Cellular Signalling 27 (2015) 1469–1477

Contents lists available at ScienceDirect

Cellular Signalling

journal homepage: www.elsevier.com/locate/cellsig

Inhibition of integrin-linked kinase expression by emodin through crosstalk of AMPKα and ERK1/2 signaling and reciprocal interplay of Sp1 and c-Jun

Qing Tang, Shunyu Zhao, Jingjing Wu, Fang Zheng, LiJun Yang, JingHeng Hu, Swei Sunny Hann *

Laboratory of Tumor Biology, Guangdong Provincial Hospital of Chinese Medicine, The Second Clinical Medical College, University of Guangzhou Traditional Chinese Medicine, Guangzhou, Guangdong Province 510120, China

ARTICLE INFO

Article history:
Received 12 March 2015
Received in revised form 4 April 2015
Accepted 9 April 2015
Available online 16 April 2015

Keywords:
NSCLC
Emodin
ILK
AMPKα
Sp1
c-Jun

ABSTRACT

Despite the anti-cancer effect of emodin observed in several cancers, the underlying molecular mechanism remains to be elucidated. In this study, we showed that emodin-inhibited NSCLC cell growth and increased phosphorylation of AMPKα and ERK1/2. In addition, emodin-inhibited ILK protein expression. The overexpression of ILK reversed the effect of emodin on cell growth inhibition. Furthermore, the blockade of AMPK by compound C abrogated, while metformin, an activator of AMPK, strengthened the effect of emodin on the inhibition of ILK expression. Interestingly, the inhibitor of MAPK extracellular signaling-regulated kinase (ERK) kinase (MEK)/ERK1/2 (PD98059) attenuated emodin-induced phosphorylation of AMPKα. Moreover, emodin reduced the protein expression of Sp1 and AP-1 subunit c-Jun. Exogenous expression of Sp1 and c-Jun diminished emodin-reduced ILK protein expression. Emodin suppressed ILK promoter activity, which was not observed in cells overexpression of Sp1 and treated with compound C. Intriguingly, exogenous expression of c-Jun overcame the emodin-inhibited Sp1 protein expression. Collectively, our results demonstrate that emodin inhibits ILK expression through AMPKα-mediated reduction of Sp1 and c-Jun. Metformin enhances the effects of emodin. Exogenous expression of Sp1 and c-Jun resists emodin-inhibited ILK promoter activity and protein expression. In addition, the overexpression of c-Jun diminishes emodin-induced AMPKα signaling. Thus, the crosstalk of AMPKα and MEK/ERK1/2 signaling and the reciprocal interaction between Sp1 and c-Jun proteins contribute to the overall responses of emodin. This novel signaling axis may be a therapeutic potential for prevention and treatment of NSCLC.

1. Introduction

Lung cancer is the leading cause of cancer-related deaths in the world, with non-small cell lung cancer (NSCLC) accounting for more than 80% of primary lung cancers [1]. Importantly, the majority of lung cancers are metastasized at the time of diagnosis, making them illegible for curative treatment by either surgery or radiochemotherapy. As such, continuous efforts in the search for new agents and treatment options to increase therapeutic efficiency and improve patient survival are highly needed. A considerable number of plant extracts and isolated compounds from traditional Chinese medicine (TCM) possess significant anti-growth or pro-apoptotic effects in various cancer types including lung [2–4]. However, the detailed mechanisms underlining these effects have not been well elucidated. Recent advances in the understanding of biological mechanisms of tumor growth have allowed for identification of novel molecular targets for cancer treatment.

Emodin (1,3,8-trihydroxy-6-methylanthraquinone), a natural anthraquinone derivative found in the roots and rhizomes of various plants, such as the genus *Polygonum*, *Rheum palmatum*, among others, showed to induce apoptosis and growth inhibition in various cancer cells, including lung tumor, through multiple mechanisms [5–7]. Emodin enhances targeted therapy-induced cytotoxicity via downregulation of repair protein Rad51 and inactivation of extracellular signal-regulated kinase 1/2 (ERK1/2) in human lung cancer cells [8]. However, the detailed molecular mechanism underlying the anti-tumor effect by this agent has not been well elucidated.

Integrin-linked kinase (ILK), an intracellular adaptor and intracellular serine/threonine kinase, has been implicated in the regulation of various cellular processes, including cell proliferation, apoptosis, differentiation and angiogenesis, and is highly expressed in several human malignancies [9–11]. High expression of ILK correlates with malignant phenotype in cancers, including NSCLC [12,13]. Furthermore, forced expression of ILK promotes invasion and migration of human cancer cells

* Corresponding author at: No. 55, Neihuan West Road, Higher Education Mega Center, Panyu District, Guangzhou, Guangdong Province, PR China, 510006. Tel.: +86 20 39318472.
E-mail address: swhan20010@live.com (S.S. Hann).

http://dx.doi.org/10.1016/j.cellsig.2015.04.005
0898-6568/

[14,15]. Thus, the inhibition of ILK is anti-tumorigenic and could be considered as a solid target for the prevention and treatment of cancers. Nevertheless, the underlying molecular mechanisms and the potential role of ILK in lung cancer growth remain to be determined.

AMP-activated protein kinase (AMPK), a central cellular energetic cascade, has been shown to regulate several cellular metabolic processes induced by nutrient deprivation, mitochondrial dysfunction, and oxidative stress, among others. The inactivation or alternation of its expression leads to a variety of metabolic disorders [16,17]. In addition, AMPK signaling also involves in growth, differentiation, and progression of cancer and has emerged as an attractive target to cancer therapy [18,19]. However, AMPK has recently been shown to promote cancer cell progression by extrinsic and intrinsic stimuli including growth factors and oncogenes [20]. Thus, the true role of AMPK acting as a tumor suppressor or an oncogene, and if AMPK should be targeted for activation/stimulation or inhibition during cancer therapeutics, requires clarification.

The aim of this study was to explore the potential mechanism by which emodin inhibits growth of NSCLC cells.

2. Materials and methods

2.1. Culture and chemicals

The human NSCLC cell lines A549, PC9, H1299, H1650, and H1975 were obtained from the Cell Line Bank at the Laboratory Animal Center of Sun Yat-sen University (Guangzhou, China) and Cell Bank of Type Culture Collection, Chinese Academy of Science (CBTCCCAS) (Beijing, China), and grown in RPMI-1640 medium (obtained from GIBCO, Life Technologies, Grand Island, NY, USA) supplemented with 10% heat-inactivated FBS, HEPES buffer, 50 IU/mL penicillin/streptomycin, and 1 μg amphotericin. All cell lines have been tested and authenticated for absence of *Mycoplasma*, genotypes, drug response, and morphology using a commercially available kit (Invitrogen, Carlsbad, CA, USA) in the Laboratory and in Animal Center at Sun Yat-sen University in August 2012 and have passed the test of DNA profiling (STR). Polyclonal antibodies specific for ILK, Sp1, c-Jun, phosphor-AMPKα (thr172), extracellular signaling-regulated kinase 1/2 (ERK 1/2) (thr202/204), and total AMPKα, ERK/1/2 were purchased from Cell Signaling Inc.(Cell Signaling, Beverly, MA, USA). Emodin was purchased from Chengdu Must Biotechnology Company (Chengdu, Sichuan, China). Metformin was ordered from Enzo Life Sciences, Inc. (Guangzhou, Guangdong Province, China). Compound C and PD98059 were purchased from Sigma Aldrich (St. Louis, MO, USA).

2.2. Western blot analysis

The experimental procedure was reported previously [21]. After measuring the protein concentrations by Bio-Rad protein assays, the cell lysates were solubilized in 5× SDS-sample buffer and separated on 10% SDS polyacrylamide gels. Membranes were incubated with antibodies against ILK, phosphor-AMPKα, ERK/12, and their total forms, Sp1 and c-Jun. The membranes were washed and incubated with a secondary antibody raised against rabbit IgG conjugated to horseradish peroxidase (Cell Signaling, Inc. Beverly, MA, USA). The membranes were washed again and transferred to freshly made ECL solution (Pierce, Rockford, IL, USA), followed by observing signals using the Molecular Imager ChemiDoc XRS Gel Imagine System (Bio-Rad, Hercules, CA, USA).

2.3. MTT cell viability assay

Cell viability was measured using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay and reported previously [21]. Briefly, NSCLC cells (5×10^3 cells/well) were seeded into a 96-well microtiterplate. The cells were treated with increasing concentrations of emodin for up to 72 h or emodin (50 μM) for 48 h in different NSCLC cell lines (1×10^4 cells/well). In separate experiments, cells were treated with compound C for 2 h or were transfected with control or Sp1, c-Jun, or ILK expression vectors for 24 h before exposure of the cells to emodin with or without metformin for an additional 24 h. After incubation, 10 μL MTT solution (5 g/L) was added to each well and incubated at 37 °C for an additional 4 h. Supernatant was removed, then DMSO (150 μL) was added and oscillated for 10 min. Absorbance at 490 nm was determined through the use of ELISA reader (Perkin Elmer, Victor X5, Waltham, MA, USA). Cell viability (% of control) was calculated as follows: (absorbance of test sample/absorbance of control).

2.4. Transient transfection assays

The human ILK promoter constructs ligated to the luciferase reporter gene was obtained from Dr. Desvergne at the University of Lausanne and has been reported previously [22]. The control or ILK overexpression vector (RG201544, pCMV6-AC-ILK-GFP) was purchased from OriGene Technologies, Inc. (Rockville, MD, USA). Briefly, cells were seeded in 6-well dishes and grown to 50–60% confluence. For each well, 2 μg of ILK DNA constructs, with or without 0.2 μg of the internal control phRL-TK Renilla Luciferase Reporter Vector, were co-transfected into the cells using Lipofectamine 3000 transfection reagent (Thermo Fisher Scientific Inc, Waltham, MA, USA). The preparation of cell extracts and measurement of luciferase activities were determined using the Dual-Luciferase Reporter Kit (Promega, Madison, WI, USA). Firefly luciferase activity was normalized with Renilla within each sample. In separate experiments, after transfection, cells were incubated for 48 h at 37 °C, then treated with emodin for an additional 24 h for all other experiments.

2.5. Electroporated transfection assays

The procedure was reported previously [21]. Briefly, after centrifugation, the medium were removed, and the NSCLC cells (5×10^7 cells/mL) were washed with 1× PBS and centrifuged again at 160×*g* rpm for 5 min. Afterward, the tubes were added Bio-Rad Gene Pulser electroporation buffer and resuspended the cells. The desired control (pCMV-Tag2A) and the c-Jun expression plasmid (Plasmid No.47443, Flag-JunWT-Myc, purchased from Addgene, Inc., Cambridge, MA, USA) [23] or control (pCMV6) and Sp1 expression vector (Sp1-Myc-DDK-tagged), purchased from OriGene Technologies, Inc. (Rockville, MD, USA) (2 μg/well, each), were added, and the electroporation plate were put in the MXcell plate chamber in Gene Pulser II Electroporation System (Bio-Rad, Hercules, CA, USA). The electroporation conditions on the plates to deliver 150 V/5 ms square wave were adjusted until reaching the optimum. After electroporation was completed, the cells were transferred to a tissue culture plate. We typically transfer each 150 μL sample to a 6-well tissue culture plate containing 2 mL RPMI1640. Cells were incubated 48 h at 37 °C, then treated with emodin and other agents for an additional 24 h, followed by performing other experiments.

2.6. Statistical analysis

Values are expressed as the mean ± SD. All experiments were performed at least three times. Differences between groups were assessed by one-way ANOVA and significance of difference between particular treatment groups was analyzed by Dunnett's multiple comparison tests or Bonferroni *t*-test using GraphPad Prism software version 5.0 (GraphPad Software, Inc. La Jolla, CA, USA). Asterisks shown in the figures indicate significant differences of experimental groups in comparison with the corresponding control ($P < 0.05$, see figure legends).