Pak1/LIMK1/Cofilin Pathway Contributes to Tumor Migration and Invasion in Human Non-Small Cell Lung Carcinomas and Cell Lines

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Squamous cell carcinoma (SCC) and adenocarcinoma (AC) are the major histological types of non-small cell lung carcinoma (NSCLC). Although both SCCs and ACs have been characterized histologically and clinically, the precise mechanisms underlying their migration and invasion are not yet known. Here, we address the involvement in NSCLC of the p21-associated kinase1 (Pak1)/LIM kinase1 (LIMK1)/cofilin pathway, which recently has been reported to play a critical role in tumor migration and invasion. The Pak1/LIMK1/cofilin pathway was evaluated in tumors from SCC (n=35) and AC (n=35) patients and in SCC- and AC-type cell lines by western blotting, immunohistochemistry, and in vitro migration and invasion assays. The levels of phosphorylated Pak1, LIMK1, and cofilin in lung tumor tissues from SCC patients were increased as compared to normal tissues. In addition, immunohistochemistry showed greater expression of phosphorylated cofilin in SCC tissues. Expression of phosphorylated Pak1 and LIMK1 proteins was also significantly higher in SCC-type cells than in AC-type cells. Moreover, migration and invasion assays revealed that a higher percentage of SCC type cells exhibited migration and invasion compared to AC type cells. Migration was also decreased in LIMK1 knockdown SK-MES-1 cells. These findings suggest that the activation of the Pak1/LIMK1/cofilin pathway could preferentially contribute to greater tumor migration and invasion in SCC, relative to that in AC.

Key Words: Pak1, LIMK1, Cofilin, Lung cancer

INTRODUCTION

Non-small cell lung cancer (NSCLC) is the leading cause of cancer-related mortality worldwide. Early stage NSCLC can be cured surgically, but despite the potential benefits of surgical resection, patients with the same pathologic stage of disease display marked variability in recurrence and survival [1]. Squamous cell carcinoma (SCC) and adenocarcinoma (AC), the principal subtypes of NSCLC, exhibit different geographical prevalence, clinical behaviors, and recurrence patterns. Tumor invasion and metastasis predict a poor prognosis in lung cancer, and are the main causes of treatment failure and lung cancer-related death [2].

Tumor cell migration is important in the formation of solid tumors and necessary for the spread of cells to distant organs [3]. The process of cancer migration and invasion involves changes in cytoskeletal signaling pathways, increased directional motility, and enhanced cell survival. Thus, the actin cytoskeleton is an important determinant of tumor cell migration and invasion [4]. It has been reported that p21-associated kinases (Paks) play an evolutionarily conserved role in regulating the actin cytoskeleton during cell motility and invasion [5]. An increase in Pak protein levels has been observed in several human tumors [6]. Among the identified targets of Pak are p-Lin-11/Isl-1/Mec-3 kinase (LIMK), myosin light chain, and cofilin [7-9]. The phosphorylation of both LIMK and cofilin is greatly enhanced in the presence of active Pak [10]. Furthermore, the level and activity of LIMK are higher in invasive breast and prostatic cancer cell lines than in less invasive cells [11]. Cofilin, which is the only known substrate for LIMK1, regulates the actin cytoskeleton [12]. Suppression of cofilin activity by LIMK overexpression abolishes lamellipodium formation and polarized cell migration [13]. In contrast,
overexpression of cofilin inhibits the invasiveness of human lung cancer H1229 cells by disrupting the actin cytoskeleton at the leading edge of the cell [14]. Therefore, it is likely that the expression status of the Pak1/LIMK1/cofilin pathway is sufficient to determine the migratory and invasive properties of lung tumor cells.

In the present study, we examined altered expressions of Pak1, LIMK1, and cofilin proteins in SCC and AC cell types of human lung cancers and cell lines. In addition, we investigated the migratory and invasive activity in SCC- and AC-type cell lines.

**METHODS**

**Patients and tissue samples**

All patients were referred to the Gyeongsang National University Hospital (Jinju, South Korea) and underwent surgery, conducted by the same surgeon (Jang I), between May 2005 and July 2009. Tumor samples from a total of 70 patients with SCC (n=35) and AC (n=35) were collected intraoperatively, snap-frozen, and stored at −80°C. Diagnoses were based on a pathological examination of tissue samples. The characteristics of the patients and tumor samples are detailed in Table 1. Patients ranged in age from 31 to 77 y; 14 were female, and 56 were male. For western blot analysis, tumor tissue and adjacent normal lung tissue were collected from SCC and AC samples. All uses of human tissue samples and experimental procedures described in this study were reviewed and approved by the Ethics Committee of Gyeongsang National University Hospital.

**Cell culture**

Four human lung cancer cell lines HCC-1588 (SCC), HCC-1171 (AC), SK-MES-1 (SCC), and A549 (AC) were used in this study. HCC-1588 and HCC-1171 were purchased from the Korean Cell Line Bank (Seoul, South Korea). SK-MES-1 and A549 were purchased from the American Type Culture Collection (Manassas, VA, USA).

Table 1. Patient characteristics

| Characteristics      | SCC | AC | p value |
|----------------------|-----|----|---------|
| Numbers of patients  | 35  | 35 |         |
| Age (y)              | 63.9±6.0 | 62.3±10.0 | 0.444 |
| Gender               |     |    | 0.484  |
| Male                 | 27  | 29 |         |
| Female               | 8   | 6  |         |
| Stage                |     |    | 0.059  |
| I                    | 16  | 21 |         |
| II                   | 14  | 14 |         |
| III                  | 5   | 6  |         |
| T stage              |     |    | 0.227  |
| T1                   | 16  | 22 |         |
| T2                   | 17  | 10 |         |
| T3                   | 2   | 3  |         |
| Lymph node           |     |    | 0.044  |
| Positive             | 16  | 8  |         |
| Negative             | 19  | 27 |         |
| Smoking              | 29  | 83 |         |
| Smoking pack years   | 45.7±25.8 | 25.0±9.4 | 0.000 |

**INHIBITION OF LIMK1 BY siRNA TRANSFECTION INTO SK-MES-1 AND A549 CELLS**

LIMK1 siRNA and control siRNA were purchased from Santa Cruz Biotec technology (CA, USA). To determine the effectiveness of the LIMK1 siRNA, SK-MES-1 and A549 cells were transfected using Lipofectamine 2,000 (Invitrogen, CA, USA). After transfection, the cells were used for Western blot and migration assay. LIMK1 levels were determined with an anti-LIMK1 antibody. Migration assay were performed after a similar transfection with LIMK1 siRNA or control siRNA.

**Antibodies**

The following primary antibodies were used in this study. Rabbit anti-phospho (p)-Pak1 (ser144, 68∼74 kDa), rabbit anti-Pak1 (68 kDa), rabbit anti-p-LIMK1 (72 kDa), and rabbit anti-LIMK1 (70 kDa) were obtained from Cell Signaling Technology; rabbit anti-p-cofilin (19∼21 kDa) and goat anti-cofilin (19 kDa) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

**Migration (wound-healing) assay**

For two-dimension migration assays, cells were seeded at a density of 1×10^5 cells/well in a 6-well culture dish. After the cultures reached confluence, a 600-μm wide scratch in the monolayer was produced with the tip of a sterile plastic pipette. Cell monolayers were washed with phosphate-buffered saline (PBS) and then incubated in fresh medium, and photomicrographs were taken at specific time points during incubation. Relative cell migration distance (wound closure) was determined by measuring the wound width, observed microscopically (Axiovert 40C, Carl Zeiss MicroImaging, Göttingen, Germany), and subtracting this value from the initial wound width at 0 h. The data acquired from three scratches in each well were converted to percentage of wound closure.

**Transwell migration assay**

Three-dimensional cell migration was assayed on polycarbonate membrane inserts (8-μm pore size) using the CytoSelect™ Cell Migration Assay Kit (Cell Biolabs, Inc., CA, USA), according to the manufacturer’s instructions. In brief, a cell suspension containing 0.5×10^5 cells/ml in serum-free medium was added to the upper chamber of each insert. The lower chamber contained medium with 10% FBS (which acts as a chemotactic stimulus). The cultures were incubated for 6 h at 37°C in a humidified 5% CO_2/95% air environment to allow cell migration through the membranes, toward the lower face of the transwell inserts. Nonmigrating cells on the inner side of the inserts were gently removed with a cotton-tipped swab. Cells that had migrated to the bottom surface were stained with 0.5% crystal violet for 10 min at room temperature. Photomicrographs were taken (Axiovert 40C microscope) of five in-
individual fields per insert, the cells that had migrated were enumerated, and the number of migratory cells per well was calculated. The stained insert was washed thoroughly and stained adherent cells were removed by incubation for 10 min in extraction solution (1% Triton X-100). The extracted cell samples were transferred to a 96-well microtiter plate and the absorbance at 560 nm was measured in a plate reader (Infinite® F200, Tecan, Männedorf, Switzerland).

Invasion assay

The invasiveness of SK-MES-1 and A549 cells was tested using the quantitatitive CytoSelect 96-well Cell Invasion Assay (Cell Bioslabs), according to the manufacturer’s instructions. The experimental apparatus and assay protocol are fundamentally similar to those used for the transwell migration assay. In brief, SK-MES-1 and A549 cells, suspended in serum-free medium at a density of 0.5×10^5 cells/ml, were seeded onto the culture inserts, which had been coated with a uniform layer of dried basement membrane matrix solution. The cells were allowed to invade the basement membrane layer for 24 h. After removing the non-invasive cells on the upper surface of the membrane by swabbing, the cells that invaded through the membrane were stained with 0.5% crystal violet, then viewed microscopically and counted. Invasiveness was quantitatively expressed as the average number of cells that had invaded.

Western blot analysis

For protein extraction from tissue, frozen lung tissues were transferred to sterile 1.5 ml microcentrifuge tubes, each containing 200 μl of lysis buffer (15 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES] pH 7.9, 0.25 M sucrose, 60 mM KCI, 10 mM NaCl, 10 mM NaF). Homogenized tissues were incubated for 10 min on ice and then disrupted by sonication. For protein extraction from cells, the cells were washed twice with PBS and then lysed in lysis buffer. The samples were stored at −80°C until analysis. Protein concentrations in tissue extracts and cell lysates were measured using the Bio-Rad Protein Assay (Bio-Rad, USA). For western blots, lung lysates (30 μg per lane) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrophoretically transferred onto a polyvinylidene difluoride membrane (Millipore, MA, USA). The membranes were probed with each antibody (diluted 1 : 1000), and the bound antibody was visualized using an enhanced chemiluminescence substrate (Pierce, Rockford, IL, USA). The membrane was reprobed with an antibody against β-actin as a control for equivalent protein loading.

Immunohistochemistry

For immunohistochemical analysis, paraffin-embedded lung tissues were cut into 5-μm sections that were then placed on gelatin-coated slides. Sections were deparaffinized, rehydrated with graded alcohol solutions, and then processed using the avidin-biotin immunoperoxidase method. In brief, sections on the slides were incubated with rabbit anti-p-cofilin antibody (diluted 1 : 500; Santa Cruz Biotechnology) overnight at 4°C. After incubation, sections were washed three times with 0.1 M PBS, and then incubated with secondary biotinylated antibodies (1 : 200) for 1 h at room temperature. After three additional washes with 0.1 M PBS, sections were incubated with avidin-biotin-peroxidase complex solution (ABC solution, Vector Laboratories, CA, USA), and then developed in a solution of 0.05% dianisobenidine (Sigma) containing 0.05% H2O2. The sections were counterstained with hematoxylin, dehydrated through graded alcohols, cleared in xylene, and then mounted on coverslips with Permount (Sigma). The sections were visualized by magnification with a BX51 light microscope (Olympus, Tokyo, Japan), and digital images were captured and documented.

Statistics

Differences between measurements of SCC and AC tumor tissues or cell lines were determined using two-tailed Student t-tests. Values are expressed as the mean±standard error of the mean (SEM). The relationships of stage, T stage, relapse, death, and smoking in the cases of SCC and AC were analyzed by Fisher’s exact test. The lymph node and pack-years were compared using the χ² and t-tests, respectively. A p-value <0.05 was considered statistically significant.

RESULTS

Patient characteristics

The clinicopathological characteristics of the 70 patients who underwent surgery for SCC or AC are summarized in Table 1. The SCC type is most commonly found in men (77%), and it is closely correlated with a smoking history (83%). Of these, 46% (n=16) and 23% (n=8) of SCC or AC patients had lymph node-positive tumors, respectively.

Expression of Pak1 and LIMK1 phosphorylation in human lung tissues and cell lines of SCC and AC

To evaluate the phosphorylated proteins of Pak1 and LIMK1, protein extracts of representative tissue samples from matched pairs (tumor and adjacent normal tissue) of surgically resected SCC and AD lungs were examined (Fig. 1A). All tumor samples from SCC were found to have increased expression of p-Pak1 and p-LIMK1 proteins compared with samples of the corresponding adjacent normal lung tissue (Fig. 1A). In addition, there was increased expression of both total Pak1 and LIMK1 in tumor tissues compared with normal tissues. In the tumor samples from AC, western blot analyses revealed p-Pak1 was not expressed in AC tumor tissues except in one sample. Total Pak1 and LIMK1 protein levels were highly increased in AC tissues compared with normal tissues. These findings showed that Pak1/LIMK1 signaling pathway is not dominant in AC as compared to SCC. In parallel with studies on human lung cancer tissues, we evaluated the expression of Pak1 and LIMK1 proteins in corresponding SCC (HCC1588 and SK-MES-1)- and AC (HCC1711 and A549)-type cell lines by western blot analysis (Fig. 1B). Consistent with results from human lung tissues, SCC type cells were found to have increased expression of p-Pak1 and total...
Pak1 proteins compared with AC type cells. However, there was no significant difference of p-LIMK1 and total LIMK1 expression in between HCC1588 and HCC1711 (Fig. 1B).

**Expression of cofilin phosphorylation in human lung tissues and cell lines of SCC and AC type**

Fig. 2A depicts SCC lung tissues, showing squamous cell differentiation with keratinization features, and AC tissues, showing poorly differentiated adenoma features with gland-like structures. To observe localization of p-cofilin in lung sections of SCC and AC, we performed immunohistochemistry. Immunohistochemical staining revealed increased p-cofilin immunoreactivity in sections of SCC lung tissues (Fig. 2B). Positive staining for p-cofilin was restricted almost exclusively to tumor cells in sections from SCC, and was localized in the cytoplasm; p-cofilin immunostaining was not detected in AC tissues. In addition to immunohistochemical study, we evaluated the expression of p-cofilin and total cofilin protein by western blot analysis (Fig. 2C). All tumor samples from SCC were found to have increased expression of p-cofilin and total cofilin proteins compared with samples of the corresponding adjacent normal lung tissue (Fig. 2C). However, p-cofilin was not expressed in AC tumor tissues except in one sample and there was no difference of total cofilin expression in between normal and ACC tissues. Consistent with results from human lung tissues, SK-MES-1 cells were found to have increased expression of p-cofilin protein compared with A549 cells (Fig. 2D).

**Migration is enhanced in SK-MES-1 cells compared to A549 cells**

To investigate possible differences in cell migration between SCC and AC cell types, we used wound-healing and transwell migration assays (Fig. 3). As shown in Fig. 3A and B, wound closure at 24 h was significantly (p<0.05) more robust in SK-MES-1 cells (65.5±8.6%) than in A549 cells (22.8±9.3%). Because wound healing is a complex process involving many cellular activities, including cell migration and proliferation, we also performed a three-dimensional migration assay to confirm the greater migratory capacity of SK-MES-1 cells (Fig. 3C, D). Using a transwell assay to monitor cell migration towards a chemotactic stimulus (10% FBS), as described in Methods, we found that the percentage of cells that migrated was significantly higher for SK-MES-1 cells (20.3±6.6%) than for A549 cells (0.06±0.02%). The migration of SK-MES-1 cells also increased by approximately 20% compared to that of A549 cells (Fig. 3D). To better assess the mechanism by which Pak1/LIMK1/cofilin pathway contributes to cell migration, we examined the effect of knocking down LIMK1 expression on cell migration. SK-MES-1 or A549 cells were transfected with LIMK1 siRNA or control siRNA. The LIMK1 siRNA substantially reduced the expression of LIMK1 in both SK-MES-1 and A549 cells, whereas the control siRNA had no effect (Fig. 3E). As shown in Fig. 1B, there was increased expression of LIMK1 in SK-MES-1 cells compared with A549 cells. Also, the LIMK1 siRNA, but not the control siRNA, dramatically decreased wound closure in SK-MES-1 cells (Fig. 3F).

**Invasiveness is enhanced in SK-MES-1 cells compared to A549 cells**

To investigate possible differences in cell invasion between SCC and AC cell types, we used a cell invasion assay (Fig. 4). Many more SCC cells (visualized by crystal violet staining) invaded through the membrane than did AC cells (Fig. 4A). The invasiveness of SK-MES-1 cells also was approximately 50% higher than that of A549 cells (Fig. 4B).
Expression of Pak1/LIMK1/Coﬁlin in Human NSCLC

Fig. 3. Migration in SCC (SK-MES-1) and AC (A549) cell lines. (A) Migration of human lung cancer cells in a two-dimensional cell migration assay system. Phases-contrast images of wound areas 0, 9, and 24 h after wounding of SK-MES-1 and A549 monolayers. (B) Wound-healing measurements are shown (percentages), where each bar is the mean±SEM of three independent experiments. (C) Migration of human lung cancer cells in a three-dimensional cell migration assay system. Representative microscopic images of migrating cells. (D) Migration measurements (percentages) are shown, where each bar represents the mean±SEM of four independent experiments. (E) Western blot analysis for LIMK1 from SK-MES-1 and A549 cells with LIMK1 siRNA or control siRNA. β-actin was used as a loading control. (F) Migration measurements (percentages) are shown, where each bar represents the mean±SEM of three independent experiments. Asterisks indicate a significant difference from control siRNA-treated cells (p < 0.05).

DISCUSSION

This is the first study showing that the Pak1/LIMK1/coﬁlin pathway is dominant in SCC compared to AC lung tumor tissues. These results were obtained from examination of lung tissue from SCC and AC patients, as well in SCC- and AC-type cell lines. In addition, migration and invasion were enhanced in SCC-type cells compared to AC type.

Paks are important regulators of the actin cytoskeleton and motility, and several studies have suggested that they are involved in cancer [15-17]. Pak1 gene amplification and increased Pak1 protein levels have been reported in ovarian, breast, and lung cancers [18-20]. Pak1 may also be involved in cancer cell survival and progression, leading to suggestions that Pak1 inhibitors may have potential as a novel oncolytic therapy [6]. Ong et al. [20] demonstrated that antagonists of Pak1 or Pak1 knockdown with siRNA oligonucleotides induces apoptosis of NSCLC cells and decreases proliferation of tumor cells. Consistent with this observation, exogenous expression of a Pak1 mutant with an inactivated kinase domain reduced the invasiveness of highly invasive breast cancer cells [21]. The increased p-Pak1 and total Pak1 protein levels in SCC tissue and SCC-type cells described here are in accord with results obtained from ovarian, breast, and lung tumors, reinforcing the view that Pak1 activity is associated with development of the SCC tumor type.

LIMK1 is upregulated in invasive carcinoma cells, and is the most abundant and dominant cofilin kinase in invasive carcinoma cells [11]. The level and activity of endogenous LIMK1 are higher in invasive breast and prostate cancer cell lines than in less invasive cells. LIMK1 is phosphorylated and activated by Pak1 [22]. The activation of LIMK1 represses cofilin activity and enhances cancer invasiveness [23]. As shown in Fig. 3, our results were consistent that the suppression of LIMK1 expression by small interfering RNA (siRNA) inhibited the motility of Jurkat cells, and the overexpression of LIMK1 increased the invasiveness of prostate epithelial cells in vitro [24]. However, there is some confusion about whether LIMK increases or decreases invasiveness. For example, other studies have reported that overexpression of LIMK suppresses motility in neuroblastoma, and dominant-negative LIMK increases motility [24]. Also, LIMK1-mediated increases in p-coﬁlin levels were shown to inhibit actin polymerization and motility in mammary carcinoma cells in response to epidermal growth factor [25]. Our results are consistent with previous studies demonstrating that inhibition of LIMK1 expression by siRNA decreases tumor motility and invasion, and suggest that Pak1 activation might contribute to LIMK phosphorylation in SCC.

Cofilin is well known to be important for cell motility by enhancing actin dynamics at lamellipodia, a process that is involved in cell invasion [26]. As for LIMK, however, there is confusion about whether cofilin increases or decreases invasiveness. Cofilin is abundantly expressed in the highly invasive C6 rat glioblastoma cell line and in human
pancreatic cancer cells [27,28]. A recent study demonstrated that NSCLC patients with high cofilin expression levels in tumors presented low overall survival rates [29]. However, other studies have found that cofilin is downregulated in cancer and that overexpression antagonizes invasion [30]. Lee et al. [14] demonstrated that overexpression of cofilin disrupts the actin cytoskeleton at the leading edge of the cell and decreases invasiveness of human H1299 cells. In MTLn3 cells, siRNA-mediated knockdown of cofilin results in cells that exhibit less directional change and higher migration velocities than control cells [31]. In the present study, we have found that Pak1/LIMK1-mediated phosphorylation of cofilin may be sufficient to determine migration and invasiveness of SCC cells, and thus confer oncogenic properties. However, it is unlikely that the expression status of cofilin alone is sufficient to determine the motility and invasion tumor cells.

ACs have been reported to have strong migratory activity [32]. However, we have found that migration and invasion were enhanced in SKMES-1 cells compared to A549 cells. The cancer cases in this study were preoperatively early stage SCC lung cancer patients with a higher percentage of lymph nodes. As shown in Table 1, AC patients had a higher percentage of lymph nodes. Our result is consistent with a recent study of Id1 expression, which belongs to the Id family of helix-loop-helix transcription factors, showing that Id1 is more highly upregulated in SCC compared to AC [33]. Expression of Id1 in NSCLS cells significantly increases tumor cell migration without affecting normal cell migration. In general, the rate of growth of an SCC-type tumor at its site of origin is usually more rapid than that of an ACC type [34]. It has been reported that the absolute levels of matrix metalloproteinase (MMP)-9 are increased in SCC, but not in AC, and subsequently it was suggested that the increased levels of MMP-9 correlate with a poor prognosis [35,36]. In addition, glycipican-3, which is overexpressed in lung carcinoma compared to normal lung, is more frequently detected in SCC than in AC [37,38]. In the present study, we observed 3 y after surgical resection that the recurrence or death associated with SCC tumors is higher than that with AC types. These data indicate that early stage SCC patients with higher percentage of lymph nodes may be prone to greater invasion and migration of their tumors compared to AC patients.

In the present study, we report for the first time that the Pak1/LIMK1/cofilin signaling pathway contributes to the migration and invasiveness of lung tumors, especially in SCC. Importantly, we found that the dominant Pak1/LIMK1/cofilin signaling pathway in the SCC tumor type is correlated with higher frequencies of recurrence within 3 y after lung tumor resection at the early stage. Thus, this study supports the idea that a balance between the contribution of cofilin and upstream molecules in the cofilin pathway, including Pak1 and LIMK1, is required for migration and invasiveness seen in tumor cells.

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