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Research Article

14-3-3ζ promotes lung cancer cell invasion by increasing the Snail protein expression through atypical protein kinase C (aPKC)/NF-κB signaling

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ABSTRACT

14–3-3ζ has been identified as a putative oncogene in several cancers, including non–small cell lung cancer (NSCLC). However, the mechanisms underlying its functions remain undefined. In this study, we show that overexpression of 14–3-3ζ was frequently detected in lung adenocarcinoma (LuAC) tissues and was significantly associated with lymph node metastasis and poor outcome. Functional studies demonstrated that 14–3-3ζ promoted migration and invasion in A549 cells, both of which were effectively inhibited when 14–3-3ζ was silenced with short hairpin RNA (shRNA). Furthermore, 14–3-3ζ-mediated invasion of cancer cells was found to upregulate Snail through the activation of atypical protein kinase C (aPKC). Activation of aPKCζ mediates this effect by stimulating NF-κB signaling. Our results identify a specific pathway by which 14–3-3ζ induces tumor invasion and provide insight into potential therapeutic approaches to target 14–3-3ζ-associated lung adenocarcinoma.

1. Introduction

Lung cancer is one of the the leading cause of cancer-related mortality worldwide. Non–small cell lung cancer (NSCLC) accounts for approximately 85% of lung cancers. The invasive properties of lung cancer underlie the urgency of exploring new prognostic markers and effective therapeutic targets in the management of this cancer [1].

The 14-3-3 proteins are a family of highly conserved regulatory molecules expressed in all eukaryotic organisms [2]. In humans, seven different isoforms (ζ, σ, β, ε, η, γ, θ) have been identified and were originally described as enzyme cofactors that affect the activity of protein kinase C and Raf-1 [3]. Several findings have indicated that the 14-3-3 family is associated with the products of oncogenes. 14-3-3ζ is a member of the 14-3-3 family widely known for its role in promoting the development and progression of human cancers [4,5]. 14-3-3ζ is one of the major proteins induced by TGF-β and can promote the epithelial-mesenchymal transition in cancer cells [6]. Furthermore, 14-3-3ζ can interact with the tumor suppressor tuberin to inhibit phosphoinositide-3′-kinase signaling downstream of Akt [7].

Snail acts as a transcriptional factor, binds to E-box sequences and represses E-cadherin expression [8]. Snail is also a crucial inducer of EMT, and the overexpression of Snail correlates with tumor grade and nodal metastasis and predicts a poor outcome in patients with various cancers [9]. Although overexpression of Snail was observed in certain cancers, the molecules involved in Snail expression are largely unknown. In the present study, we report that 14-3-3ζ overexpression is associated with lung cancer malignancy, and Snail is upregulated by 14-3-3ζ through the activation of the aPKC/NF-κB pathway.

2. Materials and methods

2.1. Cell lines and reagents

A549 and PC9 cells (American Type Culture Collection, USA) were grown in Dulbecco's modified Eagle's medium (Hyclone, USA) supplemented with 10% fetal bovine serum (Gibco, USA). Anti-Snail (ab53519), anti-Slug (ab27568), and anti-Par3 (ab64646) antibodies were purchased from Abcam (USA); anti-14-3-3ζ (sc-1019) and anti-aPKC (sc-216) antibodies were from Santa Cruz Biotechnology (Santa Cruz, USA); and anti-P-aPKC^{T560} (CG1453) antibody was purchased from Cell Applications. Anti-IκBα (4814), anti-P-IKKα/β (2697), anti-total IKKα (8943), anti-total IKKβ (11,930), anti-P-NF-κB p65 (3303), anti-NF-κB p65

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(8242), and anti-β-actin (4970) antibodies were from Cell Signaling Technology (USA). JSH-23 (4-methyl-N1-(3-phenylpropyl)-1,2-benzenediamine, Sigma-Aldrich) and CAPE (Caffeic acid phenethyl ester, Sigma–Aldrich) were from Sigma–Aldrich (USA).

2.2. Patients and sample preparation

Sixty-one specimens of surgically resected lung adenocarcinoma and corresponding adjacent normal tissues (with >5 cm distance from the tumor's edge) were collected at Union Hospital, Tongji Medical College, Huazhong University of Science and Technology from September 2014 to December 2015 after receiving the patients' informed consent and the approval of the hospital authorities. None of the patients received radiotherapy or chemotherapy prior to the pulmonary surgery. After surgical removal, all of the samples were immediately snap-frozen in liquid nitrogen or fixed with formaldehyde solution. The study was approved by the Human Research Committee of Huazhong University of Science and Technology and China Anti-Cancer Association (CACA) and was performed in accordance with the *Helsinki Declaration*.

2.3. Plasmid constructs and transfection

Full-length human 14–3-3ζ and PKCζ complementary DNA (cDNA) was amplified by PCR, cloned into a pcDNA3.1(+) expression vector (Invitrogen), and then transfected into A549 and PC9 cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Cells transfected with empty vector were used as controls. Lentiviruses containing shRNAs targeting Par3 and 14-3-3ζ were purchased from Shanghai GeneChem (GeneChem, China) and used to transfect A549 cells. The shSnail target sequence was 5′-GCAGGACTCTAATCCAGAGTT-3′, the sh14-3-3ζ target sequence was 5′-GCAATTACTGAGAGACAACTT-3′, the shIκBα target sequence was 5′-GACGAGAAAGATCATTGAAAT-3′ and the shPKCζ target sequence was 5′-GACGATGAGGATGCCATAAAG-3′. Cells transfected with empty vector or scrambled shRNA were used as controls.

2.4. Cell migration and invasion assays

Scratch wound migration assays were performed on A549 cells as previously described [10]. The cells were cultured on a 35 mm dish and then wounded using a 10 μl pipette tip and washed three times with PBS. Serum-free DMEM was then added. Triplicate wells were used for each condition, and three fields per well were captured at each time-point over a period of 24 h. An invasion activity assay was measured with growth factor-reduced Matrigel invasion chambers (BD Biosciences, USA) following the manufacturer's instructions. After incubation for 24 h, the cells were fixed and counted under a microscope.

2.5. MTT assay

For the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, cells were plated at a density of 5×10^3/well in 96-well plates and incubated in complete culture medium. After treatment with MTT (20 μl, 5 mg/ml, Sigma) and DMSO (200 μl, Sigma), the absorbance was determined using a microplate reader at 490 nm with subtraction of the baseline reading.

2.6. Western blotting

Cells were lysed and electrophoresed in a 10% SDS-PAGE gel at 100 V for 2 h and electroblotted onto a PVDF membrane (Millipore). The membrane was incubated with 5% fat-free milk in PBS for 2 h. Then, the membranes were incubated with primary and secondary antibodies, and the immune complexes were detected with a chemiluminescent reagent (Thermo).

2.7. qRT-PCR

Total RNA was extracted from normal and malignant human LuAC specimens using TRIZOL reagent (Invitrogen). cDNA was synthesized and subjected to real-time PCR using the SYBR® Premix Ex Taq™ Kit (Takara, Japan). 18S rRNA was used as an internal control. The sequences of the 14–3-3ζ primers were as follows: forward, 5′-CCTGCATGAAGTCTGTAACTGAG-3′; reverse, 5′-GACCTACGGGCTCCTACAACA-3′. The sequences of the Snail primers were as follows: forward, 5′-TCGGAAGCCTAACTACAGCGA-3′; reverse, 5′-AGATGAGCATTGGCAGCGAG-3′. The relative mRNA expression level was normalized as previously described [11].

2.8. Immunoprecipitation

A549 cells were collected in lysis buffer [20 mM Tris Cl (pH 7.5), 150 mM NaCl, 5% glycerol, 1% Nonidet P-40, 10 μg/ml Aprotinin, 10 μg/ml Leupeptin, 1 mM PMSF, 10 μg/ml Pepstatin A, 1 mM sodium orthovanadate]. The immunoprecipitates were washed three times with lysis buffer. The proteins were incubated with 2 μl of anti-Par3 antibodies and 50 μl of Protein A (Sigma) for 1 h at 4 °C. After washing, the samples were analyzed by western blotting.

2.9. Immunohistochemical and immunofluorescence staining analyses

Immunofluorescence (IF) staining was performed as described previously [12]. Cells were plated on 35 mm dish and fixed with 4% paraformaldehyde. Following fixation, the cells were permeabilized with Triton X-100, blocked with 3% bovine serum albumin (BSA) in PBS for 1 h at room temperature, stained with anti-p65 (1:600) in 0.3% BSA in PBS, and stained with FITC-conjugated secondary antibodies (1:300, Boster, China) in 0.3% BSA in PBS. Fluorescence was visualized with a microscope (Olympus, Japan).

Immunohistochemistry and the semiquantitative scoring technique were performed as previously described [13]. The scores were defined according to the cell positive staining intensity (0=negative; 1=weak; 2=moderate; and 3=strong) multiplied by the extent of positive stained cells (75–100%=4, 50–74%=3, 25–49%=2, 1–24%=1, 0%=0), leading to scores from 0 to 12.

2.10. NF-κB luciferase reporter assays

The NF-κB luciferase reporter assay was performed as described previously [14]. Cells were transfected with $3\times$ κBL and a Renilla luciferase reporter plasmid. Twenty-four hours after transfection, firefly and Renilla luciferase activities were assessed using a dual luciferase reporter gene assay kit (Beyotime, China).

2.11. Statistical analysis

Two-tailed Student's t tests were used in statistical analyses. Clinical correlations were analyzed by χ^2 test, and survival analyses were assessed using log-rank tests and Kaplan–Meier plots. Multivariate survival analyses were performed using a Cox regression model. Statistical analysis was performed using the EmpowerStats statistical software program Version 2.16.1.