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USP22 down-regulation facilitates human retinoblastoma cell aging and apoptosis via inhibiting TERT/P53 pathway

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Abstract. – OBJECTIVE: Retinoblastoma is the most common malignant intraocular tumor in childhood, and still lacks effective treatment. The immortality of tumor cell can be attributed to elevated telomerase activity, which has been considered as tumor marker and treatment get. USP22 is one of the important targets fo hibiting tumor growth, but clear illustration garding its effects of telomerase, tumor cell i mortality and retinoblastoma cell ag or apop tosis via suppressing TERT/P53 athway remains to be elusive.

MATERIALS AND METHO MTT s used for describing cell prolifera a blot was used to test tein ession lev- el of USP22, TERT an 53. RT-q was used to test USP22 mR el, followe TRAP method to detec om e activity. low cytometry and comet assay used to quantify cell apoptosi nd DNA dam Cell aging was measured p-galactosidase.

RESU . The erexpression of USP22 significant nha ed cell proliferation potency and telom activity evated TERT expression level, i ted expression and cell ag- ell as sed cell apoptosis or DNA damag Down-r ation of USP22 contributed to or effects.

NS: USP22 played an important in retinoblastoma cell proliferation/aging a poptosis. The reduction of USP22 expression ilitated human retinoblastoma cell aging or apoptosis via suppressing TERT/P53 signal pathway. USP22, thus, may work as a target for treating retinoblastoma.

Key Words:
USP22, Retinoblastoma, Telomerase, TERT/P53.

Introduction

tinoblasto is a kind of malignant tumor de l from bryonic retinoblastoma, and is the m on primary intraocular cancer associated with family inheritance. Currently, major nt approaches for retinoblastoma include rgical resection of the eyeball or conservative treatment, although both efficacy and prognosis need to be further improved. Tumor cell immortalization is one of the most distinguished features apart from normal cells. The possible reason is elevated telomerase activity, which has been realized as a tumor marker and satisfactory target of tumor treatment[1]. Telomere is a protein-DNA complex at chromosome ending, and can be shortened with repeated mitosis under normal circumstance to induce cell aging[2,3]. The inhibition of telomerase activity can impede tumor cell immortalization. Moreover, differential expression of telomerase between cancer cell and normal cell could reduce toxic effects of drugs[4,5]. Ubiquitin specific proteinase 22 (USP22) belongs to novel human de-ubiquitination gene with the function of predicting human cancer metastatic potency and treatment outcomes[6]. Previous studies[7,8] showed that abnormal expression of USP22 was correlated with prognosis of infiltrative breast cancer or colorectal carcinoma. USP22 can modulate various cellular functions including cell growth/differentiation and cycle regulation, and is correlated with cellular gene transcriptional activation and signal transduction[9-11]. Another study[12] showed that the

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silence of USP22 gene inhibited prostate cancer cell proliferation and induced cell cycle arrest. USP22 depletion also facilitated human brain glioma cell apoptosis[13]. USP22, as an important target of tumor growth inhibition, however, requires to be further studied regarding its role in facilitating human retinoblastoma cell aging or apoptosis via suppressing TERT/P53 signal pathway. This study aimed to observe the effect of USP22 on TERT/P53 signal pathway, and its effects on retinoblastoma cell DNA damage, apoptosis and telomerase activity. The knowledge of USP22 in tumor cell aging and apoptosis may provide evidence for clinical exploration of novel drug target against intraocular tumors.

Materials and Methods

Reagents and Drugs

MTT reagent was purchased from Sigma-Aldrich (St. Louis, MO, USA). One-step RT-qPCR kit was purchased from Quanshijin Bio (Beijing, China). Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS) and d... antibiotics were purchased from Gibco (R... ville, MD, USA). Human gastric cancer cell l... MGC-803 was purchased from Cell Bank ... Chinese Academy of Sciences (B... China). Anti-rabbit CDKL1 polyclonal a...body ...as purchased from Abcam (Camb...ge, M... USA). Goat anti-rabbit IgG (H+L) an... were purchased from P...eintec... Wuhan, Hubei, China). Lentivi... transfection ...ctor was purchased from J... e (Shanghai, China). Annexin V-Propidium iod... (AV-PI) staining kit was purchase...om Beyotim... Shanghai, China). Telomeras...tivity assay kit wa... purchased from Kaiji Bi...ch (Sh...ghai, China). TUNEL assay kit was ...d fro... Beyotime (Shanghai, Chi...). AV... ouble ...ining kit was purchased ...osai B... Shanghai, China). 8-OHdG assay ... was pu...ased from Huamei (Luoyang, Hen... China). HEK293T cell was purchased ... Cell B...k of Chinese Academy of Sci... (Beijing, China). Lipofectamine 2000 transfect... reagent was purchased from Invitrogen (Carlsbad, CA, USA).

Cell Culture

Human retinoblastoma cell line HXO-Rb_{44} was purchased from the Tumor Institute, Harbin Medical University. Cells were cultured in 1640 medium containing 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin in a 37°C incubator with 5% CO_2 in suspension. Cell morphology and growth status were observed under an inverted phase contrasted microscope. Those cells at log-growth phase were used for experiments.

Lentiviral Transfection

Lentiviral vector for USP22 k...kdown ... over-expression was synthesized t...d on CDKL... gene sequence (Jikai Gene, Sh...ngha...hina), an... was sub-cloned into pGCS...-GFP pl...d wi... Age I/EcoR1 sites. Whe...ell den...ty rea...0-80%, Lipofectamine ...00 in...ated lent...viral particles was used to t... HEK2...T cells. 72 h later, USP2... nockdo...nd o...expression lentivirus we...rvested fro...ure medium, enriched b...Cen...on Plus-20 ...ntrapure centrifuge, and were ke...t -80°C. Retinoblastoma cells ... inoculated ... 6-well plate. When re...ing 80% confluenc..., lentivirus was added b...d on viral t...er. On the next day, normal cult... medium w...used for observing transfection effi...cy und...luorescent microscope.

MTT Assay

...oblastoma cells at log-growth phase were ...djusted and inoculated into 96-well plate (100 μL each well). After transfection by lentiviral vector for USP22 knockdown or over expression for 6 h, the plate was centrifuged, and normal medium was added for 18 h incubation. Each group included 8 paralleled replicated wells. After 24 h incubation, each well was added with 10 μL MTT solution for 4 h continuous incubation. Under microscopic observation, violet crystal was formed. The supernatant was discarded, with addition of 150 μL triple buffer into each well for mix well to solve the crystal. After 12-15 h, absorbent values were measured at 490 nm for calculating cell viability.

Western Blot

Retinoblastoma cells after transfection were centrifuged and were mixed with 100 μL lysis buffer (including proteinase inhibitor, phenylmethylsulfonyl fluoride (PMSF), phosphatase inhibitor). After homogenization, the mixture was centrifuged at 14000 g for 10 min. The supernatant was collected to obtain the whole protein solution. After adding equal volume of loading buffer, the mixture was boiled for 5 min for denaturing. The sample was loaded into sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and was transferred to polyvinylidene fluoride (PVDF)

membrane, which was blocked in defatted milk powder for 1 h. Antibody against USP22 (1:1000), p53 (1:1000), TERT (1:1000) and GAPDH (1:1000) was used for hybridization at 4°C overnight. After Tris-buffered saline (TBS) rinsing for three times, secondary antibody diluted at 1:1000 was added for 37°C incubation for 1 h. ECL development was the performed.

Total cell RNA Extraction and Real-time qPCR

All cells were cultured for 48 h after transfection. Culture medium was removed, and cells were washed with phosphate-buffered saline (PBS) for three times. 1 mL Trizol was added to each well for 5 min iced incubation. Pipette tip was used for homogenization. Lysate from each well was collected and mixed with 200 μL chloroform for 15 s rough mixture. After room temperature incubation for 3 min, the mixture was centrifuged at 12000 g at 4°C for 15 min. The upper aqueous phase was carefully transferred and mixed with 500 μL isopropanol for 10 min room temperature incubation. By 4°C centrifugation at 12000 g, the supernatant was discarded, triple washing in 1 mL ethanol. The supernatant was carefully removed eventually, and mRNA was re-suspended in 20 μL DEPC water. USP22 primers were synthesized by Sigma-Aldrich (St. Louis, MO, USA)[14,15]. Sequence were forward primer, 5'-ATCTC CGTCC TCG TC-3'; reverse primer, 5'-ATCTC CGTC TA GAPDH was used as internal reference (forward primer, 5'-TGACT AC AGC CACCC A-3'; reverse primer CCC TGTT TGTA GCCAA A-3'. One step RT-PCR was performed in a 50 μL system following manual instruction. Reaction conditions were: 50°C for 30 min and 95°C for min, followed by 40 cycles each containing 50 s, 55°C 30 s and 72°C 50 s, with ending 72°C elongation for 5 min. After RT-amplification, amplifying curve and melting curve of RT-PCR were confirmed. Ct values of target were compared against Ct values of internal reference gene. Gene expression was then quantified by $2^{-\Delta\Delta Ct}$ method.

Cell Apoptosis Assay

Cell apoptosis was tested by AV-PI staining. Cells were inoculated into 6-well plate. After transfection using lentivirus, cells were centrifuged and changed for normal culture medium. AV and PI staining buffer were sequentially added for iced dark incubation in 10 min. Cells were then loaded into FACS Calibur flow cytometry (BD, San Jose, CA, USA) for online testing. All experiments were repeated for three times.

Telomerase Activity Assay

This study utilized telomer repeated amplification method to test telomerase activity[16]. In brief, cells were precipitated in CHAPS buffer containing nuclease inhibitor for h at 4°C 30 min. Cell extracts (1 mg) were d with telomerase substrate (TS) primer, CAPD primer and Maxima SYBR green qPCR Master M TR Peze mixture. TS prime was elongated C for 30 min, followed by PCR amplification. TRAP products from the same ent were analyzed for telomerase a ity using $^{\Delta Ct}$ m od.

Comet Assay

Comet assay kit was purchased from Kaiji (Shanghai, China). The l was paved following manual instruction, with the addition of gel containing single cell suspension. When the gel was fixed, the coverslip was carefully removed. The glass slide was immersed in pre-cold cell lysis h incubation. After balancing in PBS, the slide was placed in horizontal electrophoresis tank filled with alkaline electrophoresis buffer. The mixture was denatured in the dark for 30 min, and was separated under 25 V, 300 mA current for 25 min. EB staining dye was added followed by coverslips, and observation under fluorescent microscope.

β-galactosidase Staining

Cellular β-galactosidase staining kit was purchased from Kaiji (Shanghai, China). Cells were collected by centrifugation. After rinsing in phosphate-buffered saline (PBS), 1 mL β-galactosidase fixation buffer was added for room temperature incubation for 10 min. Cell fixation buffer was removed by centrifugation, and cells were washed with phosphate-buffered saline (PBS) for three times (3 min each). The supernatant was discarded, and each tube was added with 0.5 mL staining working solution for 37°C overnight incubation. On the second day, staining cells were added onto glass slides for observation under light field microscope.

8-OHdG Assay

Intracellular 8-OHdG assay was performed using enzyme-linked immunosorbent assay. The kit was provided by Xitang Biotech (Shanghai, China) following manual instruction.

Statistical Analysis

Experimental data were obtained from at least three independent experiments and data were presented as mean±standard deviation (SD). Comparison between two groups was performed by student *t*-test. Comparison among multiple groups was performed by analysis of variance (ANOVA), followed by SNK approach between two groups. Statistical significance was defined when $p < 0.05$.

Results

Change of USP22 RNA Level

After infection by recombinant lentivirus of over-expression or knockdown of USP22, qPCR was used to test USP22 RNA expression level. As shown in Figure 1, over-expression lentivirus vector significantly up-regulated USP22 expression, whilst USP22 knockdown lentivirus remarkably suppressed USP22 RNA expression, with significant difference compared to blank control group ($p<0.01$). These results proved that recombinant lentivirus vectors were successfully constru and effectively enhanced or suppressed US RNA level.

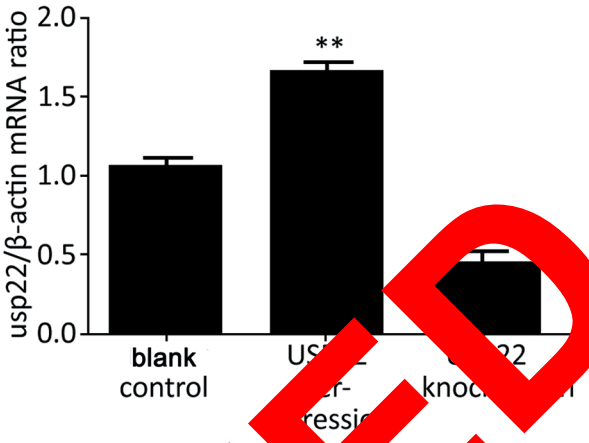

Figure 1. Effects of 22 kn wn and expression lentivirus on USP expression. < compared to blank control g

USP22, TERT and P53 Protein Expression Level

We then used Western b ng to easure USP22, TERT and P53 protein re After infected by USP over ssion lentivirus, USP22 and T protein els were significantly eleva while, exp sion of P53 was statist y down-regulated, as sh n in Figure 2. In contrast, opposite effects w found with the infection by USP22-down-re lation lenti us. These results suggested that US activat could potentiate TERT/p53 signal pa

of USP22 Up-Regulation and own-Regulation on Cell Proliferation

Subsequently, MTT assay was performed to describe cell proliferation. Results showed (Figure 3) that the increase of USP22 level promoted cell proliferation whilst knockdown of USP22 remarkably suppressed proliferation level. These results indicated that the up-regulation of USP22 was correlated with enhancing cell proliferation potency.

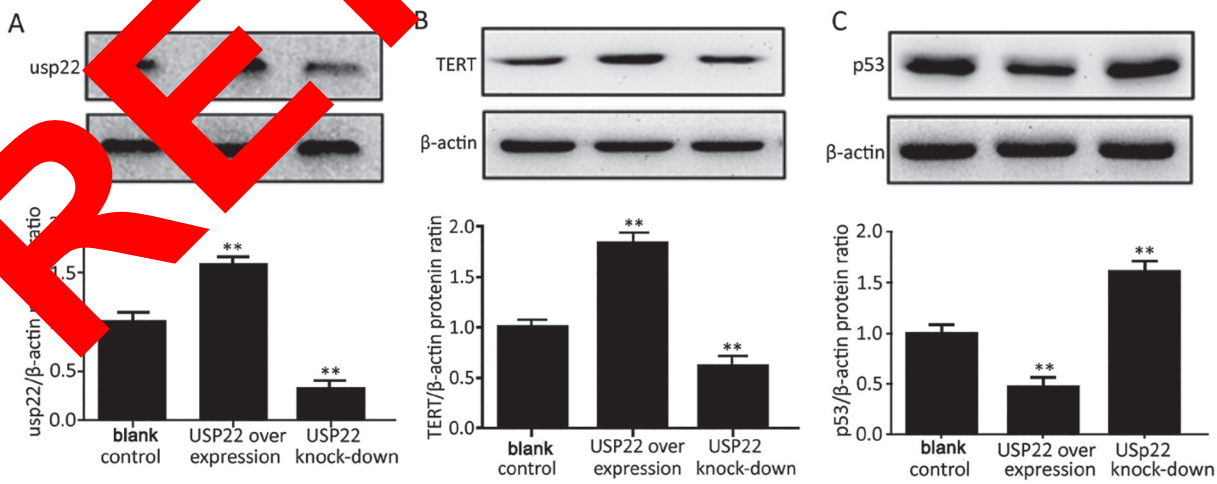

Figure 2. Effects of USP22 knockdown and over-expression on USP22, TERT and p53 protein expression. **, $p < 0.01$ compared to blank control group.

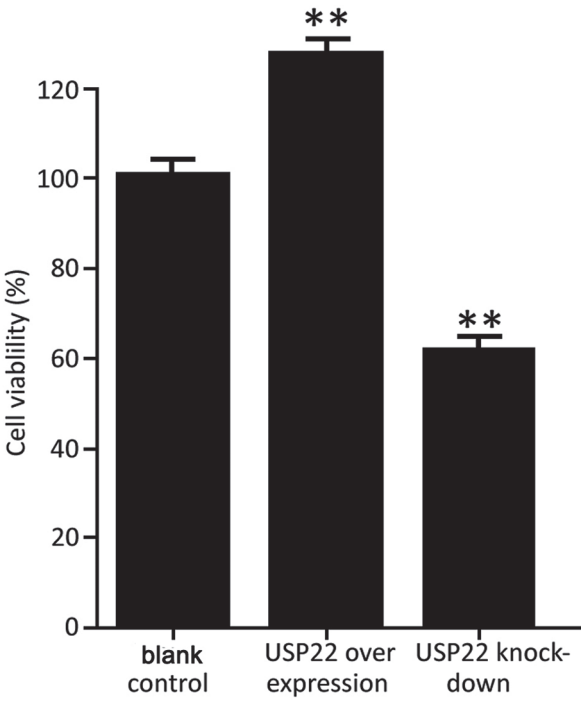

Figure 3. Effects of USP22 on cell proliferation. **, $p < 0.01$ compared to blank control group.

Telomerase Activity Change

In an assay for telomerase activity, we fou that the rise of USP22 level enhan omeras activity, whilst the decrease of 22 kened telomerase activity, which sh d signi ant difference compared to the contr ro in Figure 4.

Effects of USP2 k-Down o Over-Expression Lenti l Transfection on Cell Ap osis and D Damage

Flow c metry was used quantify the effect SP22 cell apoptosis. As shown in Figure 5 expres on of USP22 signifi- ca inhi cell a ptosis, while a low lev- SP22 ntly increased apoptotic cell n ber co ed to control group. In the test ular DNA oxidative injury produce dG le , we also found significantly de- ed level of such DNA damage products in 22 over-expression group, whilst DNA damage was elevated in USP22 knockdown group (Figure 5B). Similar results were obtained in comet assay, insignificant "tail-dragging" pattern was found in USP22 over-expression group, which was enhanced in USP22 knockdown group (Figure 5C). These results manifested that the suppression of USP22 could weaken cell proliferation and enhance cellular DNA injury, thus may exert inhibitor roles on cancer cells.

Effects of USP22 knockdown or Overexpression Lentivirus on Cell Aging

Using cellular β-galactosidase staining, the increase of USP22 weakened staini ty, whilst the reduction of USP22 sig cantly plified number of β-galactosidas sitive stai ing cells. These results illu trated t USP2 expression was correlated th cell g, a the decrease of USP22 r arkabl facilit ll aging (Figure 6).

Discussio

Retinoblastoma type of malignant tumor deri m embryo retinal cells. Current ap aches mainly incl de conservative treatm t or remov of eyeball. The prognosis is, h ver, unsa factory, leading to the requirem f identi tion of effective target and signal p cessary. An early study showed hat USP22 was a critical target for the suppres- f tumor growth in cells, and telomerase tivity was related with malignant proliferation of cancer cells. However, in retinoblastoma cell line, whether USP22 can facilitate retinoblastoma cell aging or apoptosis via suppression TERT/P53 signal pathway has not been reported. This study thus used lentiviral infection to up-regulate and down-regulate USP22 RNA and protein expression level, for the study of the role of USP22 on TERT/P53 signal pathway and on

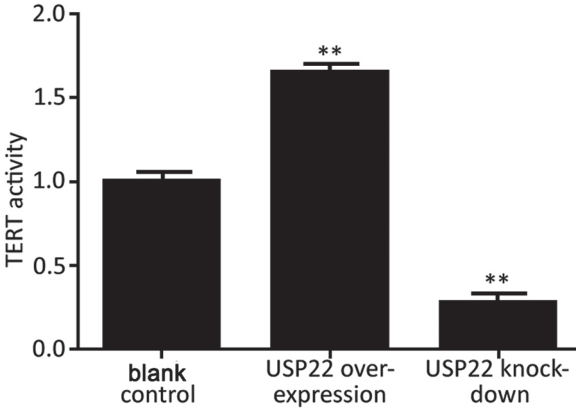

Figure 4. Effects of USP22 knockdown and over-expression lentivirus transfection on telomerase activity. **, $p < 0.01$ compared to blank control group.

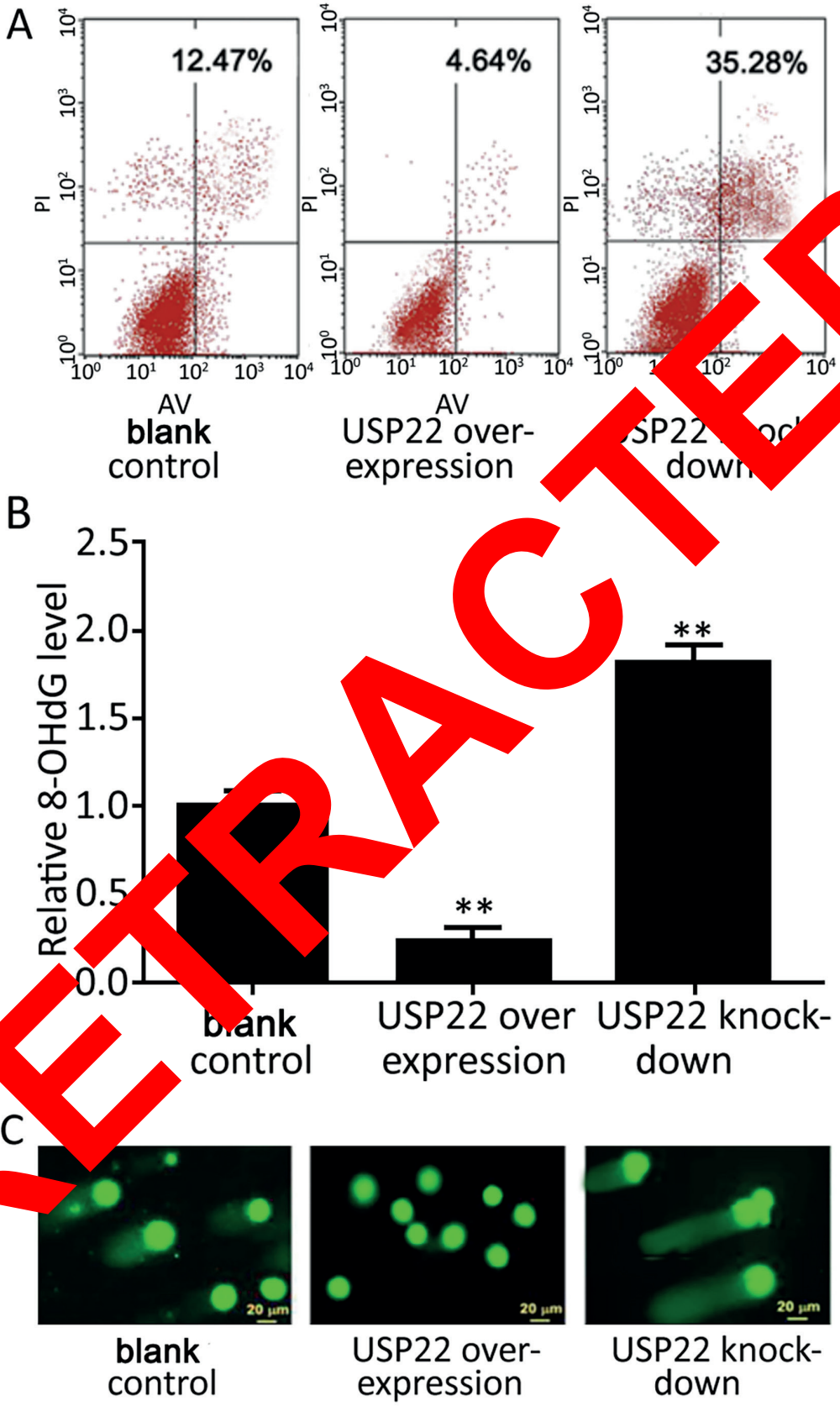

Figure 5. Effects of USP22 knockdown or over-expression lentivirus on cell apoptosis and DNA damage. **, $p < 0.01$ compared to blank control group.

Figure 6. Effects of USP22 knockdown and overexpression lentivirus on cell aging.

cancer cell behavior. Our results showed that in retinoblastoma cell line, down-regulation of USP22 inhibited TERT/P53 pathway, decreased telomerase activity, weakened cell proliferation potency, and facilitated cell aging, apoptosis or DNA damage. Increasing evidence showed critical functions of USP22 in pathology progression, and can work as markers for cancer treatment or prognosis with promising futu... In this study, we found that in retinoblast... cells, USP22 expression was correlated with ... proliferation, aging and apoptosis behavio... indicating that down-regulation of ...2 coul... suppress cancer cell prolifera..., a... facilitate cell aging or apoptosis... therefor... USP22 might be served as a diagno... an... prediction marker for ...nobla...a patients. USP22 is a novel hu... de-ubiqui...ed gene. A previous study[10] ...d that in hu...n ATC cell line 8505C, USP22 ...l was up-regulated, resulting ... G1 phase ...st and lower S or G2/M ...se with depletion ...f USP22, thus it facili...d cell ...cle progression in both cell lines. It ...ed that ...e inhibition of USP22 expression ...stively ...peded the apoptosis of ...ncer, ...tal carcinoma and thyroid cance... In the ...rent study, AV-PI staining was ...ng with flow cytometry assay, and ...ound ...favorable role of USP22 knockd... on apoptosis. Although it is still difficult to ...rate whether USP22 can directly work on apoptosis related proteins via transcriptional and translational regulation, our study still provided evidences for the activity of USP22 in retinoblastoma cell apoptosis mechanism. A previous study has shown that USP22 expression could affect cell proliferation or cycle and led to tumor cell immortalization due to elevated telomerase activity[18]. An... report[19] al... ...ed that telomerase w... ...d ... ...mor marke... and treatment target. Inhibition ... ...lomerase activity could impe... ...nor cell im... ...rtalization. Moreover, th... ...eatment of cancer cell and normal cell w... certain ...ncentration of telomerase de... ...ed drug t... ...city or adverse effects[20]. Previou... ...holars[21] ...demonstrated that activation of TER... ...al pathway could enhance telo... ...erase activity and decrease expression, thus ...ting cell proliferation via the modulation ... cell cycle. However, the exact mechanism of USP22 on signal pathway still requires further researches. Moreover, treatment effects on USP22 or TERT on retinoblastoma are still to be further elucidated.

Conclusions

USP22 played an important role in proliferation, aging and apoptosis of retinoblastoma. The decrease of USP22 expression can facilitate human retinoblastoma cell aging and apoptosis via the inhibition of TERT/P53 signal pathway. USP22 thus may work as a potential treatment target against retinoblastoma.

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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