Alpha-Actinin 4 Is Associated with Cancer Cell Motility and Is a Potential Biomarker in Non–Small Cell Lung Cancer

Ming-Chuan Wang, PhD, Ying-Hua Chang, PhD,* Chih-Chieh Wu, PhD, * Yu-Chang Tyan, PhD,†‡§ Hua-Chien Chang, MS,* Yih-Gang Goan, MD,‖ Wu-Wei Lai, MD,¶ Pin-Nan Cheng, MD,# and Pao-Chi Liao, PhD**

Background: Differential expression and secretion of alpha-actinin 4 (ACTN4) in the lung cancer cell lines CL1-0 and CL1-5 have been reported in previous proteomic studies. The aim of this study is to investigate the functional properties of the ACTN4 protein in non–small-cell lung cancer (NSCLC) cells and evaluate its clinical importance.

Methods: We used RNA interference to knock down and overexpress ACTN4 protein to evaluate the effects of this intervention on cancer cell invasion and migration, as well as on microscopic cellular morphology. Furthermore, we examined by immunohistochemistry the expression of ACTN4 protein in tissue samples at different stages of lung cancerand compared the protein levels of ACTN4 in blood plasma samples from patients with histologically confirmed lung cancer and healthy controls.

Results: CL1-5 cell motility was significantly suppressed by the knockdown of ACTN4 protein. The morphology of CL1-5 cells changed from a predominantly mesenchymal-like shape into a globular shape in response to ACTN4 protein knockdown. A quantitative immunohistochemical assessment of lung cancer tissues revealed that ACTN4 protein level was considerably higher in cancerous tissues than in the adjacent normal ones, and the area under the receiver operating characteristic curve was 0.736 ($p < 0.001$). According to an enzyme-linked immunosorbent assay, the plasma levels of ACTN4 protein were significantly different between cancer patients and healthy controls, and the areas under the receiver operating characteristic curves were 0.828 and 0.909, respectively, for two independent cohorts ($p < 0.001$).

Conclusions: We demonstrate that the knockdown of ACTN4 protein inhibited cell invasion and migration. These results suggest that ACTN4 is associated with lung cancer cell motility. Thus, the level of ACTN4 in cancerous tissue and plasma is related to the presence of lung cancer.

Key Words: ACTN4; non–small-cell lung cancers; CL1-0 and CL1-5 cell lines; siRNA

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Lung cancer is the most common cancer in the world and has been the malignancy with the highest mortality for several decades. Non–small-cell lung cancer (NSCLC) accounts for approximately 85% of all lung cancers.[1] Early NSCLC is treated with surgery, with survival rates of 35% to 85% depending on tumor size and stage.[2] However, most NSCLC patients are asymptomatic, and cancers are detected late. Moreover, the overall 5-year survival rates of patients with NSCLC are determined by the cancer stages, ranging from 49% for stage IA to only 1% for stage IV.[3]

The exceedingly low survival rates and increased mortality from cancers are believed to be due to cancer metastasis.[4] Numerous reports have shown that cancer cell–secreted proteins are related to cancer metastasis; these factors aid in cell migration/invasion and the modulation of the microenvironment.[5,6] From previous comparative secretomic analyses of two NSCLC cell lines, CL1-0 cells (which have a low invasive ability) and CL1-5 cells (which have a high invasive ability),[7] ACTN4 protein has been identified as a relatively abundant secreted candidate protein in CL1-5 cells.[8–10]

ACTN4 protein is a member of the cytoskeletal protein family; it binds to actin filaments to preserve cytoskeletal structure and cell morphology.[11–13] ACTN4 is expressed in nonmuscle cells and is commonly associated with focal adhesion contacts and migrating cells.[14] The structure of the ACTN4 protein contains conserved functional domains, including an N-terminal actin-binding domain with two highly conserved calponin homology domains, a central domain consisting of four spectrin repeats, and a calmodulin-like domain in the C-terminus.[14,15]

*Department of Environmental and Occupational Health, College of Medicine, National Cheng Kung University, 138 Sheng-Li Road, Tainan 704, Taiwan.†Department of Medical Imaging and Radiological Sciences, Kaohsiung Medical University, 100 Shi-Chuan 1st Road, Kaohsiung 807, Taiwan.‡ Translational Research Center, Kaohsiung Medical University Hospital, 100 Tzyou 1st Road, Kaohsiung 807, Taiwan.§ Institute of Medical Science and Technology, National Sun Yat-Sen University, 70 Lienhai Road, Kaohsiung 804, Taiwan.‖ Division of Thoracic Surgery, Kaohsing Veterans General Hospital, 386 Ta-Chung 1st RD. Kaohsiung 813, Taiwan.¶Department of Surgery, College of Medicine, National Cheng Kung University Hospital, National Cheng Kung University, 138 Sheng-Li Road, Tainan 704, Taiwan.#Department of Internal Medicine, College of Medicine, National Cheng Kung University Hospital, National Cheng Kung University, 138 Sheng-Li Road, Tainan 704, Taiwan.

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Address for correspondence: Pao-Chi Liao, PhD, Department of Environmental and Occupational Health, College of Medicine, National Cheng Kung University, 138 Sheng-Li Road, Tainan 704, Taiwan. E-mail: liaopc@mail.ncku.edu.tw

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Cancer cell migration in the extracellular matrix (ECM) requires that cell protrusions of lamellipodia, pseudopodia, or filopodia are formed at the leading edge of the cancer cell. The protrusion of these cells requires actin polymerization and rearrangement.[16,17] Following the formation of protrusions in cancer cell membranes, the cancer cells develop new focal adhesion complexes and secrete proteins for local ECM proteolysis. Eventually, the cancer cells release their contacts at the rear and initiate migration.[18] Therefore, ACTN4 may play an important role in cancer cell motility and may modulate cytoskeletal organization by cross-linking actin filaments.

In the current study, we utilized small interfering RNA (siRNA)–mediated ACTN4 protein knockdown, combined with migration and invasion assays, to examine the function of ACTN4 in CL1-5 cells. To investigate whether ACTN4 has the potential to be a useful biomarker for lung cancer, a tissue microarray (TMA) was used to determine the expression of ACTN4 protein at different stages of cancer in lung tissues and in adjacent normal tissues. In addition, a sandwich enzyme-linked immunosorbent assay (ELISA) was applied to measure the plasma concentration of ACTN4 protein in patients at different stages of lung cancer and in healthy controls.

MATERIALS AND METHODS

Cell Lines

The poorly differentiated CL1-0 and CL1-5 cells were provided by Dr. P.C. Yang (Department of Internal Medicine, National Taiwan University Hospital, Taiwan). The maintenance of CL1-0 and CL1-5 cells, including standardization of the media and growth conditions, was performed as described previously.[7,9] The lung cancer cell line A549 was purchased from the American Type Culture Collection and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) (Gibco BRL, Life Technologies, Carlsbad, CA) and 1% penicillin (Invitrogen, Life Technologies, Carlsbad, CA).

Conditioned Medium and Cell Extracts

CL1-0 and CL1-5 cells were cultured at 37°C with 5% carbon dioxide in RPMI-1640 medium supplemented with 10% FBS. The cells were grown to 70% confluence and washed four times with serum-free medium before they were incubated in serum-free medium for 24 hours. The collected conditioned medium (CM) was concentrated by centrifugation in Amicon Ultra-15 tubes (3-kDa cutoff; Millipore, Billerica, MA). The cell extracts (CEs) were also collected in parallel as follows. The cultured cells were washed with phosphate-buffered saline and trypsinized (Biowest, Nuaillé, France). The collected cells were resuspended in distilled water and lysed by microsonication at 4°C. The suspension was centrifuged to remove any cell debris. The Bradford assay (Bio-Rad, Hercules, CA) was used to determine the total protein concentration in the prepared CM and CE samples.

Protein Electrophoresis and Western Blot

Aliquots (10 μg) of protein from the CM and CE samples were individually separated on NuPAGE 4%–12% Bis–Tris gels (Invitrogen) using a Novex Mini-Cell electrophoresis system (Invitrogen). The proteins were transferred to an iBlot polyvinylidene fluoride membrane (Invitrogen) according to the manufacturer's protocol. The transferred membrane was blocked in nonfat milk solution. The membranes were separately probed with an anti-ACTN4 antibody (1:1000; Epitomics, Burlingame, CA), an anti-PGAM1 antibody (1:1000; Epitomics), or an anti-actin antibody (1:1000; GeneTex, San Antonio, TX). The membranes were then washed with Tris-buffered saline and Tween-20 and incubated with the appropriate secondary antibodies conjugated to horseradish peroxidase (HRP) (1:100,000; Sigma-Aldrich). The membranes were washed and developed using an enhanced chemiluminescence reagent (PerkinElmer, Waltham, MA) and photographed using a UVP BioSpectrum imaging system (UVP, Upland, CA).

Small Interfering RNA-–Mediated ACTN4 Protein Knockdown

The two siRNAs targeting ACTN4 protein were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and Life Technologies (Carlsbad, CA). The sequences for ACTN4 siRNA knockdown comprised two pools of RNA sequences; pool I of ACTN4 siRNA contained (1) 5′-CAGAGCUGAUUGAGUAUGA-3′, (2) 5′-CACUCUGUAUCUAUGCAAA-3′, and (3) 5′-CUGG UCUGGUAAAUAUGUA-3′ (Santa Cruz); and pool II of ACTN4 siRNAs sequences contained (1) 5′-GACCAGAGCU GAUUGAGUT-3′ and (2) 5′-CGCAAAUCAUCAACUCC AA-3′ (Life Technologies). The siRNA transfection process was performed using the different cell lines according to the manufacturer's instructions. Briefly, the CL1-5 cells (2×10^5 cells) were seeded in a six-well culture plate. For each transfection, the final siRNA targeting ACTN4 (30 pM siRNA) or a scrambled control siRNA was added along with siRNA transfection reagent (Santa Cruz). The reactions were mixed gently, overlaid onto the cells, and incubated for 24–48 hours.

Transient Transfection for ACTN4 Protein Overexpresssion in CL1-0 Cells

ACTN4 gene overexpression in CL1-0 cells was achieved through transfection with human ACTN4 DNA vector (Cat No. RC202873; OriGene Technologies, Inc., Rockville, MD) and pSV-β-Galactosidase control vector (Promega, MA) using the Fugene6 transfection reagent according to the manufacturer's instructions (Promega). The CL1-0 cells (1×10^5 cells) were seeded in a six-well culture plate and grown to 50%–70% confluence. Fugene 6 transfection reagent (3 μl) was directly added into serum-free medium (97 μl), and then ACTN4 DNA vector (1 μg) was added into the prepared transfection medium for each ACTN4 DNA vector transfection. The transfection reagent and DNA mixture was added into cultured cells. The reactions were incubated at 37°C in the presence of 5% carbon dioxide for 48 hours. The collected cells were washed in distilled water and lysed by microsonication at 4°C. The suspension was centrifuged to remove any cell debris. The Bradford assay was used to determine the total protein concentration. Total proteins (20 μg) from the collected CEs

