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Original Article

Overexpression of ROCK1 and ROCK2 inhibits human laryngeal squamous cell carcinoma

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Abstract: Rho-associated coiled-coil containing protein kinase (ROCK) over-expression has been implicated in the progression of many tumor types. The aim of this study was to explore the roles of ROCK1 and ROCK2 in human laryngeal squamous cell carcinoma (LSCC). ROCK1 and ROCK2 expression levels were examined in 50 cases of human LSCC samples by immunohistochemistry. Effects of ROCK1 and ROCK2 on LSCC cell proliferation and motility were investigated in the presence of the ROCK inhibitor Y-27632. The results showed that ROCK1 expression was positively correlated with tumor size and lymph node metastasis ($P < 0.05$); ROCK2 positively correlated with tumor size ($P < 0.05$). Inhibition of ROCK1 and ROCK2 by Y-27632 significantly inhibits proliferation, migration, and invasion of LSCC cells. Our data indicate that expression of ROCK1 and ROCK2 are closely associated with tumor growth and lymph node metastasis of LSCC. Thus, these two ROCK isoforms may be useful as molecular makers for LSCC diagnosis and may be useful therapeutic targets as well.

Keywords: Laryngeal squamous cell carcinoma, ROCK, Y-27632, tumor growth, lymph node metastasis

Introduction

Approximately 130,000 new cases of laryngeal cancer are diagnosed worldwide each year, and over 95% of these diagnoses are laryngeal squamous cell carcinomas (LSCC) [1]. Despite improved diagnostic methods and therapies, the cure rate of LSCC has only marginally improved over the past decade [2]. A main challenge is the lack of accurate and reliable methods for early diagnosis. Traditional methods, such as physical examination, ultrasound, and computer tomography, are often insufficient for early detection [3]. In contrast to these standard clinical methods, molecular techniques used to measure metabolic activity of cancer cells may provide more sensitive and accurate detection for patients. Such methods may also help identify new therapeutic targets for LSCC [4]. Although the best-known risk factors for LSCC, clinical TNM staging and histopathological grading, will retain their usefulness, the identification of molecular biomarkers may provide additional information for stratifying patients. Some markers, including EGFR and cyclin D1 [4], have been proposed, but none have yet translated to clinical applicability. Thus, a main objective of the work described here is to identify new biomarkers that may be useful either as prognostic markers or as therapeutic targets. Accordingly, identification of molecular markers involved in LSCC progression may improve diagnosis and treatment interventions.

Rho-associated coiled-coil containing protein kinase (ROCK) belongs to a family of serine/threonine kinases and is one of the best characterized downstream effectors of Rho GTPases [5]. Via ROCK activation, Rho GTPases have been implicated in multiple cellular processes including motility, morphogenesis, polarity, cell division, and cell adhesion [6]. In humans, there are two known ROCK isoforms-ROCK1 and ROCK2. ROCK over-expression has been implicated in the progression of many tumor types, including bladder carcinoma, hepatocellular carcinoma, and breast carcinoma [7-9]. Decr-

Table 1. Clinical characteristics of the 50 LSCC patients

Clinicopathological parameters	Number of patients	% of patients
Age (years)		
< 65	26	52%
≥ 65	24	48%
Location		
Supraglottic	25	50%
glottic	20	40%
subglottic	1	2%
Transglottic	4	8%
Lymph node metastasis		
Negative	33	66%
Positive	17	34%
Tumor size (Maximum diameter)		
≥ 2.5 cm	29	58%
< 2.5 cm	21	42%
Clinical stage		
Stage I-II	18	36%
Stage III-IV	32	64%
Histological differentiation		
Low-Mediate	25	50%
High	25	50%
Recurrence		
Yes	12	24%
No	38	76%

eased expression of ROCK1 and ROCK2 or inhibition of ROCK activity has been described as potential therapeutic options in many tumor types [10-13]. In many of these studies, ROCK has been shown to positively correlate with increased tumor grade and metastasis. However, to date, ROCK1 and ROCK2 have not been characterized in human LSCC samples. Therefore, the aim of the current study was to explore the relationship between expression of ROCK1 and ROCK2 and clinicopathological parameters of patients diagnosed with primary LSCC. Additionally, we observed the effect of Y-27632, a ROCK inhibitor [14], on the motility and proliferation of LSCC cells to preliminarily explore the mechanisms underlying the roles of ROCK1 and 2.

Materials and methods

Clinical data

The human LSCC cell line Hep-2 was obtained from the Institute of Basic Medical Sciences, Peking Union Medical College (Beijing, China). Fifty human primary LSCC samples were collected at the time of surgical resection at the Chinese PLA General Hospital & Postgraduate Medical School from May 2008 to May 2009. Patients received a detailed explanation of the study, and written informed consent was obtained from all participants. This study was approved by the Institutional Review Board of Chinese PLA General Hospital & Postgraduate Medical School.

Materials

Goat polyclonal antibodies recognizing human ROCK1 and ROCK2 were purchased from Santa Cruz Biotechnology Company (Santa Cruz, US). The ROCK inhibitor Y-27632 was purchased from Sigma-Aldrich Company (Deisenhofen, Germany). All other reagents were purchased from Zhongshan Golden Bridge Biotechnology Company (Beijing, China).

Immunohistochemistry

All tissue samples were cut into 4 μm-thick sections. To determine ROCK1 and ROCK2 expression, sections were first dried. This was followed by deparaffinization, antigen retrieval, cooling, quenching of endogenous peroxidase activity, incubating with primary antibodies (1:50 for ROCK1 goat polyclonal antibody and 1:100 for ROCK2 goat polyclonal antibody), incubating with corresponding secondary antibodies, visualizing with diaminobenzidine, counterstaining with hematoxylin, dehydrating, clearing with standard xylene, and mounting with resinous mounting medium. Stained sections were observed under a BX40 microscope (Olympus, Japan). Slides were evaluated by a blinded observer, unaware of the corresponding clinical information. “Positive expression” was defined as > 10% positively stained cells under 200 × microscopic field; ≤ 10% was defined as “negative expression”.

MTT assay

Hep-2 cells were diluted to a concentration of 1 × 10^5/ml. Diluted cells were then seeded into a 96-well plate (1.0 × 10^3/well) containing culture medium (100 μl). Cells were treated with 0, 25, 50, 75, and 100 μmol/L Y-27632. Additionally, each concentration was incubated for different lengths of time (2 h, 8 h, 12 h, 24 h, and 48 h). Following treatment, 20 μl MTT (5 mg/ml) was added to each well, and the plate was incubated for an additional 4 h. Following this incubation, medium was discarded and

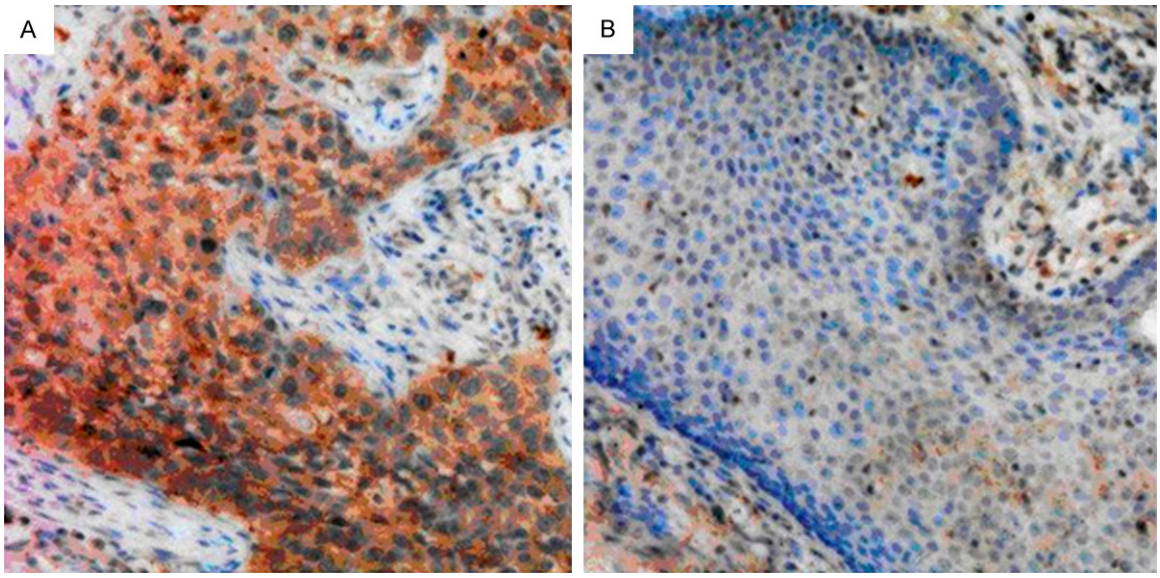

Figure 1. Localization of ROCK1 via immunohistochemistry. The positive (A) and negative (B) staining of ROCK1 in LSCC tissues (200 ×).

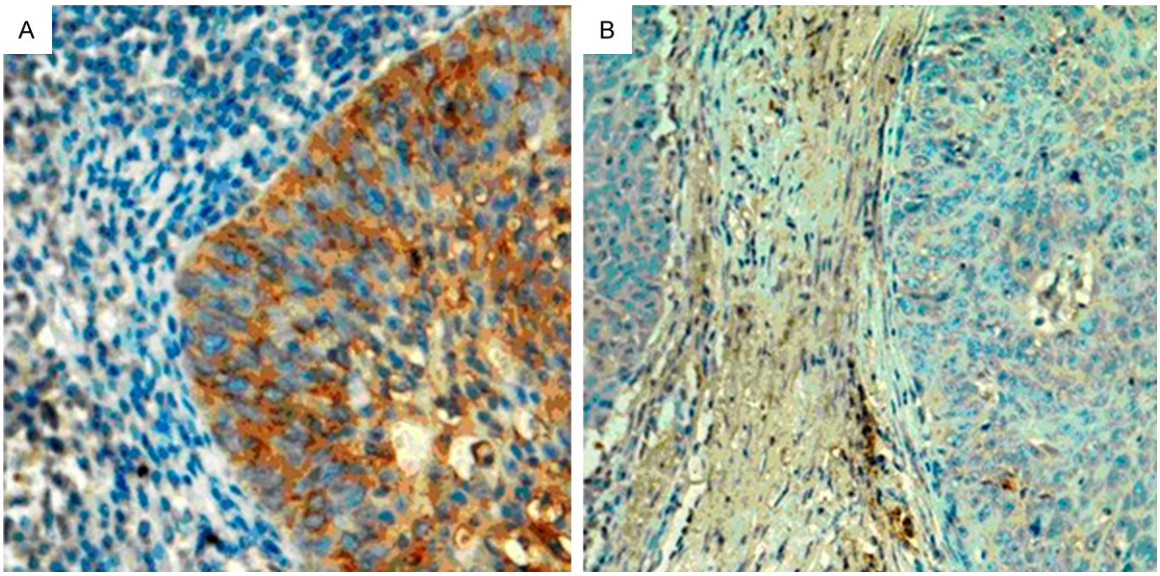

Figure 2. Localization of ROCK2 via immunohistochemistry. The positive (A) and negative (B) staining of ROCK2 in LSCC tissues (200 ×).

replaced by 150 μl DMSO. Plates were allowed to shake for 10 minutes, and the optical density of cells was measured at 490 nm using a microplate reader. The experiments were repeated 4 times.

Transwell experiments

We used Transwell migration chambers containing 8 μm-pore-size polycarbonate membranes. Hep-2 cells (1×10^5) were seeded in a total of 6 upper chambers. Cells in 3 of these chambers were treated with 25 μM Y-27632; cells in the remaining chambers served as untreated controls. After 24 h incubation, cells that had failed to migrate and that had remained on the upper side of the filter were scraped away with a moist cotton swab; additionally, PBS was used to wash the upper side of the filter. Cells that had successfully migrated through the filter were fixed, stained with crystal violet, and counted under an inverted microscope. The inhibition percentage (IP) was then calculated according to the following formula: IP (%) = (number of invaded cells in con-

Table 2. Correlation between the expression of ROCK1 and 2 in cancer and clinicopathological factors

Parameters	ROCK1 expression		*P*	ROCK2 expression		*P*
	Negative	Positive		Negative	Positive	
Tumor size						
< 2.5 cm	14	7	0.013	14	7	0.045
≥ 2.5 cm	9	20		11	18	
Lymph node metastasis						
Yes	3	14	0.006	6	11	0.136
No	20	13		19	14	
Clinical stage						
Stage I-II	11	7	0.108	12	6	0.077
Stage III-IV	12	20		13	19	
Differentiation						
Low-mediate	10	15	0.395	9	16	0.048
Well	13	12		16	9	
Recurrence						
Yes	3	9	0.094	5	7	0.508
No	20	18		20	18	

trol group-number of invaded cells in experiment group)/number of invaded cells in control group × 100%. A similar procedure was performed for invasion assays; however, for these experiments, the upper side of the polycarbonate membrane was coated with Matrigel (25 mg/cm^2). These experiments were repeated 3 times.

Statistics

All statistical analyses were performed with SPSS statistical software package (version 16.0; SPSS Inc., Chicago, IL, USA). Clinicopathological parameters were subgrouped and Chi-square with Fisher exact test was used to analyze the correlation between ROCK1 and 2 expression and clinicopathological parameters. Expression of ROCK1 and 2 in LSCC tissues was classified as 'positive' and 'negative' to analyze the overall survival (OS) and disease-free survival (DFS) of LSCC patients. Survival analysis was performed using the Kaplan-Meier method, and curves were compared using log-rank test. The statistical significance was defined as $P < 0.05$.

Results

Patient characteristics

The 50 subjects enrolled in the study included 45 men and 5 women; their mean age was 62.1 ± 10.3 years, with a range of 30-79 years. Additional clinicopathological parameters are provided in **Table 1**. The median follow-up time was 53 months, ranging from 38 to 70 months. Following surgical resection, all samples were embedded in paraffin. We evaluated and recorded several basic characteristics of each patient enrolled in the study. These included, but were not limited to, tumor stage, lymph node metastasis, and recurrence. A full description of patient characteristics is provided in **Table 1**.

ROCK expression and its correlation with clinicopathological parameters

Immunohistochemistry was performed in all LSCC tissues to assess expression of both ROCK1 and 2. While ROCK1 was primarily localized to the cytoplasm (**Figure 1**), ROCK2 was expressed both in the cytoplasm and at the membrane (**Figure 2**). ROCK1 expression significantly correlated with tumor size and lymph node metastasis ($P < 0.05$). Expression of ROCK2 significantly correlated with tumor size and differentiation ($P < 0.05$) (**Table 2**). Neither ROCK isoform correlated with clinical stage, recurrence, disease-specific survival, or overall survival (all $P > 0.05$, **Table 2**).

Survival analysis

Kaplan-Meier survival analysis showed that the OS and DFS in ROCK1 negative LSCC patients were insignificantly longer than ROCK1 positive patients ($P = 0.098$ and 0.386, respectively). The OS and DFS between the ROCK2 negative and positive LSCC patients did not show significantly difference ($P = 0.552$ and $P = 0.724$, respectively) (**Figure 3**).

ROCK inhibitor Y-27632 decreased proliferation of LSCC cells

Control LSCC cells proliferated well and were characterized by a polygonal shape with well-defined contours (**Figure 4A**). In contrast, apoptotic bodies, yellow debris, and cell necrosis were detected in cells treated with the ROCK inhibitor Y-27632; additionally, inhibitor-treated cells displayed altered morphology, becoming mostly oval in shape (**Figure 4B**). MTT assays

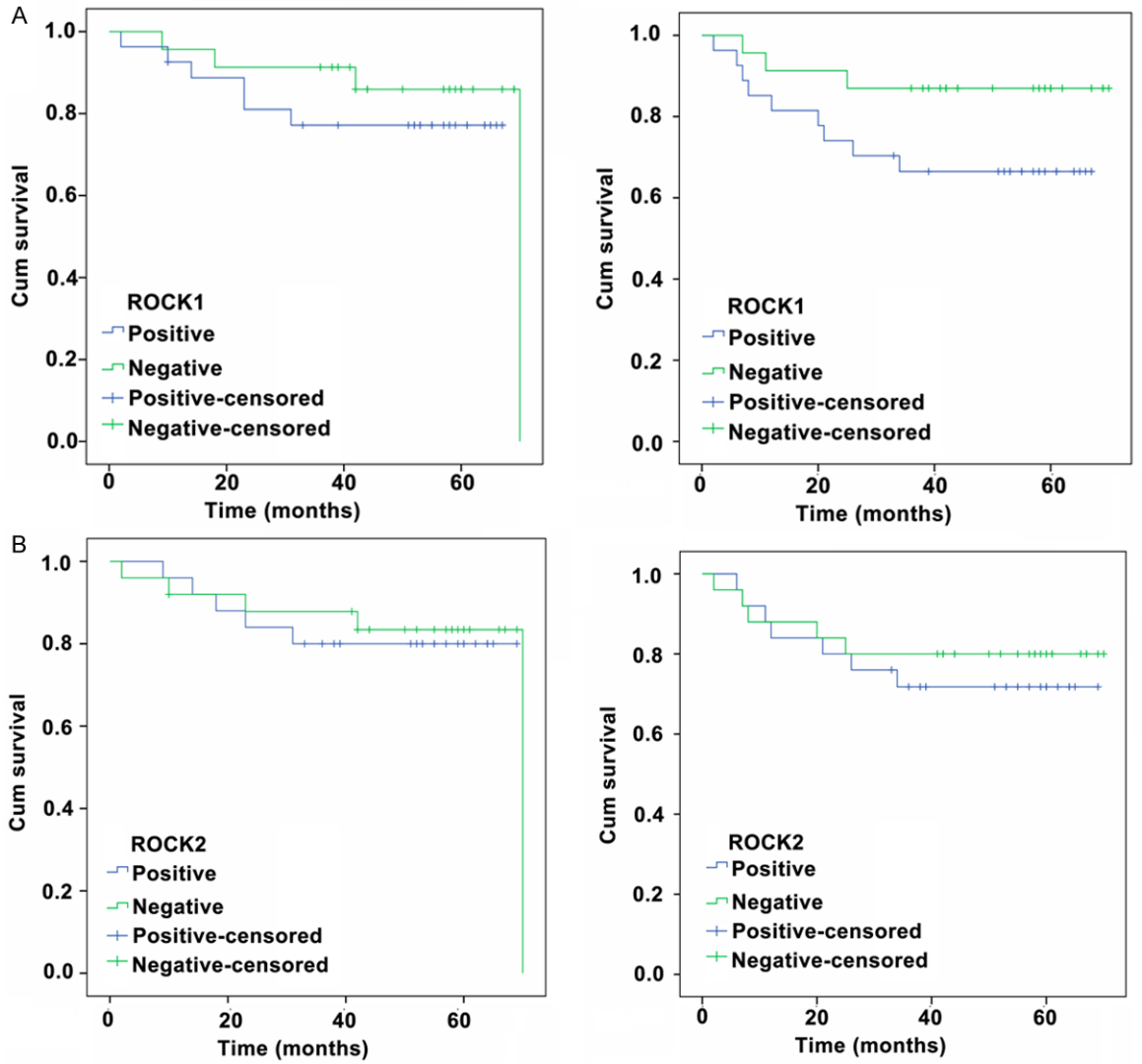

Figure 3. Kaplan-Meier survival analysis. Expression of ROCK1 and 2 in LSCC tissues was classified as 'positive' and 'negative' to analyze the expression of ROCK1 (A) and 2 (B) with OS and DFS of LSCC patients, respectively.

were performed to assess the proliferation of LSCC cells with the treatment of different concentrations of Y-27632. At 24 h of treatment, 25 μM Y-27632 had minimal effect on cellular proliferation (data not shown); thus, we selected this concentration for use in subsequent Transwell experiments.

ROCK inhibition via Y-27632 decreases both migration and invasion of LSCC cells

We performed Transwell assays to assess the effect of Y-27632 on LSCC cell migration. Compared to the control group, fewer Y-27632-treated cells migrated through the membrane (control: 84.2 ± 3.70 per field compared to Y-27632: 65.4 ± 3.65 per field, $P < 0.05$; **Figure 5**). The IP of the control group (100%) was significantly higher than that of the experimental group (23%) ($P < 0.05$).

Similar results were obtained in the invasion assay. 25 μM Y-27632 significantly decreased cellular invasion (67.0 ± 3.16 per field) compared to untreated control cells (90.2 ± 3.70 per field, $P < 0.05$; **Figure 6**). Consistently, the IP of the control group (100%) was significantly higher than that of the experimental group (21%) ($P < 0.05$).

Discussion

In this study, we explored the relationship between expression of ROCK1 and ROCK2 and

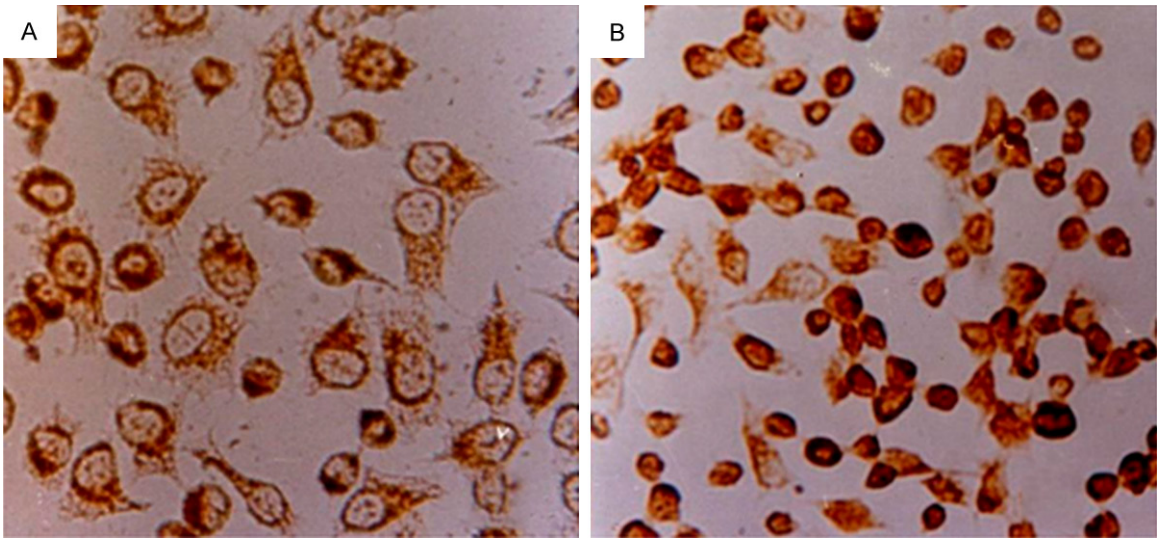

Figure 4. The effect of Y-27632 on cell proliferation. A. Untreated LSCC cells showed mainly polygonal shape and well-defined contours. B. LSCC cells treated with Y-27632 were mainly oval in shape with irregular cell membranes and atrophied cytoplasm; they also contained apoptotic bodies, yellow debris, and cell necrosis (400 ×).

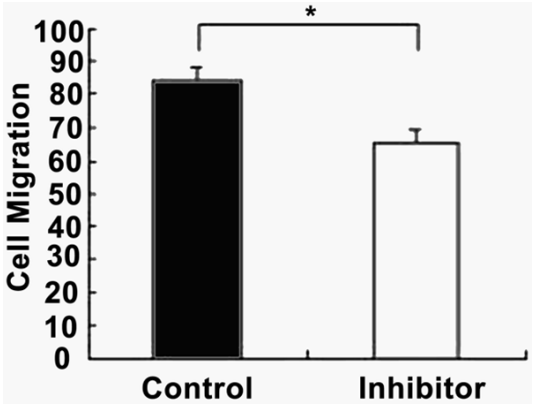

Figure 5. The effect of Y-27632 on the migration of LSCC cells. Hep-2 cells with or without treatment of 25 μM Y-27632 were tested for migration ability through a filter; cells that migrated through the matrix were imaged. (A) control group without treatment and (B) experimental group.

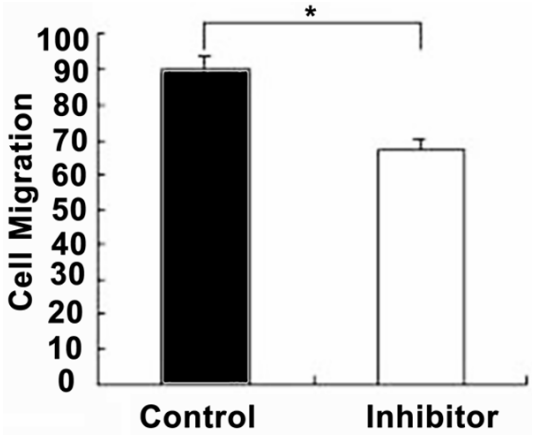

Figure 6. The effect of Y-27632 on invasion of LSCC cells. Hep-2 cells with or without treatment of 25 μM Y-27632 were tested for migration ability through a filter; cells that migrated through the matrix were imaged. (A) control group without treatment and (B) experimental group.

progression of LSCC. Expression of both isoforms was found to correlate with tumor size, and ROCK1 correlated with lymph node metastasis, while ROCK2 correlated with tumor differentiation. Treatment of LSCC cells with the ROCK inhibitor Y-27632 decreased cellular proliferation, migration, and invasion. Thus, ROCK1 and ROCK2 may play important roles in the development, progression, and metastasis of human LSCC.

Tumor growth and lymph node metastasis are hallmarks of advanced stage cancers. Based on the data we present here, expression of ROCK1 and ROCK2 in clinical samples may be informative for disease progression and metastatic potential. However, more work needs to be done to definitely establish this. Our data also suggest that ROCK1 and ROCK2 may be useful therapeutic targets in LSCC, as their inhibition reduces tumorigenic phenotypes *in vitro*.

ROCK expression has been previously correlated with metastasis of several human tumors [7-9, 15]. One study suggested that ROCK

might be involved in rearrangement of the cytoskeleton to promote tumor cell migration through the extracellular matrix [16]. Others have shown that cell motility is increased with ROCK up-regulation and that this correlates with increased metastatic potential of carcinoma cells [17, 18]. The relationship between ROCK and tumor growth has not yet been fully elucidated. Coleman *et al.* showed that ROCK activation stimulates RhoA to promote G1 to S phase transition and increase cell proliferation [19]. Another group showed that ROCK-mediated actomyosin contractility could induce tumor growth [20]. Our results are consistent with these earlier publications. Specifically, we showed that ROCK inhibition decreased LSCC proliferation, migration, and invasion. Although additional research is required to understand the molecular mechanisms governing this effect, ROCK expression may be clinically informative in terms of tumor growth and metastasis.

In this study, ROCK1 and ROCK2 showed different subcellular localization in LSCC tissues. This is consistent with data from Yoneda *et al.* [21] that showed differential ROCK isoform localization in primary rat embryonic fibroblasts. This localization difference suggests that the exact roles played by each these isoforms in LSCC progression may not be entirely the same. However, we did not further dissect the roles of each isoform in LSCC; this is because the Y-27632 inhibitor is not ROCK isoform specific but instead targets both proteins. ROCK1 and ROCK2 share 65% identity across the entire length of the protein; their kinase domains are more highly conserved, showing 92% similarity [22]. Interestingly, they show differential expression in different body tissues. ROCK1 is highly expressed in liver, testes, and kidney. In contrast, ROCK2 is most highly expressed in brain and skeletal muscle. Both isoforms are expressed in vascular smooth muscle and heart. The exact similarities and differences between the two isoforms are not completely understood. For example, Shi *et al*. showed that only under certain conditions could one isoform compensate for the other [23]. Thus, there are likely both redundant and unique properties of each protein. Hahmann and Schroeter also suggested that ROCK1 and ROCK2 might have different functions in the progression of different diseases. This lends support to the idea of developing isoform-specific ROCK inhibitors, which may help avoid unwanted side effects [24]. Improved genetic targeting approaches coupled with molecular analysis using RNA interference and gene transfer strategies may help us better understand the exact differences between ROCK1 and ROCK2 [23, 24].

The present results showed that the expression of ROCK1 and 2 was not significantly correlated with OS and DFS in LSCC patients. Expression of ROCK1 and 2 in LSCC tissues was classified as 'positive' and 'negative'; however, in some patents with positive ROCK expression in LSCC tissues, the expression was rather low. If the ROCK1 and 2 expression were classified as 'high' and 'low', a significant correlation between ROCK1 and 2 expression and survival (OS and DFS) of patients would be observed. The follow-up time (median time: 53 months, ranging 38-70 months) in this study was not long enough; with longer follow-up, the expression of ROCK1 and 2 would exhibit significant correlation with OS and DFS. In addition, there are multiple proteins and genes that influence the progression of tumors and the roles of single gene might be limited. In combination of multiple molecular markers would be much better in the prognosis of LSCC.

In conclusion, this study is the first to explore the roles of ROCK1 and ROCK2 in the progression of LSCC. Our data suggest that expression of these two proteins correlates with tumor growth and lymph node metastasis in LSCC. It is possible that patients with ROCK1- or ROCK2-positive tumors may benefit from therapeutic inhibition of these proteins for the treatment of LSCC.

Disclosure of conflict of interest

None.

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References

[1] Mirisola V, Mora R, Esposito AI, Guastini L, Tabacchiera F, Paleari L, Amaro A, Angelini G, Dellepiane M, Pfeffer U and Salami A. A prognostic multigene classifier for squamous cell

carcinomas of the larynx. Cancer Lett 2011; 307: 37-46.
[2] Siegel R, Naishadham D and Jemal A. Cancer statistics, 2012. CA Cancer J Clin 2012; 62: 10-29.
[3] Ferreira MB, De Souza JA and Cohen EE. Role of molecular markers in the management of head and neck cancers. Curr Opin Oncol 2011; 23: 259-264.
[4] Almadori G, Bussu F, Cadoni G, Galli J, Paludetti G and Maurizi M. Molecular markers in laryngeal squamous cell carcinoma: towards an integrated clinicobiological approach. Eur J Cancer 2005; 41: 683-693.
[5] Narumiya S, Tanji M and Ishizaki T. Rho signaling, ROCK and mDia1, in transformation, metastasis and invasion. Cancer Metastasis Rev 2009; 28: 65-76.
[6] Jaffe AB and Hall A. Rho GTPases: biochemistry and biology. Annu Rev Cell Dev Biol 2005; 21: 247-269.
[7] Kamai T, Tsujii T, Arai K, Takagi K, Asami H, Ito Y and Oshima H. Significant association of Rho/ROCK pathway with invasion and metastasis of bladder cancer. Clin Cancer Res 2003; 9: 2632-2641.
[8] Wong CC, Wong CM, Tung EK, Man K and Ng IO. Rho-kinase 2 is frequently overexpressed in hepatocellular carcinoma and involved in tumor invasion. Hepatology 2009; 49: 1583-1594.
[9] Lane J, Martin TA, Watkins G, Mansel RE and Jiang WG. The expression and prognostic value of ROCK I and ROCK II and their role in human breast cancer. Int J Oncol 2008; 33: 585-593.
[10] Somlyo AV, Bradshaw D, Ramos S, Murphy C, Myers CE and Somlyo AP. Rho-kinase inhibitor retards migration and in vivo dissemination of human prostate cancer cells. Biochem Biophys Res Commun 2000; 269: 652-659.
[11] Vigil D, Kim TY, Plachco A, Garton AJ, Castaldo L, Pachter JA, Dong H, Chen X, Tokar B, Campbell SL and Der CJ. ROCK1 and ROCK2 are required for non-small cell lung cancer anchorage-independent growth and invasion. Cancer Res 2012; 72: 5338-5347.
[12] Ohta T, Takahashi T, Shibuya T, Amita M, Henmi N, Takahashi K and Kurachi H. Inhibition of the Rho/ROCK pathway enhances the efficacy of cisplatin through the blockage of hypoxia-inducible factor-1alpha in human ovarian cancer cells. Cancer Biol Ther 2012; 13: 25-33.
[13] Oku Y, Tareyanagi C, Takaya S, Osaka S, Ujiie H, Yoshida K, Nishiya N and Uehara Y. Multimodal effects of small molecule ROCK and LIMK inhibitors on mitosis, and their implication as anti-leukemia agents. PLoS One 2014; 9: e92402.
[14] Ishizaki T, Uehata M, Tamechika I, Keel J, Nonomura K, Maekawa M and Narumiya S. Pharmacological properties of Y-27632, a specific inhibitor of rho-associated kinases. Mol Pharmacol 2000; 57: 976-983.
[15] Torka R, Thuma F, Herzog V and Kirfel G. ROCK signaling mediates the adoption of different modes of migration and invasion in human mammary epithelial tumor cells. Exp Cell Res 2006; 312: 3857-3871.
[16] Wyckoff JB, Pinner SE, Gschmeissner S, Condeelis JS and Sahai E. ROCK- and myosin-dependent matrix deformation enables protease-independent tumor-cell invasion in vivo. Curr Biol 2006; 16: 1515-1523.
[17] Wyckoff JB, Jones JG, Condeelis JS and Segall JE. A critical step in metastasis: in vivo analysis of intravasation at the primary tumor. Cancer Res 2000; 60: 2504-2511.
[18] Wang W, Wyckoff JB, Frohlich VC, Oleynikov Y, Huttelmaier S, Zavadil J, Cermak L, Bottinger EP, Singer RH, White JG, Segall JE and Condeelis JS. Single cell behavior in metastatic primary mammary tumors correlated with gene expression patterns revealed by molecular profiling. Cancer Res 2002; 62: 6278-6288.
[19] Coleman ML, Densham RM, Croft DR and Olson MF. Stability of p21Waf1/Cip1 CDK inhibitor protein is responsive to RhoA-mediated regulation of the actin cytoskeleton. Oncogene 2006; 25: 2708-2716.
[20] Kumper S and Marshall CJ. ROCK-driven actomyosin contractility induces tissue stiffness and tumor growth. Cancer Cell 2011; 19: 695-697.
[21] Yoneda A, Ushakov D, Multhaupt HA and Couchman JR. Fibronectin matrix assembly requires distinct contributions from Rho kinases I and -II. Mol Biol Cell 2007; 18: 66-75.
[22] Ishizaki T, Maekawa M, Fujisawa K, Okawa K, Iwamatsu A, Fujita A, Watanabe N, Saito Y, Kakizuka A, Morii N and Narumiya S. The small GTP-binding protein Rho binds to and activates a 160 kDa Ser/Thr protein kinase homologous to myotonic dystrophy kinase. EMBO J 1996; 15: 1885-1893.
[23] Shi J and Wei L. Rho kinase in the regulation of cell death and survival. Arch Immunol Ther Exp (Warsz) 2007; 55: 61-75.
[24] Hahmann C and Schroeter T. Rho-kinase inhibitors as therapeutics: from pan inhibition to isoform selectivity. Cell Mol Life Sci 2010; 67: 171-177.