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Polyoxometalate SbW9 regulates proliferation and apoptosis of NSCLC cells *via* PTEN-dependent AKT signaling pathway

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Abstract. – OBJECTIVE: To explore the influence of polyoxometalate SbW9 on proliferation and apoptosis of non-small cell lung cancer (NSCLC) cells and its mechanism.

MATERIALS AND METHODS: NSCLC cell lines A549 and PC9 were treated with 50 μM polyoxometalate. Then, the proliferation of NSCLC cells was detected *via* 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-tetrazolium hydroxide (XTT) assay and [illegible] formation assay; the apoptosis of NSCLC [illegible]s was detected *via* flow cytometry and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL); and the expression of apoptosis-related proteins, B-cell lymphoma-2 (Bcl-2) and Bcl-2 associated X protein (Bax), were detected *via* Western blotting. Moreover, the protein expression levels of phosphatase and tensin homolog deleted on chromosome ten (PTEN), phosphorylated-protein kinase B (p-AKT) and total AKT (T-AKT) were detected *via* Western blotting.

RESULTS: The polyoxometalate inhibited the proliferation of A549 and PC9 cells in a concentration-dependent manner (5[illegible]μM) ($p<0.05$), and it (50 μM) also inhibited the proliferation of both cells in a time-dependent manner (0-72 h) ($p<0.05$). The results of colony formation assay revealed that the polyoxometalate (50 μM) could significantly inhibit the colony formation of A549 and PC9 cells ($p<0.05$). The results of flow cytometry and TUNEL staining showed that the polyoxometalate (50 μM) significantly induced the apoptosis of A549 and PC9 cells ($p<0.05$). According to further studies, the polyoxometalate (50 μM) inhibited the expression of anti-apoptotic gene Bcl-2 and promoted the expression of pro-apoptotic gene Bax. Besides, the Western blotting results manifested that the polyoxometalate could activate the expression of PTEN and inhibit the phosphorylation of downstream AKT ($p<0.05$).

CONCLUSIONS: The polyoxometalate can activate the expression of PTEN to inhibit the phosphorylation of AKT, ultimately inhibiting the proliferation and inducing the apoptosis of NSCLC cells. Therefore, the polyoxometalate is expected to become a novel drug for the clinical treatment of NSCLC.

Key words
Non-small cell lung cancer, Proliferation, Apoptosis, Polyoxometalate.

Introduction

Lung cancer is one of the most common causes of cancer death in the world[1,2]. The number of deaths from non-small cell lung cancer (NSCLC) accounts for about 85-90% in the total from all types of lung cancer[3]. The therapeutic strategies of NSCLS include operation, radiotherapy, chemotherapy, targeted therapy, and combined therapy[4]. Although the potential chemotherapeutic efficacy of novel compounds on NSCLC has been confirmed in many studies, the sensitivity of high-grade NSCLC to chemotherapeutic drugs remains poor, and the exact mechanism of such a phenomenon has not been fully clarified[5].

The expression of phosphatase and tensin homolog deleted on chromosome ten (PTEN) is inhibited in a variety of tumor tissues, and it has been proved to be a cancer suppressor gene[6]. PTEN is a major inhibitor of phosphoinositide 3-kinase (PI3K), which, therefore, exerts an important regulatory effect on the PI3K/AKT signaling pathway and subsequent cellular biological behaviors[7]. The PI3K/AKT signaling pathway plays an important role in regulating cell survival, growth, differentiation, apoptosis, and autophagy[8]. The activated PI3K can mediate the phos-

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phorylation of AKT Thr 308 (catalytic domain) and Ser 473 (regulatory domain), and once AKT is activated, it can regulate a variety of important life activities, such as apoptosis and proliferation[9].

The polyoxometalate, as a transition metal-oxygen cluster, has the structural charges and size that can be regulated and possesses the potential to produce organic-inorganic hybrids[10]. Based on these chemical structures, researchers combined some biomolecules with polyoxometalate to form specific biomacromolecules, obtaining good effects in the treatment of disease. In this study, the effects of polyoxometalate SbW9 on proliferation and apoptosis of NSCLC cells were detected, and the molecular mechanism of SbW9 in affecting the proliferation and apoptosis of NSCLC was explored.

Materials and Methods

Materials and Cells

NSCLC cell lines A549 and PC9 were purchased from Shanghai Kanglang Biological Technology Co., Ltd. (Shanghai, China), and SbW9 was synthesized by the Chemical Research I tute of the Chinese Academy of Science bovine serum (FBS) was purchased from (Rockville, MD, USA). Roswell Park Mem Institute-1640 (RPMI-1640) medium contain penicillin and streptomycin w sed fro Gibco (Rockville, MD, USA e ce ere cultured in the medium at 37

Cell Intervention

NSCLC cells and PC9 inoculated into a 96-well 10^4/mL) a ted with SbW9 in diff con ations (0.1, , 5, 10, 20, 50 and 10 M) after 4 After treatment for differen e, 50 mL 2,3-b methoxy-4-nitro-5-su enyl) [(phenylam o)carbonyl]-2H-t etr m h ide (XTT) was added for incubatio or an 2 h, and the absorbance rea 50 nm

W ern Bl g

treatment of the two kinds of NSCLC SbW9 for 72 h, the total protein was xtracted, and the specific steps are as follows: (1) culture solution in the medium was discarded f and cells were washed with PBS for 3 times. (2) 1000 μL lysis buffer was added into every dish and fully vibrated for 20 min. (3) The cells at the bottom of the dish were scraped off using a brush and placed into the Eppendorf (EP) tube. (4) The cells collected were lysed using an ultrasonic pyrolyser for about 15 s. (5) After standing for 15 min, the cells were centrifuged at 12000 rpm for 0.5 h. (6) The supernatant was taken and placed into the EP tube, the protein concentration was detected *via* ultraviolet spectrometry, and all the pr ples were quantified to be the same c itration. (7) The protein was sub-packaged placed in the refrigerator at -80°C. After the protein was extracted from NSCLC lls, S Do-decyl Sulfate-Polyacrylami el Electro (SDS-PAGE) was perfo Then e prot the gel was transferre o a n inylidene di-fluoride (PVDF) m bra e, Bas Switzerland) and inc ed wit prima tibody at 4°C overni en, it was i gain with the goat a o condary an dy in a dark place for 1 h. The p n band was scanned and quanti ing the Od scanner, and the level of n to be detected w orrected using glyce ehyde-3-phosphate dehydrogenase (GAPDH).

C ny Forma n Assay

ells in n group were cultured till logarithm phase, and digested with 0.25% trypsin into single cell suspension (the proportion le cells >95%). Then, the suspension was d into a 6-well plate (about 500 cells/well) and added with 2 mL 1640 medium per well, and the medium was replaced once every 48 h. After 10 d, the cells were fixed with formaldehyde and stained with crystal violet, and the number of colonies in each well was counted.

Detection of Apoptosis Via Flow Cytometry

The cells in the logarithmic growth phase were taken, digested with 0.25% trypsin-EDTA (ethylenediaminetetraacetic acid) into the cell suspension, and inoculated into the 6-well plate. The sample was loaded and the apoptosis rate was detected according to the operation steps of the Annexin V-FITC PI (Propidium Iodide) apoptosis assay kit (Beyotime, Shanghai, China).

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End Labeling (TUNEL) Staining

The cells on the slide were fixed in fixing solution for 1 h and washed with PBS for 3 times. After transparentization, TUNEL reagent was prepared and two negative controls were set. After staining, the cells were observed, photographed and counted under a fluorescence microscope.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 software (IBM, Armonk, NY, USA) was used for the analysis of all data. Measurement data were expressed as mean ± standard deviation, and the *t*-test was used for the comparison of data between the two groups. $p<0.05$ suggested that the difference was statistically significant.

Results

Polyoxometalate SbW9 Inhibited Proliferation of NSCLC Cells in a Concentration-Dependent Manner

After treatment of two kinds of NSCLC cell lines with SbW9 in different concentrations (0.1, 1, 5, 10, 20, 50, and 100 μM) for 72 h, the cell proliferation in each group was detected *via* XTT assay. As shown in Figure 1, SbW9 (5, 10, 20, 50, and 100 μM) could significantly inhibit the proliferation of A549 and PC9 cells in a concentration-dependent manner ($p<0.05$). SbW9 in a concentration of 0.1 and 1 μM had no influence on the proliferation of NSCLC cells ($p>0.05$). Ther[illegible] in the subsequent experiments, SbW9 in [illegible] centration of 50 μM was selected for verific[illegible].

Polyoxometalate SbW9 Inhibited Proliferation of NSCLC Cel[illegible] a Time-Dependent Man[illegible]

As shown in Figure [illegible]e prol[illegible]ation of A549 cells significantl[illegible] d[illegible]d a[illegible] with SbW9 (50 μM) [illegible]4, 3[illegible]d 72 h com-pared with that in [illegible]trol group[illegible].05). SbW9 (50 μM) could [illegible]hibit the p[illegible]ration of PC9 cells at 12, 24, 36, 48, and 72 h. Therefore, SbW9 in a concentration of 50 μM was selected in the subsequent experiments, and NSCLC cells were stimulated for 72 h.

Polyoxometalate SbW9 Inhibited [illegible] Formation of NSCLC Cells

Furthermore, the proliferation [illegible] kinds of NSCLC cell lines was detected *via* [illegible]ny formation assay. The results re[illegible]ed tha[illegible] A549 cell lines, the number of c[illegible]ies in SbW[illegible] (44.82±6.32) was signifi[illegible]ly sm[illegible]r than in control group (22[illegible]11.46[illegible]<0.05). The same was true in P[illegible] c[illegible](40.05±[illegible]) *vs.* (199.83±10.32)] ([illegible].05) (F[illegible]3).

Polyoxo[illegible]SbW9 Ina[illegible] Apoptosis of NS[illegible]Cells

Th[illegible]lts of flo[illegible]tometry showed that the [illegible]yoxometalate Sb[illegible] exerted a signifi-c[illegible]pro-apoptotic effect on both A549 and PC9 c[illegible] The apop[illegible]s rate in control group and S[illegible] group in [illegible] two kinds of cell lines was (1.6[illegible]4)% [illegible]4.78±2.11)% and (1.49±0.34)% *vs.* (4[illegible]%, respectively ($p<0.05$) (Figure 4), indicating that the polyoxometalate SbW9 can [illegible]te the apoptosis of NSCLC cells.

Influence of Polyoxometalate SbW9 on Apoptosis-Related Proteins in NSCLC Cells

The results of Western blotting showed that after treatment with polyoxometalate SbW9, the expression of pro-apoptotic gene Bax was up-regulated, while the expression of anti-apoptotic gene Bcl-2 was significantly inhibited in the two

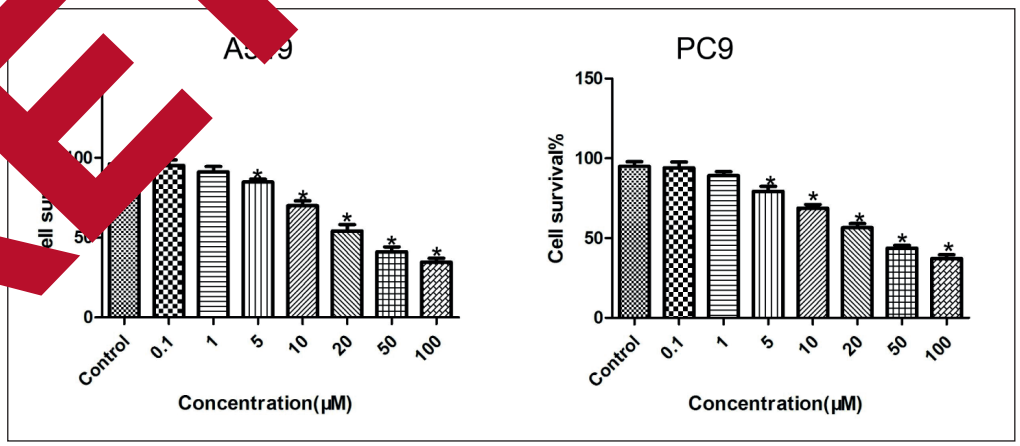

Figure 1. Polyoxometalate SbW9 inhibits proliferation of NSCLC cells in a concentration-dependent manner. Control: control group, *$p<0.05$: There is a statistically significant difference compared with control group.

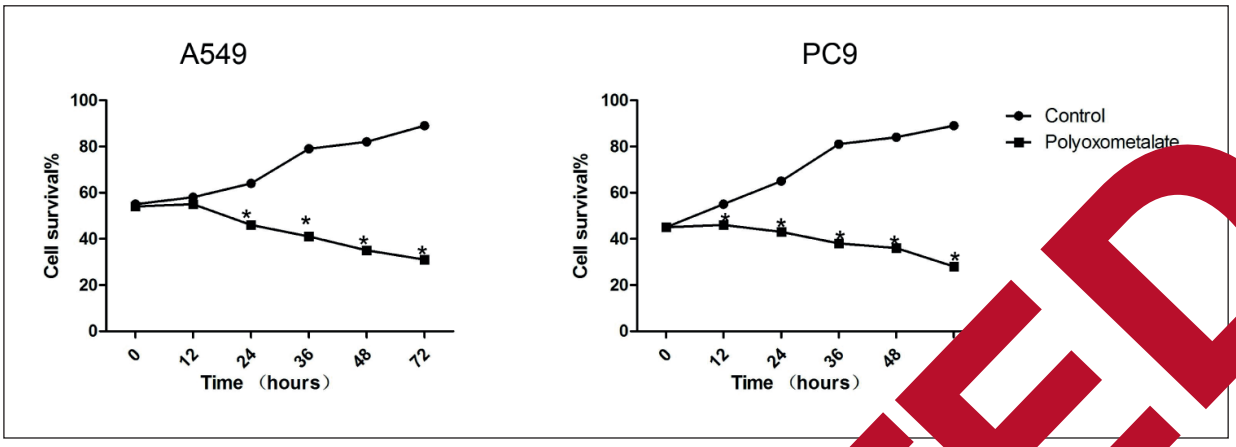

Figure 2. Polyoxometalate SbW9 inhibits proliferation of NSCLC cells in a time-dep manner. C cont up, Polyoxometalate: polyoxometalate SbW9 group, *p<0.05: There is a statistically significa nce compare l group.

kinds of cell lines (p<0.05) (Figure 5), suggesting that the inducible effect of polyoxometalate SbW9 on apoptosis of NSCLC cells is dependent on the Bcl-2 pathway.

TUNEL Staining of NSCLC Cells in Each Group

To further detect the apoptosis in each g more intuitively, TUNEL staining was per for cells in each group and the TUNEL-po e cells were counted. As shown in Figure 6,

apoptosis r e in co group and SbW9 group in A54 PC9 cell was (2.34±0.77)% *vs.* (18 .32)% and (3.81±1 % *vs.* (24.77±1.92)%, r ctively (p<0.05).

I ence of P oxometalate Sb n PT AKT Signaling Path SCLC

Considering the important role of the PTEN/ signaling pathway in the tumor, whether the e of polyoxometalate SbW9 on biological

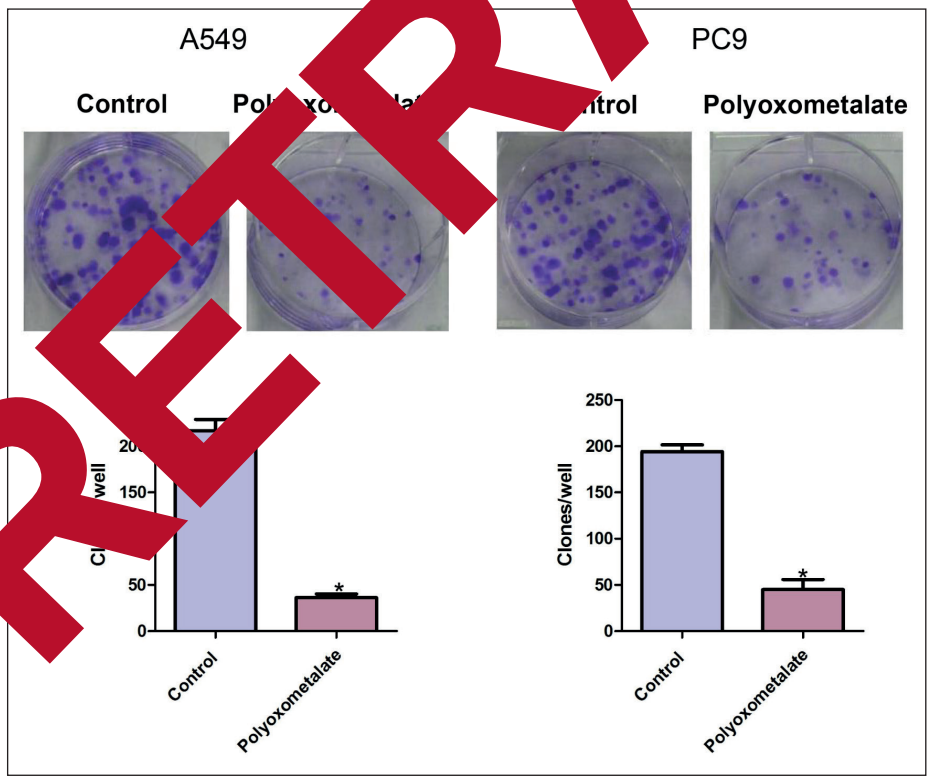

Figure 3. Polyoxometalate SbW9 inhibits colony formation of NSCLC cells. Control: control group, Polyoxometalate: polyoxometalate SbW9 group, *p<0.05: There is a statistically significant difference compared with control group.

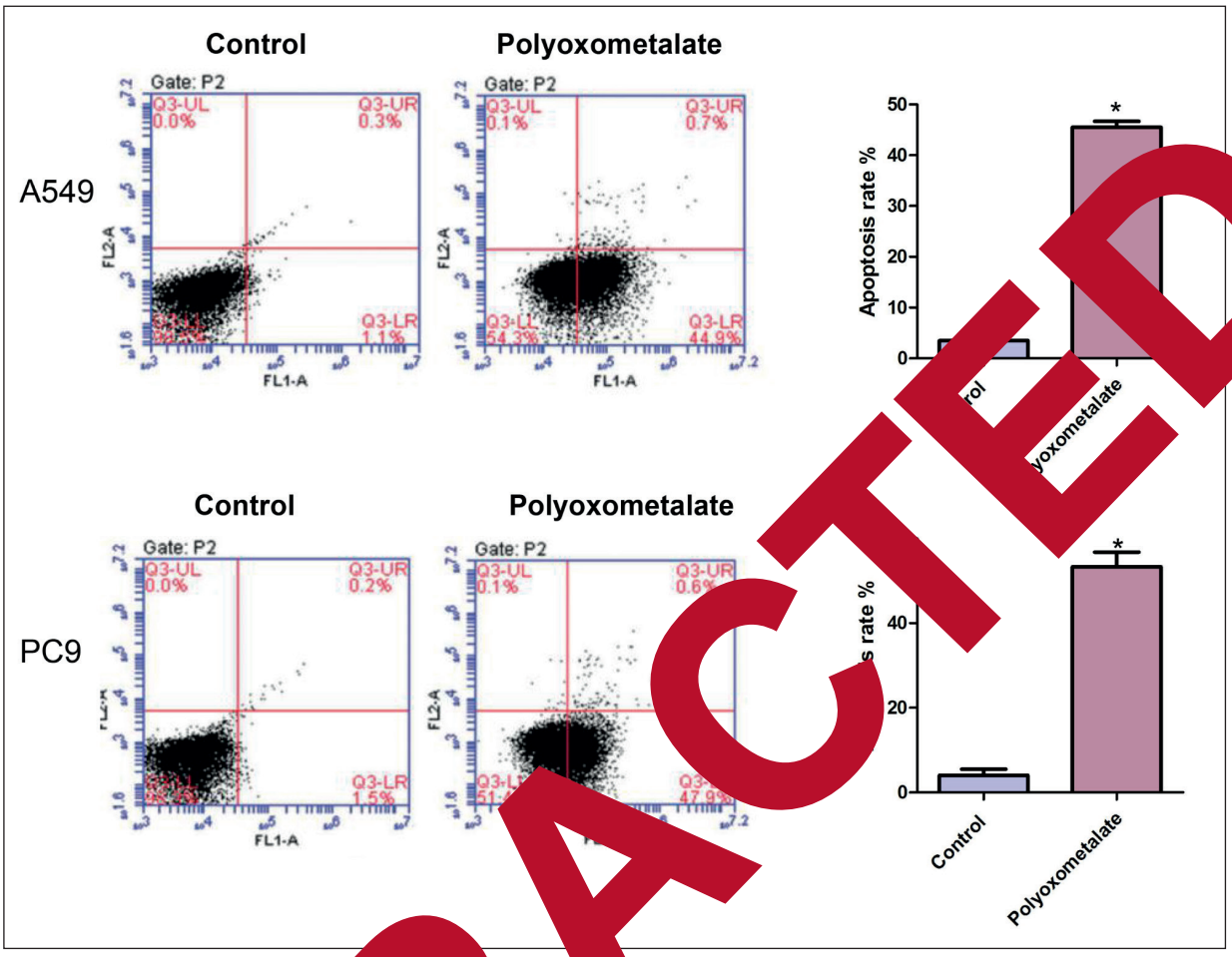

Figure 4. Polyoxometalate SbW9 ... notes a ... tosis of N... C cells. Control: control group, Polyoxometalate: polyoxometalate SbW9 group, *p<0.05: T... a statist... y significa... ifference compared with control group.

behaviors of N... ells is dep... t on the PTEN/AKT s...aling ...way was de...ected. The expression ...PTEN and ...T in each group was detected ...d it was found ...the polyoxometalate ...9 cou...activate the PTEN expression an...bit th...osphorylation level of AKT in the t... of cel...es (p<0.05) (Figure 7), ...catin... the ...N/AKT signaling pathway p...an im...e in the anti-proliferative and pro...optotic ...ts of polyoxometalate SbW9.

Discussion

...ung cancer is one of the most common and severe cancers, and the number of deaths from lung cancer is larger than the total number of deaths from breast cancer, colon cancer, and prostate cancer every year[11]. SCLC and NSCLC are two major types of lung cancer. Despite the great progress in the diagnosis and treatment of lung cancer, neither treatment nor prognosis of lung cancer is satisfactory, which is related to the high recurrence and mortality rates[12,13]. There is increasing evidence that the proliferation and apoptosis of lung cancer cells have a great influence on the incidence and prognosis of lung cancer[14]. Therefore, inhibiting the proliferation and promoting the apoptosis of lung cancer cells in time and effectively are of great significance in delaying the progression of lung cancer.

Apoptosis is mediated through the death receptor pathway (exogenous) or the mitochondrial pathway (endogenous), thereby eliminating the damaged cells and keeping the homeostasis[15]. In both pathways, poly ADP-ribose polymerase-1 is cleaved and caspase 3/7 is activated to respond to DNA damage stress in cells[16]. Therefore, target-

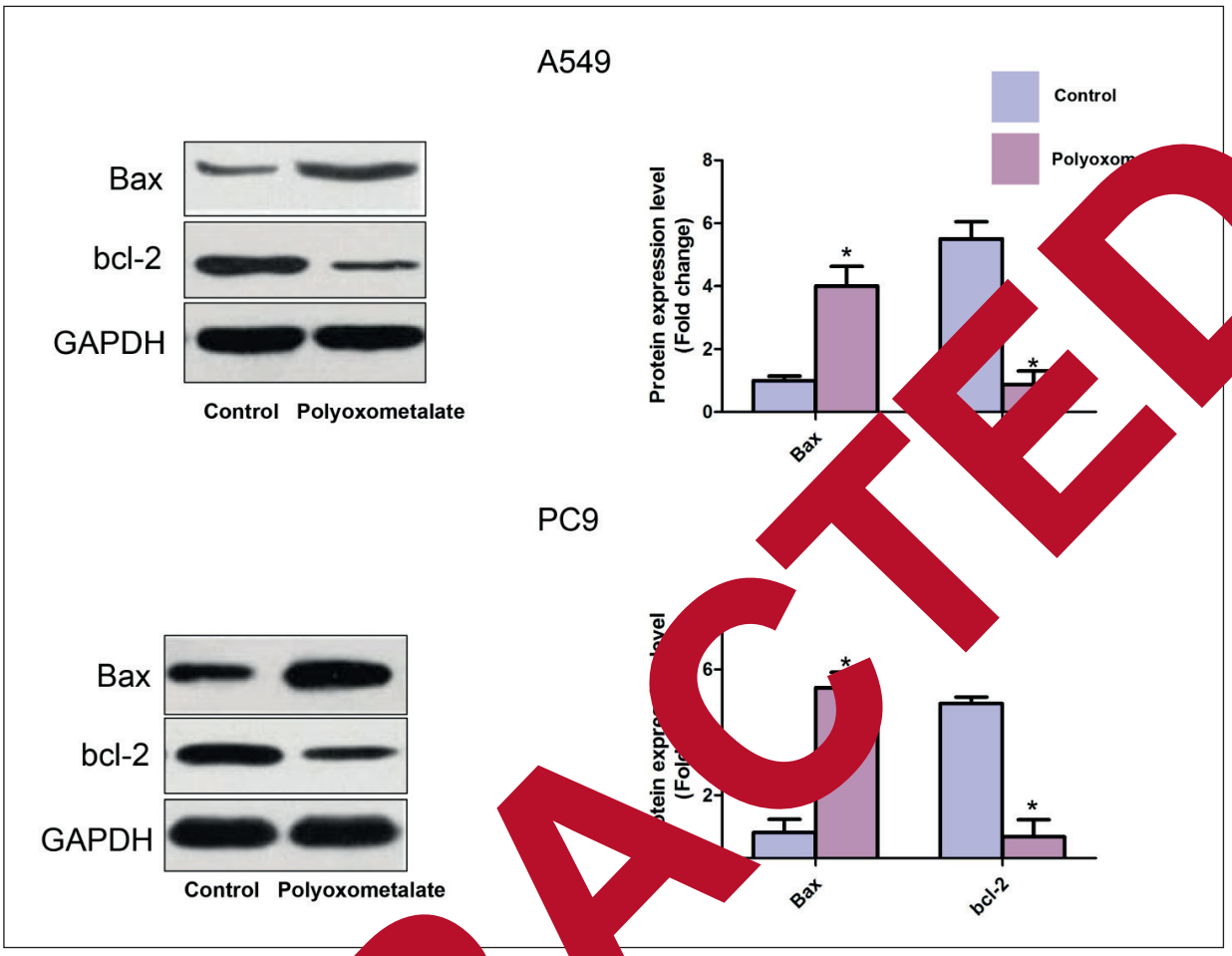

Figure 5. Influence of polyoxom te SbW apoptosis ed proteins in NSCLC cells. Control: control group, Polyoxometalate: polyoxometalate Sb oup, *p There is a tatistically significant difference compared with control group.

ing the program death thro egulating apoptosis and toph as become one of the promising thods in c therapy. The Bcl-2 family ein is the regula factor of apoptosis, many mbers can egulate apoptosis, su Bax Bak, important pathways for caspa ed cell th. The Bcl-2/Bax ratio im t det nant of apoptosis[17,18]. Cur- the activation of the PI3K/AKT sig ng pati has been observed in various t d the activation of this pathway can mote survival and proliferation of cancer cells[19]. PTEN negatively regulates the AKT aling pathway through dephosphorylation o 3 to produce PIP2. There is often a genetic mutation in the PTEN gene in cancer tissues, and its protein expression is inhibited. Besides, PTEN can also negatively regulate the cell proliferation[20]. For example, in prostate cancer cells, PTEN can inhibit the cell proliferation and promote the apoptosis through down-regulating the IGF-IR expression on cell membrane[21]. In addition, in pancreatic cancer cells, IGF-1-mediated inhibition on PTEN can enhance the cell invasion and proliferation through activating the PI3K/AKT signaling pathway[22]. In NSCLC, the low expression of PTEN and excessive phosphorylation of AKT have close correlations with the poorer prognosis of NSCLC patients[23]. In this study, two kinds of NSCLC cell lines were stimulated with polyoxometalate SbW9 in different doses for different time in *in-vitro* experiments. It was found in XTT assay and colony formation assay that the polyoxometalate SbW9 (50 μM, 72 h) significantly inhibited the proliferation and colony formation of the two kinds of NSCLC cell lines. At the same time, the apoptosis in each group was further detected *via* flow cytometry and TUNEL

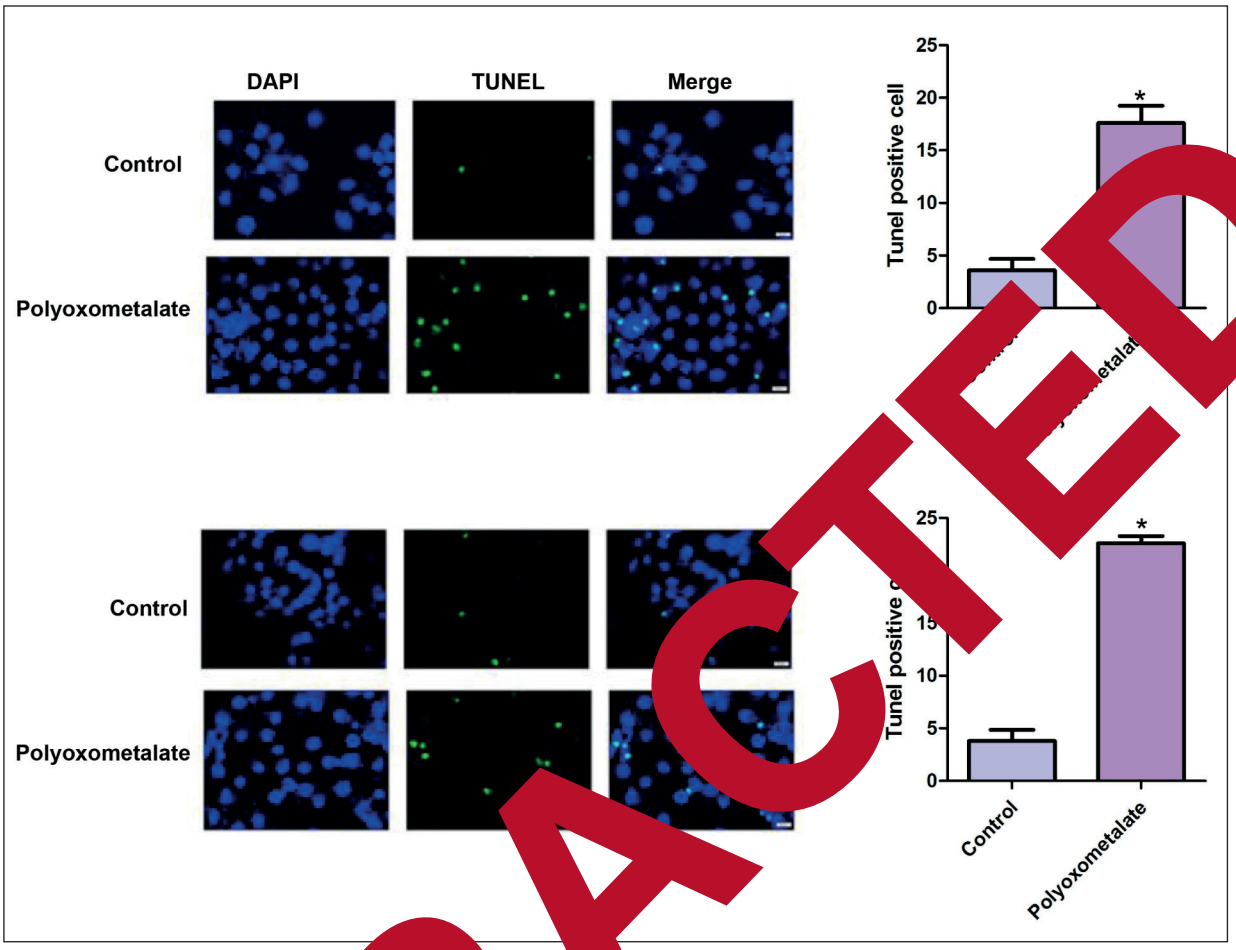

Figure 6. TUNEL staining of NS cells ch group trol: control group, Polyoxometalate: polyoxometalate SbW9 group, *p<0.05: There is a statis y signifi difference pared with control group.

staining, and the ts showed the polyoxometalate SbW vell induce apoptosis of NSCLC ce Fur ore, the W stern blotting show hat the ind e effect of polyoxometalat W9 on apoptosi SCLC cells may be d dent o he Bcl-2 pa way. Finally, the ex on of classical PTEN/AKT signaling pathw etected d it was found that the oxo ate Sb could effectively enhance t otein n of PTEN and significantly inh the ph orylation of AKT, thus explaini lecular mechanism of anti-proliferative ect W9 on lung cancer cells. Moreover, e experiments have revealed that the reason for low expression of PTEN is closely related to t ethylation in its promoter region[24]. Therefore, it is speculated that the regulatory effect of polyoxometalate SbW9 on proliferation and apoptosis of NSCLC cells may be related to its inhibition on PTEN gene methylation, and more in-depth experiments are needed for verification. However, there were still some deficiencies in this experiment: 1) The animal experiments were not designed, and 2) the anti-cancer effect of the polyoxometalate SbW9 was not verified using PTEN or AKT inhibitors.

Conclusions

This investigation revealed the anti-proliferative and pro-apoptotic effects of polyoxometalate SbW9 on NSCLC cells for the first time, whose mechanism may be mediated by the PTEN/AKT signaling pathway.

Conflict of Interests

The Authors declare that they have no conflict of interests.

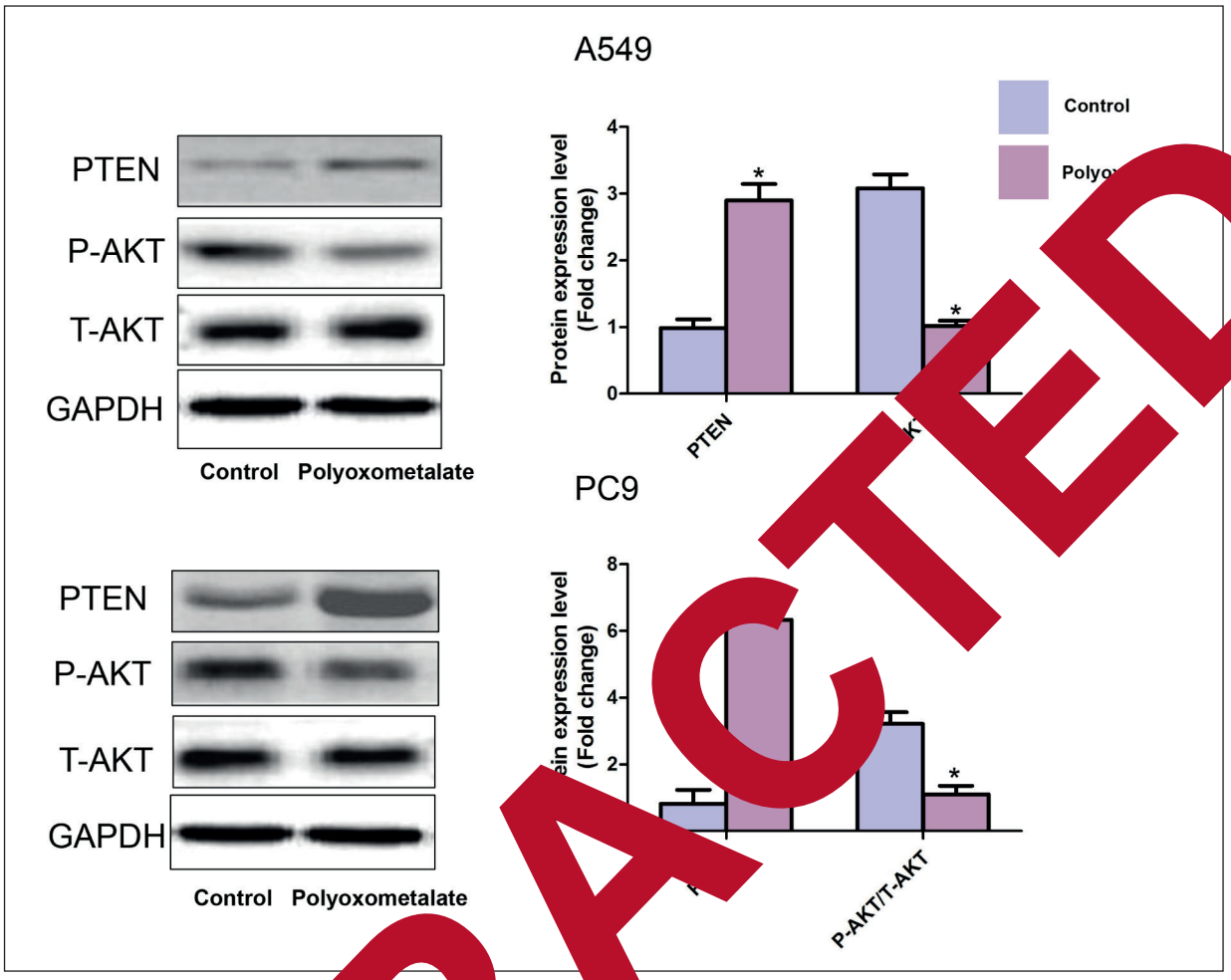

Figure 7. Influence of polyoxo[illegible]ate SbW[illegible] PTEN/AK[illegible] signaling pathway in NSCLC. Control: control group, Polyoxometalate: polyoxometalate Sb[illegible]up, *[illegible]a statistically significant difference compared with control group.

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