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The role of ZNRF2 in the growth of non-small cell lung cancer

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Abstract. – OBJECTIVE: ZNRF2 belongs to ubiquitin ligases of the RING superfamily, and has been recently shown to be regulated by Akt to interact with a Mechanistic target of rapamycin (mTor). Nevertheless, a role of ZNRF2 in tumorigenesis, especially in non-small cell lung cancer (NSCLC), is unknown. Here, we assessed ZNRF2 expression in NSCLC.

PATIENTS AND METHODS: We examined ZNRF2 levels by Western blot using NSCLC specimens, compared to the paired non-tumor controls. We also examined the effects of ZNRF2 on cell growth and cell survival in the presence of fluorouracil.

RESULTS: We detected significant higher levels of ZNRF2 and mTor in NSCLC tissues, compared to the paired non-tumor controls. Moreover, ZNRF2 and mTor levels strongly correlated in NSCLC tissues. High ZNRF2 levels were correlated with poor prognosis of the NSCLC patients. *In vitro*, overexpression of ZNRF2 increased NSCLC cell growth and suppressed apoptotic cell death in the presence of Fluorouracil, while depletion of ZNRF2 decreased NSCLC cell growth and increased apoptotic cell death in the presence of fluorouracil. ZNRF2 appeared to augment mTor and its downstream targets CyclinD1 and CDK in NSCLC cells.

CONCLUSIONS: ZNRF2 may be a promising target for treating NSCLC.

Key Words:
NSCLC, ZNRF2, Prognosis, Tumor growth, mTor.

Introduction

Non-small cell lung cancer (NSCLC) is a malignant lung cancer of high prevalence in humans[1-4]. Fluorouracil (5-FU) treatment has been used as a supplementary treatment to surgical removal of the primary tumor, which improves the survival of the patients[5,6]. However, some NSCLCs have been shown to be resistant to 5-FU treatment and thus, understanding the molecular mechanisms underlying this phenomenon appears to be critical for improving NSCLC therapy[7-9].

During digestion, proteins are broken down into amino acids. Amino acids are transported in the bloodstream and are used to build up new cells and repair old ones. Optimal regulation of the cellular rates of amino acid uptake and protein synthesis is critical to the overall health of our bodies. Inside each of our cells is a molecule called mammalian target of rapamycin (mTor), which acts as a controller that receives information about amino acid availability[10-13]. mTor also senses how much of each amino acid the cell needs and calibrates the cell's amino acid uptake and protein synthesis machinery accordingly. mTor is a conserved serine/threonine protein kinase of the phosphatidylinositol 3-kinase (PI3K)-related kinase family (PIKK), and the aberrant expression of mTor has been shown to be essential for the tumorigenesis of the majority of all cancers[1-4].

The membrane-associated E3 ubiquitin ligase ZNRF2 has been shown to be involved in the activation and regulation of mTor pathway[14]. Specifically, activation of Akt phosphorylates both ZNRF2 and mTor, which interact with each other on membranes to induce the amino acid-stimulated translocation of mTORC1 to lysosomes and activation. ZNRF2 also interacts with the V-ATPase and preserves lysosomal acidity[15]. Moreover, knockdown of ZNRF2 has been shown to decrease cell size and cell proliferation. These findings reveal ZNRF2 as a component of the amino acid sensing machinery that acts upstream of Rag-GTPases and the V-ATPase to activate mTORC1[15].

Here, we investigated the clinical correlation of ZNRF2 expression in NSCLC and the underlying molecular signaling pathways. We detected significant higher levels of ZNRF2 and mTor in NSCLC tissues, compared to the paired non-tumor controls. Moreover, ZNRF2 and mTor levels

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strongly correlated in NSCLC tissues. High ZNRF2 levels were correlated with poor prognosis of the NSCLC patients. *In vitro*, overexpression of ZNRF2 increased NSCLC cell growth and suppressed apoptotic cell death in the presence of fluorouracil, while depletion of ZNRF2 decreased NSCLC cell growth and increased apoptotic cell death in the presence of fluorouracil. ZNRF2 appeared to augment mTor and its downstream targets CyclinD1 and CDK in NSCLC cells. Together, our data suggest that ZNRF2 may be a promising target for treating NSCLC.

Patients and Methods

Patient Specimens

Resected fresh NSCLC specimens were collected from 30 patients (all at stage IV). NSCLC specimens were compared with the paired adjacent non-tumor bladder tissue (NT) from the same patient. All specimens were histologically and clinically diagnosed at Putuo Hospital from 2009 to 2014. NSCLC *vs.* NT was determined based on pathological and cytological evidence. For the use of these clinical materials for research purposes, prior patient's consents and approval from the Institutional Research Ethics Committee were obtained.

Cell Line Culture, Transfection and Reagents

A human lung cancer cell lines A549 (origin from carcinoma) was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA), and cultured in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 15% fetal bovine serum (FBS; Sigma-Aldrich, St Louis, MO, USA) in a humidified chamber with 5% CO_2 at 37°C. Fluorouracil (5-FU; Sigma-Aldrich) was prepared in a stock of 1mmol/l and applied to the cultured cells at 10μmol/l. ZNRF2, scrambled control (scr) and short hairpin small interfering RNA for ZNRF2 (shZNRF2, sequence: 5'- TACGTCCAAGGTCGGGCAGGAAGA-3') were cloned into pCMV-luciferase-2A-GFP vector (Clontech, Mountain View, CA, USA) to replace the GFP to generate pCMV-luciferase-2A-transgene. Sequencing was performed to confirm the correct orientation of the plasmids, which were then used to transfect the cells at a concentration of 50 nmol/l using Lipofectamine 2000, according to the manufacturer's instructions (Invitrogen). The transfection efficiency was more than 95%.

Quantitative PCR (RT-qPCR)

Total RNA was extracted from the cultured cells using RNeasy kit (Qiagen, Hilden, Germany). For cDNA synthesis, complementary DNA (cDNA) was randomly primed from 2μg of total RNA using the Omniscript reverse transcription kit (Qiagen). RT-qPCR was subsequently performed in triplicate with a 1:4 dilution of cDNA using the Quantitect SyBr Green PCR system (Qiagen). All primers were purchased from Qiagen. Data were collected and analyzed using the 2-ΔΔ Ct method. Values of genes were first normalized against α-tubulin, and then compared to the experimental controls.

Western Blot

For analysis of total protein, the protein was extracted from the tissue specimens or the cultured cells, and homogenized in RIPA lysis buffer (Sigma-Aldrich, St. Louis, MO, USA) on ice. Protein concentration was determined using a BCA protein assay kit (Bio-Rad, Beijing, China), and then lysates were mixed with 4×SDS loading buffer (Bio-Rad) at a ratio of 1:3 for routine blotting. The primary antibodies for Western Blot are anti-ZNRF2, anti-mTor, anti-cleaved caspase 3 (Casp3), anti-CyclinD1, anti-CDK4 and α-tubulin (all from Cell Signaling, San Jose, CA, USA). α-tubulin was used as a protein loading control. The secondary antibody is HRP-conjugated anti-rabbit (Jackson ImmunoResearch Labs, West Grove, PA, USA). Images shown in the figures were representative of 5 individuals. Densitometry of Western blots was quantified with NIH ImageJ software. The protein levels were first normalized to α-tubulin, and then normalized to experimental controls.

Cell Viability Assay

The CCK-8 detection kit (Sigma-Aldrich, St. Louis, MO, USA) was used to measure cell viability according to the manufacturer's instructions. Briefly, cells were seeded in a 96-well microplate at a density of $5X10^4$/ml. After 24h, cells were treated with resveratrol. Subsequently, CCK-8 solution (20 ml/well) was added, and the plate was incubated at 37°C for 2 h. The viable cells were counted by absorbance measurements with a monochromator microplate reader at a wavelength of 450 nm. The optical density value was reported as the percentage of cell viability in relation to the control group (set as 100%).

Cell Growth Assay

A diphenyltetrazolium bromide (MTT) assay was performed to determine cell growth. Five

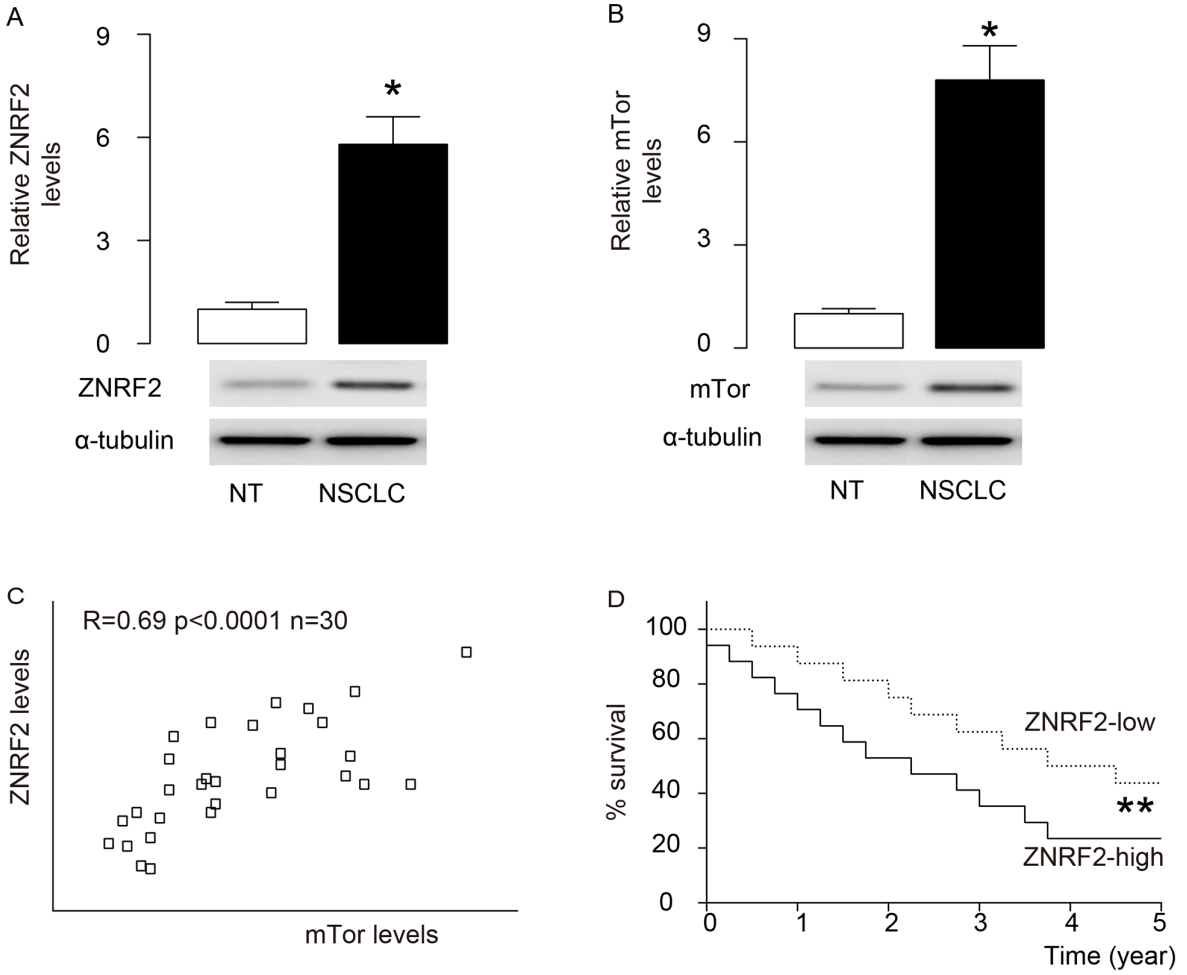

Figure 1. Increased ZNRF2 in NSCLC is associated with poor prognosis. We examined the ZNRF2 and mTor levels in NSCLC specimens, compared to paired non-tumor bladder tissue. ***(A-B)*** We detected significant increases in ZNRF2 (A) and mTor (B) levels in NSCLC specimens by Western blot. ***(C)*** Correlation between the levels of ZNRF2 and mTor in NSCLC specimens. ***(D)*** The survival of the 30 patients all diagnosed Stage IV were followed for 5 years. The median value of all 30 cases was chosen as the cutoff point for separating ZNRF2-high cases (n=15) from ZNRF2-low cases (n=15). Kaplan-Meier curves were performed. $^{*}p<0.05$. $^{**}p<0.01$. N=30.

thousand cells per well were seeded in a 96-well plate to allow the cells to grow. Then the media were removed and washed with PBS, after which 5 g/l of thiazolyl tetrazolium (Ameresco, Indianapolis, IN, USA) was added to each well. Four hours later, MTT was removed and 150µl of dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO, USA) was added. The viability of the cells was calculated from the absorption at 570/630 nm with an enzyme-linked immunosorbent assay reader.

Statistical Analysis

All statistical analyses were carried out using the SPSS 18.0 statistical software package (SPSS Inc., Chicago, IL, USA). All values in cell and animal studies are depicted as mean ± standard deviation and are considered significant if $p < 0.05$. All data were statistically analyzed using one-way ANOVA with a Bonferroni correction, followed by Fisher' Exact Test for comparison of two groups. Kaplan-Meier curves were used to analyze the patient survival by ZNRF2 levels.

Results

Increased ZNRF2 in NSCLC is Associated with Poor Prognosis

We examined the ZNRF2 and mTor levels in NSCLC specimens, compared to paired non-tu-

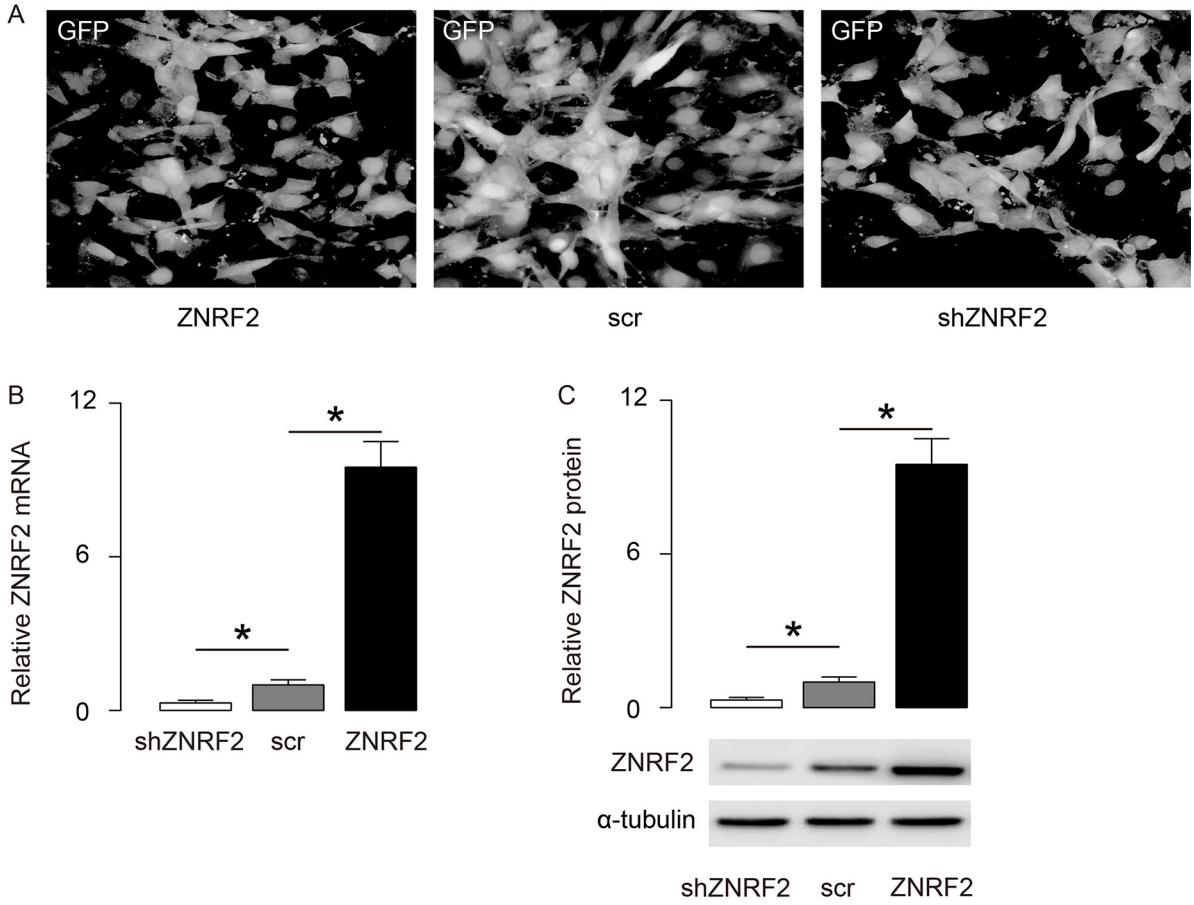

Figure 2. Modulation of ZNRF2 levels in NSCLC cells. To study the effects of ZNRF2 on NSCLC cell growth, we used a human NSCLC cell line, A549. We transfected the A549 cells with either a ZNRF2 overexpressing plasmid (ZNRF2), or a small short hairpin interfering RNA for ZNRF2 (shZNRF2). The A549 cells were transfected with a scrambled sequence as a control (scr). ***(A)*** These plasmids all carried a GFP reporter to allow determination of the transfection efficiency and purification of the transfected cells shown in culture. ***(B-C)*** We confirmed the modulation of ZNRF2 levels in these cells by RT-qPCR (B), and by Western blot (C). $^{*}p<0.05$. N=5.

mor bladder tissue. We detected significant increases in ZNRF2 levels in NSCLC specimens by Western blot (Figure 1A). We also detected significant increases in mTor levels in NSCLC specimens by Western blot (Figure 1B). Notably, the levels of ZNRF2 and mTor strongly correlated in NSCLC specimens (Figure 1C). Next, we investigated whether the levels of ZNRF2 may correlate with overall survival of NSCLC patients. The survival of the 30 patients all diagnosed Stage IV were followed for 5 years. The median value of all 30 cases was chosen as the cutoff point for separating ZNRF2-high cases (n=15) from ZNRF2-low cases (n=15). Kaplan-Meier curves were performed, showing that ZNRF2-high NSCLC patients had a significantly worse survival, compared to ZNRF2-low NSCLC patients (Figure 1D). Thus, increased ZNRF2 in NSCLC is associated with poor prognosis.

Modulation of ZNRF2 Levels in NSCLC Cells

To study the effects of ZNRF2 on NSCLC cell growth, we used a human NSCLC cell line, A549. We transfected the A549 cells with either a ZNRF2 overexpressing plasmid (ZNRF2), or a small short hairpin interfering RNA for ZNRF2 (shZNRF2). The A549 cells were transfected with a scrambled sequence as a control (scr). These plasmids all carried a GFP reporter to allow determination of the transfection efficiency and purification of the transfected cells (Figure 2A). We confirmed the modulation of ZNRF2 levels in these cells by RT-qPCR (Figure 2B), and by Western blot (Figure 2C).

ZNRF2 Enhances NSCLC Cell Growth

Then we examined cell growth in an MTT assay. We found that overexpression of ZNRF2

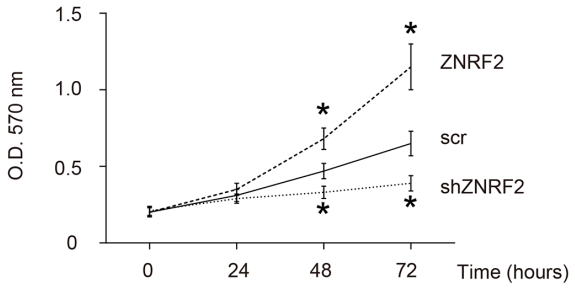

Figure 3. ZNRF2 enhances NSCLC cell growth. An MTT assay to study the growth of ZNRF2-modifed A549 cells. $^{*}p<0.05$. N=5.

in A549 cells significantly increased cell growth, while ZNRF2 depletion significantly decreased cell growth (Figure 3). These data demonstrate that ZNRF2 may increase NSCLC cell growth *in vitro*.

ZNRF2 suppresses apoptotic cell death of NSCLC cells upon 5-FU treatment

Next, we examined NSCLC cell survival in a CCK-8 assay in the presence of 5-FU. We found that overexpression of ZNRF2 in A549 cells significantly increased cell survival, while ZNRF2 depletion significantly decreased cell survival (Figure 4A), possibly through modulation of cell apoptosis (Figure 4B). These data demonstrate that ZNRF2 may suppress apoptotic cell death of NSCLC cells upon 5-FU treatment.

ZNRF2 Enhances NSCLC Cell Growth Through mTor

Finally, we examined how ZNRF2 enhances NSCLC cell growth. As above mentioned evidence from literature and our clinic data, we examined the levels of mTor and its downstream factors CyclinD1 and CDK4 in the ZNRF2-modified A549 cells. We found that overexpression of ZNRF2 in A549 cells increased mTor, CyclinD1 and CDK4 levels, while ZNRF2 depletion decreased mTor, CyclinD1 and CDK4 levels (Figure 5). Thus, ZNRF2 may enhance NSCLC cell growth through mTor.

Discussion

We observed that ZNRF2 expression may be correlated with poor prognosis in NSCLC patients. Moreover, we showed that ZNRF2 activation enhanced the NSCLC cell growth and resistance to chemotherapy *in vitro*. These observations provided strong evidence that ZNRF2 served as a critical mediator for the tumorigenesis of NSCLC, in a collaborative manner with mTor. Hence, inhibition of ZNRF2 can be potentially used as a novel strategy to improve the prognosis of NSCLC patients.

Although the expression of ZNRF2 in NSCLCs remained unknown previously, previous studies have demonstrated a molecular interaction between ZNRF2 and other factors in the PI3k/Akt/mTor pathway.

Hoxhaj et al[15] has shown that when cells are provided with amino acids and growth-stimulating hormones, mTOR is activated and attaches a phosphate group to ZNRF2, resulting in the release of ZNRF2 from membranes and separation of ZNRF2 from mTOR. In contrast, when cells are starved of amino acids, the dephosphorylation of ZNRF2 renders it to return to the membranes. On

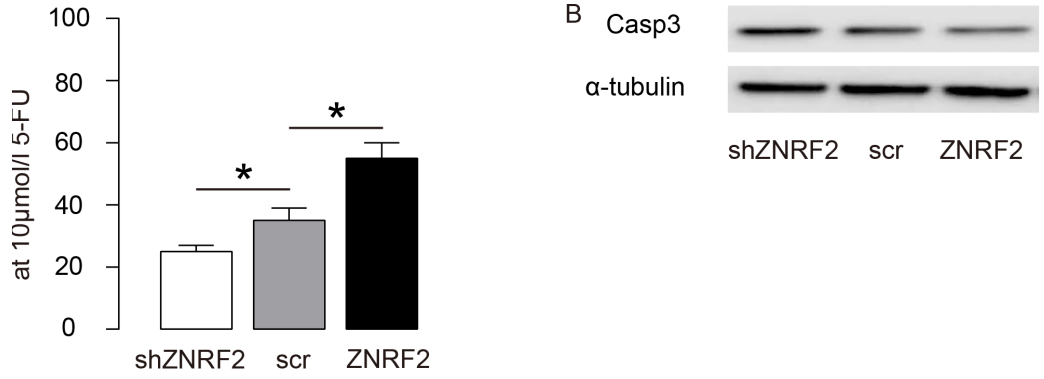

Figure 4. ZNRF2 suppresses apoptotic cell death of NSCLC cells upon 5-FU treatment. ***(A)*** ZNRF2-modified A549 cell survival in a CCK-8 assay at the presence of 5-FU. ***(B)*** Western blot for cleaved caspase 3 (Casp3). $^{*}p<0.05$. N=5.

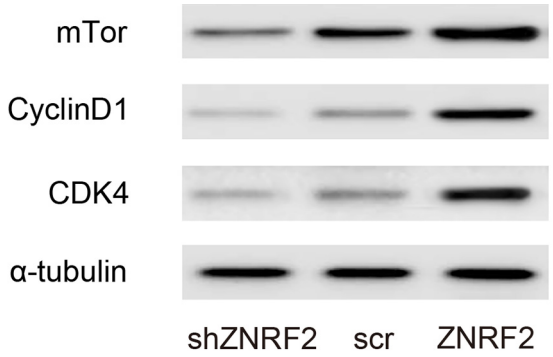

Figure 5. ZNRF2 enhances NSCLC cell growth through mTor. Western blot for mTor and its downstream factors CyclinD1 and CDK4 in the ZNRF2-modified A549 cells. N=5.

membranes, ZNRF2 also influences the activity of a pump called V-

ATPase, which controls the internal acidity of the membrane-enclosed vesicles named lysosomes for recycling amino acids. The action of ZNRF2 on the pump may help to prime mTOR so that it is ready to sense amino acids. These findings suggest that ZNRF2 and mTOR may modulate each other, making delicate adjustments to cell growth. However, new questions about ZNRF2 thus arise, e.g. how is ZNRF2 expressed in cancers and whether it is related to tumorigenesis?

Here we addressed this question. We not only detected high ZNRF2 in NSCLC, but also found an association between ZNRF2 levels and patients' prognosis. To understand the underlying mechanisms, we used several human NSCLC cell lines to examine their interactions. Since we got similar results, only data from A549 cells were shown here. The detailed molecular signaling should be explored in the future studies.

Conclusions

Although we did not analyze *in vitro* cell invasiveness regulated by ZNRF2, the adaption of the cell growth may partially result from the changes in cell invasiveness, suggesting this possibility. Further delineation of the precise molecular mechanisms underlying the regulation of tumor behavior by ZNRF2 as well as the relationship between the control of cell growth and cell invasion may substantially improve our understanding of the NSCLC cell growth and may provide novel therapeutic strategies for NSCLC treatment.

Conflicts of interest

The authors declare no conflicts of interest.

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