

Reduced apurinic/apyrimidinic endonuclease activity enhances the antitumor activity of oxymatrine in lung cancer cells

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Abstract. Lung cancer is the leading cause of cancer-related deaths worldwide and is associated with a very poor outcome. Oxymatrine exerts antitumor effects by inducing apoptosis and inhibiting the proliferation of different cancer cells; however, the anticancer effects and mechanism of action of oxymatrine have not been evaluated sufficiently in human lung cancer cells. Thus, the present study aimed to investigate the anticancer effects of oxymatrine in human lung cancer cells and identify the molecular mechanisms underlying these effects. MTT assays demonstrated that oxymatrine significantly inhibited the proliferation of A549 and H1299 cells in a time- and dose-dependent manner. In addition, flow cytometry and terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling assays suggested that oxymatrine treatment may induce lung cancer cell apoptosis in a dose-dependent manner. Furthermore, we detected that oxymatrine induced a significant increase in DNA damage and the expression of PARP and phosphorylated H2AX, and a significant decrease in that of nuclear APE1 and AP endonuclease activity in A549 cells. APE1 knockdown cells (APE1^{shRNA}) plus oxymatrine treatment reduced cells proliferation and induced apoptosis more seriously than control shRNA cells. This appeared to be a consequence of an increase in the number of apurinic/apyrimidinic (AP) sites, DNA damage, PARP and H2AX phosphorylation, which together resulted in the induction of apoptosis. In contrast, the sensitizing effects of APE1 overexpression plus oxymatrine treatment did not occur in APE1^{OE} cells. These findings reveal a potential mechanism of action for oxymatrine-induced apoptosis and suggest that oxymatrine

is a promising potential therapeutic agent for the treatment of lung cancer.

Introduction

The clinical outcome of patients with advanced-stage lung cancer remains poor due to intrinsic or acquired chemoresistance to platinum-based chemotherapy and severe dose-limiting organ toxicities (1). Therefore, new and effective clinical agents are urgently needed to improve the outlook of these patients. Traditional Chinese herbs are sources of compounds that may have potential as therapeutic drugs for cancer (2). Oxymatrine is an alkaloid present in Kushen, which is widely used as a traditional Chinese medicine. It exhibits anti-inflammatory, anti-allergic, antiviral, antifibrotic and cardioprotective properties (3-5). Furthermore, oxymatrine has antitumor properties, including inhibiting cancer cell proliferation, cell cycle progression, and angiogenesis, promoting cellular apoptosis and reversing multidrug resistance in patients with cancer (6-8). A recent study demonstrated that oxymatrine inhibited the proliferation and induced apoptosis in A549 cells by regulating the expression of Bcl-2 and Bax (9); however, a more detailed mechanism of action remains elusive.

A previous study showed that modulating DNA repair pathways can sensitize a number of cancers to DNA damage-based cancer treatments (10). Consequently, targeting DNA repair systems is a promising strategy for the development of a novel lung cancer therapy. Human apurinic/apyrimidinic endonuclease 1 (APE1, also known as REF-1) is an essential base-excision repair (BER) enzyme that is responsible for the repair of DNA damage resulting from oxidative stress, chemotherapy and radiotherapy (11). APE1 is essential, since the deletion of both alleles (Apex^{-/-}) results in early-stage embryonic lethality in animals (12). Cell lines with the complete lack of APE1 are also non-viable, further indicating its significance in maintaining cell survival (12). APE1 has received significant attention as an attractive target for the pharmacological treatment of certain types of cancer.

Apurinic/apyrimidinic (AP) sites can form spontaneously or in response to DNA damage, and early studies showed that ~10,000 depurination/depyrimidination events occur spontaneously in the mammalian genome per day (13). If

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left unrepaired AP sites can block DNA synthesis and lead to mutation during semiconservative replication (14). AP sites can also occur as intermediates in BER, which is initiated by a DNA glycosylase.

The mechanism of action of oxymatrine in human lung cancer is largely unknown. To investigate the role and mechanism of oxymatrine in human lung cancer we hypothesized that the cytotoxic effects of oxymatrine may be exerted by inhibiting the function of APE1. To test this hypothesis APE1^{shRNA} (APE1 knockdown) and APE1^{OE} (APE1 overexpression) cells were used to investigate the effects of APE1 knockdown and overexpression in oxymatrine-treated A549 cells. Several cellular parameters were measured, including proliferation, survival and the induction of DNA damage and apoptosis.

Materials and methods

Reagents. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and antibiotics (penicillin and streptomycin) were purchased from Invitrogen-Life Technologies (Carlsbad, CA, USA). Antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Oxymatrine was obtained from Shanghai Chemical Technology Co., Ltd., (Shanghai, China) and dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) at a stock concentration of 500 mg/ml; it was then diluted further in culture medium. Each experiment was repeated at least three times and new dilutions were prepared for each experiment. Thiazolyl blue tetrazolium bromide (MTT) was obtained from Sigma-Aldrich.

Cell culture and transfections. Human lung carcinoma cell lines A549 and NCI-H1299 were purchased from the Shanghai Cell Institute Country Cell Bank (Shanghai, China). The A549 and NCI-H1299 cell lines were cultured in DMEM or RPMI-1640 and supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin G and 100 µg/ml streptomycin at 37°C in a humidified 5% CO₂ atmosphere.

Plasmid construction. To construct shRNA-expressing lentiviral vectors for silencing of APE1, the targeting sequence 5'-TGACAAAGAGGCAGCAGGA-3' and stable short hairpin RNA (shRNA) expression cassettes were cloned into the RNAi-Ready pSIREN-RetroQ vector containing puromycin as a screening marker, which was purchased from Clontech Laboratories, Inc. (Mountain View, CA, USA). The primer sequences were as follows: forward, 5'-GATCCGTGACAAA GAGGCAGCAGGATTCAAGAGATCCTGCTGCCTCTTT GTCATTTTTTG-3' and reverse, 5'-AATTCAAAAAATGA CAAAGAGGCAGCAGGATCTCTTGAATCCTGCTGCCT CTTTGTACAG-3'. The APE1 gene was amplified from cDNA isolated from K562 cells using polymerase chain reaction (PCR), and was then cloned into pOZN-HA vector using the primers: APE1-*Xho*I-forward, 5'-CGTTCGTA CTGAGATG CCGAAGCGTGGGAAAAAG-3' and APE1-*Not*I-reverse, 5'-CTTTTCCTTTTGCGGCCGCTCACAGTGCTAGGTAT AGGG-3'.

Generation of stable cell lines. Recombinant replication-deficient VSV lentiviruses were used and were propagated and purified and the titer was determined. A549 cells were

then infected by collected virus supernatant. A549 cell infections were carried out at MOIs of 100 and 200 for 16 h, and the transfected cells with APE1^{shRNA} were screened with puromycin for 2-5 days. The transfected cells with pOZN-HA-APE1 were screened by IL-2 receptor magnetic activated cell sorting. To determine the effect of APE1 knock-down in APE1^{shRNA} or overexpression in APE1^{OE} cells, western blotting was used to measure APE1 expression levels. Lentivirus production and cell infections were performed according to the manufacturer's instructions.

Immunofluorescence. Cells grown on coverslips were fixed in 4% paraformaldehyde, permeabilized with PBS containing 0.1% Triton X-100, and blocked in 3% normal donkey serum in PBS for 30 min at room temperature. Rabbit anti-human 8-OHdG (dilution 1:1,000) and p-H2AX (dilution 1:1,000) antibodies were diluted in 3% normal donkey serum in PBS and applied at 4°C overnight. After rinsing three times with PBS and incubating for 1 h with donkey anti-rabbit secondary antibody (dilution 1:1,000), slides were washed three times in PBS and the cell nuclei were stained with DAPI for 10 min at room temperature. Images were acquired on a Leica TCS SP5 confocal fluorescence microscope.

TUNEL staining. Terminal deoxynucleotidyl dUTP nick end labelling (TUNEL) assays were performed to measure apoptosis in cultured cells using the DeadEnd colorimetric TUNEL system (Promega, Madison, WI, USA) according to the manufacturer's instructions. Briefly, cells were grown on coverslips in 6-well plates after oxymatrine treatment; the coverslips were then removed from the media at the designated time points, fixed and permeabilized. The cells were incubated in rTdT reaction mixture for 60 min at 37°C. The slides were stained with 4',6-diamidino-2-phenylindole (DAPI) and mounted using anti-fade solution with a coverslip and nail polish. Images were captured using a confocal laser microscope (TCS SP5; Leica Microsystems, Wetzlar, Germany).

Protein extraction. Cells were treated with different concentrations of oxymatrine (2, 4 and 6 mg/ml) for 48 h, and washed twice with cold 1X PBS. The cells were then lysed using a Teflon-glass homogenizer in lysis buffer containing 0.25 M sucrose, 10 mM Tris-HCl (pH 7.5), 3 mM MgCl₂, 0.1 mM EDTA, 0.05% NP40, and a mix of protease and phosphatase inhibitors (Beyotime Institute of Biotechnology, Shanghai, China). The homogenates were centrifuged at 1,000 x g for 10 min at 4°C, and the supernatants were retained for protein quantitation and western blotting. For localization studies the total cellular extracts were fractionated into nuclear and cytosolic fractions using a nuclear protein extraction kit (Beyotime Institute of Biotechnology).

Western blotting. Cells were harvested and re-suspended in an SDS buffer (Beyotime Institute of Biotechnology) for preparation of total protein extracts. Western blot analysis was performed according to the antibody (Cell Signaling Technology) and to the manufacturer's protocols.

MTT assays. Cell lines were seeded into 96-well plates at a density of 3x10³ cells/well, incubated overnight at 37°C in 5%

CO₂, and treated with oxymatrine (0, 2, 4 and 6 mg/ml) for indicated time (24, 48 and 72 h). Cell viability was determined using MTT assays (Sigma-Aldrich). Briefly, MTT (5 mg/ml) was added and the plates were incubated at 37°C for 4 h in the dark. The absorbance was measured at a wavelength of 490 nm using a microplate reader (FluoDia T70; Photon Technology International, Lawrenceville, NJ, USA).

Colony formation assays. A549 cells (500 cells/well) were seeded into a 60-mm plate in triplicate. After incubation for 10 days the plates were washed gently with PBS and stained with 0.1% crystal violet. Colonies containing >50 cells were counted manually. The plating efficiency was calculated by dividing the number of colonies formed in the treated group by that in the control group.

Apoptosis analysis. Cells were seeded in 6-well plates (1.5x10⁵ cells/well), treated with different concentrations (0, 2, 4 or 6 mg/ml) of oxymatrine for 48 h, harvested and washed with cold PBS. The amount of cell surface phosphatidylserine in apoptotic cells was estimated quantitatively using an Annexin V-APC/7-AAD or Annexin V/PI double staining apoptosis detection kit (Liankebio, Shanghai, China) according to the manufacturer's instructions. The percentage of apoptotic cells was analyzed using flow cytometry. Triplicate experiments with triplicate samples were performed.

AP endonuclease activity assay for APE1/Ref-1. To assess the ability of oxymatrine to inhibit AP endonuclease activity an oligonucleotide cleavage assay was designed. The pair of oligonucleotides used in the fluorescence-based AP endonuclease assay was 890-FAM GGAAGGCCGCTGACAGTTT TTCTGTCAGCGG and its complementary oligonucleotide. If APE1 cleaves the DNA at the abasic site at position 7 from the 5' end the 6mer fluorescein-containing molecule can dissociate from the complementary strand by thermal melting. As a result, the quenching effect of 3'-dabcyl (which absorbs the fluorescence emitted by fluorescein when in close proximity) is lost, and APE1 activity can be measured indirectly as an increase in fluorescence signal.

For fluorescence-based AP endonuclease assays the single-stranded oligonucleotides (10 M) were dissolved and annealed in annealing buffer (25 mM Tris, pH 7.5, 1 mM EDTA and 50 mM NaCl) at 95°C for 5 min at a 1:1 ratio and allowed to cool to room temperature overnight. The DNA was diluted as appropriate with assay buffer (20 mM HEPES-KOH, 0.5 mM MgCl₂, 50 mM KCl, 0.1 mg/ml BSA), aliquoted, and stored at -20°C (15). The protein samples (8 ng/μl) and 20 μl/well hAPE1 standard (diluted to 0.05 ng/μl with assay buffer; recombinant human APE1; Sino Biological, Inc., North Wales, PA, USA) were added to each well followed by 20 μl oligonucleotide probe (500 nM) and then mixed. Fluorescence readings were taken continuously during 30-min incubation at 37°C using a LB-940 microporous multifunction analyzer in kinetic mode with excitation at 495 nm and emission at 530 nm.

Abasic site determination. Genomic DNA was purified from cells treated with oxymatrine using a Takara, MiniBest Universal Genomic DNA Extraction kit ver. 5.0 (Takara, Shiga, Japan). The abasic (AP) sites in genomic DNA were identified

using Nucleostain-DNA Damage Quantification kit-AP Site Counting (Dojindo Molecular Technologies, Kumamoto, Japan) following the manufacturer's instructions (15).

Statistical analysis. Each experiment was repeated at least three times and the new dilutions were prepared for each experiment. The results are presented as means ± standard error of the mean (SEM). Differences between means were analyzed using Student's t-test and were considered statistically significant at P<0.05. When more than one group was compared with control, significance was evaluated according to one-way analysis of variance (ANOVA). All statistical analysis was performed using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA).

Results

Oxymatrine induces apoptosis and inhibits the proliferation of lung cancer cells. Oxymatrine is a promising anticancer drug that inhibits the proliferation and growth of various cancer cells *in vitro* and *in vivo* (16-18). To investigate the effects of oxymatrine on the viability of lung cancer cells, A549 and H1299 cells were treated with different concentrations of oxymatrine (0, 2, 4 or 6 mg/ml) for the indicated times (24, 48 or 72 h). Compared with the PBS group (0 mg/ml, 0 h), treatment with 4 and 6 mg/ml oxymatrine inhibited the viability of A549 and H1299 cells, significantly (P<0.05 or P<0.01; Fig. 1A and B). Next, Annexin V-APC/7-AAD staining and flow cytometry was performed to quantify cellular apoptosis. Forty-eight hours after treatment, apoptosis was significantly higher in the oxymatrine group compared with the control group (P<0.05) (Fig. 1C and D). These observations were confirmed by investigating whether oxymatrine promoted A549 cell death using TUNEL staining. The number of TUNEL-positive cells was significantly higher in the 4 and 6 mg/ml oxymatrine groups than in 0 and 2 mg/ml oxymatrine groups (P<0.05; Fig. 1E and F). These results suggest that oxymatrine induced apoptosis in lung cancer cells.

Oxymatrine induces DNA damage in lung cancer cells. To explore the mechanism responsible for oxymatrine-induced apoptosis, DNA damage was evaluated using 8-OHdG and p-H2AX immunofluorescent staining and western blotting in A549 and H1299 cells that had been treated with oxymatrine for 48 h. Fig. 2A shows markedly increased 8-OHdG-positive areas in cells treated with both 2 and 4 mg/ml oxymatrine compared with control cells (P<0.05; Fig. 2A and C).

The H2A histone family member X (H2AX) is a key factor in the repair of damaged DNA. The phosphorylation of H2AX is an early event during the formation of double-stranded DNA breaks and the response to DNA damage (19). Therefore, the expression of p-H2AX was detected by immunofluorescence staining and western blotting. The expression of p-H2AX was increased significantly in cells treated with both 2 and 4 mg/ml oxymatrine compared with control (P<0.05; Fig. 2B, D, E and H).

PARP is a nuclear enzyme that is activated by DNA damage. Following genotoxic stress PARP synthesizes a branched polymer of poly(ADP-ribose) or PAR that participates in the regulation of nuclear homeostasis (20-22). Many different cellular insults that cause DNA damage activate PARP and induce PARP-dependent cell death. As expected, treatment

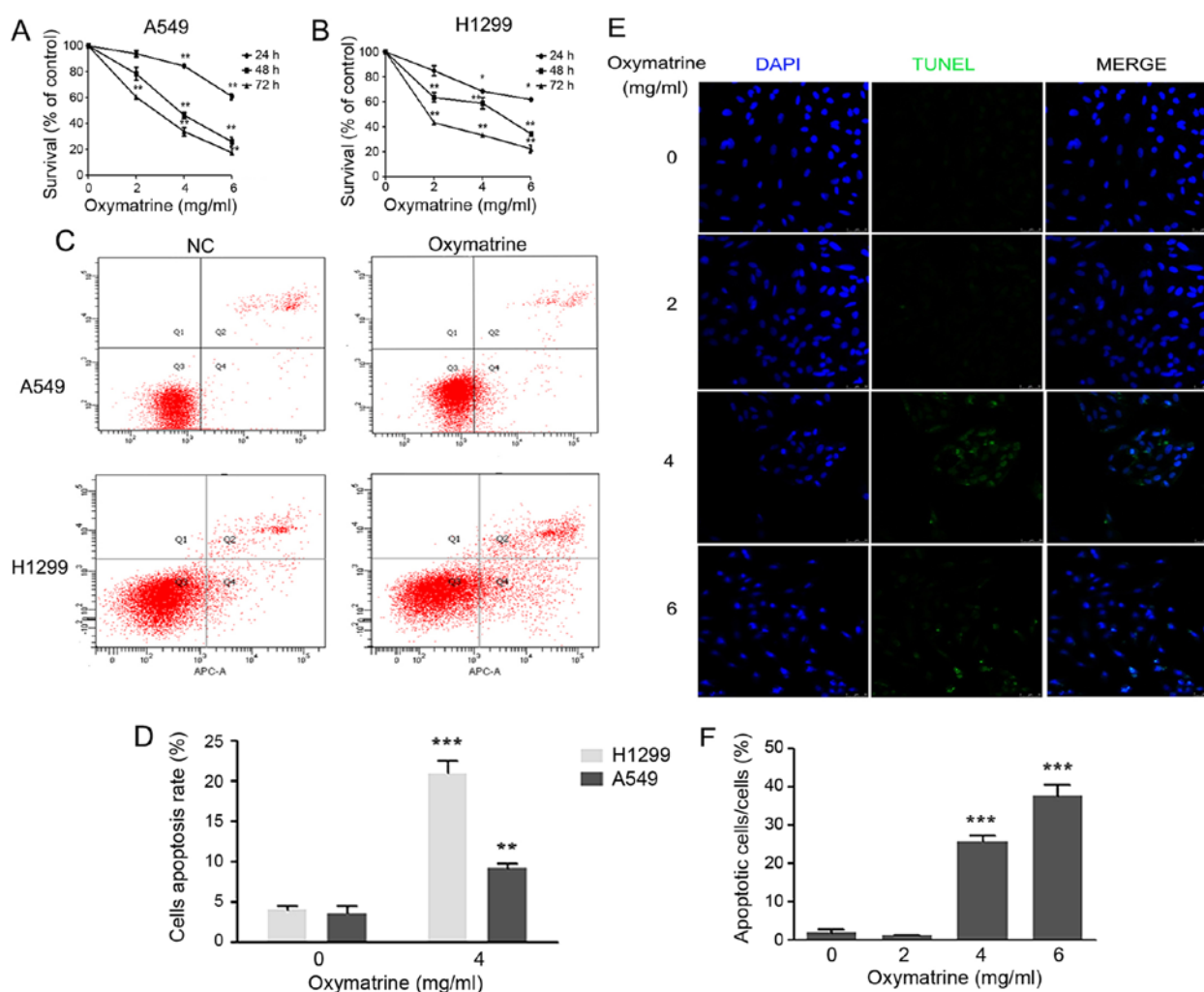


Figure 1. Oxymatrine induces apoptosis in lung cancer cells. (A and B) A549 and H1299 cells were treated with different concentrations of oxymatrine (0, 2, 4 or 6 mg/ml) for the indicated times (24, 48 or 72 h). Cell viability was determined using MTT assays. Data are presented as the means \pm SEM of three independent experiments. * P <0.05, ** P <0.01 and *** P <0.001 vs. control. (C) A549 and H1299 cells were treated with 4 mg/ml oxymatrine and apoptosis was analyzed using flow cytometry. (D) Quantification of the apoptosis images. The amount of apoptosis was defined as the percentage of early apoptotic cells plus the percentage of late apoptotic cells. Data are presented as the means \pm SEM of three independent experiments. ** P <0.01 and *** P <0.001. (E) Apoptosis was induced by different concentrations of oxymatrine (0, 2, 4 or 6 mg/ml) for 48 h in A549 cells, and TUNEL assays were performed. Images are shown at $\times 200$ magnification; blue, DAPI; green, TUNEL; merged image of TUNEL (apoptosis) and DAPI (nuclei) staining. (F) Quantification of TUNEL positive cells showing that treatment with 4 and 6 mg/ml oxymatrine increased the number of TUNEL-positive cells compared with control. Data are presented as the means \pm SEM of three independent experiments. *** P <0.001.

with oxymatrine induced the activation of PARP. H1299 and A549 cells were treated with different concentrations of oxymatrine for 48 h, and analysis revealed that oxymatrine induced PARP activation in a dose-dependent manner and oxymatrine can induced PARP cleavage in H1299 (Fig. 2E-G). This suggests that the activation of these proteins occurs as a result of apoptosis.

Oxymatrine inhibits the protein expression and AP endonuclease activity of APE1. Human APE1 is an essential enzyme in the BER pathway, where it plays a role in repairing abasic sites. APE1 is responsible for 95% of the endonuclease activity in cells (23), and is a critical part of both the short-patch and long-patch BER pathways (24). Western blotting revealed that oxymatrine inhibited the expression of APE1 protein in both H1299 and A549 cells compared with control (Fig. 3A and B). The nuclear localization of APE1/Ref-1 was also decreased

significantly by oxymatrine (6 mg/ml; P <0.05) in A549 cells (Fig. 3C).

A fluorescence-based AP endonuclease assay described by Madhusudan *et al* (25) was adapted. Oxymatrine inhibited APE1 and AP endonuclease activity in A549 cell nuclear cell extracts in a dose-dependent manner (Fig. 3D). These results suggest that oxymatrine is an inhibitor of the DNA repair activity of APE1/Ref-1 in lung cancer cell lines.

Downregulation and upregulation of APE1 expression in A549 cells. The reduction of APE1 protein expression by APE1 shRNA vector transfection in A549 cells (also called APE1^{shRNA} stable A549 cell lines) was confirmed by western blotting. Transfection with APE1 shRNA reduced APE1 expression by 47% compared with vector control transfected cells (control; Fig. 4A). In contrast, the expression of APE1 was increased markedly in A549 cells transfected with APE1-HA

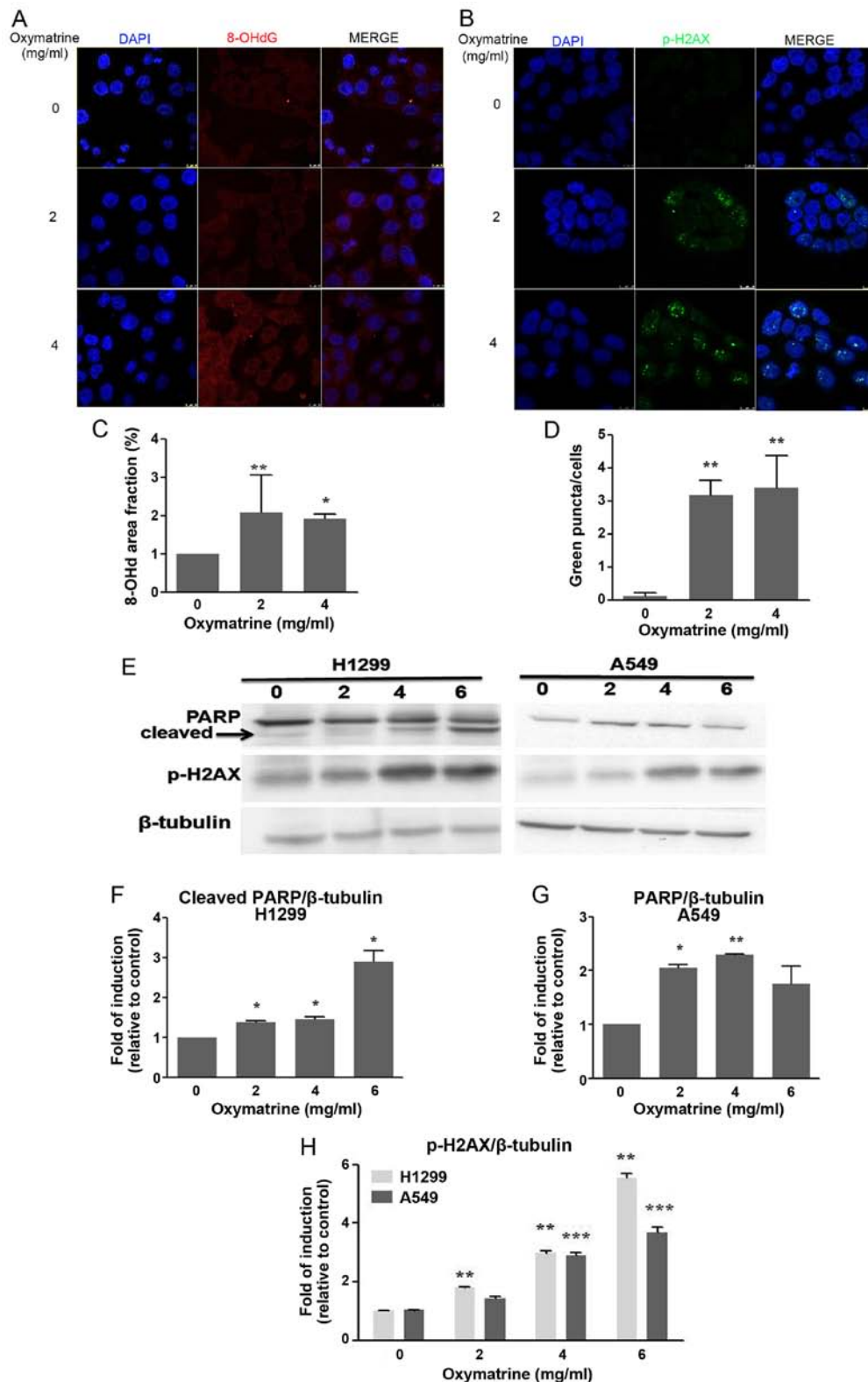


Figure 2. Effects of oxymatrine on DNA damage in A549 and H1299 lung cancer cells. A549 and H1299 cells were treated with different concentrations of oxymatrine (0, 2 or 4 mg/ml) for 48 h. (A) The expression of 8-OHdG was assessed in A549 cells using immunofluorescence. Merged images of 8-OHdG (green) and DAPI (nuclei) staining are shown. (B) The expression of p-H2AX in A549 cells was assessed using immunofluorescence. Merged images of p-H2AX (green) staining and DAPI (nuclei) staining are shown. Images are shown at x400 magnification. (C) Quantification of the 8-OHdG-positive area from A. (D) Quantification of the green puncta/cells from B. (E) Western blot analysis of PARP and p-H2AX levels. Representative blots of PARP and p-H2AX are shown; β -tubulin was used as a loading control. (F-H) Quantification of the western blots. The number below the bar shows the ratio of cleaved PARP or p-H2AX to β -tubulin in each sample. Data are presented as the means \pm SEM of three independent incubations. * P <0.05, ** P <0.01, *** P <0.001 vs. control cells.

(also called APE1^{OE} stable A549 cell lines); HA was used as a fusion tag for detection. Specifically, the expression of APE1

was increased 4-fold in APE1^{OE} cells compared with control vector (Fig. 4B).

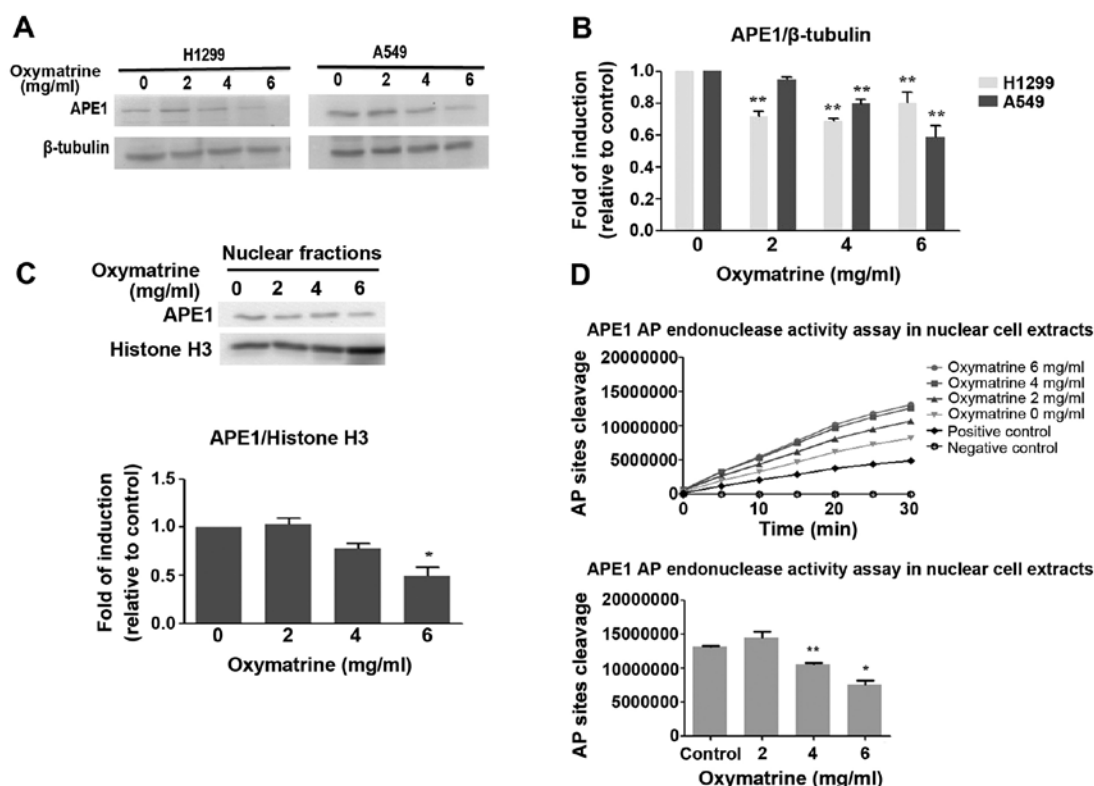


Figure 3. The downregulation of APE1 is associated with reduced AP endonuclease activity in oxymatrine-treated lung cancer cells. A549 and H1299 cells were treated with different concentrations of oxymatrine (0, 2, 4 or 6 mg/ml) for 48 h. (A) Representative images of APE1 protein expression, as determined using western blotting. β -tubulin was used as a loading control. (B) Quantification of APE1 expression. (C) Western blot analysis of APE1 protein expression in the nuclear fraction of oxymatrine-treated A549 cells; histone H3 was used as a loading control. APE1 expression was quantified and normalized to H3 expression in the nuclear fraction. All error bars represent the standard error of the mean (SEM). (D) AP endonuclease activity assay demonstrating that oxymatrine inhibits APE1 activity. The graphs are representations of nuclear protein extracts from A549 cells treated with different concentrations of oxymatrine (hAPE1 as positive control) plotted with their inhibition of the activity of APE1 AP endonuclease activity. Data are presented as the means \pm SEM of three independent experiments. * $P < 0.05$, ** $P < 0.01$, vs. control.

Knocking down APE1 enhances oxymatrine-induced DNA damage and apoptosis and reduces cell proliferation and clonogenic survival. Oxymatrine treatment significantly reduced the proliferation of APE1^{shRNA} cells, as measured by MTT and colony formation assays after 48 h ($P < 0.001$). Furthermore, APE1^{shRNA} alone inhibited cell growth by 20% ($P < 0.05$). However, oxymatrine treatment did not induce a significant reduction in proliferation in APE1^{OE} cells, suggesting that these cells are resistant to oxymatrine (Fig. 4C-E).

To determine if oxymatrine inhibits APE1 directly in APE1^{shRNA} and APE1^{OE} cells, AP site formation was measured using ARP assays. A significant increase in the number of AP sites in APE1^{shRNA} cells were observed with oxymatrine. However, there was no significant change in AP sites in APE1^{OE} cells treated with oxymatrine compared with control. The assay was performed four times, each in triplicate, and the data presented show the mean of the four experiments with standard errors (Fig. 4F).

Compared with the APE1^{shRNA} cells, oxymatrine treatment caused a significant increase in apoptosis using Annexin V/PI staining and flow cytometry. In contrast, APE1^{OE} cells presented low apoptosis induction with statistically significant difference between the groups APE1^{shRNA} oxymatrine-treated cells and APE1^{OE} oxymatrine-treated cells ($P < 0.05$) (Fig. 5A and B). Consistent with these results there were higher levels of

PARP and phosphorylated H2AX_[Ser139] in oxymatrine-treated APE1^{shRNA} cells compared with untreated group (Fig. 5C-E). In contrast, these changes of phosphorylated H2AX_[Ser139] were not observed in oxymatrine-treated APE1^{OE} cells, but PARP expression decreased significantly. These results suggest that APE1 plays an important role in regulating oxymatrine-induced apoptosis.

Discussion

Oxymatrine is an alkaloid that is derived from Kushen and is a potential treatment for a number of cancers, including pancreatic (6), gastric (26) and breast cancer (27). However, the effects of oxymatrine on lung cancer and the underlying molecular mechanisms of these effects have not yet been investigated. Previous studies demonstrated that oxymatrine markedly inhibited cell proliferation in dose-dependent manner, induced cell apoptosis in a dose- and time-dependent manner, upregulated cleaved caspase-3 and -9, and downregulated Bax/Bcl-2 in human osteosarcoma MG-63 cells (17). These proteins play a pivotal role in regulating apoptosis. In the present study, we hypothesized that the increased apoptosis elicited by oxymatrine is associated with the inhibition of APE1 function. To test this hypothesis, two stable A549 cell lines (APE1^{shRNA} and APE1^{OE}) were developed (Fig. 4A and B). Oxymatrine

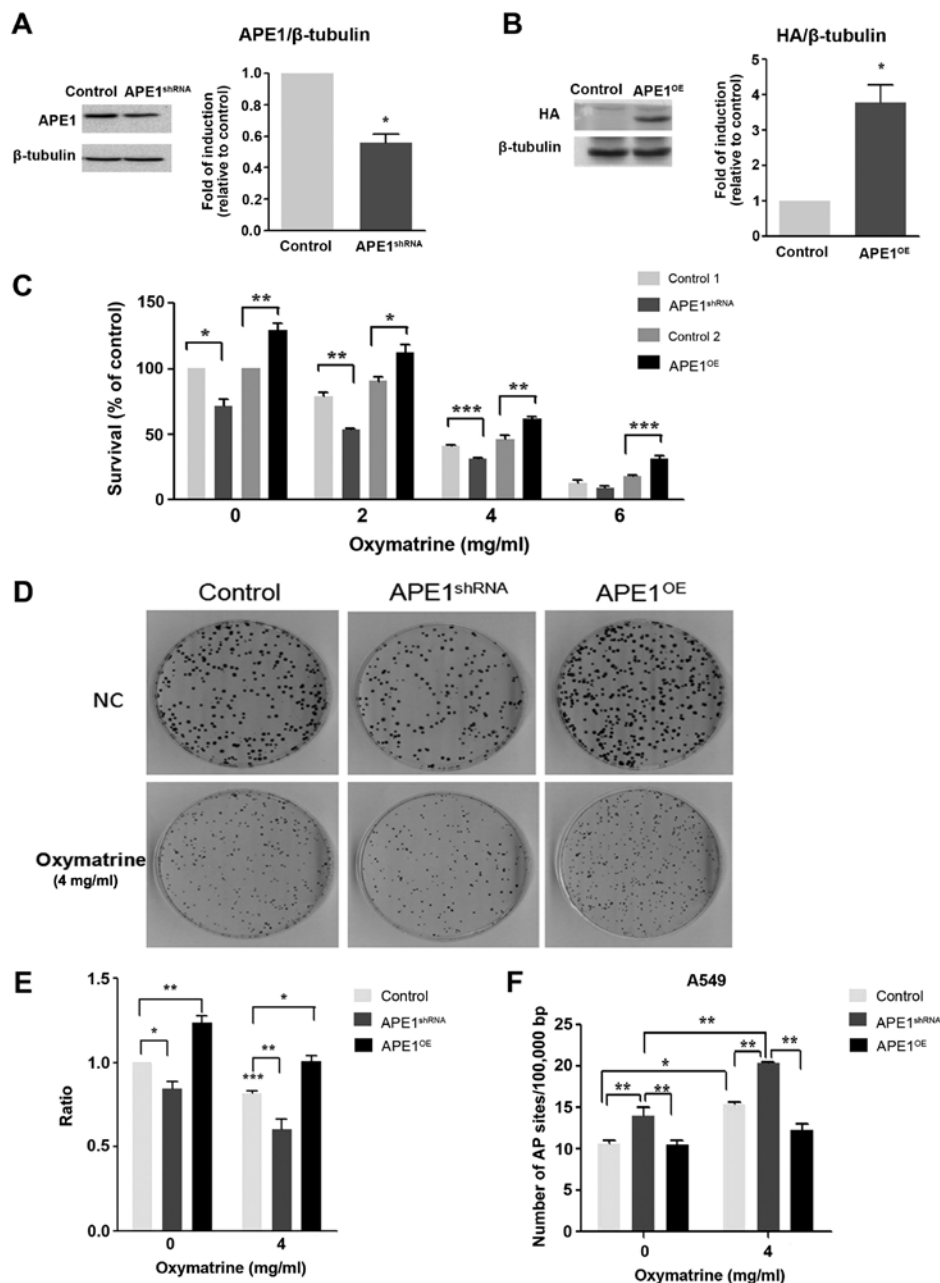


Figure 4. Silencing APE1 restored the survival of lung cancer cells exposed to oxymatrine. (A) The knockdown efficiency of shRNA against APE1 in APE1^{shRNA} A549 stable cells was validated by western blotting; β -tubulin was used as a loading control. APE1 levels were quantified and normalized to the expression of β -tubulin. (B) The overexpression of APE1 in APE1^{OE} A549 stable cells was validated using an HA-tag antibody by western blotting. HA-APE1 levels were quantified and normalized to the expression of β -tubulin. (C) Control vector, APE1^{shRNA} (APE1 knock down), and APE1^{OE} (APE1 overexpression) cells were treated with different concentrations of oxymatrine (0, 2, 4 or 6 mg/ml) for 48 h. Cell viability was determined using MTT assays. Data are presented as the means \pm SEM of three independent experiments. * P <0.05, ** P <0.01 and *** P <0.001. (D) Control vector, APE1^{shRNA} and APE1^{OE} cells were treated with 4 mg/ml oxymatrine for 10 days and allowed to form colonies. The colonies were fixed with methanol for 30 min and stained with crystal violet for 20 min. Crystal violet-stained cells from a representative experiment are shown (n=3). (E) The number of colonies. The data are presented as the means of triple assays with standard error of the mean * P <0.05, ** P <0.01 and *** P <0.001. (F) Quantitation of the number of AP sites on control vector, APE1^{shRNA} and APE1^{OE} cells treated with 4 mg/ml oxymatrine. The data are presented as the means \pm SEM of three independent experiments. * P <0.05, ** P <0.01.

significantly promoted lung cancer cell apoptosis, which was associated with the reduction of APE1 AP endonuclease activity; therefore, the association between APE1 enzymatic activity and oxymatrine dose was examined in lung cancer cells. Oxymatrine reduced APE1 activity in a dose-dependent manner (Fig. 3D). To the best of our knowledge, this is the first study that clearly identifies APE1 as a potential target for oxymatrine-induced apoptosis in lung cancer cells. This is consistent with anticancer strategies that propose inhibiting

BER as a principle for anticancer chemotherapy (28). However, increased APE1 expression was also reported to cause drug resistance in lung cancer patients (29).

Cellular APE1 levels might be critical for the repair of DNA strand breaks induced by ROS (30). This study suggests that APE1 is a promising target for cancer treatment. This protein has been targeted using antisense oligonucleotides, RNA interference, and natural and chemical agents, which sensitizes tumor cells to radiotherapy or chemotherapeutic

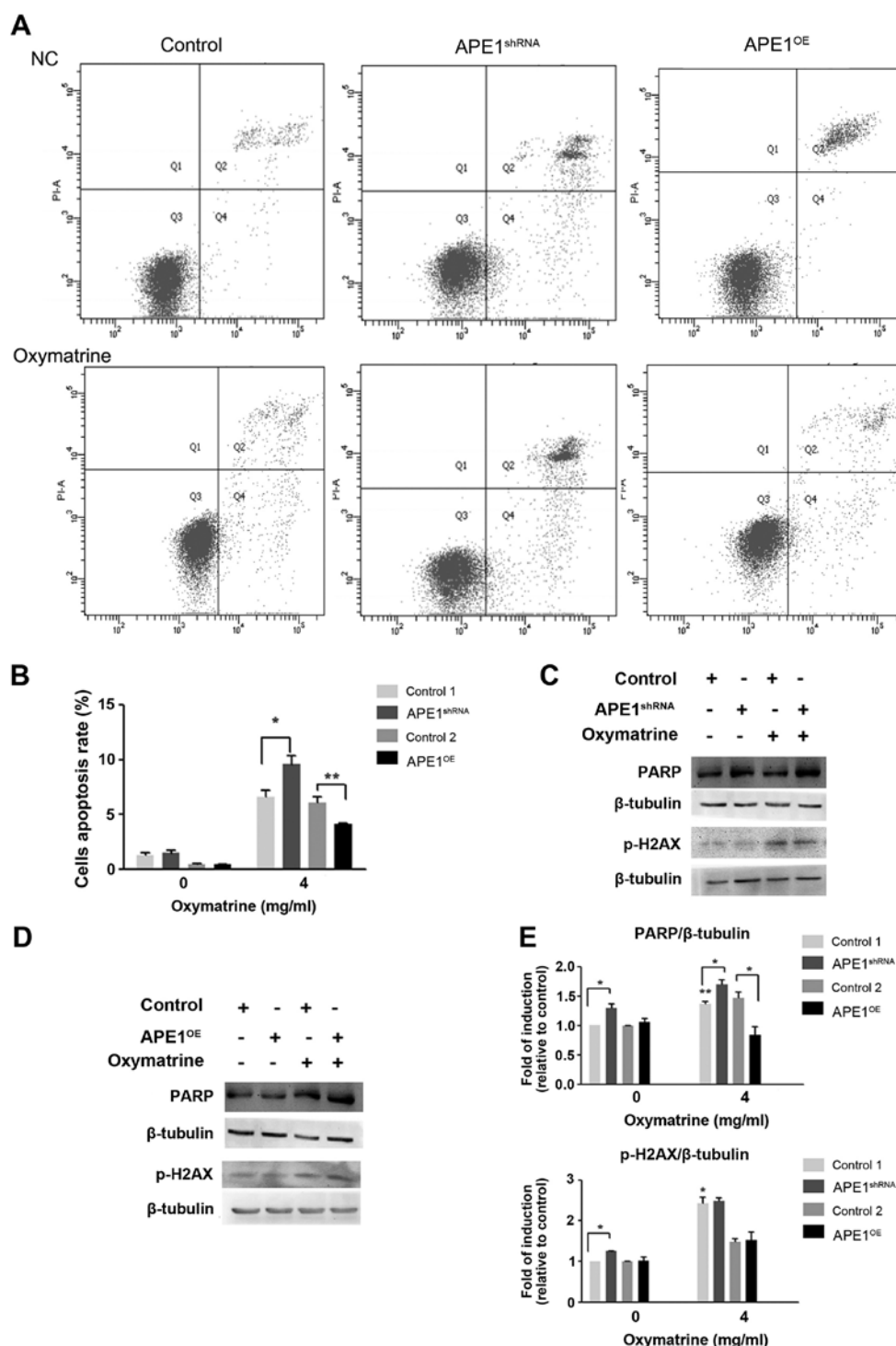


Figure 5. Effects of oxymatrine on APE1^{shRNA} and APE1^{OE} cells. Control, APE1^{shRNA} and APE1^{OE} cells were treated with 4 mg/ml oxymatrine for 48 h. (A) Apoptosis was assessed using flow cytometry. (B) Quantification of the apoptotic images. The amount of apoptosis was calculated as the percentage of early apoptotic (Annexin V⁺/PI⁻) cells plus the percentage of late apoptotic (Annexin V⁺/PI⁺) cells. Data are presented as the means \pm SEM of three independent experiments. * P <0.05, ** P <0.01. (C and D) Representative western blots of PARP and p-H2AX expression; β -tubulin was used as a loading control. (E) Quantification of PARP and p-H2AX expression. APE1 levels were quantified and normalized to the expression of β -tubulin. Data are presented as the means \pm SEM of three independent experiments. * P <0.05, ** P <0.01.

drugs (31). For example, the present study revealed that APE1 is a target of oxymatrine, since the protein expression and AP endonuclease activity of APE1 were reduced by oxymatrine (Fig. 3). Earlier studies revealed that \sim 10,000 abasic sites are generated in a human cell every day, and that these AP sites are the most commonly generated lesions in DNA (32). AP

sites can lead to error-prone bypass synthesis and thus cause mutagenesis (33). AP sites and 5-foU are the most common lesions in genomic DNA (34). If an AP site is present within a double-stranded clustered lesion it would be repaired first (35). It is assumed that oxymatrine increased DNA damage and associated with increasing AP site, since APE1 activity was

inhibited when DNA repair is hindered. The repair of AP sites requires an AP endonuclease or AP lyase. In the present study, oxymatrine increased the number of AP sites in APE1 knockdown stable cell lines (APE1^{shRNA}) compared with control (Fig. 5A and B). This suggests that oxymatrine can increase DNA damage in APE1 knockdown cells.

8-OHdG levels were significantly higher in oxymatrine-treated cells compared with control (Fig. 2A and C). 8-OHdG is used widely as a biomarker for measuring endogenous oxidative DNA damage; it reflects the oxidative damage induced by free radicals to nuclear and mitochondrial DNA (36,37).

The phosphorylation of histone H2AX on serine-139 (p-H2AX) is a sensitive marker for DNA DSBs. In this study oxymatrine induced the phosphorylation of H2AX at the sites of DNA DSBs, which could be observed as fluorescent foci using immunostaining (Fig. 2B and D). In addition, western blotting showed an increase in p-H2AX levels after oxymatrine. Finally, phosphorylated H2AX levels were significantly associated with the dose of oxymatrine in H1299 cells (Fig. 2E and H). PARP cleavage was observed in oxymatrine-treated H1299 cells in a dose-dependent manner (Fig. 2E-G).

Although the present study revealed that oxymatrine induced apoptosis at least in part by inhibiting APE1 activity, the mechanisms for the proapoptotic actions of oxymatrine in cancer cells are complex and they may include several other pathways. Further studies are needed to determine the molecular mechanism of APE1 in anticancer drug targets.

In conclusion, the present study revealed that treatment with oxymatrine significantly induced apoptosis and DNA damage in lung cancer cells. Oxymatrine also inhibited APE1 activity and protein expression significantly. The selective inhibition of the repair activity of APE1 is a promising target for developing novel cancer therapeutics (38). This study provides evidence supporting the hypothesis that APE1 is a target for oxymatrine and contributes to oxymatrine-induced apoptosis in lung cancer cells.

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References

- Dasari S and Tchounwou PB: Cisplatin in cancer therapy: Molecular mechanisms of action. *Eur J Pharmacol* 740: 364-378, 2014.
- Liu YH, Li ML, Hsu MY, Pang YY, Chen IL, Chen CK, Tang SW, Lin HY and Lin JY: Effects of a Chinese herbal medicine, Guan-Jen-Huang (*Aeginetia indica* Linn.), on renal cancer cell growth and metastasis. *Evid Based Complement Alternat Med* 2012: 935860, 2012.
- Huang M, Hu YY, Dong XQ, Xu QP, Yu WH and Zhang ZY: The protective role of oxymatrine on neuronal cell apoptosis in the hemorrhagic rat brain. *J Ethnopharmacol* 143: 228-235, 2012.
- Chai NL, Fu Q, Shi H, Cai CH, Wan J, Xu SP and Wu BY: Oxymatrine liposome attenuates hepatic fibrosis via targeting hepatic stellate cells. *World J Gastroenterol* 18: 4199-4206, 2012.
- Hong-Li S, Lei L, Lei S, Dan Z, De-Li D, Guo-Fen Q, Yan L, Wen-Feng C and Bao-Feng Y: Cardioprotective effects and underlying mechanisms of oxymatrine against ischemic myocardial injuries of rats. *Phytother Res* 22: 985-989, 2008.
- Ling Q, Xu X, Wei X, Wang W, Zhou B, Wang B and Zheng S: Oxymatrine induces human pancreatic cancer PANC-1 cells apoptosis via regulating expression of Bcl-2 and IAP families, and releasing of cytochrome c. *J Exp Clin Cancer Res* 30: 66, 2011.
- Liu Y, Xu Y, Ji W, Li X, Sun B, Gao Q and Su C: Anti-tumor activities of matrine and oxymatrine: Literature review. *Tumour Biol* 35: 5111-5119, 2014.
- Song G, Luo Q, Qin J, Wang L, Shi Y and Sun C: Effects of oxymatrine on proliferation and apoptosis in human hepatoma cells. *Colloids Surf B Biointerfaces* 48: 1-5, 2006.
- Wang D, Xiang DB, Yang XQ, Chen LS, Li MX, Zhong ZY and Zhang YS: APE1 overexpression is associated with cisplatin resistance in non-small cell lung cancer and targeted inhibition of APE1 enhances the activity of cisplatin in A549 cells. *Lung Cancer* 66: 298-304, 2009.
- Helleday T, Petermann E, Lundin C, Hodgson B and Sharma RA: DNA repair pathways as targets for cancer therapy. *Nat Rev Cancer* 8: 193-204, 2008.
- Kelley MR, Georgiadis MM and Fishel ML: APE1/Ref-1 role in redox signaling: Translational applications of targeting the redox function of the DNA repair/redox protein APE1/Ref-1. *Curr Mol Pharmacol* 5: 36-53, 2012.
- Xanthoudakis S, Smeyne RJ, Wallace JD and Curran T: The redox/DNA repair protein, Ref-1, is essential for early embryonic development in mice. *Proc Natl Acad Sci USA* 93: 8919-8923, 1996.
- Lindahl T: Instability and decay of the primary structure of DNA. *Nature* 362: 709-715, 1993.
- Dianov GL, Sleeth KM, Dianova II and Allinson SL: Repair of abasic sites in DNA. *Mutat Res* 531: 157-163, 2003.
- Bapat A, Glass LS, Luo M, Fishel ML, Long EC, Georgiadis MM and Kelley MR: Novel small-molecule inhibitor of apurinic/apyrimidinic endonuclease 1 blocks proliferation and reduces viability of glioblastoma cells. *J Pharmacol Exp Ther* 334: 988-998, 2010.
- Wu C, Huang W, Guo Y, Xia P, Sun X, Pan X and Hu W: Oxymatrine inhibits the proliferation of prostate cancer cells in vitro and in vivo. *Mol Med Rep* 11: 4129-4134, 2015.
- Wei J, Zhu Y, Xu G, Yang F, Guan Z, Wang M and Fang Y: Oxymatrine extracted from *Sophora flavescens* inhibited cell growth and induced apoptosis in human osteosarcoma MG-63 cells in vitro. *Cell Biochem Biophys* 70: 1439-1444, 2014.
- Wang L, Hou X, Fu H, Pan X, Xu W, Tang W and Fang H: Design, synthesis and preliminary bioactivity evaluations of substituted quinoline hydroxamic acid derivatives as novel histone deacetylase (HDAC) inhibitors. *Bioorg Med Chem* 23: 4364-4374, 2015.
- Valdiglesias V, Giunta S, Fenech M, Neri M and Bonassi S: γ H2AX as a marker of DNA double strand breaks and genomic instability in human population studies. *Mutat Res* 753: 24-40, 2013.
- Schreiber V, Dantzer F, Ame JC and de Murcia G: Poly(ADP-ribose): Novel functions for an old molecule. *Nat Rev Mol Cell Biol* 7: 517-528, 2006.
- Muñoz-Gómez JA, Rodríguez-Vargas JM, Quiles-Pérez R, Aguilar-Quesada R, Martín-Oliva D, de Murcia G, Menissier de Murcia J, Almendros A, Ruiz de Almodóvar M and Oliver FJ: PARP-1 is involved in autophagy induced by DNA damage. *Autophagy* 5: 61-74, 2009.
- Krishnakumar R and Kraus WL: The PARP side of the nucleus: Molecular actions, physiological outcomes, and clinical targets. *Mol Cell* 39: 8-24, 2010.
- Scott TL, Rangaswamy S, Wicker CA and Izumi T: Repair of oxidative DNA damage and cancer: Recent progress in DNA base excision repair. *Antioxid Redox Signal* 20: 708-726, 2014.
- Evans AR, Limp-Foster M and Kelley MR: Going APE over ref-1. *Mutat Res* 461: 83-108, 2000.
- Madhusudan S, Smart F, Shrimpton P, Parsons JL, Gardiner L, Houlbrook S, Talbot DC, Hammonds T, Freemont PA, Sternberg MJ, et al: Isolation of a small molecule inhibitor of DNA base excision repair. *Nucleic Acids Res* 33: 4711-4724, 2005.
- Song MQ, Zhu JS, Chen JL, Wang L, Da W, Zhu L and Zhang WP: Synergistic effect of oxymatrine and angiogenesis inhibitor NM-3 on modulating apoptosis in human gastric cancer cells. *World J Gastroenterol* 13: 1788-1793, 2007.

27. Zhang Y, Piao B, Zhang Y, Hua B, Hou W, Xu W, Qi X, Zhu X, Pei Y and Lin H: Oxymatrine diminishes the side population and inhibits the expression of β -catenin in MCF-7 breast cancer cells. *Med Oncol* 28 (Suppl 1): S99-S107, 2011.
28. Fishel ML and Kelley MR: The DNA base excision repair protein Ape1/Ref-1 as a therapeutic and chemopreventive target. *Mol Aspects Med* 28: 375-395, 2007.
29. Li Z, Qing Y, Guan W, Li M, Peng Y, Zhang S, Xiong Y and Wang D: Predictive value of APE1, BRCA1, ERCC1 and TUBB3 expression in patients with advanced non-small cell lung cancer (NSCLC) receiving first-line platinum-paclitaxel chemotherapy. *Cancer Chemother Pharmacol* 74: 777-786, 2014.
30. Izumi T, Hazra TK, Boldogh I, Tomkinson AE, Park MS, Ikeda S and Mitra S: Requirement for human AP endonuclease 1 for repair of 3'-blocking damage at DNA single-strand breaks induced by reactive oxygen species. *Carcinogenesis* 21: 1329-1334, 2000.
31. Silber JR, Bobola MS, Blank A, Schoeler KD, Haroldson PD, Huynh MB and Kolstoe DD: The apurinic/apyrimidinic endonuclease activity of Ape1/Ref-1 contributes to human glioma cell resistance to alkylating agents and is elevated by oxidative stress. *Clin Cancer Res* 8: 3008-3018, 2002.
32. Kunkel TA: The high cost of living. American Association for Cancer Research Special Conference: Endogenous sources of mutations, Fort Myers, Florida, USA, 11-15 November 1998. *Trends Genet* 15: 93-94, 1999.
33. Loeb LA and Preston BD: Mutagenesis by apurinic/apyrimidinic sites. *Annu Rev Genet* 20: 201-230, 1986.
34. Bjelland S, Anensen H, Knaevelsrud I and Seeberg E: Cellular effects of 5-formyluracil in DNA. *Mutat Res* 486: 147-154, 2001.
35. Georgakilas AG, O'Neill P and Stewart RD: Induction and repair of clustered DNA lesions: What do we know so far? *Radiat Res* 180: 100-109, 2013.
36. Prabhulkar S and Li CZ: Assessment of oxidative DNA damage and repair at single cellular level via real-time monitoring of 8-OHdG biomarker. *Biosens Bioelectron* 26: 1743-1749, 2010.
37. Kim J, Cha YN and Surh YJ: A protective role of nuclear factor-erythroid 2-related factor-2 (Nrf2) in inflammatory disorders. *Mutat Res* 690: 12-23, 2010.
38. Bapat A, Fishel ML and Kelley MR: Going ape as an approach to cancer therapeutics. *Antioxid Redox Signal* 11: 651-668, 2009.