Original Article
Rho-associated protein kinase inhibitor Y-27632 increases the sensitivity of lung adenocarcinoma A549 cells to matrine via the Rad51/ERCC1 signaling pathway

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Abstract: *Purpose:* Non-small cell lung carcinoma (NSCLC) is one of the most lethal types of cancer. The aim of this study was to investigate the potential of Y-27632, which is a selective inhibitor of ROCK/Rho-kinase, to enhance the antitumor effects of matrine, a major alkaloid component extracted from the traditional Chinese herb *Sophora flavescens* Ait, on lung adenocarcinoma A549 cells. *Methods:* A549 cells were treated with different concentrations of matrine alone or in combination with Y-27632 for 48 h and cell viability, migration, and apoptosis were assessed. The mRNA and protein levels of the DNA repair-related molecules Rad51 and ERCC1 were determined by qRT-PCR and Western blot analysis, respectively. *Results:* Matrine inhibited A549 cell viability in a dose- and time-dependent manner. However, when A549 cells were treated with matrine combined with Y-27632, the decreases in cell viability and migration were greater than those observed following treatment with matrine alone. Matrine in combination with Y-27632 induced apoptosis more effectively than matrine alone. Notably, the mRNA and protein levels of Rad51 and ERCC1 were also suppressed by treatment with the combination of matrine and Y-27632. *Conclusion:* Y-27632 increases the sensitivity of A549 cells to matrine in regulating cell proliferation, migration and apoptosis. The effect of the combination of matrine and Y-27632 on A549 cells is likely to be related to Rad51/ERCC1 signaling.

Keywords: Matrine, Y-27632, lung cancer, ERCC1, Rad51

Introduction

Lung cancer is the most frequent cause of cancer-related mortality worldwide, with approximately 1.6 million new cases and 1.4 million deaths each year [1, 2]. Non-small cell lung cancer (NSCLC), the most common form of lung cancer, is further subtyped into adenocarcinoma, squamous cell carcinoma, and large cell carcinoma [3]. Significant advances have been made in NSCLC therapy since the discovery of activating EGFR mutations and the development of specific EGFR tyrosine kinase inhibitors [4, 5]. However, acquired resistance to these therapies has become a significant challenge and more specific and more potent inhibitors of relevant signaling pathways are urgently needed.

Matrine ($C_{15}H_{24}N_2O$, **Figure 1A**) is a natural alkaloid component of *Sophora flavescens* with various biological activities including anti-inflammatory, antiviral, and anticancer effects [6-8]. Matrine has been reported to exert antitumor effects on A549 human non-small lung cancer cells through different signaling pathways including the anti-apoptotic pathway involving the BCL-2 members [9-11]. However, the curative effect of matrine alone on lung cancer is limited. Moreover, it is not entirely clear whether the efficacy of matrine alone as a lung cancer therapy can be improved by use in combination with other inhibitors.

Y-27632 (**Figure 1B**), (R)-(+)-trans-4-(1-aminoethyl)-N-(4-pyridyl)cyclohexanecarboxamide dihydrochloride, was first identified as a specific
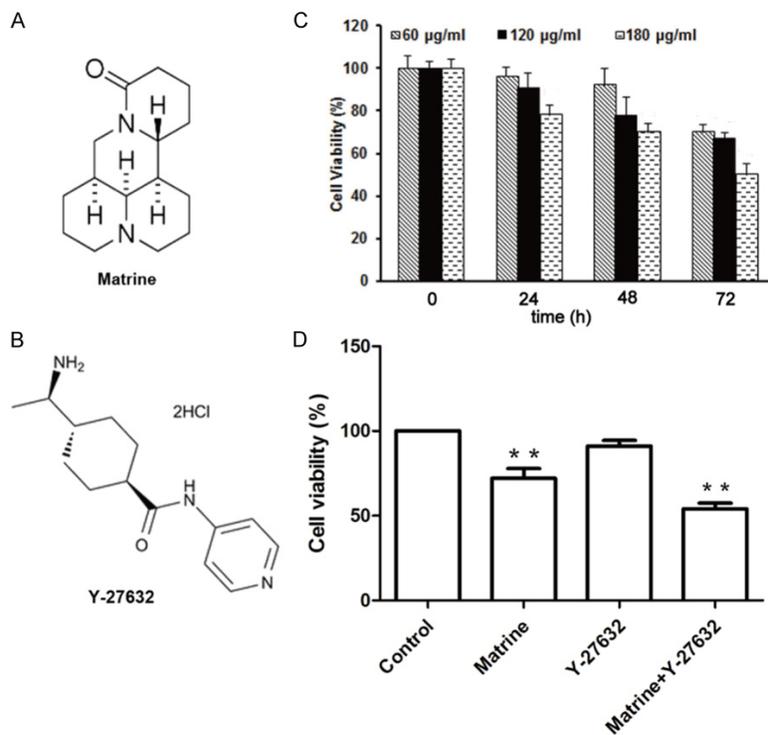
Figure 1. Effect of the combination of matrine and Y-27632 on A549 cell proliferation. A, B. Chemical structure of matrine and Y-27632. C. A549 cells were treated with matrine (60, 120 and 180 µg/ml) for 24, 48 and 72 h. Cell viabilities were then measured using the MTT assay. D. A549 cells were treated with DMSO, matrine (180 µg/ml), Y-27632 (20 µM), and the combination of matrine (180 µg/ml) and Y-27632 (20 µM). Cell viabilities were then measured using the MTT assay. Data represent the means ± S.E.M of three independent experiments. [**]$P < 0.05$ compared with the control, matrine, or Y-27632 groups.

(NER), which involves the recognition of DNA damage and removal of the damaged nucleotides [21]. The DNA recombinase, Rad51, plays a key role in the homologous recombination repair (HRR) pathway [22]. Overexpression of ERCC1 and Rad51 induces resistance to chemotherapeutic agents that promote double-stranded DNA breaks. Moreover, inhibition of Rad51 and ERCC1 expression has been shown to sensitize NSCLC cells to chemotherapeutic agents [23-25].

In the present study, we investigated the capacity of Y-27632 to sensitize A549 cells to the effects of matrine in regulating cell proliferation, migration and apoptosis. Furthermore, we examined the correlation between the expression of the DNA damage repair proteins, Rad51 and ERCC1, and the effects of matrine alone or in combination with Y-27632.

Materials and methods

Reagents and cell line

Matrine, Y-27632 dihydrochloride, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Corning® Transwell® polycarbonate membrane cell culture inserts and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Annexin V Apoptosis Kits and SYBR green qPCR Kits were obtained from Thermo Fisher Scientific (China) Co., Ltd (Shanghai, China). Primary antibodies for the detection of Rad51and ERCC1 were purchased from Santa Cruz Biotechnology (Shanghai, China). The human lung adenocarcinoma cell line A549 was purchased from the Cell Bank of Chinese Academy of Sciences (Shanghai, China).

Cell culture

Cells were grown in the RPMI-1640 medium (Gibco, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS), penicillin

inhibitor of the Rho-associated coiled-coil forming protein serine/threonine kinase (ROCK) family of protein kinases in 1997 by Uehata [12]. Emerging evidence [13] indicates that the closely related ROCK1 and ROCK2 kinases, known regulators of actin cytoskeletal organization that play an important role in cellular contractility and adhesion [14], migration and morphogenesis, support the invasive and metastatic phenotype of a variety of cancers [15] including lung cancer. Y-27632 is widely used in the treatment of cardiovascular disease, inflammation, and cancer [16-18]. Although Y-27632 inhibits the growth and invasion of NSCLC cells (such as H1299 and H358), it shows weak effects on A549 cell proliferation and apoptosis because of the loss of the DLC1 (deleted in liver cancer 1) gene [19, 20]. However, the mechanism of the effects of Y-27632 on NSCLC is not yet clear.

Excision repair cross-complementary 1 (ERCC1) is a leading protein in nucleotide excision repair

Table 1. Primer sequences

Gene	Primer sequences
β-actin forward	5'-CCCATCTATGAGGGTTACGC-3'
β-actin reverse	5'-TTTAATGTCACGCACGATTTC-3'
Rad51 forward	5'-GTGGAGCTAATGGCAATGCAG-3'
Rad51 reverse	5'-TTAGCTCCTTCTTTGGCGCA-3'
ERCC1 forward	5'-GTCTCCCGGTCTCTGGAACA-3'
ERCC1 reverse	5'-GCTCGTGCAGGACATCAAAC-3'

(100 U/mL) and streptomycin (100 U/mL) at 37°C under 5% CO_2 in air and at 100% humidity.

MTT assays

Since there were no significant differences in the viability of the cells exposed to Y-27632 at concentrations ranging from 0.01 to 50 µM for 48 h [20], Y-27632 was used in experiments at 20 µM, which does not affect cell viability. A549 cells (4×10^3 per well) were seeded into 96-well plates (Corning, NY, USA). Cells were treated with matrine (60, 120, and 180 µg/ml) for 24, 48 and 72 h, or Y-27632 (20 µM), and matrine (180 µg/ml) combined with Y-27632 (20 µM) for 48 h. MTT solution (5 mg/ml) was added to the culture medium and incubated at 37°C for 4 h. After removal of MTT, dimethyl sulfoxide (DMSO) solution was added to dissolve the formazan crystals. Absorbance was measured at a wavelength of 570 nm. Cell viability was expressed as a percentage of the control without matrine treatment.

Transwell migration assay

A549 cells were detached with phosphate-buffered saline (PBS) containing EDTA and then resuspended in serum free RPMI 1640. The cells (1×10^5) were placed in the upper chamber of a transwell filter. Drugs (DMSO, 180 µg/ml matrine, 20 µM Y-27632, and 180 µg/ml matrine + 20 µM Y-27632) were added into the upper medium and cells were incubated for 10 h. The surface of the cell membrane in the upper chamber was then scraped carefully with a cotton swab to remover the cells. After fixation and staining, cells were counted and the cell migration rate was calculated according to the following formula: cell migration rate = [1 - (number of migrated cells in the intervention group/number of migrating cells in the control group)] × 100%.

Apoptosis analysis

A549 cells were treated with DMSO, 180 µg/ml matrine, 20 µM Y-27632, and 180 µg/ml matrine + 20 µM Y-27632 for 48 h. Apoptosis was detected using an Annexin V-fluorescein isothiocyanate/propidium iodide (Annexin V-FITC/PI) affinity assay. Briefly, 1×10^5 cells were harvested and washed twice with pre-cold PBS and then resuspended in 500 µl binding buffer. 5 µl of Annexin V-FITC (KeyGen Biotechnology, Nanjing, China) and 5 µl of Propidium Iodide (PI) were added to each sample and then incubated at room temperature in dark for 20 minutes. The stained cells were analyzed by flow cytometry (BD Biosciences, San Jose, CA, USA).

Quantitative real-time RT-PCR

A549 cells were treated with DMSO, 180 µg/ml matrine, 20 µM Y-27632, and 180 µg/ml matrine + 20 µM Y-27632 for 48 h. Cells were then collected and total RNA was extracted with Trizol. Real-time PCR was performed using SYBR® green PCR master mix with the PCR primer sequences described in **Table 1** on a Bio-Red CFX Manager. Data were normalized to β-actin expression and further normalized to the negative control. Relative mRNA was calculated using the formula [26], 2-ΔΔCt.

Western blot

A549 cells were seeded into 10 cm plates (1×10^6 cells per plate), and then treated with DMSO, 180 µg/ml matrine, 20 µM Y-27632, and 180 µg/ml matrine + 20 µM Y-27632 for 48 h. Cells washed twice with PBS, and total proteins were extracted in 500 µl lysis buffer. Aliquots of whole-cell lysates were added for separation of total proteins by SDS-PAGE and then transferred to PVDF membranes (Millipore, Bedford, MA, USA). The blots were blocked with 5% dried non-fat milk/TBS-0.1% Tween and then incubated with primary antibodies for the detection of Rad51 (H-92; 1:200), ERCC1 (FL-297; 1:200), and β-actin (1:500). After washing with TBST, the membrane was incubated for 2 h at 37°C with the horseradish peroxidase-conjugated affinipure goat anti-rabbit IgG (H+L) (1:2000 dilution, ZSGB-BIO, Beijing, China).

Bands were monitored using Western blot chemiluminescence reagents (Millipore).
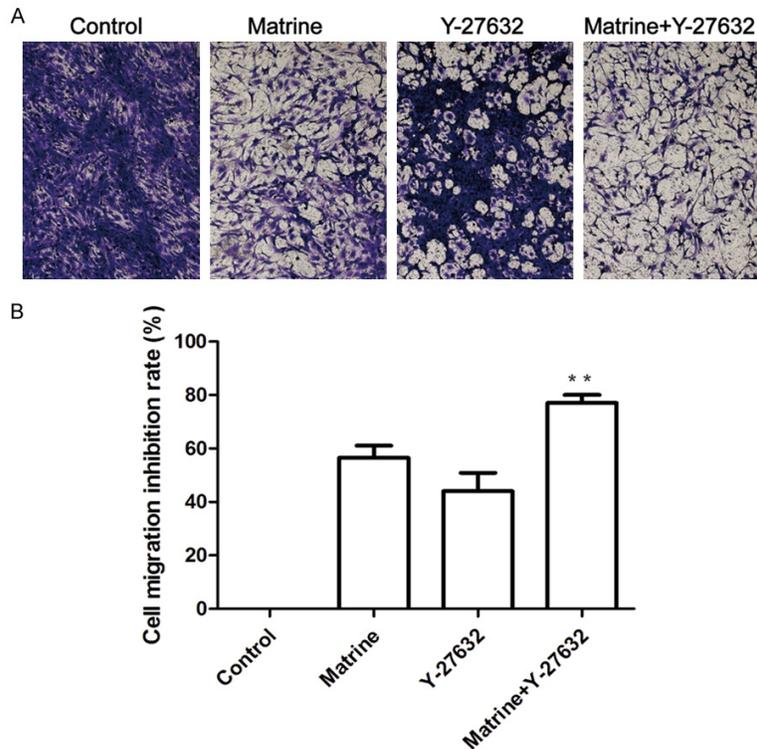
A

Control | Matrine | Y-27632 | Matrine+Y-27632

B

Figure 2. Effect of the combination of matrine and Y-27632 on A549 cell migration. A549 cells were treated with DMSO, matrine (180 μg/ml), Y-27632 (20 μM), and the combination of matrine (180 μg/ml) and Y-27632 (20 μM) for 10 h. Migration was assessed using transwell assays. A. The percentage migration rate is expressed as a percentage of the control. B. Represent images of migration assays. Data represent the means ± S.E.M of three independent experiments. **P < 0.05 compared with the control, matrine, or Y-27632 groups.

Statistical analysis

Data from three independent experiments are expressed as mean ± S.E.M. and one-way ANOVA followed by Dunnett's method was used for the statistical analyses. A *P*-value of < 0.05 was considered to indicate statistical significance (**P < 0.05).

Results

Y-27632 augments the antiproliferative effect of matrine in A549 cells

The effect of matrine on the A549 cell proliferation was evaluated by MTT assay. Different concentrations of matrine (60, 120, and 180 μg/ml) inhibited cell viability in a dose- and time-dependent manner (**Figure 1C**). Matrine at 180 μg/ml inhibited A549 cells viability by 78% (Compared with control, t = 3.118, **P < 0.05) (**Figure 1D**). And matrine (180 μg/ml) combined

with Y-27632 (20 μM) inhibited A549 cells viability by 64% (Compared with matrine, t = 2.939, **P < 0.05) (**Figure 1D**). In contrast, Y-27632 (20 μM) failed to inhibit the viability of A549 cells. These results suggested that Y-27632 enhances the sensitivity of A549 cells to matrine and suppresses cell proliferation.

Y-27632 improved the anti-migration effect of matrine in A549 cells

The effect of matrine on the A549 cell migration in the presence of Y-27632 was evaluated using transwell migration assays (**Figure 2A**). Following treatment with the combination of matrine (180 μg/ml) and Y-27632 (20 μM), cell migration were significantly decreased when compared with that of the untreated A549 control (Compared with control, t = 26.065, **P < 0.05), and the cells treated with matrine (180 μg/ml) (Compared with control, t = 3.791, **P < 0.05) or Y-27632 (20 μM) (Compared with Y-27632, t = 4.424, **P < 0.05) alone (**Figure 2B**). These data suggested that A549 cell migration is inhibited by treatment with the combination of matrine and Y-27632.

Y-27632 enhanced matrine-induced apoptosis in A549 cells

The effect of Y-27632 on matrine-induced apoptosis in A549 cells was further investigated using Annexin V-FITC/PI assays (**Figure 3A**). A549 cell apoptosis reached 9.9% and 4.4% following treatment with matrine (180 μg/ml) or Y-27632 (20 μM) alone compared to 1.9% in the untreated control group. However, A549 cell apoptosis was significantly increased to 24.9% following treatment with the combination of matrine (180 μg/ml) and Y-27632 (20 μM) [(Compared with control, t = 20.852, **P < 0.05); (Compared with matrine, t = 12.358, **P
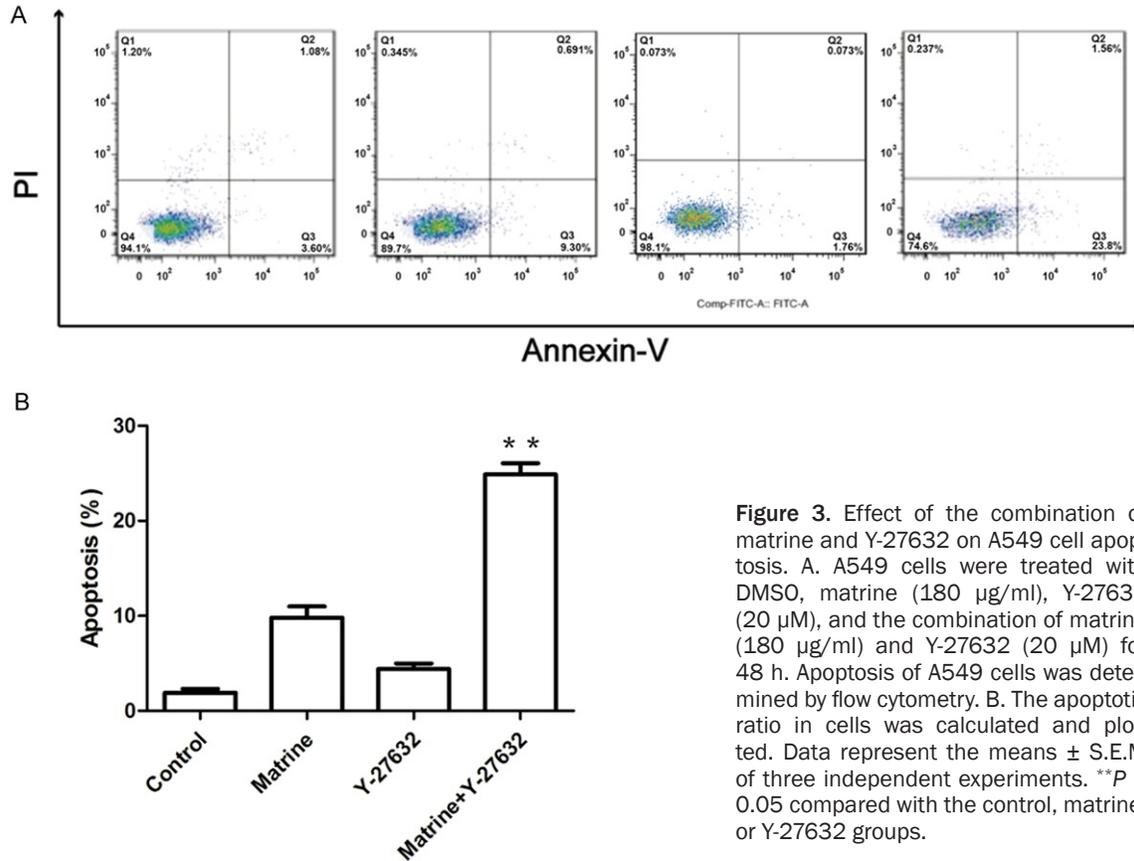
Figure 3. Effect of the combination of matrine and Y-27632 on A549 cell apoptosis. A. A549 cells were treated with DMSO, matrine (180 μg/ml), Y-27632 (20 μM), and the combination of matrine (180 μg/ml) and Y-27632 (20 μM) for 48 h. Apoptosis of A549 cells was determined by flow cytometry. B. The apoptotic ratio in cells was calculated and plotted. Data represent the means ± S.E.M of three independent experiments. **$P < 0.05$ compared with the control, matrine, or Y-27632 groups.

< 0.05); (Compared with Y-27632, t = 9.253, **$P < 0.05$)] (**Figure 3B**).

The combination of matrine and Y-27632 decreased the expression of Rad51 and ERCC1 at the mRNA and proteins levels

Rad51 and ERCC1 overexpression [27, 28] causes drug resistance. Therefore, we finally investigated the capacity of Y-27632 to sensitize A549 cells to matrine by inhibiting Rad51 and ERCC1 expression. The expression of Rad51 and ERCC1 genes was determined by qRT-PCR. The amplification and melting curves of Rad51 and ERCC1 are shown in **Figure 4A** and **4B**. Significant changes in the expression of Rad51 and ERCC1 were observed in A549 cells treated with the combination of matrine (180 μg/ml) and Y-27632 (20 μM) in comparison to the cells treated with matrine (180 μg/ml) or Y-27632 (20 μM) alone (**Figure 4C**). Treatment with Y-27632 combined with matrine caused a sharp decreased (approximately 50%) in gene expression of Rad51 (Compared with control, t = 6.491, **$P < 0.05$) and ERCC1 (Compared with control, t = 3.062, **$P < 0.05$)

compared to the levels detected following treatment with matrine or Y-27632 alone.

Furthermore, the expression of Rad51 and ERCC1 proteins was determined by Western blot analysis (**Figure 5B**). Similarly, treatment with Y-27632 (20 μM) combined with matrine (180 μg/ml) treatment significantly suppressed the expression of Rad51 (Compared with control, t = 5.634, **$P < 0.05$) and ERCC1 (Compared with control, t = 2.817, **$P < 0.05$) proteins in A549 cells, while only a slight decrease was observed following treatment with matrine (180 μg/ml) or Y-27632 (20 μM) alone (**Figure 5A**).

Discussion

In the current study, we demonstrated that Y-27632 sensitized the NSCLC cell line A549 to the inhibitory effects of matrine on cell proliferation and migration. Furthermore, the combination of Y-27632 and matrine promoted apoptosis. Our results indicate that the Y-27632-induced sensitization is related to the decreased expression of Rad51 and ERCC1.
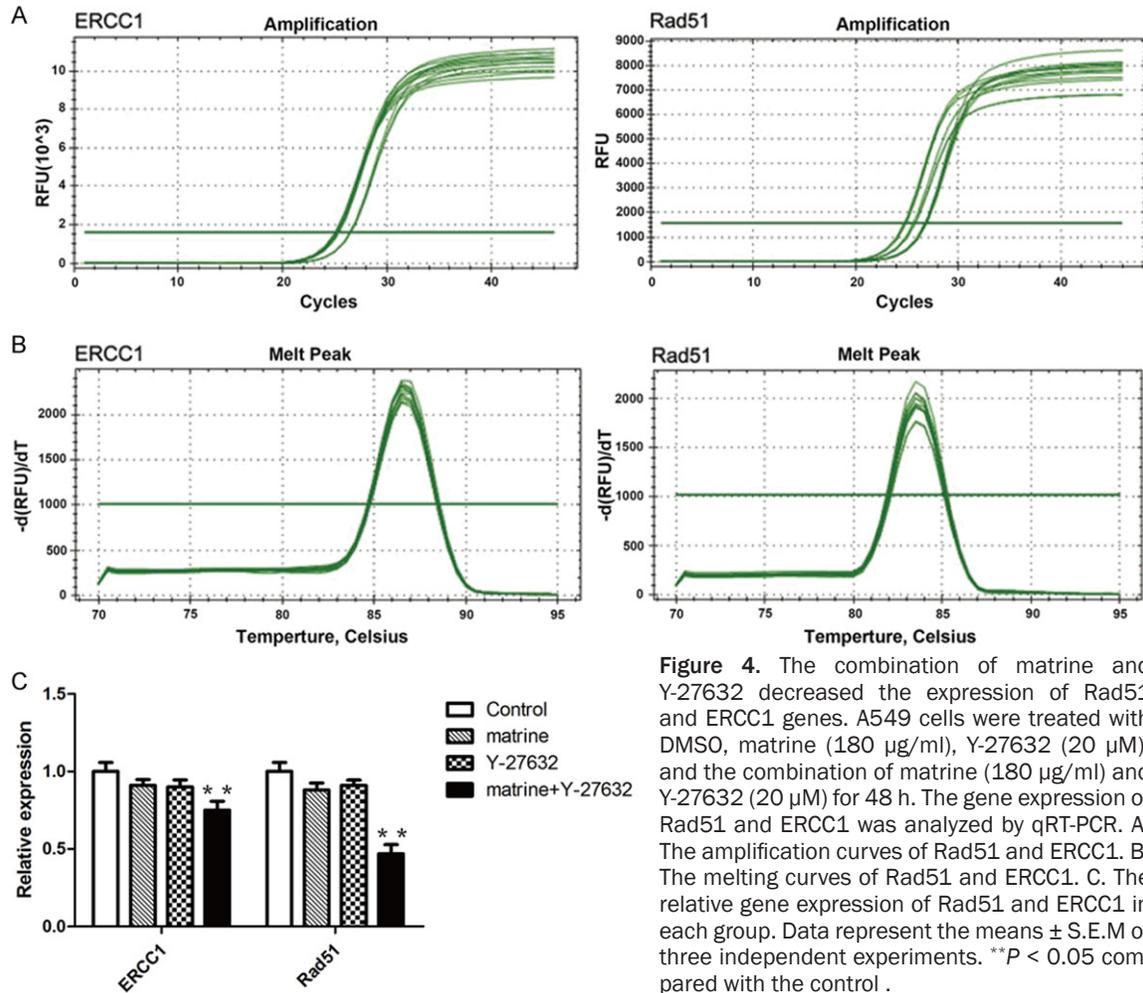
Figure 4. The combination of matrine and Y-27632 decreased the expression of Rad51 and ERCC1 genes. A549 cells were treated with DMSO, matrine (180 µg/ml), Y-27632 (20 µM), and the combination of matrine (180 µg/ml) and Y-27632 (20 µM) for 48 h. The gene expression of Rad51 and ERCC1 was analyzed by qRT-PCR. A. The amplification curves of Rad51 and ERCC1. B. The melting curves of Rad51 and ERCC1. C. The relative gene expression of Rad51 and ERCC1 in each group. Data represent the means ± S.E.M of three independent experiments. **$P < 0.05$ compared with the control .

Lung cancer is a major cause of deaths from cancer worldwide, with an extremely poor 5-year survival rate (< 15%), accentuating the need for more effective therapeutic strategies [29]. Naturally occurring phytochemicals are an important source of anticancer agents and are usually associated with low toxicity and side-effects [30]. Matrine is an alkaloid that possesses a variety pharmacological and biological activities [31-33]. The results of our study show that matrine inhibits the proliferation and migration of A549 cells, and also induces apoptosis. Therefore, we hypothesized that the anticancer effects of matrine may be enhanced when combined with other agents.

Rho is a small GTPase, which is involved in the processes of cell adhesion, formation of actin stress fibers, and cell contraction [34-36]. Rho-associated coiled-coil forming protein kinase (ROCK) is the best characterized Rho effector and has been implicated in Rho-mediated cell adhesion and smooth muscle contraction [37, 38]. Y-27632 is a selective inhibitor of ROCK-1 and ROCK-2 (K_i values of 0.22 µmol/L and 0.30 µmol/L, respectively) [39]. In the present study, we evaluated the anticancer effect of matrine combined with Y-27632 in NSCLCs. Interestingly, Y-27632 treatment for 48 h did not significantly inhibit A549 cell proliferation over the concentration range from 0.01 µM to 50 µM [20]. Our results show that Y-27632 enhances the anti-proliferative effect of matrine on A549 cells when treated at 20 µM for 48 h. Furthermore, the effects of matrine in regulating migration and apoptosis of A549 cells were enhanced when treated in combination with Y-27632.

The DNA damage repair enzymes, Rad51 and ERCC1, are both involved in homologous recombination and DNA replication pathways [40]. The expression of ERCC1 and Rad51 proteins is closely related to tumor response and
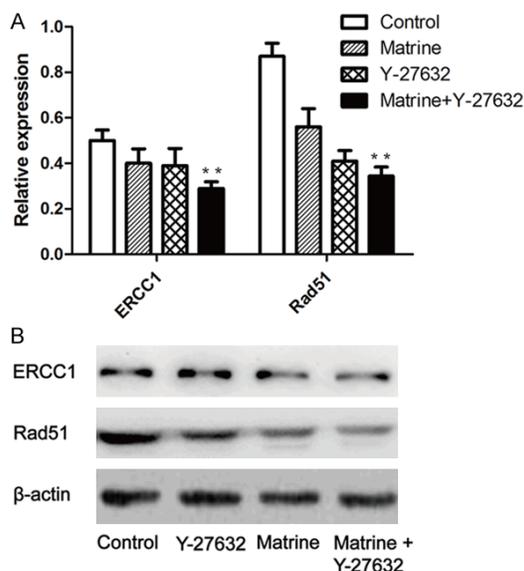
Figure 5. The combination of matrine and Y-27632 suppressed the expression of Rad51 and ERCC1 proteins. A549 cells were treated with DMSO, matrine (180 μg/ml), Y-27632 (20 μM), and the combination of matrine (180 μg/ml) and Y-27632 (20 μM) for 48 h. The expression of Rad51 and ERCC1 proteins was determined by Western blot analysis. A. Relative protein expression of Rad51 and ERCC1. B. Representative blots are shown in lower panels. Data represent the means ± S.E.M of three independent experiments. $^{**}P < 0.05$ compared with the control.

survival of stage III/IV NSCLC patients treated with chemotherapy [41]. Moreover, ERCC1 and Rad51 expression plays a key role in regulating the sensitivity of NSCLC cells to chemotherapeutic agents. Therefore, we evaluated the expression of ERCC1 and Rad51 in the cells treated with matrine combined with Y-27632 or matrine alone. The combination of matrine and Y-27632 significantly decreased the expression of ERCC1 and Rad51 at both the mRNA and protein levels. These data suggest that Y-27632 sensitizes A549 cells to matrine by inhibiting the expression of ERCC1 and Rad51. The synergistic effect of the combination of matrine and Y-27632 on NSCLC provides new and useful information for its application in lung cancer therapy.

Acknowledgements

Disclosure of conflict of interest

None.

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