Original Article
NRSN2 promotes non-small cell lung cancer cell growth through PI3K/Akt/mTOR pathway

Xin-Yi Zhang[1], Jiu-Long Kuang[1], Chun-Song Yan[1], Xiao-Yun Tu[1], Ji-Hua Zhao[1], Xiao-Shu Cheng[2], Xiao-Qun Ye[1]

[1]Department of Respiratory Diseases, The Second Affiliated Hospital of Nanchang University, Nanchang 330006, China; [2]Department of Cardiology, The Second Affiliated Hospital of Nanchang University, Nanchang 330006, China

Abstract: Neurensin-2 (NRSN2), a small neural membrane protein which localized in small vesicles in neural cells. Recent report suggested that Neurensin-2 might play a suppressive role in tumor. While the biological functions and molecular mechanisms in cancer progression remain unknown. We retrieved Oncomine Database and found that NRSN2 is commonly highly expressed in non-small cell lung cancer (NSCLC). We examined the levels of NRSN2 in 18 pairs of NSCLC and adjacent tissues and found that NRSN2 was overexpressed in malignant tissues. Both loss and gain of function experiments in NSCLC cell lines suggest that NRSN2 promotes cell growth, but no effects in cell invasion. Further investigation show that NRSN2 could affect phosphatidylinositol 3 kinase/protein kinase B/mammalian target of rapamycin (PI3K/Akt/mTOR) signaling. Taken together, our findings suggest that NRSN2 promotes non-small cell lung cancer cell growth through PI3K/Akt/mTOR pathway.

Keywords: Neurensin-2, NSCLC, cell proliferation, PI3K/Akt/mTOR pathway

Introduction

Non-small cell lung cancer (NSCLC) remains the leading cause of cancer death in the world [1]. Statistical results suggest that less than 15% of NSCLC patients surviving beyond 5 years [2]. Cytotoxic chemotherapy remains the main treatment for every stage of NSCLC, although newer molecularly targeted treatments have shown activity in advanced stages, but many patients do not respond to those treatments for NSCLC [3-5]. So, newly predictive and prognostic biomarkers in NSCLC should be excavated for Effective treatment [6].

NRSN2, a newly isolated gene that encodes a small neuronal membrane protein which was characterized showing high sequence homology to NRSN1, a protein exclusively localized to small vesicles [7]. As NRSN1 may play an important role in neuronal organelle transport and in conduction of nerve signals [8], we proposed that NRSN2 might own a similar function in neuromodulation for the similar structure they possessed. Katsuyuki et al. found that NRSN2 in neurons was also mainly localized in

small vesicles. Compared to NRSN1, their localization in the vesicles is not always accordance [7]. Recently Yu et al. reported that NRSN2 is a candidate gene that associated with development delay [9]. As we know, NSCLC, especially lung adenocarcinoma is closely related to the dysregulation of neuroendocrine. We suspect that NRSN2 might relate to NSCLC.

Interestingly, in a previous publication, Zender et al. reported that NRSN2 might be a tumor suppressive gene in HCC development [10]. They combined genome-wide short hairpin RNAs (shRNAs) and a mosaic mouse model functionally identified and validated 12 tumor suppressor genes in which had not reported in cancer and NRSN2 ranked top three among those genes. We hypothesized that NRSN2 might have the same role in NSCLC.

In this study, we retrieved Oncomine Database and found that NRSN2 was highly expressed in NSCLC compared to normal lung tissues. Then we focused to explore the biological functions of NRSN2 in NSCLC and contradictorily found that NRSN2 might play an oncogenic role in
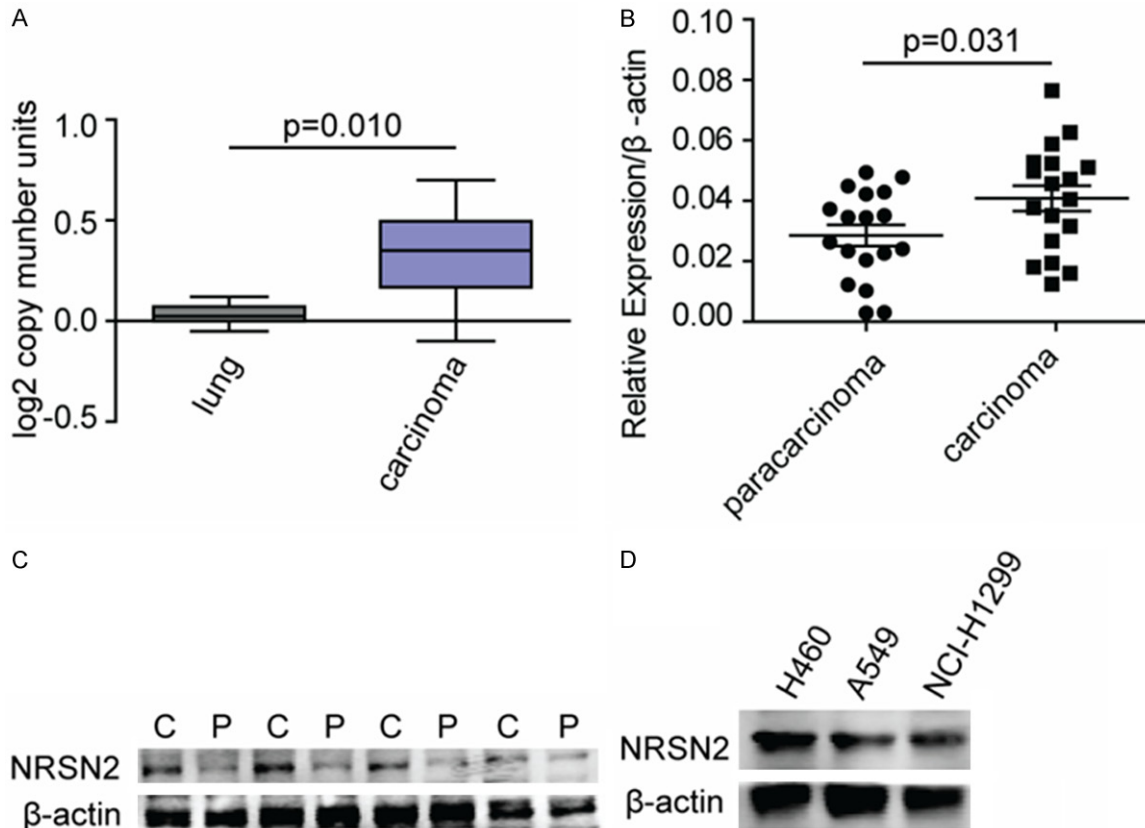
Figure 1. NRSN2 expression is increased in NSCLC. A. Illustrate the Oncomine Database analyzed the NRSN2 copy number alteration. Compared to normal lung, the copy number of NRSN2 significantly gained in NSCLC (P = 0.010). B. The mRNA level of NRSN2 is significantly elevated in NSCLC compared to paired adjacent tissues (P = 0.031). C. The protein level of NRSN2 in NSCLC is higher than paracarcinoma tissues in four representative patients. (P: Paracarcinoma, C: cancer). D. The expression of NRSN2 in three NSCLC cell lines, β-actin was used as a loading control.

NSCLC for it promoting NSCLC cell growth. Taken together, NRSN2 obtains a complex role in tumor biology for the opposite role of NRSN2 in hepatocellular carcinoma and NSCLC.

Materials and methods

Patient samples

All samples were collected in Department of Respiratory Diseases, The Second Affiliated Hospital of Nanchang University. Fresh samples, including tumor tissues and adjacent were obtained from NSCLC patients during tumor resection. All human samples were obtained with informed consent, and protocols were approved by the ethical review committee of the World Health Organization Collaborating Center for Research in Human Production.

Cell culture

The non-small cell lung cancer (NSCLC) cell lines H460, A549 and NCI-H1299 were pur-

chased from American type culture collection (ATCC). A549 and cells were grown in DMEM supplemented with 10% FBS and penicillin/ streptomycin at 37°C in a humidified incubator under 5% CO_2 condition.

Quantitative real-time PCR

Total RNA were extracted from patient tissues and cell lines using Trizol reagent (Takara, China) and reversely transcribed using PrimeScript RT-PCR kit (Takara) according to the manufacturer's instruction. Quantitative real-time PCR analyses were performed with SYBR Premix Ex Taq (Takara) on ABI 7500 (Applied Biosystems Inc., USA). Primers for this study as follows: β-actin, forward, 5'-GCACA-GAGCCTCGCCTT-3'; Reverse, 5'-CCTTGCACA-TGCCGGAG-3'. NRSN2, forward, 5'-GATGGCA-AGTGGTATGGGGTC-3'; Reverse, 5'-CGAGGAC-AGGCTGATCTTCC-3'. The relative expression of NRSN2 was analyzed by the comparative cycle
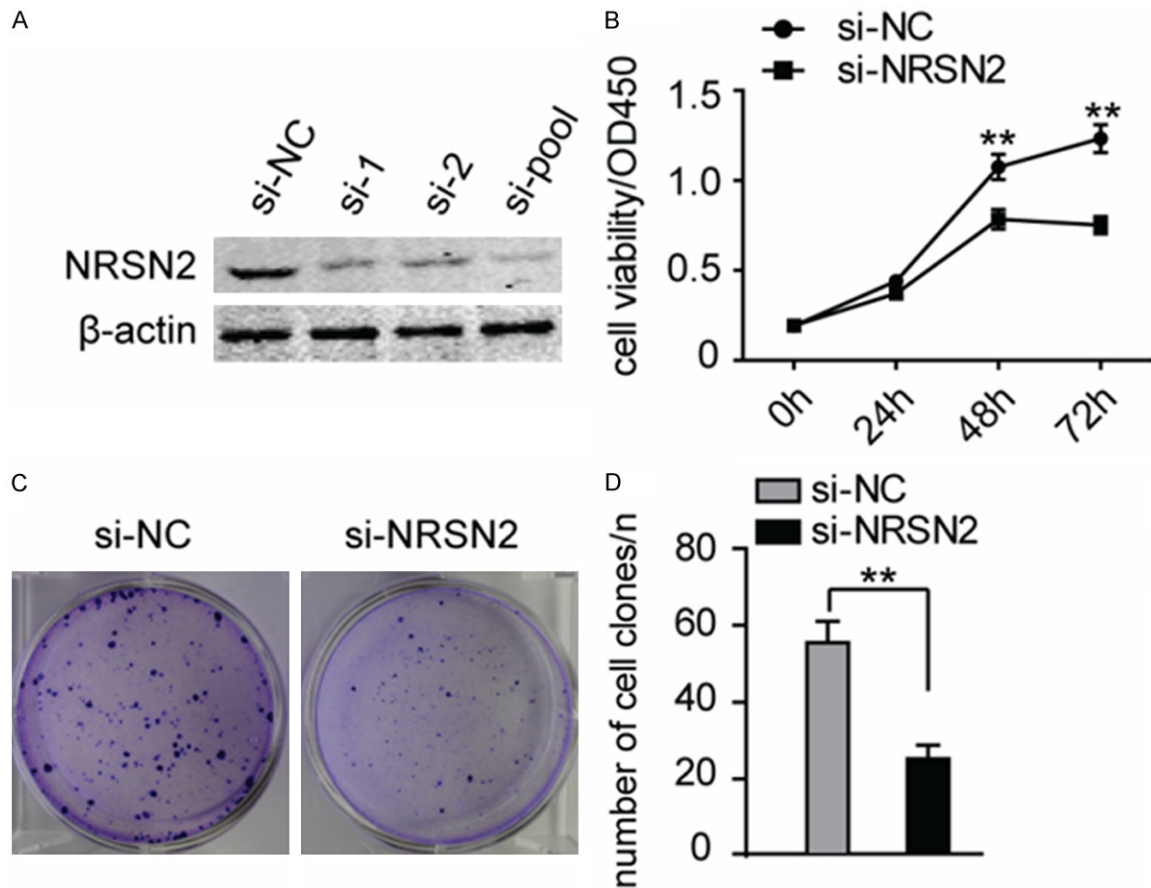
Figure 2. Silencing NRSN2 inhibits cell viability and proliferation. A. The level of NRSN2 in A549 was significantly decreased after specific siRNA and pooled siRNAs treated 48 hours. B. Pooled siRNAs were used to interfering NRSN2 in A549 cells. After transfection 48 hours, cells were plated into 96 well, and cell viability was significantly inhibited following NRSN2 knockdown in indicated days. C. Representative image disclose the ability of clone formation significantly reduced when NRSN2 knockdown. D. Statistical analysis of the clone formation assay (**, $P < 0.01$). Data presented here are representative of three to four independent experiments.

threshold method (ΔΔCT method), which was normalized to β-actin.

Western blotting

Total protein extracted from patient tissues and cell lines using RIPA lysis buffer (Beyotime, China) followed the protocol and 30-50 μg those proteins were separated by reduced SDS-PAGE, and transferred onto nitrocellulose membrane then the membrane was blocked in TBS buffer containing 5% BSA (Sangon, China) for 1 hour. The membrane was incubated with primary antibodies for NRSN2 (1:1000, protein tech, USA), AKT (1:1000, Cell Signaling Technology, USA), p-AKT (1:1000, Cell Signaling Technology, USA), p-mTOR (1:1000, Cell Signaling Technology, USA), mTOR (1:1000, Cell Signaling Technology, USA), and β-actin

(1:5000, Cell Signaling Technology, USA) overnight, and then followed by horseradish peroxidase (HRP)-linked secondary antibody (Cell signaling, USA). ImmobilonTM Western chemiluminscent HRP substrate kit (Millipore, Germany) was used for detection. The optical density quantification was analyzed by Image J software.

siRNA treatment

The NRSN2 siRNA sequences were employed in this study. The sequences for NRSN2 siRNAs as follows: si-1: 5'-CAATCTTCTGTGCAGACTA-TC-3', si-2: 5'-CTCTTCCAAGATACCAGCATT-3'. To elevate the interfering efficiency, we pooled these 3 different siRNAs to transfect cells. The A549 cells were transfected with 100 nM siRNAs or with 100 nM RNAi negative control using Lipofectamine 2000 (Invitrogen, USA).
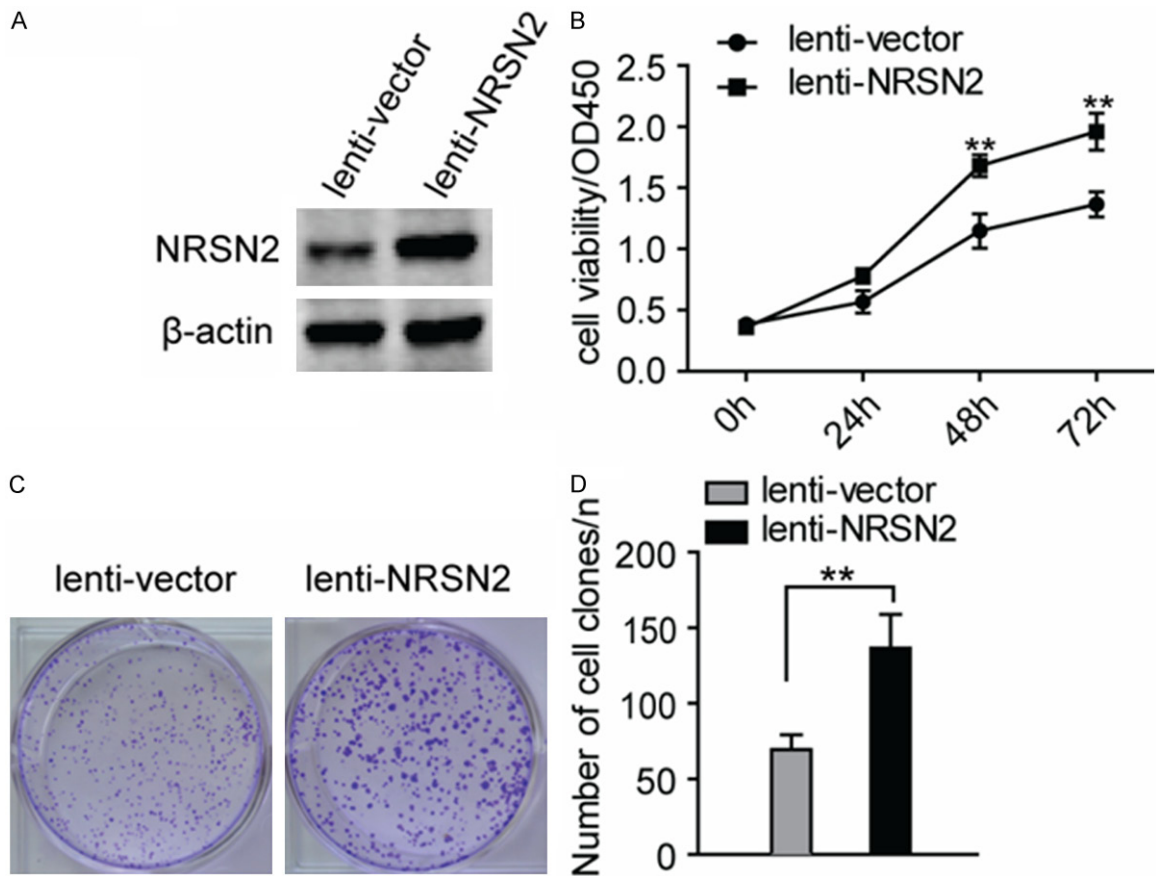
Figure 3. Over-expression of NRSN2 promotes cell viability and proliferation. A. The level of NRSN2 in A549 was significantly increased after over-expression. B. A549 cells which stable over-express NRSN2 were subject to perform CCK-8 cell viability assays and cell viability remarkably elevated after NRSN2 heightened. C. Representative image reveal the ability of clone formation significantly increased when NRSN2 over-expressed. D. Statistical analysis of the clone formation assay (**, $P < 0.01$). Data presented here are representative of three to four independent experiments.

Over-expression of NRSN2

The open reading frame of NRSN2 was amplified by PCR using the forward primer: 5'-CTAGCTAGCATGATGCCGAGCTGCAATC-3' and the reverse primer: 5'-CGCGGATCCTCAGG-AGTCCCTCTTGGG-3'. The amplified fragment was cloned in Nhe I and BamH I sites of pCDH-CMV-MCS-EF1-Puro cDNA Cloning and Expression Vector to obtain the NRSN2 over-expression vector and its identity was confirmed by DNA sequencing. 40×104 A549 cells seeded in 6-well plate were transfe(cted with 2 μg DNA using Lipofectamine 2000 Reagent (Invitrogen, USA). After 48 h incubation, stably transfected cells were selected by administration of 2 μg/ml puromycin for 21 days. The puromycin -resistant colonies were isolated and they were expanded and then maintained in regular growth medium containing 2 μg/ml puromycin.

Cell viability assay

Cells were seeded into a 96-well plate at 4×103 cells per well with 100 μl complete medium and cultured at 37°C. The cell viability was quantified by addition 10 μl of cell counting kit (CCK8, Dojindo, Japan). After 2 hours incubation, the plates were monitored by Power Wave XS micro-plate reader (BIO-TEK, USA) at an absorbance 450 nm.

Clone formation assay

Cells were trypsin digested and then seeded into a 6-well plate at 500 cells per well with 1.5 ml complete medium cultured at 37°C incubator for three weeks. After incubation, cells were
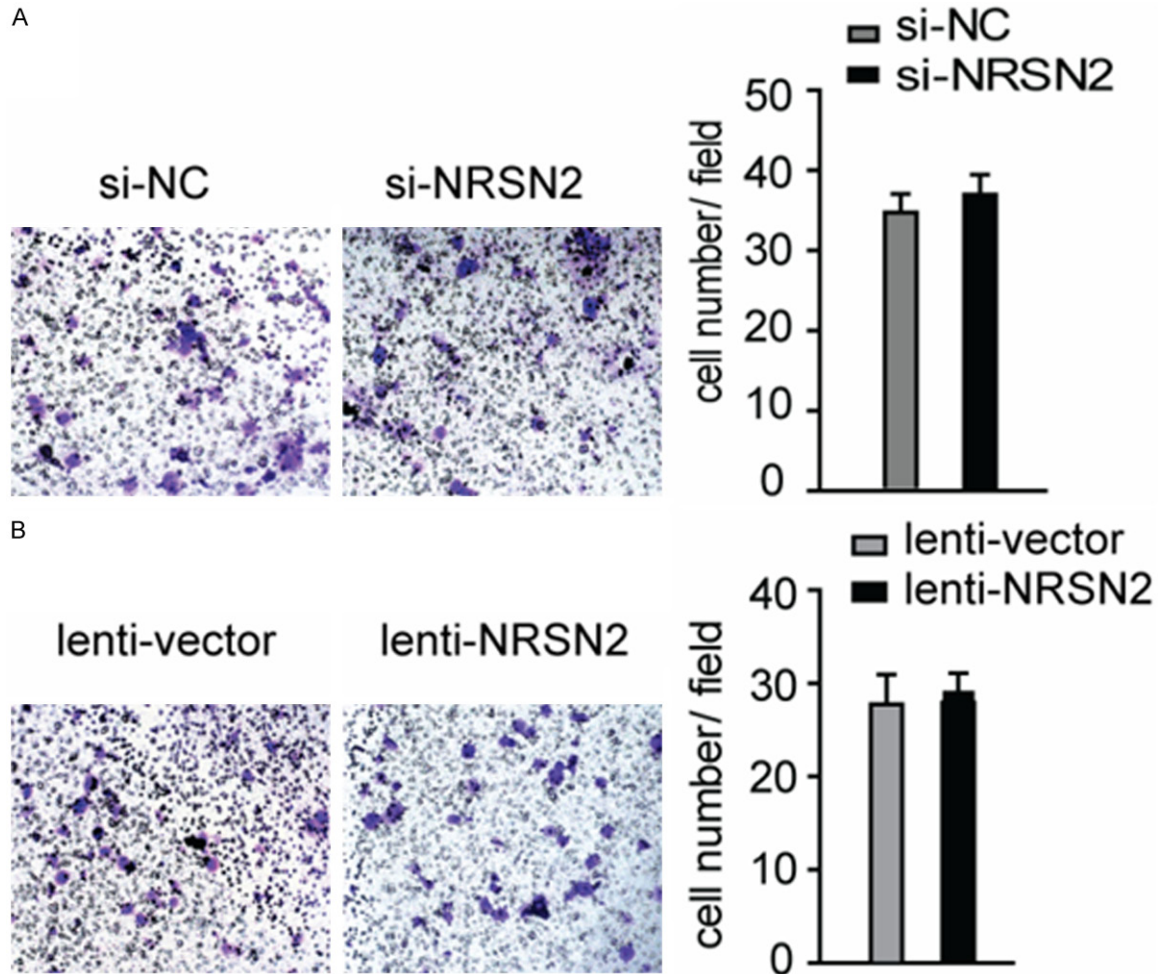
A

B

Figure 4. NRSN2 does not affect the ability of cell invasion. A. Representative images of A549 transwell invasion assays after silencing NRSN2, statistical analysis show no difference between control and treated group (right panel). B. Representative images of A549 transwell invasion assays after over-expressing NRSN2, statistical analysis show no difference between control and treated group (right panel). Data presented here are representative of three to four independent experiments.

fixed by 2.5% Glutaraldehyde for 15 min and then stained by 0.1% Crystal Violet for 30 min. washed and statistic results.

Transwell cell migration assay

To assess whether NRSN2 involved in the metastatic potential of A540 cells in vitro, we performed transwell cell migration assay using transwell chamber (BD Biosciences, USA). In brief, cells were seeded onto the upper chamber at a density of 4×104 cells per 200 μl per chamber and maintained in serum-free medium, and lower chambers were filled with 700 μl complete medium. Cells were incubated for 14 h at 37°C in a 5% CO_2 incubator. Non-invaded cells retaining on the upper surface were

removed by scrubbing with a cotton swab. The invaded cells were fixed and then stained with 0.1% crystal violet.

Statistical analysis

Data were presented here as the means ± SD. the student's t-test was used for comparison between groups. Values of *P* < 0.05 were considered statistically significant.

Results

Elevated NRSN2 expression in human non-small cell lung carcinoma

We retrieved the Oncomine Database and found that NRSN2 was more commonly elevat-
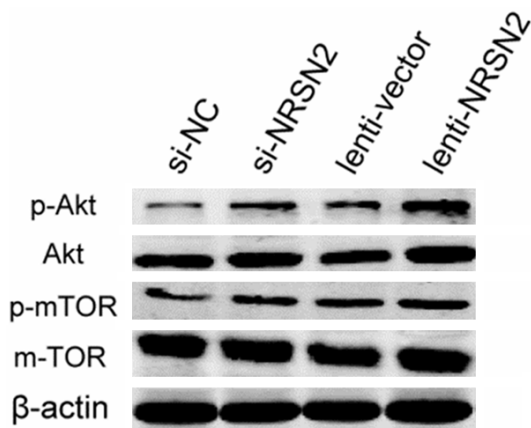
Figure 5. NRSN2 influence PI3K/Akt/mTOR pathway. Whole cell protein extracted from A549 which treated with control and pooled siRNAs 72 hours and stable cell lines. Proteins were examined with indicated antibodies. Data presented here are representative of three independent experiments.

ed expressed in non-small cell lung carcinoma (NSCLC). As shown in **Figure 1A**, the copy number of NRSN2 was significantly gained in NSCLC compared to normal lung tissues. We further detected the expression of NRSN2 in fresh NSCLC and paired adjacent tissues. The results shown in **Figure 1B** and **1C** indicated that the expression of NRSN2 does heightened in NSCLC.

Silencing NRSN2 inhibits cell viability and proliferation

We detected the level of NRSN2 in three NSCLC cell lines (**Figure 1D**), and found that NRSN2 was relative moderately expressed in A549 cell lines compared to H460 and NCI-H1299. We then selected A549 to investigate the biological functions of NRSN2 in NSCLC. Specific siRNAs were employed to silence NRSN2. We found pooled siRNAs exhibit more effective to knock-down NRSN2 compared with single siRNA (**Figure 2A**). Then pooled siRNAs were used to conduct following cellular assays. The CCK-8 cell viability assays disclosed that NRSN2 plays a positive role in cell viability. Silencing NRSN2 the cell viability significantly decreased compared to control (**Figure 2B**). The results from cell clone formation assays suggested that knockdown NRSN2 could also inhibit the ability of cell proliferation (**Figure 2C**, and **2D**).

Over-expressing NRSN2 promotes cell viability and proliferation

To confirm the cellular functions of NRSN2 identified by NRSN2 silencing, we constructed the stable A549 cell lines which heightened express NRSN2. We found that cell viability and ability to proliferate enhanced by elevated the levels of NRSN2, as **Figure 3** indicates. Taken together, we confirmed that NRSN2 plays a role in cell viability and proliferation.

NRSN2 does not affect cell invasion

Considering NRSN2 might involve in multi cellular processes in NSCLC progression, we also performed transwell cell invasion assays in both NRSN2 knockdown and over-expressed A549 cell lines. While the results, as illustrate in **Figure 4**, suggested that NRSN2 does not affect the ability of cell invasion.

NRSN2 affect cell viability and proliferation through PI3K/AKT/mTOR pathway

As PI3K/Akt/mTOR pathway plays a key role in NSCLC cell growth [11], we examined the effect of NRSN2 silencing and over-expressing on the expression of phosphorylated Akt and phosphorylated mammalian target of rapamycin (mTOR) in A549 cells. As shown in **Figure 5**, silencing NRSN2 reduced phosphorylation levels of Akt and mTOR, while increased when NRSN2 over-expression. In summary, these clues indicate that NRSN2 in some way to influence PI3K/Akt/mTOR pathway, thereby affecting cell viability and proliferation.

Discussion

This is the first report focus on the biological functions and associated mechanisms of NRSN2 in NSCLC. NRSN2, previously reported as a tumor suppressive gene in a Genome-wide shRNAs screen experiment during HCC progression [10]. To our knowledge, NRSN2 is a poorly studied gene, either in nerve system or cancer. And the cellular functions of NRSN2 haven't been reported in tumor to date. Here, we enlarge the understanding of the biological functions of NRSN2 in NSCLC.

Ma et al. investigate the clinical significance of NRSN2 in HCC tissues and found that patients with higher NRSN2 expression more commonly

indicate a relatively better prognosis while lower with unfavorable [12]. In this study, we retrieved Oncomine Database and examined the expression of NRSN2 in fresh NSCLC tissues and found that NRSN2 is highly expressed in cancer compared to normal tissues. The contradictory finding between HCC and NSCLC reminds us to investigate what roles NRSN2 plays in NSCLC.

In this study, we examined the level of NRSN2 in NSCLC tissues and the results confirmed to the database, NRSN2 highly expressed in malignant tissues. In the following studies, we silenced and over-expressed NRSN2 in NSCLC cell line A549 which relative moderately expressed Neurensin-2, and then employed these cells to conduct the cellular function assays *in vitro.* The findings in A549 suggest that NRSN2 promotes cancer cell growth but has no effect in cell invasion. Those results indicated that NRSN2 might play a role in the initiation stage of NSCLC development.

Here, we report NRSN2 play an oncogenic role in cancer progression, the underlying mechanisms are really deserved to investigate. We further found that PI3K/Akt/mTOR pathway which play a centre role in regulating cell proliferation and growth [13, 14], and angiogenesis [15], was activated and inactivated after NRSN2 over-expressing or silencing, respectively. These findings suggest that NRSN2 promotes cell growth by activated PI3K/Akt/mTOR pathway.

NRSN2 which encodes a small neuronal membrane protein and it is localized in small vesicles in neural cells [7], and its expression is limited in brain tissues. However, the role of NRSN2 in the nervous system is currently unknown, not to mentioned in tumor. Factors which involved in nerves have been found could exert some effects on cancer [16]. For example, MAOA, a catecholamine neurotransmitter degrading enzyme, which reported could suppress HCC metastasis by inhibiting the adrenergic system and its trans-activation of EGFR signaling [17]. The role of NRSN2 which might regulate nerve signaling like NRSN1, in current study, has been found could promote cancer cell growth not invasion, suggests that in might involve in the tumor initiation. Further study focus on the mechanisms remains further study in the future.

However, the clinical significance of NRSN2 still remains examined. In conclusion, NRSN2, a novel transport vesicle protein expressed in neural cells was exhibits oncogenic functions in NSCLC and as it is located in the cell membrane, it might be a novel target for NSCLC treatment.

Acknowledgements

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Xiao-Qun Ye, Department of Respiratory Diseases, The Second Affiliated Hospital of Nanchang University, Nanchang 330006, China. E-mail: yxq-li@tom.com; Xiao-Shu Cheng, Department of Cardiology, The Second Affiliated Hospital of Nanchang University, Nanchang 330006, China. E-mail: xiaoshumenfan@126.com

References

[1] Jemal A, Bray F, Center MM, Ferlay J, Ward E and Forman D. Global cancer statistics. CA Cancer J Clin 2011; 61: 69-90.

[2] Ettinger DS, Akerley W, Bepler G, Blum MG, Chang A, Cheney RT, Chirieac LR, D'Amico TA, Demmy TL and Ganti AKP. Non–small cell lung cancer. J Natl Compr canc netw 2010; 8: 740-801.

[3] Cisplatin-Based Adjuvant Chemotherapy in Patients with Completely Resected Non–Small-Cell Lung Cancer. New Engl J Med 2004; 350: 351-360.

[4] Winton T, Livingston R, Johnson D, Rigas J, Johnston M, Butts C, Cormier Y, Goss G, Inculet R, Vallieres E, Fry W, Bethune D, Ayoub J, Ding K, Seymour L, Graham B, Tsao MS, Gandara D, Kesler K, Demmy T and Shepherd F. Vinorelbine plus Cisplatin vs. Observation in Resected Non–Small-Cell Lung Cancer. New Engl J Med 2005; 352: 2589-2597.

[5] Douillard YJ, Rosell R, De Lena M, Carpagnano F, Ramlau R, Gonzáles-Larriba JL, Grodzki T, Pereira JR, Le Groumellec A, Lorusso V, Clary C, Torres AJ, Dahabreh J, Souquet PJ, Astudillo J, Fournel P, Artal-Cortes A, Jassem J, Koubkova L, His P, Riggi M and Hurteloup P. Adjuvant vinorelbine plus cisplatin versus observation in patients with completely resected stage IB–IIIA non-small-cell lung cancer (Adjuvant

Navelbine International Trialist Association [ANITA]): a randomised controlled trial. The Lancet Oncol 2006; 7: 719-727.

[6] Coate LE, John T, Tsao MS and Shepherd FA. Molecular predictive and prognostic markers in non-small-cell lung cancer. Lancet Oncol 2009; 10: 1001-1010.

[7] Nakanishi K, Ida M, Suzuki H, Kitano C, Yamamoto A, Mori N, Araki M and Taketani S. Molecular characterization of a transport vesicle protein Neurensin-2, a homologue of Neurensin-1, expressed in neural cells. Brain Res 2006; 1081: 1-8.

[8] Araki M, Nagata K, Satoh Y, Kadota Y, Hisha H, Adachi Y and Taketani S. Developmentally regulated expression of Neuro-p24 and its possible function in neurite extension. Neurosci Res 2002; 44: 379-389.

[9] An Y, Amr SS, Torres A, Weissman L, Raffalli P, Cox G, Sheng X, Lip V, Bi W, Patel A, Stankiewicz P, Wu BL and Shen Y. SOX12 and NRSN2 are candidate genes for 20p13 subtelomeric deletions associated with developmental delay. Am J Med Genet B Neuropsychiatr Genet 2013; 162b: 832-840.

[10] Zender L, Xue W, Zuber J, Semighini CP, Krasnitz A, Ma B, Zender P, Kubicka S, Luk JM, Schirmacher P, McCombie WR, Wigler M, Hicks J, Hannon GJ, Powers S and Lowe SW. An oncogenomics-based in vivo RNAi screen identifies tumor suppressors in liver cancer. Cell 2008; 135: 852-864.

[11] Fumarola C, Bonelli MA, Petronini PG and Alfieri RR. Targeting PI3K/AKT/mTOR pathway in non small cell lung cancer. Biochem Pharmacol 2014; 90: 197-207.

[12] Ma HQ, Liang XT, Zhao JJ, Wang H, Sun JC, Chen YB, Pan K and Xia JC. Decreased expression of Neurensin-2 correlates with poor prognosis in hepatocellular carcinoma. World J Gastroenterol 2009; 15: 4844-4848.

[13] Xing X, Zhang L, Wen X, Wang X, Cheng X, Du H, Hu Y, Li L, Dong B, Li Z and Ji J. PP242 suppresses cell proliferation, metastasis, and angiogenesis of gastric cancer through inhibition of the PI3K/AKT/mTOR pathway. Anticancer Drugs 2014; 25: 1129-1140.

[14] Arcaro A. Targeting PI3K/mTOR Signaling in Cancer. Molecular Cell Oncol 2014; 4: 84.

[15] Karar J and Maity A. PI3K/AKT/mTOR pathway in angiogenesis. Front Mol Neurosci 2011; 4: 51.

[16] Romon R, Adriaenssens E, Lagadec C, Germain E, Hondermarck H and Le Bourhis X. Research Nerve growth factor promotes breast cancer angiogenesis by activating multiple pathways. Mol Cancer 2010; 9: 11.

[17] Li J, Yang XM, Wang YH, Feng MX, Liu XJ, Zhang YL, Huang S, Wu Z, Xue F, Qin WX, Gu JR, Xia Q, Zhang ZG. Monoamine oxidase a suppresses hepatocellular carcinoma metastasis by inhibiting the adrenergic system and its transactivation of egfr signaling. J Hepatol 2014; 60: 1225-34.