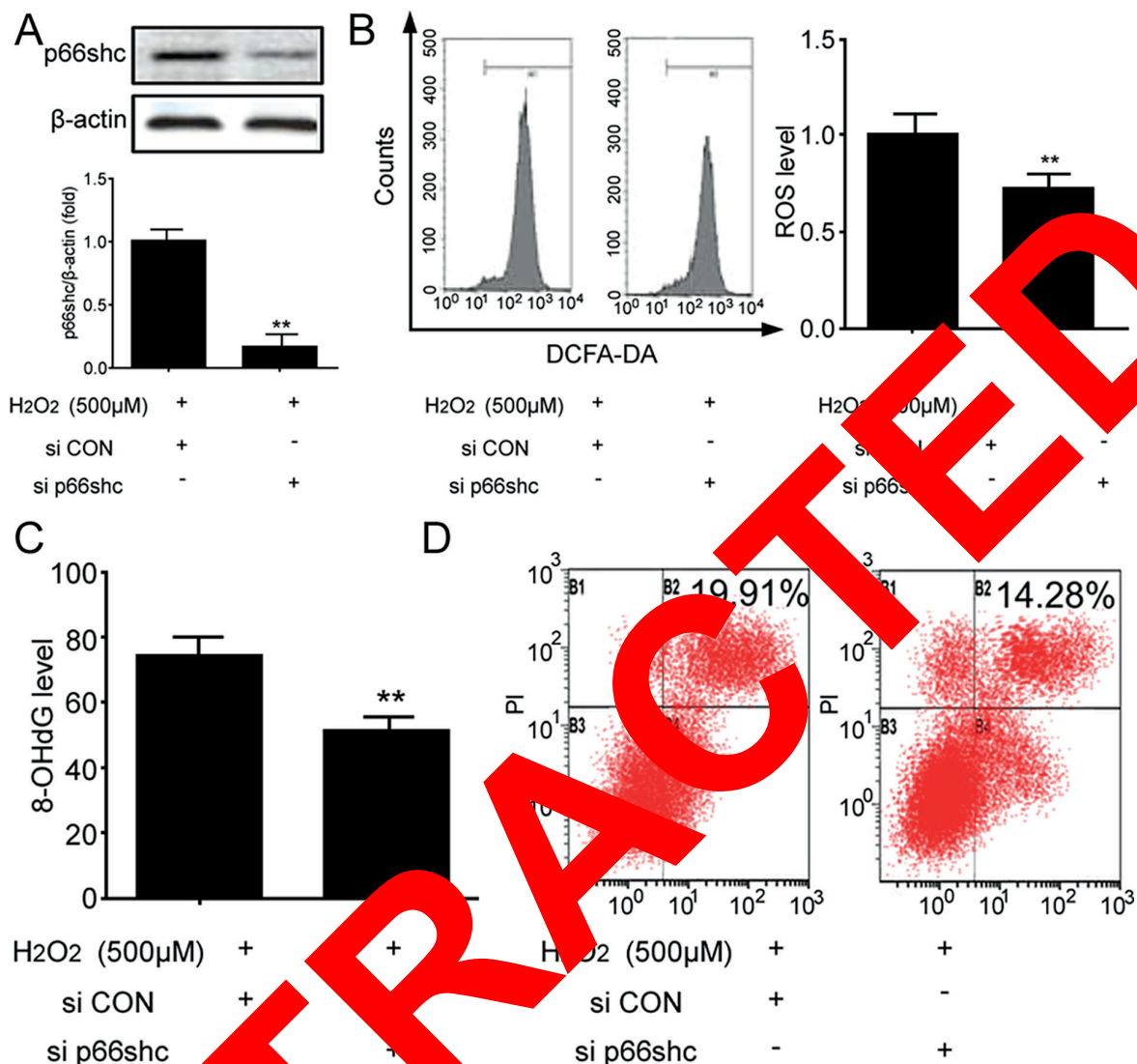


Figure 4. The effect of p66shc knockdown on ROS, DNA damage, and cell apoptosis in B lymphocytoma cell line. **A**, p66shc protein level; **B**, ROS level; **C**, 8-OHdG level; **D**, Cell apoptosis. ** $p < 0.05$, compared with control.

and cell apoptosis were significantly attenuated after intervention, suggesting that p66shc upregulation induced ROS release, aggravated DNA damage, and induced cell apoptosis. Therefore, p66shc could be treated as the target for the inhibition of cell apoptosis.

The Influence of p66shc Knockdown together with NAC on p66shc Expression, DNA Damage, and Cell Apoptosis in B Lymphocytoma Cell Line

Next, we measured the role of ROS elevation and p66shc overexpression in B lymphoma cell H₂O₂ damage. We used NAC and siRNA p66shc to determine their impact on B lymphoma cell.

As shown in Figure 6A and B, ROS level was not changed significantly after siRNA p66shc transfection in B lymphoma cell line treated by NAC. On the contrary, p66shc level was markedly declined. As shown in Figure 6C and D, compared with single H₂O₂ treatment group, DNA damage and cell apoptosis were significantly declined after NAC and siRNA p66shc treatment compared with single intervention.

Discussion

Cell oxidative stress level contributes to the chromosome stability¹⁵ and is closely associat-

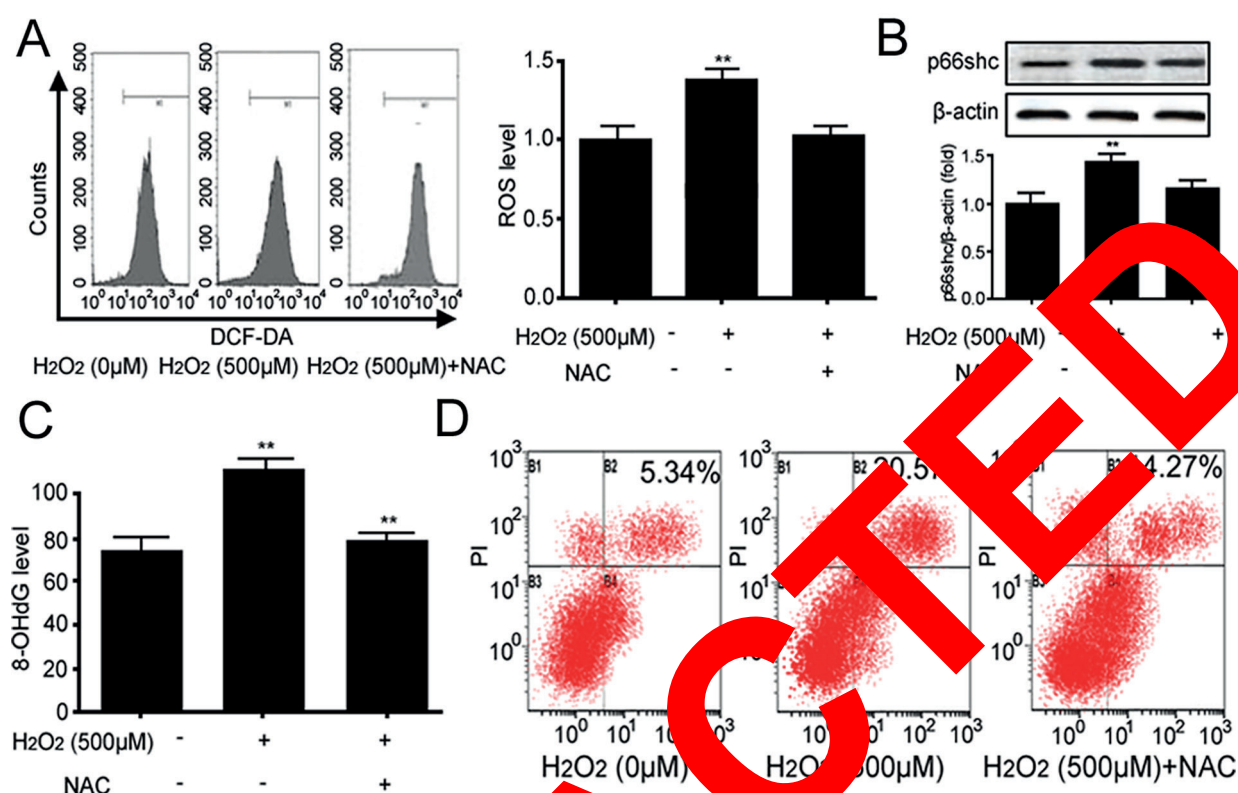


Figure 5. The impact of NAC on ROS, p66shc expression, DNA damage, and cell apoptosis in B lymphocytoma cell line. **A**, ROS level; **B**, p66shc protein level; **C**, 8-OHdG level; **D**, cell apoptosis. ** $p < 0.05$, compared with control.

ed with cell senescence and apoptosis. However, the mechanism remained to be studied. Recent researches revealed that NF- κ B, Ras gene mutation^{19,20} and inflammatory cell infiltration are related with oxidative stress in B lymphoma tissue, leading to the decrease of antioxidant enzymes such as H₂O₂, along with the induction of anaerobic metabolism and ROS expression. Therefore, lymphocytoma is generally in a state of low antioxidant and high oxidative stress. It was also pointed out that the survival of lymphoma cells under high ROS stress status depended on the extent of the oxidative damage and the sensitivity to oxidative stress. The elevated showed selectivity of killing effect on tumor cells²¹.

The main function of p66shc is to regulate cell oxidative stress reaction and the signal transduction of life cycle²². It is the key protein in recent research about cell oxidative stress, and also participates in the modulation of mitochondrial activity, oxygen metabolism, and cell oxidative damage²³. Current researches indicated that the expression of p66shc was associated

with tumor proliferation and migration. p66shc was found decreased expression in B lymphoma cells, which was similar to our results²⁴.

Meanwhile, we also found that after ROS production was increased by H₂O₂ intervention, the level of p66shc was elevated, and presented killing effect to the tumor cells. However, ROS production was decreased, DNA damage and cell apoptosis were attenuated after p66shc was knocked down by siRNA, indicating that p66shc overexpression killed cells via the production of ROS, which was in agreement with the previous finding of the effect of oxidative stress on cancer²⁵. At the same time, we further found that NAC also reduced p66shc expression, attenuated cell injury and apoptosis to a certain extent. The killing effect of H₂O₂ was further alleviated after NAC intervention and p66shc knockdown together, confirming that H₂O₂ mainly played its role through ROS-p66shc pathway. Therefore, the activation of ROS-p66shc in tumor cells was one of the possible anti-tumor strategies. However, its efficacy in animal experiment still needs further investigation.

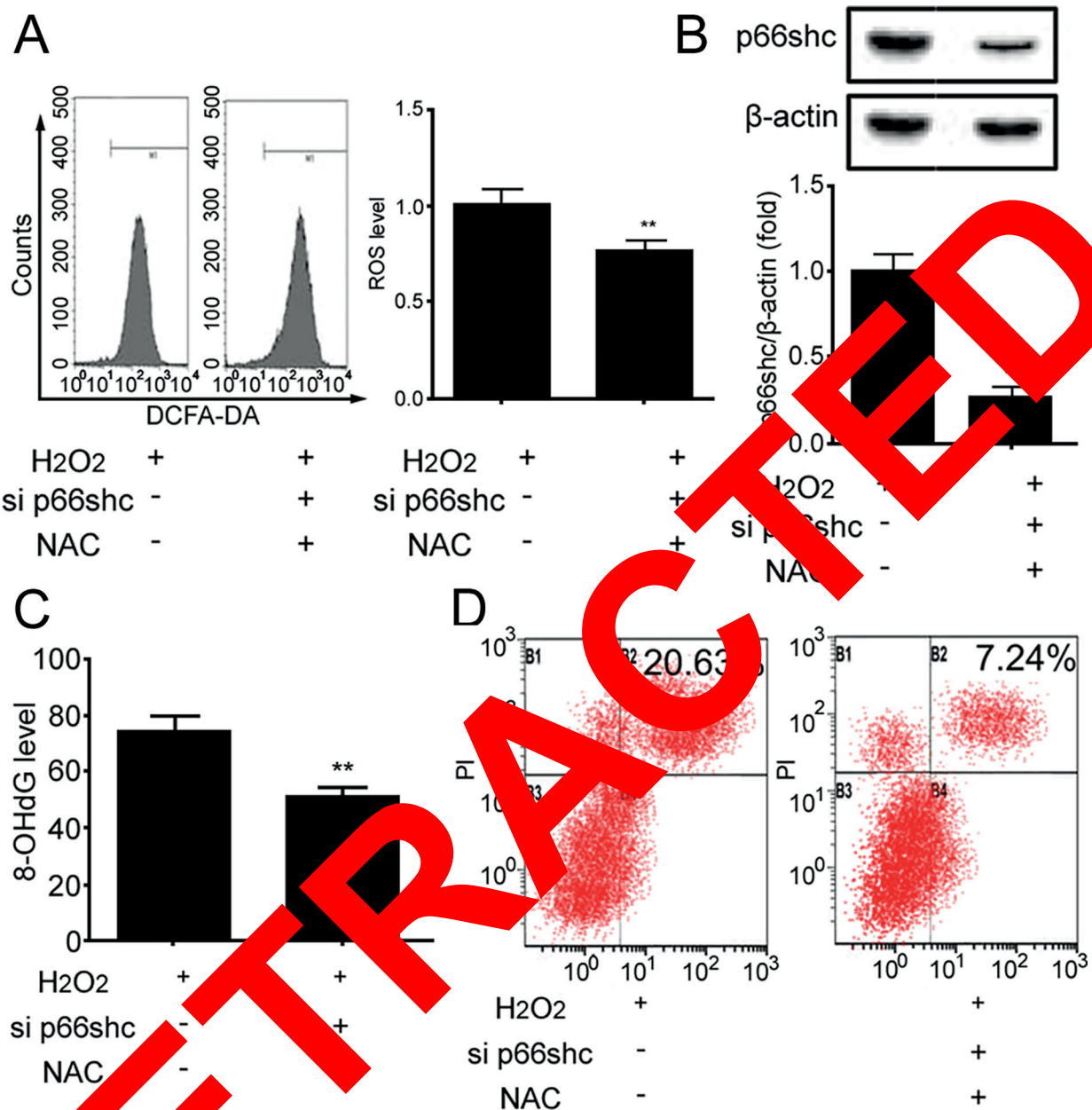


Figure 6. Effect of p66shc knockdown together with NAC on p66shc expression, DNA damage, and cell apoptosis in B lymphocytoma cell line. **A**, ROS level; **B**, p66shc protein level; **C**, 8-OHdG level; **D**, Cell apoptosis. ** $p < 0.05$, compared with control.

Conclusions

ROS promoted p66shc expression, induced DNA damage, and facilitated cell apoptosis. Up-regulation of p66shc could be treated as a new therapeutic target for the treatment of B lymphoma.

Acknowledgements

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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