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In vitro study of FUZ as a novel potential therapeutic target in non-small-cell lung cancer

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ABSTRACT

FUZ is regarded as a planar cell polarity effector that controls multiple cellular processes during vertebrate development. However, the role of FUZ in tumor biology remains poorly studied. Our purpose of this study is to discover the physiological effects and mechanism of FUZ in non-small-cell lung cancer (NSCLC) *in vitro*. With the help of bioinformatics analysis, we noticed that the expression level of FUZ negatively correlates with prognosis of NSCLC patients. Exogenous FUZ expression markedly promoted cell proliferation of NSCLC cells. The phosphorylation of Erk1/2, STAT3 and related signaling molecules were induced activated after FUZ over-expression. FUZ also plays an important role in cell motility by regulating cell signaling pathways and inducing epithelial to mesenchymal transition (EMT). FUZ promotes EMT along with the up-regulation of N-cadherin, vimentin, Zeb1, Twist1 and decreased level of E-cadherin. Furthermore, we also carried out FUZ directed siRNA treatments to prove the above observations. Knockdown of FUZ resulted in delayed cell growth as well as impaired cell migration and reversed EMT phonotype. Importantly, we reported for the first time that FUZ is a BNIP3-interacting protein. Loss of FUZ resulted in decreased BNIP3 protein level, but no influence on BNIP3 mRNA level, suggesting weakened stability of BNIP3 protein. Overall, our results *in vitro* show that FUZ is responsible for NSCLC progression and metastasis, suggesting that FUZ can be a potential therapeutic target for NSCLC.

1. Introduction

Lung cancer has become the leading form of cancer in terms of both incidence and cancer related deaths [1]. Non-small-cell lung cancer (NSCLC) accounts for about 85% of all lung cancers [2], among all histological types of NSCLC, lung adenocarcinoma is the most common one that accounts for approximately 50% [3]. Chemotherapy was previously the conclusive recommendations, but it had a low cure rate and brought patients bad side effects and miserable experiences. Even worse, compared to small cell carcinoma, NSCLC relatively lacks sensitivity to chemotherapy. With the accumulation of our knowledge about tumor driver genes and promoter genes, targeted therapies against these genes have provided a better choice for advanced patients, which has much better treatment effects and lower side effects. Thus, the study about tumor driver or promoter genes has been a crucial breakthrough to solve these problems [4].

In the post-genome era, addressing gene function is an important and arduous task. While for most of the human encoding genes, their functions in specific cases are yet to know. In cancer diagnosis and treatment, many tumor-related genes are regarded as markers as they are involved in the selection of therapy as well as the prognosis of the patient. To our knowledge, highly activated oncogenes such as c-myc [5] and STAT3 [6] suggest poor prognosis of patient while an increased level of tumor suppressor genes such as p53 [7] and PTEN [8] indicated a better outcome. On the basis of this theory, it is vital for us to discover more potential markers to make clear of the mechanism of tumorigenesis and development as well as to assist diagnosis and treatment.

The *Drosophila melanogaster* homolog of FUZ is associated with planar cell polarity (PCP) and Hedgehog signaling [9,10]. In *Deosophila*, Fuz was reported to regulate hair polarity in a restricted set of fly tissues [11–13]. Fuz is a critical regulator of cilia structure and function in *Xenopus laevis* and mice. In *Xenopus laevis*, knockdown of Fuz (XFy) results in an open neural tube along the entire spinal axis, associated with body shortening, disruption of the cortical actin network and sparse rudimental cilia in skin cells [14]. A recently published study revealed that Fuz knockout mouse exhibits severe cranial neural tube defects (NTD) [15,16]. Actually, the functions of FUZ have been quite well elucidated, but limited to neural system development. Few reports

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of FUZ in tumor biology have been published yet, including in NSCLC. Planar polarity signaling is involved in the elongation of the body axis during development, a process that involves the coordination of multiple cell behaviors including cell rearrangement, cell division and cell-shape changes [17]. Interestingly, these are exactly what happen during tumorigenesis. As a PCP effector, dysregulation of FUZ could result in overactive PCP signaling pathway followed by subsequent uncontrollable cell division and cell-shape changes, which suggests FUZ as a potential tumor promoter.

BNIP3 is described as a pro-cell death protein, and its activity is dependent on the BH3 domain as well as on the transmembrane domain [18–20]. Unlike most BH3-only members that induce acute apoptotic cell death upon overexpression, transient expression of BNIP3 results in delayed cell death [21]. BNIP3 has also been implicated in autophagic cell survival possibly by facilitating removal of damaged mitochondrial [22,23]. High-level expression of BNIP3 is also reported in several human carcinomas compared with normal tissues [24]. Notably, recent studies suggest that BNIP3 plays an essential role in solid tumor development, including NSCLC [25].

In this study, we investigated the effects and mechanism of FUZ in NSCLC cells *in vitro*. Our results indicated that FUZ promotes cell proliferation along with the up-regulation of the phosphorylation of Erk1/2 and STAT3. Besides, FUZ accelerates the migration of NSCLC cells. From bio-informatics analysis, the expression of FUZ is found negatively correlated with the predicted overall survival of patients. FUZ is a BNIP3-interacting protein and the interaction enhances their stability. These results *in vitro* all support FUZ as an oncogene and a potential therapeutic target in NSCLC.

2. Materials and methods

2.1. Cell culture and reagents

A549 and H1299 cells were obtained from the American Type Culture Collection (ATCC) and routinely cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA) and 100 mg/ml penicillin-streptomycin in a humidified 5% CO_2 at 37 °C. Lipofectamine™ 3000 was purchased from Life Technologies (USA). All transfections were performed under the manufacturer's instructions. SKL2001 and KYA1797K reagents were purchased from Selleck. The MG-132 was purchased from EMD Millipore (Billerica, MA, USA). Antibodies against Flag-tag, Erk1/2, phosphor-Erk1/2 (Thr202/Tyr204), STAT3, phosphor-STAT3 (Tyr705), Ras, Raf1, p21, CyclinB, E-Cadherin, N-cadherin, Snail, TCF8/ZEB1, Twist1, Smad2/3, p-Smad3, Vimentin were purchased from Cell Signaling technology (USA). Anti-GAPDH and anti-β-actin (Proteintech group), anti-FUZ antibody (ab122742), anti-BNIP3 antibody was purchased from Abcam. Adenoviruses carrying the FUZ gene (AdFUZ) and empty adenovirus (AdNC) were packaged by Vigene Biosciences. The siRNAs were synthesized by GenePharma (Shanghai, China). The following sequences were used:

siFUZ-1:5′CCCUCAAUGGAGUCCACAUTT3′(sense), 5′AUGUGGACUCCAUUGAGGTT3′(antisense), siFUZ-2:5′GGUCCUUCUUGUGGGACUUTT3′(sense), 5′AAGUCCCACAAGAAGGACCTT3′(antisense); siBNIP3-1:5′AAGGAACACGAGCGUCAUGAATT3′(sense), 5′UUCAUGACGCUCGUGUGUUCCUUTT3′(antisense); siBNIP3-2:5′GUUCCAGCCUCGGUUUCUATT3′(sense), 5′UAGAAACCGAGGCUGGAACTT3′(antisense).

2.2. Cell proliferation assays

A549, H1299 cells infected with AdNC, AdFUZ or siNC, siFUZ were seeded in 96-well plates at a density of 2500 cells per well, cells were then incubated routinely. Cell proliferation was detected by using Cell Counting Kit-8 (CCK8) (Dojindo Molecular Technologies, Japan). 10 μl CCK-8 reagent was diluted in each well and incubated for 2 h at 37 °C. The absorbance as 450 nm was measured by a spectrophotometer. Results from three independent experiments were presented as the means ± standard deviation (SD).

2.3. Colony formation assay

1000 A549 cells that were equally treated in CCK-8 assay were plated in 6-well culture plates. 2 weeks later, cells were fixed with paraformaldehyde for 10 min and then stained with 0.5% crystal violet for 10 min at room temperature. Colonies were defined as a minimum of 50 cells in a group and counted with the image analysis software (IPP6). Results were obtained from two or three independent experiments.

2.4. Cell cycle analysis

A549 cells were harvested at 48 h after Ad-FUZ infection. Cells were washed with PBS for 3 times and then fixed in pre-cooled 70% ethanol overnight at −20 °C. Fixed cells were then pelleted through centrifugation, washed with PBS and incubated with 500 mg/ml RNase A (Sigma-Aldrich) in PBS at 37 °C for 30 min. Cells were then analyzed on BD FACS Calibur (BD bioscience, San Jose, CA, USA) after stained with 10 mg/ml PI (Sigma-Aldrich) in 0.1% Triton X-100. Cell cycle distribution was analyzed with the ModFit LT software (Verity Software House, Topsham, ME).

2.5. Western blotting analysis

Cells were lysed in RIPA (Sigma-Aldrich, St Louis, MO, USA) supplemented with 1% protease inhibitor cocktail and 5% phosphatase inhibitor (Roche, Basel, Switzerland). Protein concentrations were determined using BCA protein assays (Pierce, Rockford, IL, USA). Cell lysates were then fractionated using 12.5% SDS-PAGE gels and electrotransferred onto polyvinylidene difluoride (PVDF) membranes (GE Healthcare, Buckinghamshire, UK), then probed with primary antibodies and subsequently HRP-labeled secondary antibodies. Signals were detected by LAS500 Quant Image (GE, New York City, NY, USA). GAPDH was used as a lysate loading control.

2.6. Cell migration assay

Transwell assay. 24 h after infection, A549 or H1299 cells were serum starved for 6 h, 3×10^4 cells (for A549 cells) or 5×10^4 cells (for H1299 cells) in 200 μl serum-free medium were seeded into the upper chamber of a Transwell chamber with a fibronectin-coated filter (8 mm pore size, Corning Life Sciences, NY, USA). Bottom chamber contained medium supplemented with 10% FBS. After a 24-hour incubation at 37 °C, non-migrated cells were scraped off the filter by using a wet cotton swab and migrated cells were stained with crystal violet following fixation with 4% paraformaldehyde. Number of cells was counted in 6 randomly chosen fields. Triplicate wells were performed in each assay and the assay was repeated for 3 times.

Wound healing assay. H1299 cells were infected with AdNC/AdFUZ or transfected with siNC/siFUZ for 48 h, then cells were moved into serum-free medium. At each indicated time point, photographs of wound closure were taken utilizing a Motic AE31E microscope (×40 magnification) and subsequently analyzed by Imagej.

2.7. Yeast two-hybrid

The FUZ and BNIP3 cDNA was fused to the GAL4 binding domain (BD) (pGBK-T7 vector) and activation domain (AD) (pGAD-T7 vector) respectively. The BD and AD plasmids were co-transformed into yeast AH109 strains using His (+) [SD (Leu-, Trp-)] plates. The transformants with the desired protein expression were plated on His (+) and His (−) [SD (Leu-, Trp-, His-)] plates. The strains were incubated at 30 °C till visible colonies appeared. Protein expression was analyzed by Western-