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ORIGINAL ARTICLE

ROCK inhibitor Y-27632 inhibits the growth, migration, and invasion of Tca8113 and CAL-27 cells in tongue squamous cell carcinoma

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Abstract The objective of this study is to determine the effects of Rho-associated coiled-coil containing protein kinase (ROCK) inhibitor Y-27632 on the growth, invasion, and migration of Tca8113 and CAL-27 cells in tongue squamous cell carcinoma (TSCC). The methods of the study are as follows: After being routinely cultured for 24 h, Tca8113 and CAL-27 cells were treated with Y-27632 solution. The morphological change of Y-27632-treated cells was observed under an optical microscope and an inverted microscope; MTT assay was performed to measure the optical density (OD) of cells and calculate cell growth inhibition rate; the change of apoptosis was detected by AnnexinV-FITC/PI assay; cell invasion and migration were measured by Transwell assay. The results were as follows: (1) With increasing concentration of Y-27632, cell morphology changed and cell apoptosis appeared; (2) MTT assay showed that inhibition effect of Y-27632 on Tca8113 and CAL-27 cells was enhanced with increasing concentrations and time (all $P<0.01$); (3) Apoptosis showed that, compared with controls, the number of apoptosis cells in experimental groups was significantly increased (all $P<0.01$). Apoptosis rate was elevated with increasing concentrations of Y-27632; (4) Transwell assay showed, after a treatment with Y-27632, the number of migrated and invaded Tca8113 and CAL-27 cells in each group was statistically different (all $P<0.01$); compared with controls, the number of migrated cell in groups treated with Y-27632 was decreased and less Tca8113 and CAL-27 cells in experimental groups passed through polycarbonate membrane (all $P<0.05$). The study concludes that Y-27632 can inhibit the growth, invasion, and migration of Tca8113 and CAL-27 cells, suggesting that Y-27632 may be therapeutically useful in TSCC.

Keywords ROCK inhibitor · Y-27632 · Tca8113 · CAL-27 · Tongue squamous cell carcinoma · Cell growth · Cell invasion · Cell migration

Introduction

Tongue squamous cell carcinoma (TSCC) remains one of the most aggressive types of oral squamous cell carcinoma in terms of cell spreading and local invasion [1]. It is reported that an estimated 10,990 new cases of TSCC are developed each year around the world, accounting for about 30 % of all pharynx and oral cavity cancers [2]. TSCC is more aggressive than other types of oral cancer, with a propensity for rapid local spread and invasion together with a high rate of recurrence. As a matter of fact, the main causes of TSCC-related death are local relapse and metastasis. At the time of diagnosis, 20~40 % of TSCC patients in stage I and II have occult nodal metastasis; 40 % of TSCC patients have neck metastasis [1]. Despite of a variety of improvements in surgery, chemotherapy, and radiotherapy for the treatment of TSCC, the 5-year survival rate for early-stage (I–II) TSCC patients remains modest and the cure rate of TSCC has only marginally enhanced [3]. Hence, identification of molecular markers

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involved in TSCC progression may be useful as therapeutic targets to improve treatment interventions.

Rho-associated coiled-coil containing protein kinase (ROCK) is regarded as one of the best characterized downstream effectors of Rho GTPases which entail various cellular functions, such as cell apoptosis, invasion, proliferation, as well as metastasis [4, 5]. Elevated expression of ROCK has been implicated in the progression of many tumors, such as breast and hepatocellular carcinomas [6, 7]. Previous study demonstrated that ROCK level was associated with cell migration, invasion, and the reduction in stress fiber formation in TSCC [8]. Decreased ROCK expression and inhibition of ROCK activity had been introduced as potential tumor therapeutic options for TSCC [9, 10]. Y-27632, a protein kinase inhibitor selective for ROCK, involves various cellular functions, including actin cytoskeleton organization, cell motility, cell adhesion, and anti-apoptosis and binds to the Rho-kinase ATP binding pocket in an ATP-competitive manner [11]. Previous study had demonstrated that the expression of the dominant-negative ROCK or the ROCK inhibitor Y-27632 could massively inhibit in vitro cancer cell invasion/migration [12]. Recent reports have also revealed that ROCK inhibitor could block cell invasion, cell migration, proliferation, or metastasis, such as in human laryngeal squamous cell carcinoma cells, human mesenchymal stem cells, and human prostate cancer cells [13, 14]. However, to date, ROCK inhibitor Y-27632 has not been characterized in human TSCC samples. The aim of the current study was to determine the effects of ROCK inhibitor Y-27632 on the growth, invasion, and migration of human Tca8113 and CAL-27 TSCC cell lines.

Methods and materials

Cell culture and group design

Human tongue squamous cell carcinoma cell line (Tca8113) was purchased from Shanghai Institute for Cellular Biology, Chinese Academy of Sciences and CAL-27 cell line was purchased from Shanghai Ninth People's Hospital Affiliated to Shanghai Jiaotong University. Tca8113 and CAL-27 cell lines were cultured in RPMI-1640 medium supplemented with 10 % fetal bovine serum (FBS), 100 μ/mL penicillin G, and 100 μ/mL streptomycin, routinely incubated at 37 °C in 5 % CO_2. The growth condition of cells was observed under an inverted microscope with the medium passaged every other day. The cells were digested in 0.25 % trypsin and subcultured until the density of proliferation reached 80 %. Cells in logarithmic growth phase were collected and divided into experimental group and control group. Y-27632 (10 mM, Alexis Biochemicals, USA) treated by 0.01 M phosphate-buffered saline (PBS) solution was added to the experimental group for 24 h for three-group design: Y-27632 10 μmol/L group; Y-27632 20 μmol/L group, and Y-27632 40 μmol/L group, while the control group was treated with equal amounts of PBS solution.

Morphology observation of Y-27632-treated Tca8113 and CAL-27 cells

The morphology and growth condition of Tca8113 and CAL-27 cells were observed under an inverted microscope. Cells in logarithmic growth phase were digested for single cell suspension and seeded to petri dish equipped with sterilized slide with the concentration of 5×10^5/mL. After being cultured for 24 h, medium and design groups were changed. The slides with cells were collected and fixed with 95 % ethanol for 15 min followed by hematoxylin-eosin (HE) staining. The cell morphology was observed under an optical microscope.

MTT assay for measurement of cell proliferation

The cell suspension was seeded to a 96-well plate (200 μL per well) with the adjusted concentration 2.5×10^4 cell/mL, immediately incubated overnight at 37 °C with 5 % CO_2 and saturated humidity. The medium and design groups were replaced; in each experimental group (Y-27632 10 μmol/L group; Y-27632 20 μmol/L group and Y-27632 40 μmol/L group), 6 duplications were set. Cell proliferation was assessed 12, 24, 48, 72, and 96 h later. MTT solution (20 μL) was added to each well and the plate was further incubated for 4 h. Removing the medium, dimethyl sulfoxide (DMSO) was supplemented with oscillation for 10 min. The optical density (OD) was measured with a spectrophotometer (Olympus, Japan) at 492 nm. OD value of cells was drawn with time as x axis and absorbance as y axis. Proliferation inhibition rate=(1−OD of experiment group/OD of control group)×100 %.

Annexin V-FITC/PI staining for apoptosis evaluation

Cultured cells were suspended in PBS and counted. Cells were centrifuged for 5 min and re-suspended in Annexin V-FITC binding buffer (195 μL). Annexin V-FITC (195 μL) was supplemented and the cells were incubated away from light at 20~25 °C for 10 min. Propidium iodide (PI) (10 μL) was further added, followed by incubation in the darkness. Apoptosis was quantified by using flow cytometry. CellQuestPro software was used for analyzing apoptosis condition.

Cell migration and invasion assay

Transwell assay was used for measuring the migration and invasion ability change of Tca8113 and CAL-27 cells. For cell

migration assays, Tca8113 and CAL-27 cells were cultured in a serum-free medium for 24 h and then digested. Regular cell culture medium was used for adjusting the cell concentration to 1×10^5 cells/mL. Totally, 100-μL cell suspension with 10 % FBS was added to the upper compartment of the invasion chamber and 500-μL medium supplemented with 20 % FBS was used to the lower compartment. The 24-well plate Transwell system was incubated at 37 °C with 5 % CO_2 and taken out 48 h later. After wiping up the cells in Transwell chamber, the Transwell chamber was washed by 1×PBS (5 min×3 times). The Transwell chamber with cells were immersed in 95 % ethanol for 20 min and washed by 1×PBS (5 min×3 times) and immersed again in crystal violet solution for 5 min and washed with 1×PBS (2 min×3 times). The number of cells migrating through polycarbonate membrane was counted under an inverted microscope. Each cell line was seeded to six Transwell chambers with six high-magnification visual field. The average value was calculated as the number of migrated cells. Mean±standard deviation (Mean±SD) was calculated by using the six migrated number of each cell line. For cell invasion assays, Transwell chamber was not coated by Matrigel and hydrated directly. Other procedures were treated as migration assays.

Statistical analysis

SPSS version 19.00 software for Windows (SPSS, Inc., Chicago, IL, USA) was performed for statistical analysis. The results were presented as Mean±SD. The comparison for average values between two groups was performed using *q* test or Dunnett-*t* test. The inhibition effect of Y-27632 on the proliferation of Tca8113 and CAL-27 was analyzed by applying one-way analysis of variance (ANOVA) test with repeated measurement data. The value of *P* less than 0.05 was considered as statistically significant.

Results

Morphology change of Y-27632-treated Tca8113 and CAL-27 cells

Observation under an inverted microscope: Tca8113 cells (control) were adherent cells, polygon-shaped or fusiform, with clear morphology and a trend toward arrangement in nest, as well as good growth condition (Fig. 1A (a)). The growth of Y-27632-treated Tca8113 cells was significantly inhibited, with reduced projections in some cells. Cells shrank and cytoplasmic granules increased. Some cells presented pyknotic and a few cells cracked and died. The change was increasingly obvious with increasing dose (Fig. 1A (b, c, d)). In control group, larger Tca8113 cells were found under an optical microscope: irregular nuclear shape, prominent nucleolus, and multiple nucleoli could be observed. With increasing concentrations of Y-27632, shrank and small cells, decreased nucleoplasm ratio, reduced number of nucleoli, vacuole formation in cytoplasm, and apoptotic cells were observed (Fig. 1B). CAL-27 cells in control group were adherent cells, polygon-shaped or oval, with good growth, different size, and even distribution (Fig. 1C (a)). Y-27632-treated CAL-27 cells showed budding, foaming, or sunflower-shaped cell membrane; cell nucleus was pyknotic, horseshoe-like, or crescent-shape or showed karyorrhexis; with the increase of concentration, cells changed obviously (Fig. 1C (b, c, d)). HE staining results showed aligned and clear CAL-27 cells with good growth during logarithmic phase and lots of mitotic figures were seen; Y-27632-treated CAL-27 cells were swollen, with bubbles in cells; some cells showed unclear cell outline; few cells had cytoplasm pyknosis and apoptotic cells appeared (Fig. 1D).

Y-27632 inhibits the proliferation of Tca8113 and CAL-27 cells

Figure 2a, c showed the change of OD value for Y-27632-treated Tca8113 and CAL-27 cells by using MTT assay. With the increase of Y-27632 concentrations and time, proportion of the increase in living cells was gradually reduced; cell growth was significantly inhibited. The growth inhibition effect is positively related with concentration and time. Figure 2b, d showed the inhibition ratio of Y-27632 on in vitro proliferation of Tca8113 and CAL-27 cells. Results suggested that inhibition effect of Y-27632 on Tca8113 and CAL-27 cells was enhanced with increasing concentrations at the same time point (all $P<0.01$); the inhibition effect of Y-27632 with the same concentration on Tca8113 and CAL-27 cells was improved with increasing time (all $P<0.05$). The inhibition ratio of Y-27632 on in vitro proliferation of Tca8113 and CAL-27 cells positively increased with time and dose. Variance analysis revealed that Y-27632 with different concentrations delivered different growth inhibition effect on cells (all $P<0.05$); different time also had different growth inhibition effect (both $P<0.01$); there is a significant interaction between treatment time and concentration of Y-27632 (all $P<0.05$).

Y-27632 induces apoptosis of Tca8113 and CAL-27 cells

The apoptosis of Tca8113 and CAL-27 cells in each group was detected by Annexin V-FITC Apoptosis Detection Kit. Apoptosis ability of cells in four groups was measured by flow cytometry. Results showed that the proportions of Tca8113 apoptotic cells in each group were the following: control group: 0 %; Y-27632 10 μmol/L group: (3.86±0.23)%; Y-27632 20 μmol/L group: (7.17±0.64)%; Y-27632 40 μmol/L group: (15.25±0.87)%. Compared with controls, apoptosis

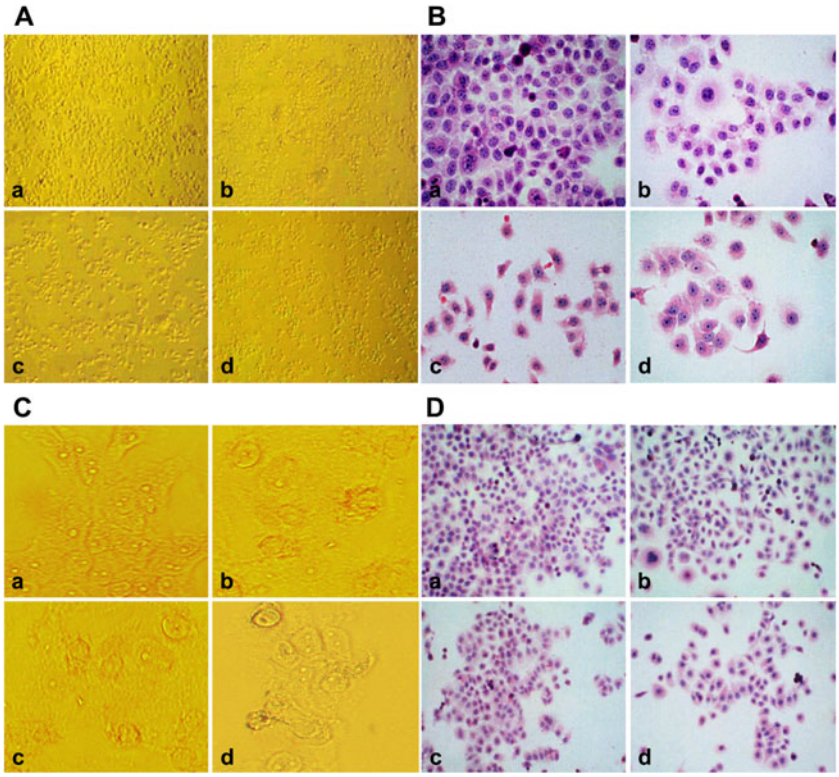

Fig. 1 Morphological change of Y-27632-treated Tca8113 and CAL-27 cells. **A** Observation under an inverted microscope: the growth of Y-27632-treated Tca8113 cells was significantly inhibited, with obviously reduced projections in some cells. Cells became crenated with increased cytoplasmic granules. Some cells presented pyknotic. The crack and death of a few Tca8113 cells were observed, and the change was increasingly significant with the increasing dose. **B** With the increasing concentrations of Y-27632, alterations of Tca8113 cell morphology, crenation, wane, decreased nucleoplasm ratio, reduced number of nucleoli, vacuole formation in cytoplasm, and apoptotic cells were observed. **C** Y-27632-treated CAL-27 cells showed budding, foaming, or sunflower-shaped cell membrane; cell nucleus was pyknotic, horseshoe-like, or crescent-shape or showed karyorrhexis. **D** Y-27632-treated CAL-27 cells were swollen, with bubbles in cells; some cells showed unclear cell outline; few cells had cytoplasm pyknosis and apoptotic cells appeared. *a* control, *b* Y-27632 10 μmol/L, *c* Y-27632 20 μmol/L, *d* Y-27632 40 μmol/L

cells were significantly increased in experimental groups (all $P<0.01$). Additionally, the apoptosis rate was getting higher with increasing concentrations of Y-27632. The apoptosis rate was significantly higher in 40 μmol/L group than in 10 and 20 μmol/L group (both $P<0.01$), which indicated that Y-27632 induces apoptosis of Tca8113 cells in a dose-dependent manner (Fig. 3a). As shown in Fig. 3b, with respect to CAL-27 cells, apoptotic cells were significantly higher in experimental groups than in control group (all $P<0.05$); the apoptosis rate was raised with the increase of Y-27632 concentration; apoptosis rate of CAL-27 cells was significantly higher in 40 μmol/L group than in 10 and 20 μmol/L group (both $P<0.05$), suggesting that Y-27632 induces apoptosis of CAL-27 cells in a dose-dependent manner.

Y-27632 inhibits migration of Tca8113 and CAL-27 cells

After Tca8113 and CAL-27 cells were incubated in Boyden Chamber for 24 h, cells transferred from the top to the bottom through pores on the membrane (Fig. 4a, c). One-way ANOVA showed that homoscedasticity and the number of migration cells in each group was statistically different (all $P<0.001$). Dunnett-*t* test between groups indicated that, compared with controls, numbers of Tca8113 and CAL-27 cell migration in each Y-27632-treated group were decreased. Y-27632-treated 10 μmol/L group had difference as compared with control group (all $P<0.05$). Moreover, significant difference was seen between Y-27632-treated 20/40 μmol/L group and control group (all $P<0.001$) (Fig. 4b, d).

Y-27632 inhibits invasion of Tca8113 and CAL-27 cells

As shown in Fig. 5a, c, high-migration cells could degrade extracellular matrix components to pass through artificially reconstructed basement membrane. Y-27632 could inhibit the invasion of Tca8113 and CAL-27 cells in a dose-dependent manner. One-way ANOVA showed homoscedasticity, and the number of invasion cells in each group was statistically different (all $P<0.001$). Compared with controls, a less number of Tca8113 cells in experimental groups passed through polycarbonate

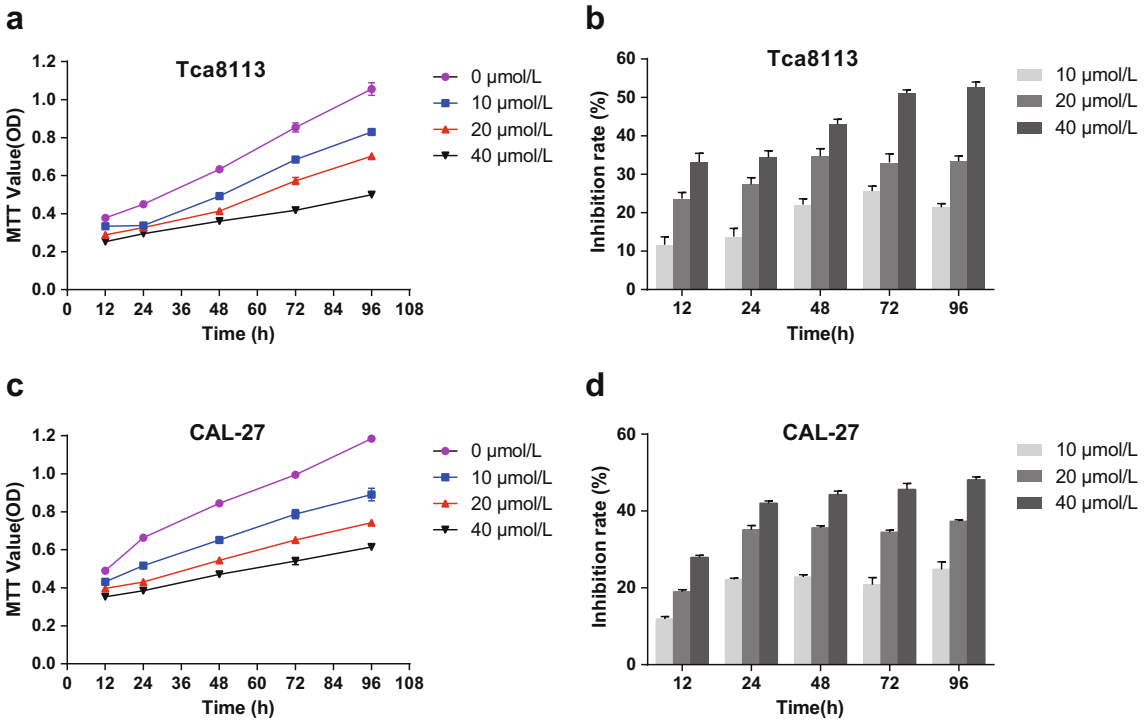

Fig. 2 Anti-proliferative inhibition effect of Y-27632 on Tca8113 and CAL-27 cells proliferation. **a**, **c** The change of OD value after MTT detection. **b**, **d** Inhibition rate of Y-27632 on Tca8113 and CAL-27 cell proliferation

membrane. Dunnett-*t* test between groups indicated that there were differences between Y-27632-treated group (three groups with different concentration) and control group (all $P<0.05$). Treated with the concentration of 10, 20, and 40 μmol/L Y-27632, the numbers of Tca8113 cells passing through artificial basement membrane were 132.71±6.03 ($P=0.032$), 68.33±6.52 ($P<0.001$), and 34.73±4.51 ($P<0.001$), respectively (Fig. 5b). As shown in Fig. 5d, after being treated with the concentration of 10, 20, and 40 μmol/L Y-27632, the

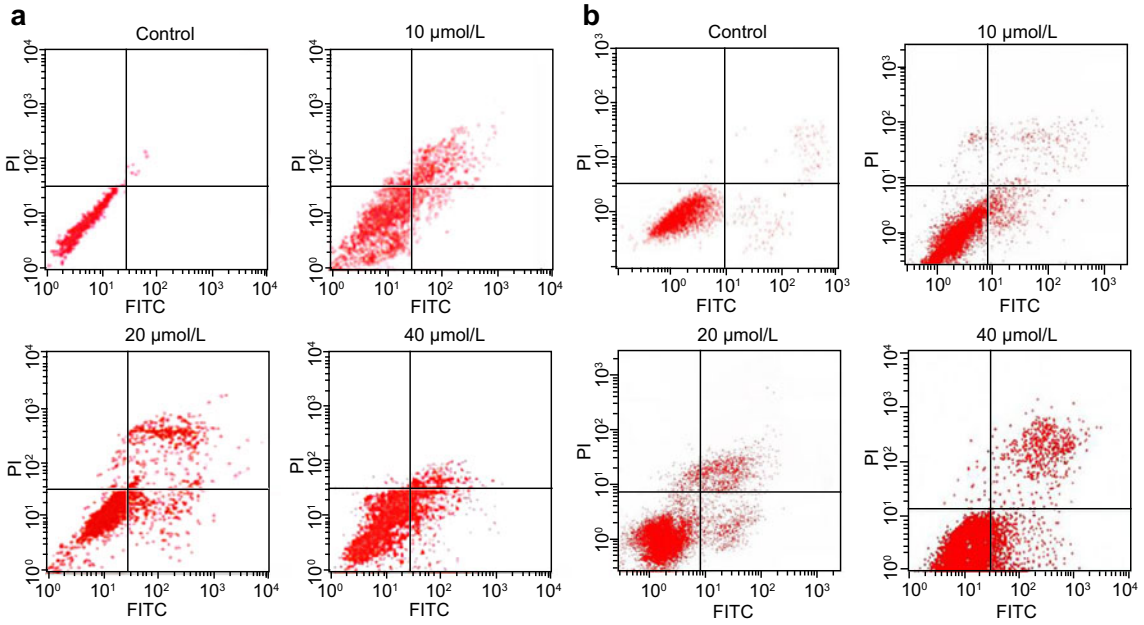

Fig. 3 Apoptosis results of Tca8113 (**a**) and CAL-27 (**b**) cells detected by flow cytometry

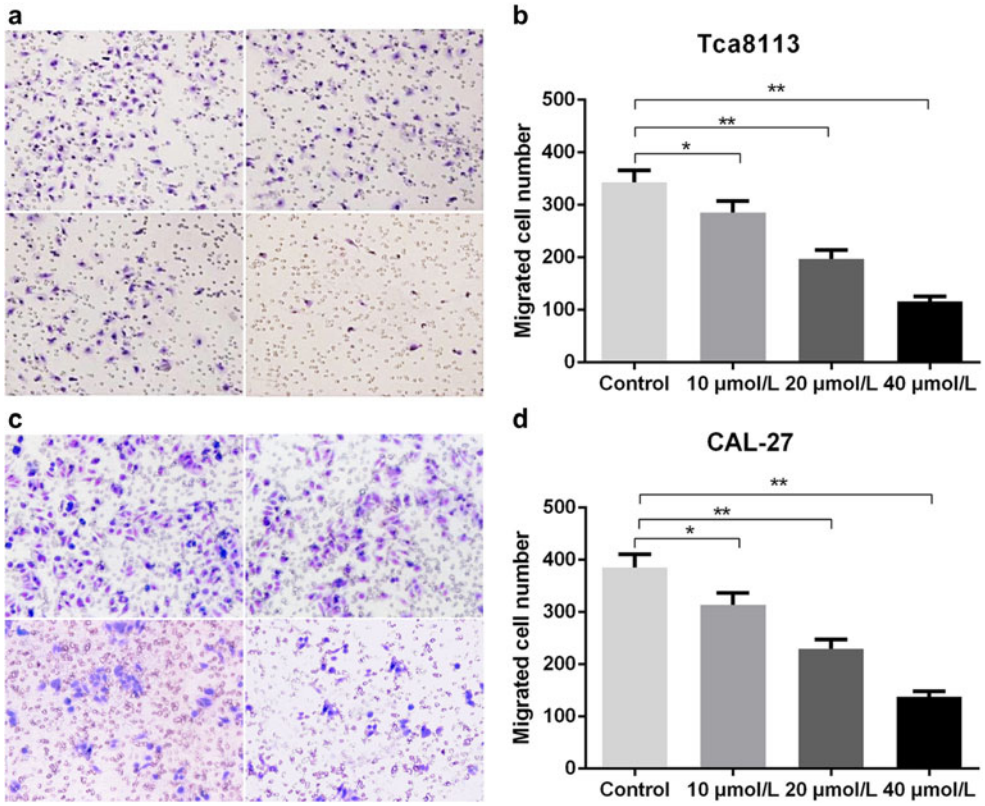

Fig. 4 Staining (×100) of adherent cells in Transwell migration assay and statistical graph of adherent cells in Transwell migration assay. **a** Tca8113 cell migration. *Upleft*, control group; *upright*, Y-27632 10 μmol/L; *left below*, Y-27632 20 μmol/L; *right below*, Y-27632 40 μmol/L. **b** Statistical graph of Tca8113 cell migration. **c** CAL-27 cell migration. *Upleft*, control group; *upright*, Y-27632 10 μmol/L; *left below*, Y-27632 20 μmol/L; *right below*, Y-27632 40 μmol/L. **d** Statistical graph of CAL-27 cell migration. **P*<0.05 was statistically significant different as compared with controls. ***P*<0.01 was extremely significant different as compared with controls

number of CAL-27 cells passing through artificial basement membrane was significantly lower as compared with the control group; the number of migrated cells decreased with the increase of concentration (all $P<0.05$).

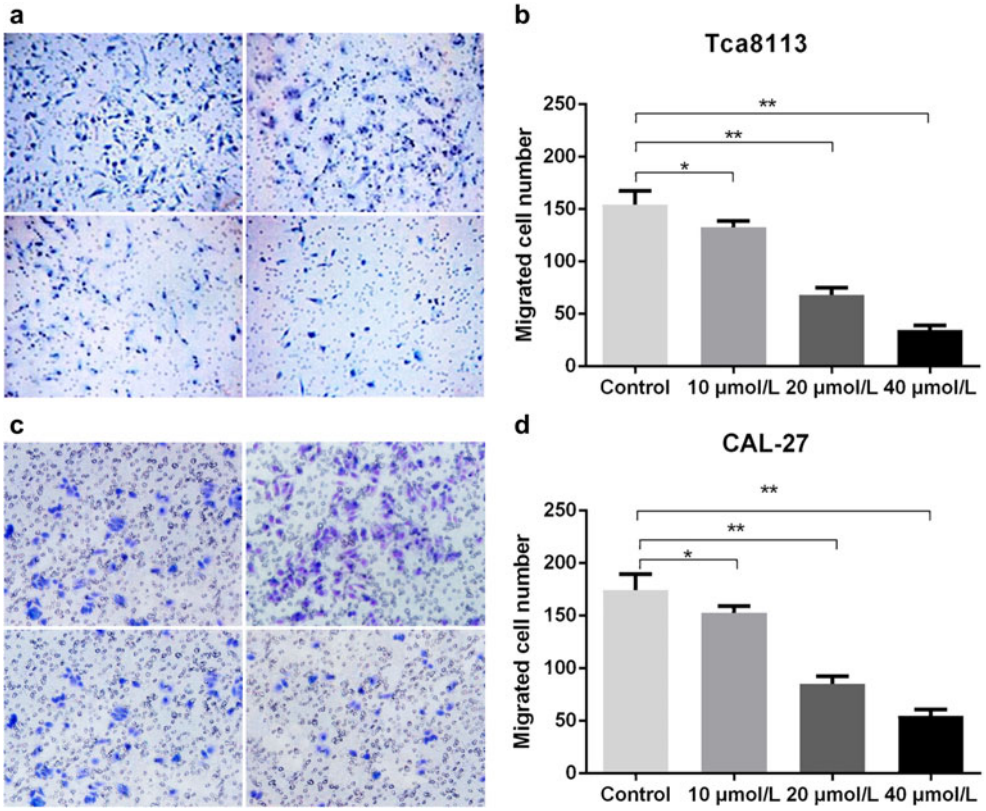

Fig. 5 Staining (×100) of adherent cells in Transwell invasion assay and statistical graph of adherent cells in Transwell invasion assay. **a** Tca8113 cell invasion. *Upleft*, control group; *upright*, Y-27632 10 μmol/L; *left below*, Y-27632 20 μmol/L; *right below*, Y-27632 40 μmol/L. **b** Statistical graph of Tca8113 cell invasion. **c** CAL-27 cell invasion. *Upleft*, control group; *upright*, Y-27632 10 μmol/L; *left below*, Y-27632 20 μmol/L; *right below*, Y-27632 40 μmol/L. **d** Statistical graph of CAL-27 cell invasion. **P*<0.05 was statistically significant different as compared with controls. ***P*<0.01 was extremely significant different as compared with controls

Discussion

Previous study demonstrated that ROCK level was associated with cell migration, invasion, and reduction in stress fiber formation in TSCC [8]. Inhibition of ROCK activity has been introduced as potential tumor therapeutic options, such as in ovarian cancer and non-small cell lung cancer, etc. [9, 10]. Hence, this study was performed with the aim to determine the effects of ROCK inhibitor Y-27632 on the growth, invasion, and migration of Tca8113 and CAL-27 cells in TSCC. Our results revealed that Y-27632 could promote apoptosis and inhibit proliferation, invasion, and migration of Tca8113 and CAL-27 cells in TSCC.

Our study showed that Y-27632-treated Tca8113 and CAL-27 cells had morphology change in cell shapes and size and apoptotic cells appeared. Apoptosis detection results demonstrated that ROCK inhibitor Y-27632 could induce apoptosis of Tca8113 and CAL-27 cells in a dose-dependent manner. In this study, MTT assay results revealed that with increasing concentrations and time of Y-27632, proportion of the increase in living cells was gradually reduced and cell growth was significantly inhibited, which suggested that ROCK inhibitor Y-27632 could suppress Tca8113 and CAL-27 cell proliferation and cell growth in a dose- and time-dependent manner. Rho-family GTPases influence a plethora cellular activity, including cell invasion and migration, as well as cancer cell growth and apoptosis [10]. ROCK, a member of the protein kinase family, is an important effector of Rho family [15]. ROCK is mainly to alter the cytoskeleton and the cell polarity and influence the stability of actin and contraction of myosin; additionally, the cellular adhesion and structure of cytoskeleton had feedback with RhoA/ROCK signaling pathway [16]. ROCK activation also could induce cell growth by mediating actomyosin contractility and promote morphological changes of cells [13]. Previous study also reported that ROCK signaling pathway could induce apoptosis in prostate cancer cells through regulating membrane androgen receptor [17]. Kinetic analysis revealed that Y-27632 inhibits ROCK by competing with ATP for binding to the catalytic site, which potently inhibited ROCK-mediated cellular responses at micromolar concentrations [17]. ROCK activation could increase cell proliferation via stimulating RhoA to promote G1 to S phase transition [18]. Y-27632 could inhibit the ROCK family of kinases 100 times more potently than other kinases including protein kinase C, myosin light chain kinase, and cAMP-dependent kinase [19]. Yang et al. reported that ROCK could induce the growth and proliferation of NCI-H446 small cell lung cancer cells, while the use of Fasudil, a Rho-kinase inhibitor, could induce apoptosis of NCI-H446 small cell lung cancer cells by inhibiting the Rho/Rho-kinase pathway [20]. In this regard, the use of ROCK inhibitor Y-27632 could effectively suppress cell proliferation and growth, which has been reported by a series of studies [4, 11, 14].

Accumulating reports support a critical role of the Rho/ROCK pathway in cancer cell invasion and metastasis [6, 21]. Cell motility is increased with the up-regulation of ROCK, which is correlated with increased metastatic potential of cancer cells [13]. One of the potential mechanisms is through increased polymerization of the actin microfilament cytoskeleton and enhancing actomyosin contractility [22]. Previous study showed that ROCK might be entailed in the rearrangement of the cytoskeleton to promote tumor cell migration via extracellular matrix [23]. In our study, the cell invasion and migration assay by performing Transwell chambers revealed that compared with controls, the number of cell migration and invasion in each Y-27632-treated group was decreased. Y-27632 could inhibit the invasion and migration ability of Tca8113 and CAL-27 cells in a TSCC in dose-dependent manner.

Taken together, the findings described in this study demonstrate that ROCK inhibitor Y-27632 can induce apoptosis and inhibit the proliferation, invasion, as well as migration of Tca8113 and CAL-27 cells in TSCC. In this regard, inhibition of ROCK by using ROCK inhibitor Y-27632 is a promising target for the treatment of TSCC.

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Conflicts of interest None

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