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Effects of ROCK inhibitor, Y-27632, on adhesion and mobility in esophageal squamous cell cancer cells

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Abstract Rho-associated protein kinase (ROCK), a molecular switch, modulates cellular functions in many cancers, such as hepatocellular, breast, colon cancers, etc. However, little is known the effect of ROCK on cell adhesion and mobility in esophageal squamous cell cancer (ESCC), one of the most diagnosed cancers in China. In this study, Y-27632 was used to specifically block ROCK activity in ESCC cells. Adhesion of ESCC cells was detected by homotypic and heterotypic adhesion assay together with examination of E-cadherin expression. Motility of ESCC cells changes were examined by detection of phosphorylated cofilin and observed under confocal microscopy, respectively. We found that Y-27632 increased both heterotypic and homotypic adhesion, and the expression of E-cadherin; decreased phosphorylated cofilin resulting in actin rearrangement in ESCC cells. All these findings indicate that ROCK signaling pathway plays an important role in cell adhesion and mobility, suggesting that it may be used as a potential target for therapy of ESCC.

Keywords Rho-associated protein kinase · Y-27632 · Esophageal squamous cell cancer · Adhesion · Motility

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Introduction

Invasion and metastasis of cancer cells are involved in multiple-step process including cell spreading, adhesion, proliferation and migration [1]. The changes of mobility and focal adhesion are among the initial events of invasion and metastasis [2]. Dynamic reorganization of the actin and tubulin cytoskeleton provides the driving force for cell movement [3]. Among the signaling pathways participating in regulating invasion and metastasis of cancer cells, Rho-associated kinase (ROCK) signaling pathway plays a key role in the process [4].

ROCK is the main downstream effector of Ras-homologous (Rho) family of GTPases which are involved in many cellular functions, such as cell proliferation, apoptosis, invasion and metastasis [5]. Overexpression of ROCK promotes invasion and metastasis in many solid tumors, such as hepatocellular, breast and colon cancers [6–9]. In human breast cancer, ROCK knockdown cells exhibited significantly decreased invasiveness and reduced motility compared with control cells [9]. ROCK signaling pathway also induced apoptosis in prostate cancer cells through regulating membrane androgen receptor [10], and participated in the inhibition of proliferation and promotion of differentiation of colon cancer cells induced by vitamin D [11]. Tumor-derived endothelial cells expressing a constitutively high level of ROCK exhibited aberrant mechanosensing and abnormal angiogenesis in vitro [12]. So, inhibition of ROCK becomes a more attractive strategy for the treatment of cancers.

Esophageal squamous cell carcinoma (ESCC) is one of the most frequently diagnosed cancers in developing countries [13, 14], especially in China. Although therapy strategies have been improved, the prognosis of patients with ESCC is still poor owing to early invasion and

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metastasis. Because of lacking knowledge of etiology and mode of carcinogenesis of this disease [15], a better understanding of the molecular mechanisms of progression of ESCC will help to improve the prognosis of patients with ESCC. In this study, we questioned the mechanism by which ROCK pathway plays an important role in adhesion and mobility of ESCC cells. The adhesion and mobility of ESCC cells were examined after Y-27632, [(R)-(+)-trans-N-(4-pyridyl)-4-(1-aminoethyl)-ciclohexanecarboxamide], was used, which can specific and effectively inhibits the activity of ROCK both in vitro and in vivo [16].

Materials and methods

Cell lines and cell cultures

ESCC cell line EC9706 was provided by the State Key Laboratory of Molecular Oncology, Chinese Academy of Medical Sciences (Beijing, China). The cell line was cultured in RPMI 1640 medium(Gibco-BR2, Rockville, USA) supplemented with 10% fetal bovine serum (FBS) (HyClone Laboratories, Logan, USA), 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in the presence of 5% CO_2, as described in the previous study from our laboratory [17].

Treatment of Y-27632

EC9706 cells were incubated with medium containing Y-27632 in 10, 20 and 30 μM, respectively, for 1 h at 37°C. Then, the treated cells were harvested for further experiments.

Western blots

EC9706 cells untreated and treated with 10, 20 and 30 μM of Y-27632 at 37°C for 1 h were harvested and lysed for 30 min in cold lysis buffer. After centrifugation at 12,000 rpm for 5 min, the supernatant was collected as the total cellular protein extracts. The protein concentration was determined using Bradford method [18]. The total cellular protein extracts were separated on 10% SDS–PAGE. Proteins were electrotransferred to supported nitrocellulose membranes (Amersham, Uppsala, Sweden) by a semi-dry transferor. The membranes were blocked in 5% skimmed milk in TBS-T containing 0.05% Tween 20 at room temperature (RT) for 2 h, and then incubated at RT for 2 h with antibodies to cofilin, p-cofilin, E-cadherin, and β-actin (Santa Cruz, USA) diluted in 1% skimmed milk in TBS-T, respectively, followed by incubating with the appropriate HRP-linked secondary antibodies. Finally, the bands of specific proteins on the membranes were developed with Western Blotting Luminal Reagent (Santa Cruz, USA) according to manufacturer's instructions [19].

Heterotypic adhesion assay

The heterotypic adhesion assay was performed as previously described [20]. Ninety-six-well plates were coated with 10 mg/l of fibronectin (FN) (Sigma, USA), and the wells coated with the same volume of bovine serum albumin (BSA) at 10 g/l were served as control wells. After the wells were rehydrated with BSA at 37°C for 1 h, a 100 μl of cell suspension (1×10^5/ml) treated with and without Y-27632 at 10, 20 and 30 μM, respectively, was seeded in each well and incubated at 37°C for 1 h. The unattached cells were then gently washed away with phosphate-buffered saline (PBS, pH 7.4). After being dyed with 10 μl WST-8 dye (Beyotime Inst Biotech, China) at 37°C for 1 h, the adhesion cells were finally determined at 450 nm by using a microplate reader. The rate of Y-27632 on cell adhesion to FN was calculated with the following formula: Cell adhesion rate = (average OD of Y-27632 treated wells—average OD of control wells)/(average OD of control wells) × 100%.

Homotypic adhesion assay

The homotypic adhesion assay was performed as reported previously [21]. Briefly, Monolayer of EC9706 cells incubated as mentioned above on a 24-well plate was gently washed three times with PBS. And then, 1×10^5 cells in 1 ml of medium with and without Y-27632 at 10, 20 and 30 μM, respectively, was seeded to each well. The 24-well plate was incubated in a horizontal shaker at 70 rpm at 37°C. The unattached cells were dropped out to calculate under a microscopy after being incubated for 10, 30, 60 min, respectively. The attached cells were calculated with the following formula: the number of adhesion cells = 1×10^5—the number of unattached cells.

Confocal microscopy

According to previously reported, F-actin of cells was observed by confocal microscopy [22]. The EC9706 cells plated on several sterile slides were incubated at 37°C for 24 h and treated with and without Y-27632 at 10, 20 and 30 μM for a further 1 h. The slides were rinsed three times in PBS and fixed with 4% polyformaldehyde at RT for 10 min. After rinsed three times in PBS, the cells were stained with FITC-phalloidin (Sigma, USA) for 1 h at 37°C and PI (Sigma, USA) for 5 min. Slides were then washed three times with PBS and observed by confocal microscopy (Leica SP2, German).

Statistical analysis

All experiment results were performed by one-way analysis of variance using SPSS version 13.0 (SPSS, Chicago,

USA). Summary statistics were expressed as means ± standard deviations, except as otherwise stated. In all statistical analyses, a P value < 0.05 was considered statistically significant, and all P values were two-sided.

Results

Effects of ROCK on adhesion capacity of EC9706 cells

Adhesion is one of the main steps that are involved in tumor metastasis in vivo. To investigate the effect of ROCK signaling pathway on adhesion of ESCC, we examined E-cadherin, one of the cellular adhesion molecules (CAMs), in EC9706 cells treated with Y-27632 at different concentrations by Western blots. As is shown in Fig. 1a and b, the expression of E-cadherin was increased in the three groups of cells treated with Y-27632 compared to untreated cells ($P < 0.05$), and there were also statistical differences between cells treated with Y-27632 in 10, 20 and 30 μM ($P < 0.05$). To further ascertain the change of cell–cell adhesion influenced by Y-27632 in EC9706, the homotypic adhesion assay was performed. There were statistical differences between the untreated cells and cells treated with 10, 20 and 30 μM Y-27632, respectively, both in 30 and 60 min ($P < 0.05$) but not in 10 min, and the increase of adhesion cells was a time and dose-dependent of Y-27632 (Fig. 1c).

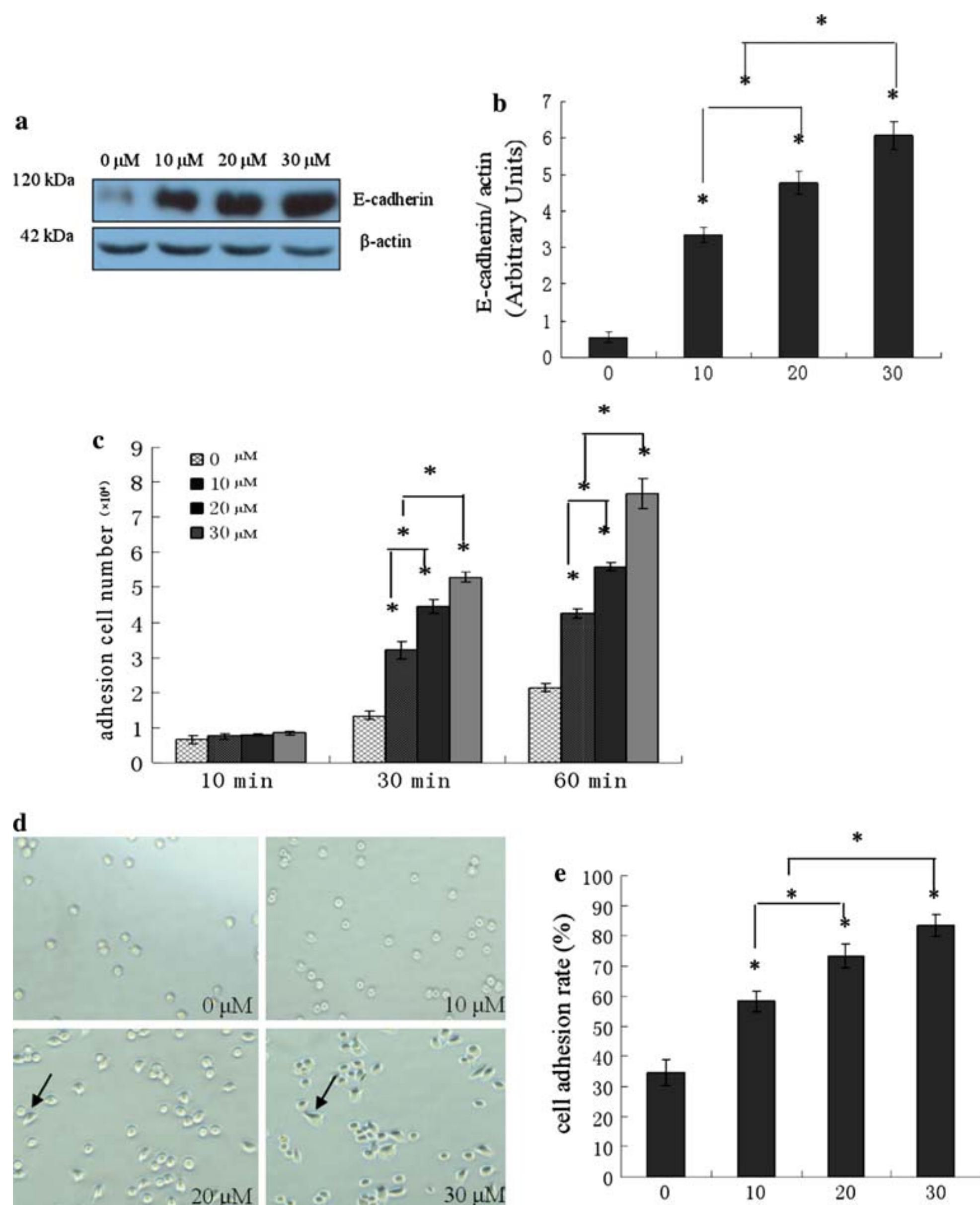

Fig. 1 Effects of Y-27632 on adhesion of EC9706 cells EC9706 cells were treated with Y-27632 at concentrations of 10, 20 and 30 μM for 1 h. **a** The cell extracts were subjected to Western blots with antibodies to E-cadherin and β-actin (loading control). **b** Semi-quantitative values of three independently repeated experiments, which were statistically analyzed by densitometry using TotalLab 2.0 software, are expressed as means ± SD. *$P < 0.05$, compared to those of EC9706 cells treated without Y-27632. **c** The numbers of adhesion cells of three independently repeated experiments are expressed as means ± SD in homotypic adhesion assay. *$P < 0.05$, compared to those of EC9706 cells treated without Y-27632. **d** EC9706 cells adhered to FN were observed under a microscopy (100×). **e** Cell adhesion rates are expressed as means ± SD in heterotypic adhesion assay. *$P < 0.05$, compared to those of EC9706 cells treated without Y-27632

FN is an important adhesion molecule in the extracellular matrix. The heterotypic adhesion assay was performed to observe the effects of ROCK on adhesion to FN in EC9706 cells. The adhesion rate was increased from 34.67 ± 4.16 to 58.33 ± 3.51, 73.33 ± 4.04 and 83.33 ± 3.51 (%) in the untreated cells and cells treated with 10, 20 and 30 μM Y-27632 ($P < 0.05$), respectively (Fig. 1e). EC9706 cultured in vitro is an anchorage-dependent cell which shows petal-like shape of Chrysanthemum. The trypsinized cell becomes a sphaerocyst. When observed under a microscopy with 100 magnification, the adhesion cells still were sphaerocysts in the untreated cells and cells treated with 10 μM Y-27632, while there were some cells stretched to petal-like shape of Chrysanthemum in the groups treated with 20 μM Y-27632 and more cells showed the petal-like shapes in the groups treated with 30 μM Y-27632 (Fig. 1d).

Effects of ROCK on mobility of EC9706 cells

Cofilin, an actin-binding protein, can polymerize actin and change the structure of cytoskeleton which is important in migration of cancer cells. To investigate the effect of ROCK on the phosphorylation level of cofilin, the proteins of cofilin and phosphorylated cofilin were examined by Western blots. As is shown in Fig. 2a and b, the declination of phosphorylated cofilin was $\sim$0.37, $\sim$0.53 and $\sim$0.68 fold ($P < 0.05$) in the cells treated with 10, 20 and 30 μM Y-27632, respectively, compared to untreated cells. There were no statistical differences in the expression of cofilin between the four groups ($P > 0.05$). The aggregation of F-actin stained by FITC-phalloidin was observed using confocal microscopy (Fig. 2c). F-actin in the untreated cells tended to assemble to bundles while it dispersed in the three groups of cells treated with Y-27632.

Discussion

Adhesion of cancer cells plays an important role in invasion and metastasis which are the main cause of death in patients with cancers. The change of cell adhesion capacity is always accompanied with the expression changes of CAMs. As one of the main CAMs, E-cadherin is a transmembrane glycoprotein which mediates homophilic calcium-dependent intercellular adhesion and plays a principal role in maintaining normal epithelial cell morphology [23]. The integrity of epithelium which depends on maintenance of cell–cell adherence junctions mediated by homotypic interactions between E-cadherin glycoproteins on adjacent cells disrupted in Crohn's Disease patients due to disfunction of E-cadherin [24]. Previous studies also showed that cancer cells with the loss or reduction of E-cadherin expression are frequently associated with acquisition of an invasive phenotype [25], such as in gastric, hepatocellular and oral squamous cell cancers [26–28]. In this study, to investigate whether ROCK could affect cell–cell adhesion in ESCC, we examined E-cadherin expression in EC9706 cells treated with Y-27632. As shown above, the expression of E-cadherin increased in a dose-dependent manner of Y-27632, indicating the aberrant activation of ROCK signaling pathway may promote invasion and metastasis of ESCC by reducing cell–cell adhesion. We further ascertained the elevated cell–cell adhesion by the homotypic adhesion assay, the adhesion EC9706 cells were in a dose and time dependent manner of Y-27632. But unlike previously reported, we also observed an enhanced adhesion of EC9706 cells to FN examined by the heterotypic adhesion assay. FN is a multifunctional glycoprotein in the extracellular matrix and plays a role in cell adhesion, migration, and maintenance of normal cell morphology [29], and adhesion to FN has been thought to facilitate metastasis of cancer cells [30, 31]. Gill et al. [32] has reported that inhibition of Rho and ROCK in chondrocytes increased cell spreading on bone sialoprotein and membrane protrusiveness on FN but did not affect cell adhesion. But in this study, we found that Y-27632 not only increased cell adhesion rate to FN in a dose dependent manner, but also promoted spreading of EC9706 cells on the plate to show their normal morphology (Fig. 1d, e). Our findings are accord with the research by Honjo et al. [33] who demonstrated that Y-27632 promoted adhesiveness of human Tenon fibroblast to both fibronectin and collagen type I. So, we suppose that there may be different dominant pattern of adhesion regulation in different type of cancer cells, because of the complexity of mechanism in adhesion regulation. ROCK signaling pathway has a multifunction in regulating adhesion capacity of EC9706 and the adhesion of cells to FN regulated by ROCK may not play a main role in the promotion of metastasis in ESCC. The elevated spreading of EC9706 on FN may be due to the enhancement of E-cadherin expression by Y-27632 which is important in maintenance of cell morphology. Whatever, the mechanism is not well understood yet and needs to be further investigated.

Furthermore, we investigated the mobility changes of ESCC cells treated with Y-27632. Autonomous motility ability of tumor cells is involved strongly in local invasion and distant metastasis [34]. Cofilin, a microbullin combined protein, plays an important role in cell mobility through polymerizing actin and changing the structure of cytoskeleton [35]. In this study, downregulation in a dose-dependent manner of phosphorylated cofilin in the cells treated with Y-27632 was observed, and the rearrangement of F-actin was ascertained by observation under the confocal microscopy. The findings suggest that mobility of ESCC cells were elevated due to the change of its actin

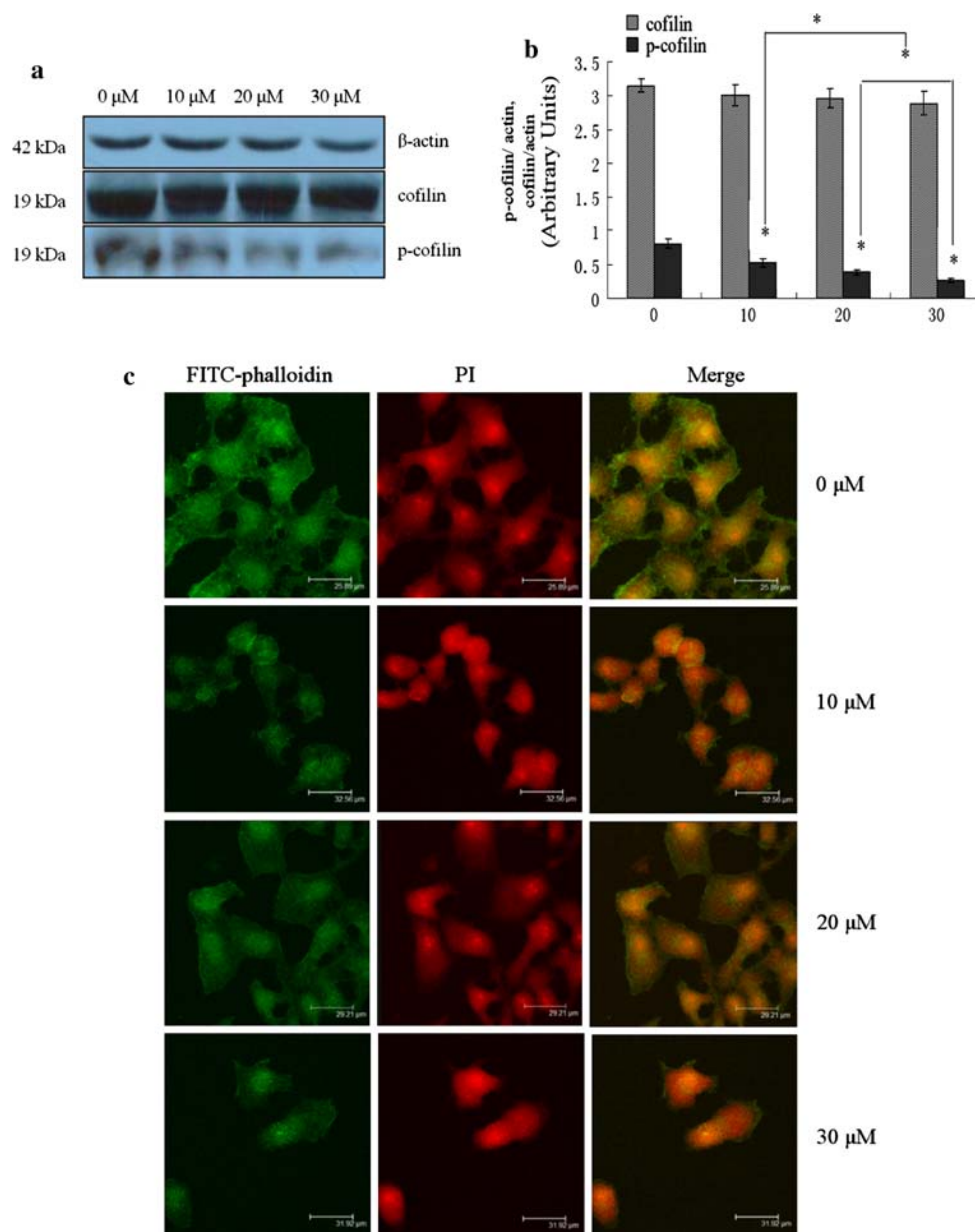

Fig. 2 Effects of Y-27632 on mobility of EC9706 cells EC9706 cells were treated with Y-27632 at concentrations of 10, 20 and 30 μM for 1 h. **a** The cell extracts were subjected to Western blots with antibodies to cofilin, p-cofilin and *β*-actin (loading control). **b** Semi-quantitative values of three independently repeated experiments, which were statistically analyzed by densitometry using TotalLab 2.0 software, are expressed as means ± SD. $*P < 0.05$, compared to those of EC9706 cells treated without Y-27632. **c** EC9706 cells stained with FITC-phalloidin and PI were observed by a confocal microscopy. F-actin in the untreated cells tended to assemble to bundles while it dispersed in the cells treated with Y-27632 at different levels

arrangement, which is resulted from high level of phosphorylated cofilin by activated ROCK.

Taken together, Y-27632 not only increases cell–cell adhesion by upregulating E-cadherin, but also reduces cell mobility by downregulating phosphorylated coffilin, suggesting that the ROCK signaling pathway might be one responsible for the invasion and metastasis of ESCC cells and the pathway may be used as a potential target for the therapy of ESCC.

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