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Original Article

Caveolin-1 promotes an invasive phenotype and predicts poor prognosis in large cell lung carcinoma

Fei Han, Jie Zhang, Jinchen Shao, Xianghua Yi*

Department of Pathology, Shanghai Tongji Hospital Affiliated to Shanghai Tongji University Shanghai, 389 Xincun Road, Shanghai, China

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ABSTRACT

Purpose: This study investigated the relationships of caveolin-1 expression with clinical pathologic parameters and the prognosis of patients with large cell lung carcinoma. This study also explored the roles of caveolin-1 in cell invasiveness, matrix metalloproteinase (MMP) expression, and non-small cell lung carcinoma activity *in vitro*.
Methods: A total of 120 tissue samples were immunohistochemically analyzed for caveolin-1 expression. Cell invasion ability was measured by a Transwell invasion assay. Protein expression was assessed by Western blotting. MMP activity was detected by gelatin zymography.
Results: Caveolin-1 was expressed in 54 of 120 (45.0%) cases of large cell lung carcinoma. Caveolin-1 expression was significantly correlated with node status (N0 *vs.* N1, N2, and N3; $P = 0.005$) and advanced pTNM stage (Stages I and II *vs.* Stage III, $P < 0.001$). Patients with caveolin-1-positive expression had a poorer prognosis than did those with caveolin-1-negative expression ($P < 0.001$). The knockdown of caveolin-1 in H460 and 95D cells reduced the invasive ability of the cells and the expression of phosphorylated epidermal growth factor receptor (EGFR), phospho-extracellular signal-regulated kinases 1 and 2, MMP2, and MMP9; the protein level and activity of MMP2 and MMP9 were also decreased by the inhibition of EGFR activity in H460 and 95D cells.
Conclusions: The expression of caveolin-1 was positively correlated with an advanced pathologic stage. Thus, caveolin-1 could act as a predictor of a poor prognosis in patients with large cell lung carcinoma. In addition, the downregulation of caveolin-1 reduced both the invasive ability of tumor cells and the protein and activity levels of MMP2 and MMP9 *in vitro*, suggesting the involvement of EGFR/MMP signaling in this process.

Introduction

Lung cancer is associated with a low survival rate and is one of the leading causes of cancer death worldwide. Among lung cancers, non-small cell lung cancer (NSCLC) accounts for approximately 80% of cases of primary lung cancer. Patients with NSCLC often relapse and develop metastases after surgery, resulting in a dismal 5-year survival rate of only 15%. As one of the most poorly differentiated carcinomas, large cell carcinoma comprises nearly 9% of cases of NSCLC and commonly metastasizes to distant organs such as the brain, adrenal glands, liver, and bone with a poor clinical outcome [1].

Caveolin-1 (Cav1), a principal component of flask-shaped invaginations in the plasma membrane called caveolae, is a 22- to 24-kDa membrane protein with a scaffolding domain. It has a membrane-spanning hairpin loop structure with N- and C-terminal hydrophilic segments. Cav1 has been implicated in diverse cellular processes, including vesicular transport, cholesterol homeostasis, cellular proliferation, and the regulation of signal transduction [2–4]. It also regulates the activity of many signaling molecules such as Src tyrosine kinase, H-Ras, extracellular signal-regulated kinases (Erk), protein kinase C, and endothelial nitric oxide synthase by binding to its scaffolding domain [5–9]. The biological function of Cav1 in malignant tumors is controversial. For example, the Cav1 level is reportedly downregulated in a variety of human cancer cell lines, including colon cancer, breast cancer, and ovarian cancer, and the re-expression of Cav1 inhibits cellular proliferation and invasion [10–12]. However, the Cav1 level is elevated and positively

Abbreviations: Cav1, caveolin-1; FBS, fetal bovine serum; EGFR, epidermal growth factor receptor; Erk1/2, extracellular signal-regulated kinases 1 and 2; MMPs, matrix metalloproteinases; NSCLC, non-small cell lung cancer; siRNA, small interfering RNA.

* Corresponding author. Tel.: +86 21 66111162; fax: +86 21 66111160.
E-mail address: yixhxf@163.com (X. Yi).

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correlated with the tumor stage and a poor prognosis in tissue specimens of prostate cancer, bladder cancer, and esophageal or oral squamous cell carcinoma [13–16]. In lung cancer, the expression and roles of Cav1 vary according to the histological type. Kato et al. [17] reported that Cav1 serves as a tumor suppressor gene in patients with lung adenocarcinoma and that its absence resulted in tumor extension and dedifferentiation. In contrast, Cav1 overexpression was reportedly correlated with tumor extension in patients with squamous cell carcinoma. Moreover, Song [18] reported that the knockdown of Cav1 inhibited the proliferation of NCI-H460 cells and promoted cell migration and invasion, suggesting that Cav1 acts as a proproliferative and antimetastatic gene. However, the roles of Cav1 in the prognosis and cell invasion ability of large cell lung carcinoma remain unclear.

Thus, in this study, we investigated the expression of Cav1 in 120 tissue samples from large cell lung carcinomas by immunohistochemistry *in vivo*. The relationships of Cav1 expression with clinicopathologic parameters and patient survival were analyzed using statistical software. In addition, the effects of the knockdown of Cav1 on cell invasiveness and matrix metalloproteinase (MMP) expression and activity were studied in NSCLC cell lines *in vitro*.

Materials and methods

Patients and samples

A total of 120 patients with primary large cell lung carcinoma (endocrine carcinoma subtype excluded) who underwent complete surgical resection from 1 January 2006 to 1 December 2009 at Shanghai Chest Hospital (Shanghai, China) were included in the current retrospective study. None of the patients received chemotherapy or radiation therapy before surgery. The 7th Edition of the AJCC TNM Classification and Stage Grouping System was used for pathological TNM staging [19]. An additional 20 normal lung tissue specimens were obtained from deceased donors. Informed consent was obtained, and the study was approved by the local ethics committee. All patients were followed after surgery until 31 July 2013 with detailed and complete clinicopathological data. The follow-up process ranged from 7 to 72 months (average, 37 months).

Immunohistochemistry and evaluation of the results

Briefly, slides were dehydrated in xylene and graded alcohol solutions. All specimens underwent heat-induced antigen retrieval (0.01 M citrate buffer at pH 6.0) and were immunostained by the EnVision method. They were then incubated with rabbit polyclonal anti-Cav1 antibodies (1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h at 37 °C. Capillary endothelial cells were used as internal positive controls for Cav1 immunostaining. Replacement of the primary antibody with phosphate-buffered saline was used as a negative control. All slides were reviewed by two pathologists who were not aware of the patients' clinical information. Five visual fields were chosen, and the percentages of staining and intensity of the immunoassayed cells were evaluated at a power of 100× using an Olympus microscope (Olympus Optical Co., Ltd., Tokyo, Japan). The intensity of Cav1 staining was categorized into four grades (−, +, ++, and +++). Cav1-negative expression was defined as negative/weak (−/+) staining for all tumor cells and <30% staining for those exhibiting moderate (++) or strong staining (+++). Cav1-positive expression was defined as >30% staining for cells exhibiting moderate (++) or strong staining (+++) [17].

Materials, cell culture, and the transfection of synthetic small interfering RNA (siRNA)

AG1478, an epidermal growth factor receptor (EGFR) inhibitor, was purchased from Cell Signaling Technology (Beverly, MA, USA). The human NSCLC cell lines H460 and 95D were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). H460 and 95D cells were cultured in RPMI-1640 medium (GIBCO, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS) and 1% antibiotics (100 μg/ml penicillin and 100 μg/ml streptomycin; Sigma–Aldrich, St. Louis, MO, USA). The siRNA targeting Cav1 (siCav1) was designed and prepared as described previously [20]. The siRNA sequences against Cav1 were: for siCav1-1, 5′-AGACGAGCUGAGCGAGAAGCA-3′ (sense) and 5′-CUUCUCGCUCAGCUCGUCUGC-3′ (antisense); and for siCav1-2, 5′-CAUCUACAAGCCCAACAACTT-3′ (sense) and 5′-GUUGUUGGGCUUGUAGAUGTT-3′ (antisense). The siCav1 and control siRNA (Con siRNA) oligonucleotides were obtained from Shanghai GenePharma Co., Ltd. (Shanghai, China). Cav1 siRNA (100 nM) and Con siRNA (100 nM) were transfected into H460 and 95D cells, respectively, using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA). The transfected cells were harvested after 72 h for further analysis.

Cell invasion assay

The ability of cells to invade through a Matrigel-coated filter was measured in Transwell chambers (Corning Inc., Corning, NY, USA). Polyvinylpyrrolidone-free polycarbonate filters (pore size, 8 μm) were coated with basement membrane Matrigel (50 μl/filter) (BD, Bedford, OH, USA). Briefly, the lower chamber was filled with 0.5 ml of RPMI-1640 medium containing 10% FBS. Cells were serum-starved overnight (0.5% FBS), harvested with trypsin/EDTA, and washed twice with serum-free RPMI-1640 medium. The cells were then seeded at a density of 1×10^5 cells/well into the upper chamber. After 48 h, the cells on the upper surface of the membrane were removed using a cotton swab. The migrant cells attached to the lower surface were fixed in methanol for 10 min at room temperature and stained with hematoxylin and eosin. The number of invasive cells was counted under a microscope at 100× magnification.

Western blot analysis

Cells were cultured to 80% confluence then harvested and lysed in ice-cold lysis buffer. The amount of protein was quantified according to the instructions included with the BCA protein assay kit (Pierce, Rockford, IL, USA). An equal amount of protein was separated on an SDS-polyacrylamide gel and electroblotted to polyvinylidine difluoride membranes (Invitrogen). Blocking solution (5% milk) was loaded over the membrane and incubated for 1 h at room temperature. The membranes were then incubated with rabbit anti-human Cav1 antibodies (1:1000 dilution, sc-894; Santa Cruz Biotechnology), mouse anti-human MMP2 antibodies (1:300; Abcam, Burlingame, CA, USA), rabbit anti-human phospho-Erk1/2 and Erk1/2 antibodies (1:1000 dilution, nos. 4370S and 4695S; CST, Beverly, MA, USA), rabbit anti-human phospho-EGFR and EGFR antibodies (1:1000 dilution, nos. 3777S and 2085S; CST), rabbit anti-human MMP9 antibodies (1:1000 dilution, no. 2270S; CST), and mouse anti-human β-actin antibodies (1:5000, A5441; Sigma–Aldrich) overnight at 4 °C. After washing three times, the membranes were incubated with IRDye™ 700-conjugated goat anti-mouse IgG or IRDye™ 800-conjugated goat anti-rabbit IgG (Rockland Immunochemicals, Gilbertsville, PA, USA) for 2 h at room temperature. The protein bands were visualized with an Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA); the