Oncology Research, Vol. 27, pp. 987–995
Printed in the USA.

0965-0407/19 $90.00 + .00
DOI: https://doi.org/10.3727/096504018X15424918479652
E-ISSN 1555-3906
www.cognizantcommunication.com

Nutlin-3-Induced Sensitization of Non-Small Cell Lung Cancer Stem Cells to Axitinib-Induced Apoptosis Through Repression of Akt1/Wnt Signaling

Meng Wang,*[1] Xin Wang,†[1] Yuan Li,‡[1] Qiang Xiao,§ Xiao-Hai Cui,* Guo-Dong Xiao,* Ji-Chang Wang,¶ Chong-Wen Xu,# Hong Ren,* and Dapeng Liu*

*Department of Thoracic Surgery and Oncology, the Second Department of Thoracic Surgery, Cancer Center, First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, Shaanxi Province, P.R. China
†Department of Gastroenterology, the First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, Shaanxi Province, P.R. China
‡School of Humanities and Social Sciences, Xi'an Jiaotong University, Xi'an, Shaanxi Province, P.R. China
§Department of Medical Oncology, Cancer Center, First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, Shaanxi Province, P.R. China
¶Department of Vascular and Endovascular Surgery, First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, Shaanxi Province, P.R. China
#Department of Otorhinolaryngology, First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, Shaanxi Province, P.R. China

The aim of this study was to investigate the potential biological activities of nutlin-3 in the regulation of growth and proliferation of non-small cell lung cancer (NSCLC) stem cells (CSCs), which may help in sensitizing to axitinib-induced apoptosis. Nutlin-3 induction of p53 expression was used to test its role in controlling the cell division pattern and apoptosis of NSCLC cells. A549 cells and H460 cells were pretreated with nutlin-3 and then treated with either an Akt1 activator or shRNA-GSK3β, to investigate the potential role of p53 sensitization in the biological effects of axitinib. We also determined the expression levels of GSK3β and p-Akt1 in patients with NSCLC and determined their potential association with survival data using Kaplan–Meier plots and CBIOTAL. Increased p53 expression stimulated the induction of apoptosis by axitinib and promoted asymmetric cell division (ACD) of NSCLC CSCs. The repression of Akt phosphorylation induced by nutlin-3 promoted the ACD of lung CSCs, decreasing the proportion of the stem cell population. In addition to the induction of apoptosis by axitinib through inhibition of Wnt signaling, nutlin-3 treatment further enhanced axitinib-induced apoptosis by inhibiting Akt1/GSK3β/Wnt signaling. The low expression of GSK3β and increased expression of p-Akt in patients with NSCLC were closely associated with the development of NSCLC. TP53 stimulates the induction of apoptosis in NSCLC by axitinib and the ACD of lung CSCs through its regulatory effects on the p53/Akt/GSK3β pathways.

Key words: Non-small cell lung cancer (NSCLC); Nutlin-3; Axitinib; Cancer stem cells (CSCs); Wnt signaling; Apoptosis; Akt; Asymmetric cell division (ACD)

INTRODUCTION

It is well established that TP53 (p53) has a wide range of biological activities, including regulation of tumor formation and development, suppression of tumor recurrence, and inhibition of the self-renewal process of cancer stem cells (CSCs)[1–3]. Akt is an important regulatory protein of cell growth, proliferation, and survival[4–5]. Nutlins are *cis*-imidazoline analogs that inhibit the interaction between Mdm2 and the tumor suppressor p53, and they were initially discovered by screening a chemical library. There are three family members, and nutlin-3 is the agent most commonly used in anticancer studies. The nutlins exert their biological activity by inhibiting the interaction between Mdm2 and p53, which leads to stabilization of p53. This then results in a growth-inhibiting state of senescence in cancer cells. These compounds are therefore

[1]Co-first authors.

Address correspondence to Dapeng Liu, M.D., Associate Professor, Director, Department of Thoracic Surgery and Oncology, The First Affiliated Hospital of Medical College, 277 Yanta West Road, Xi'an Jiaotong University, Xi'an, Shaanxi Province 710061, P.R. China. Tel: (86) 13609181418; Fax: 86 (029)-85323473; E-mail: prof_ldp_oncology@126.com *or* Ren Hong, M.D., Professor, Chief Physician, Department of Thoracic Surgery and Oncology, the Second Department of Thoracic Surgery, Cancer Center, The First Affiliated Hospital of Medical College, 277 Yanta West Road, Xi'an Jiaotong University, Xi'an, Shaanxi Province 710061, P.R. China. Tel: 86 (029)-85324616; Fax: 86 (029)-85323473; E-mail: renhongs2000@126.com

thought to be most effective in tumors that express normal or "wild-type" p53.

Recent studies have shown that Akt, the most important molecule of the PI3K pathway, plays an important role in stem cell renewal and in regulating mitochondrial function[6]. Phosphorylation at Ser473 and Thr308 is necessary for Akt activation[7], which can promote or inhibit the expression of its downstream target proteins through phosphorylation, causing a cascade of reactions in the signaling pathway. For example, Akt was shown to regulate the self-renewal of stem cells by promoting the expression of KLF3 and Kbx3[8]. GSK3β is a multifunctional kinase that regulates the growth of cancer cells and modulates apoptotic signaling pathways. Most importantly, its activation can inhibit the Wnt signaling pathway and therefore control the renewal of stem cells. Moreover, studies have shown that there is crosstalk between the PI3K/Akt and Wnt/β-catenin signaling pathways, which implies that they can potentially synergistically regulate the self-renewal of stem cells[9]. Activated forms of Akt can inhibit GSK3β and reduce the interaction between p53 and GSK3β, suggesting a potential role of Akt/Wnt signaling in the development of cellular drug resistance. Previous studies have shown that nutlin-3 is able to promote the expression of Notch signaling in TP53 wild-type cells[10], but the relationship between nutlin-3 and Wnt signaling was not elucidated. Many p53-related genes such as PTEN and HIF-1α were demonstrated to be able to regulate both pathways[11,12]. Based on this, we propose that nutlin-3 may be a potential regulator of Wnt signaling.

The process of asymmetric cell division (ACD) is based on the premise that human stem cells divide to create one daughter cell of the same type to maintain their own numbers[13–16], while the other daughter cell enters the active mitotic cycle[14,17–19]. In contrast, the stem cells undergoing symmetric cell division (SCD) divide into two identical daughter cells, which results in excessive expansion of stem cells. This means that symmetric cell division, as opposed to ACD, can lead to uncontrolled tumor cell growth. Under normal circumstances, tumor cells undergoing rapid proliferation are sensitive to radiation and chemotherapy treatment. CSCs largely retain features of normal human stem cells[20], and after the rapidly proliferating tumor cells are killed, the CSCs in stationary phase are able to reenter the cell cycle and undergo cell division, leading to the formation of new tumors[21–23]. Lung CSCs appear to play a critical role in mediating drug resistance and cancer recurrence. It is clear that targeting this specific subpopulation of lung CSCs may provide a novel approach to more effectively treat lung cancer[24].

The activation of p53 in adjuvant chemotherapy has emerged as a promising strategy and was tentatively applied in clinical trials[25]. Moreover, therapies targeting p53 and Akt1 are both significant for improving cancer patients' prognosis[4,26,27]. Axitinib has been confirmed to be an inhibitor of Wnt signaling[28]. However, it is not currently used to treat lung cancer, and its effects on the self-renewal capacity of lung CSCs are unknown[15,28]. We hypothesized that nutlin-3 might be able to sensitize the tumor cells to anticancer treatment by repressing Akt/Wnt signaling activity, and the combined activities of axitinib and nutlin-3 were subsequently explored in this study.

MATERIALS AND METHODS

Patients and Materials

A total of 28 patients who underwent radical lung cancer surgery at the First Affiliated Hospital of Xi'an Jiaotong University and 10 healthy individuals were evaluated and enrolled from July 2014 to November 2016. Histopathologic evaluation was confirmed by two separate pathologists, and the patients were not diagnosed with any other cancers. Written informed consent was obtained from each individual prior to study enrollment in accordance with the Declaration of Helsinki. The study protocol was approved and supervised by the ethics committee of the First Affiliated Hospital of Xi'an Jiaotong University.

A549 and H460 NSCLC cell lines were purchased from ATCC (Manassas, VA, USA), and their identity was confirmed by short tandem repeats polymerase chain reaction (STR-PCR) genotyping. Cells were cultured in medium comprising DMEM 1X (Cellgro; Corning, NY, USA), 10% fetal calf serum, and 1% penicillin/streptomycin solution (Invitrogen, Carlsbad, CA, USA). PKH26 and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Nutlin-3 was purchased from Invitrogen. Fumonisin B1 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Axitinib was purchased from AbMole (Houston, TX, USA). The accutase enzyme was obtained from Gibco (Invitrogen). The culture medium used for CSCs was composed of DMEM/Ham's F-12, 10 ng/ml of epidermal growth factor (EGF), 10 ng/ml of human basic fibroblast growth factor (HbFGF), 1 μg/ml of hydrocortisone, 4 μg/ml of insulin, and 1% penicillin/streptomycin solution. All cells were incubated in a humidified atmosphere comprising 5% CO_2 at 37°C. Antibodies against p53 (sc-6243) and vinculin (sc-7649), and the horseradish peroxidase-conjugated secondary antibody of mouse origin were purchased from Santa Cruz Biotechnology.

Detection of Cell Proliferation Inhibition and Apoptosis

Cells were seeded in 96-well plates with 200 μl of DMEM medium per well and treated with different

concentrations of nutlin-3. Cell viability was measured using the MTT assay by adding 20 µl of a 5-mg/ml MTT solution per well and incubating at 37°C in the dark. After 4 h, the supernatants were discarded, 150 µl of DMSO was added per well, and the absorbance at 490 nm was measured using a plate reader. Each experiment was repeated three times. Apoptosis levels were determined using the Annexin V–FITC kit (Invitrogen). Nutlin-3 (10 µM) and axitinib (10 nM) were used to treat the cells. After discarding the supernatant, the cells were collected and resuspended, subjected to centrifugation at 1,200 rpm for 5 min, then mixed with 5 µl of Annexin V–FITC, and 10 µl of PI (20 µg/ml) before uploading into the flow cytometry instrument.

Detection of Cell Division Patterns

Serum-free suspension medium was used to suspend and filter cells, and we used the mixture of Diluent C and PKH26 ethanolic dye solution to incubate and stain, terminating with 2 ml of serum. On the fourth day, 1 ml of fresh stem cell culture medium was added. The cell spheres were counted, and the sphere formation efficiency (MFE) was calculated on the seventh day. For EdU dye staining, cells of different groups were incubated with 10 µM EdU for 30 min at room temperature, and then seeded on new plates containing fumonisin B1 (FB1) or axitinib. After 48 h, cells were seeded onto coverslips and were visualized using the EdU Alexa Fluor 488 Imaging Kit (C10637; Life Technologies; New York, NY, USA) 1 day later[28,29]. The division of stem cells was observed, counted, and summed into asymmetric division (AD), symmetric division (SD), and undefined. Subsequently, the stem cell spheres were digested to yield single cells with pancreatin and accutase.

Protein Extraction and Western Blotting

For determination of protein concentrations, 1 ml of cell lysis buffer and phosphotransferase inhibitor were added to the reaction system and quantified using the Bradford method. Protein bands in the gel were transferred to a cellulose acetate membrane using the semi-dry transfer method. Monoclonal antibodies against vinculin (1:5,000; Cell Signaling Technology, Danvers, MA, USA), p53 (1:2,000), p-Akt (Thr308; 1:2,000; Cell Signaling Technology), and p-GSK3β (serine 9; phosphorylated Akt activation site; 1:1,500; Cell Signaling Technology) were individually incubated with the membrane at 4°C overnight. On the following day, the membrane was rinsed three times with PBST, incubated for 1 h at room temperature with the secondary antibody (1:5,000 HRP labeling), rinsed three times with TBST, and the bands were then detected in a darkroom.

Immunohistochemistry

The high-pressure method was chosen for antigen retrieval, and a 3% hydrogen peroxide solution was used to block the endogenous peroxidase, and nonspecific binding was blocked with goat serum. The slides were refrigerated overnight at 4°C, and on the following day Reagents B and C were added. After rinsing, DAB dyeing was used, and the slides stained with hematoxylin showed purple.

Statistical Analysis

The results were shown as means ± SD. Comparisons between two groups were conducted using Student's *t*-test. Data of different groups (≥) were analyzed using ANOVA. Multiple comparisons between groups were performed using the S-N-K method. The inspection level was $\alpha = 0.05$; if $p < \alpha$, we considered that the difference had statistical significance (i.e., $p < 0.05$).

RESULTS

Nutlin-3 Promoted the Induction of Apoptosis by Axitinib in Lung Cancer Cells

We chose A549 and H460 cells as our experimental systems because these two cell lines express wild-type TP53 according to the cancer cell line database (http://TP53.free.fr/Database/Cancer_cell_lines/NSCLC.html). We found that a concentration of 10 µM nutlin-3 effectively inhibited the proliferation of A549 and H460 cells (Fig. 1A and B) in a dose- and time-dependent manner. Moreover, axitinib promoted apoptosis of lung cancer cells effectively, and nutlin-3 enhanced the apoptotic effects of axinitib (Fig. 1C and D). FACS analysis confirmed these findings (Fig. 1E).

Nutlin-3 and Axitinib Both Promoted the ACD

Both the PKH26-stained spheres and EdU-marked dividing stem cells revealed that 10 µM nutlin-3 effectively increased the ACD proportion of CSCs. In the PKH dye-stained group, nutlin-3 effectively increased the ratio of ACD in CSCs (Fig. 2A and B) and decreased the ratio of SCD (Fig. 2C), decreasing the amounts of CSCs. Axitinib also increased the ratio of ACD in CSCs to some extent (Fig. 2A and B). In the EdU-stained group, similar results were observed as in the PKH group, and nutlin-3 effectively increased the ratio of ACD in CSCs (Fig. 2A and B).

Nutlin-3-Induced Sensitization to Axitinib Reduced the Sphere-Forming Efficiency by Inhibiting Akt Activity

Axitinib decreased the levels of TCF-4, the key activator of Wnt signaling (Fig. 3A). Nutlin-3 increased the expression of p53 in lung cancer cells, which consequently

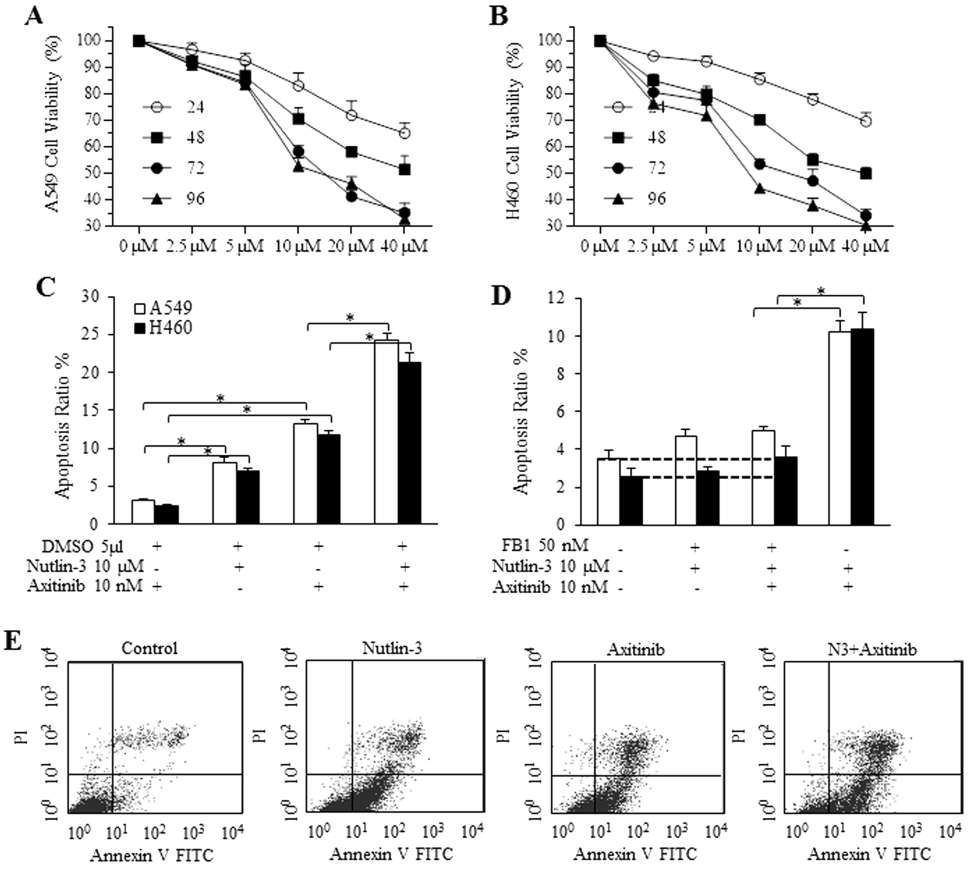

Figure 1. Nutlin-3 stimulated the apoptosis-promoting effect of axitinib in non-small cell lung cancer (NSCLC) cells. Inhibition of proliferation under different concentrations of nutlin-3 at different times in A549 cells (A) and H460 cells (B). (C) Axitinib, 10 nM, promoted lung cancer cell apoptosis, and 10 μM nutlin-3 exerted a synergistic effect. (D) Fumonisin B1 (FB1) treatment offset the nutlin-3-induced sensitization to axitinib promoted apoptosis. (E) Flow cytometry measurements of apoptosis. The data are shown as means ± SD from three independent experiments. $*p < 0.01$.

inhibited the phosphorylation of Akt-1 and expression of GSK3β (Fig. 3A). Nutlin-3 enhanced the ability of axitinib to inhibit the renewal of CSCs ($p < 0.05$) (Fig. 3B and C). FB1 stimulated the activity of Akt1, which greatly increased the number of spheres compared to the control group (Fig. 3D). Akt inhibition by Akti-1/2 did not enhance the suppressive effect of combined nutlin-3 and axitinib treatment on sphere formation (Fig. 3D). Further studies revealed that Akti-1/2-mediated Akt inhibition decreased the number of spheres, and no significant difference was detected between the nutling-3 + axitinib and Akti-1/2 groups. These findings suggested that the nutlin-3-induced sensitization to axitinib was dependent on Akt inhibition.

Nutlin-3 Increased the Ratio of Axitinib-Induced Apoptosis

Axitinib promoted the apoptosis of lung CSCs by inhibiting Wnt signaling and demonstrated an inhibitory effect on stem cell self-renewal. Nutlin-3 enhanced the tumor suppressor effect of axitinib as evidenced by reduced sphere formation (Fig. 3E), which further increased the apoptosis induced by the addition of axitinib (Fig. 1D).

Axitinib Exerted Much Stronger Inhibition of the Wnt-Supported Self-Renewal Ability of CSCs in Synergy With Nutlin-3

TP53 and Akt1 form complex interacting loops, and the genes involved in the downstream pathway of p53 control the division pattern of CSCs, which influences the molecular targeted therapy strategy for the ablation of CSCs. Several previous studies have shown that Ser-9 of GSK3β can be phosphorylated by activated Akt[30–32]. In this study, shRNA-GSK3β and FB1 were used to control the activity of Akt1 and GSK3β. FB1 treatment abolished the apoptosis-promoting effect of nutlin-3 and at the same time offset the nutlin-3-mediated sensitization of lung cancer cells to axitinib-induced apoptosis (Fig. 1D).

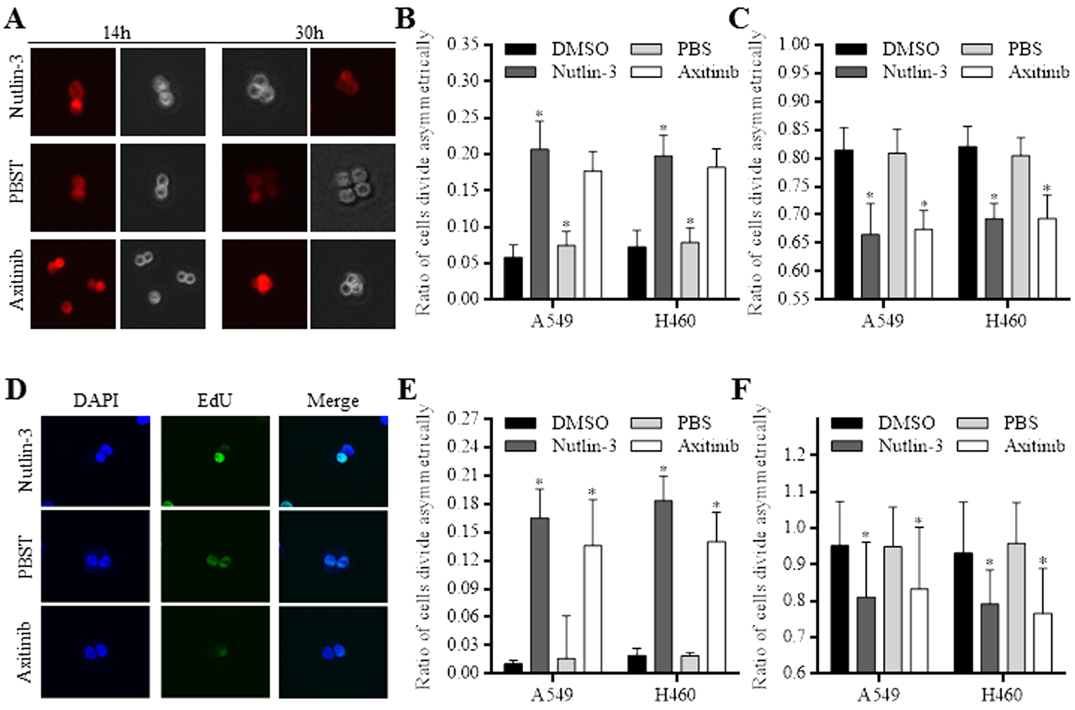

Figure 2. Nutlin-3 and axitinib promote ACD of NSCLC cancer stem cells (CSCs). (A) Nutlin-3 and axitinib induced stem cells to divide asymmetrically as revealed by PKH staining. Treatment with nutlin-3 and axitinib increased the asymmetric cell division (ACD) of stem cells (B) and decreased the ratio of symmetric cell division (SCD) (C) compared to the control group. *$p<0.01$ versus dimethyl sulfoxide (DMSO; vehicle control). (D) Division pattern defined by EdU DNA staining. Treatment with nutlin-3 and axitinib increased the ACD of stem cells (E) and decreased the ratio of SCD (F), similar to the results of PKH dyeing. *$p<0.01$ versus DMSO (vehicle control).

FB1 therefore abated the inhibition of CSC self-renewal by nutlin-3, and nutlin-3 lost the sensitizing effect on axitinib-induced apoptosis. Moreover, FB1 reduced the inhibition of CSCs by nutlin-3 and markedly reduced the inhibition of CSC self-renewal by nutlin-3 and axitinib (Fig. 3D). Inhibition of GSK3β by shRNA led to similar results as nutlin-3 treatment, reducing the sensitization for axitinib (Fig. 3E). Upregulated p53 inhibited CSC self-renewal by decreasing TCF-4 activity and increasing GSK3β expression, which consequently blocked Wnt signaling (Fig. 3F).

Low Expression of GSK3β and Increased p-Akt Levels in Clinical Tissue Samples Associated With Advanced Lung Cancer

We measured the protein levels of GSK3β and p-Akt in clinical tissue samples from 28 patients with NSCLC and in tissues from 10 healthy controls. The results of immunohistochemical staining showed that GSK3β expression was lower in lung cancer tissues than in normal control tissues (Fig. 4A) and was even lower in lung cancers from more advanced disease. In contrast, expression of p-Akt1 was higher in NSCLC than that in control tissues (Fig. 4B), and higher levels of p-Akt1 were correlated with later clinical stages. Data from Kaplan–Meier plots (http://kmplot.com/analysis) showed that high levels of GSK-3b correlated with better outcomes and longer overall survival (OS) (Fig. 4C), while lower levels of Akt1 were correlated with longer OS (Fig. 4D). Heat map analysis (http://www.cbioportal.org/) further suggested a possible mechanism of nutlin-3 action in NSCLC and cancer stem cells dominated by p53/Akt/Wnt signaling (Fig. 4E).

DISCUSSION

Genetic therapy targeting p53 has high safety and efficiency in tumor treatment. Therapeutic vectors incorporating the p53 gene and adenovirus genes can specifically kill tumor cells through various mechanisms[10,33]. The drug can be used directly for thoracic and peritoneal perfusion to enhance its lethality and promote the apoptosis of cancer cells. This is one example of using p53 as a target in clinical treatment. Axitinib is a small molecule inhibitor that is commonly used in clinical practice, and it can induce significant amounts of tumor apoptosis. However, the development of resistance often results in treatment failure or tumor recurrence[34], and strategies to improve the efficacy of axitinib in clinical cancer treatment will be

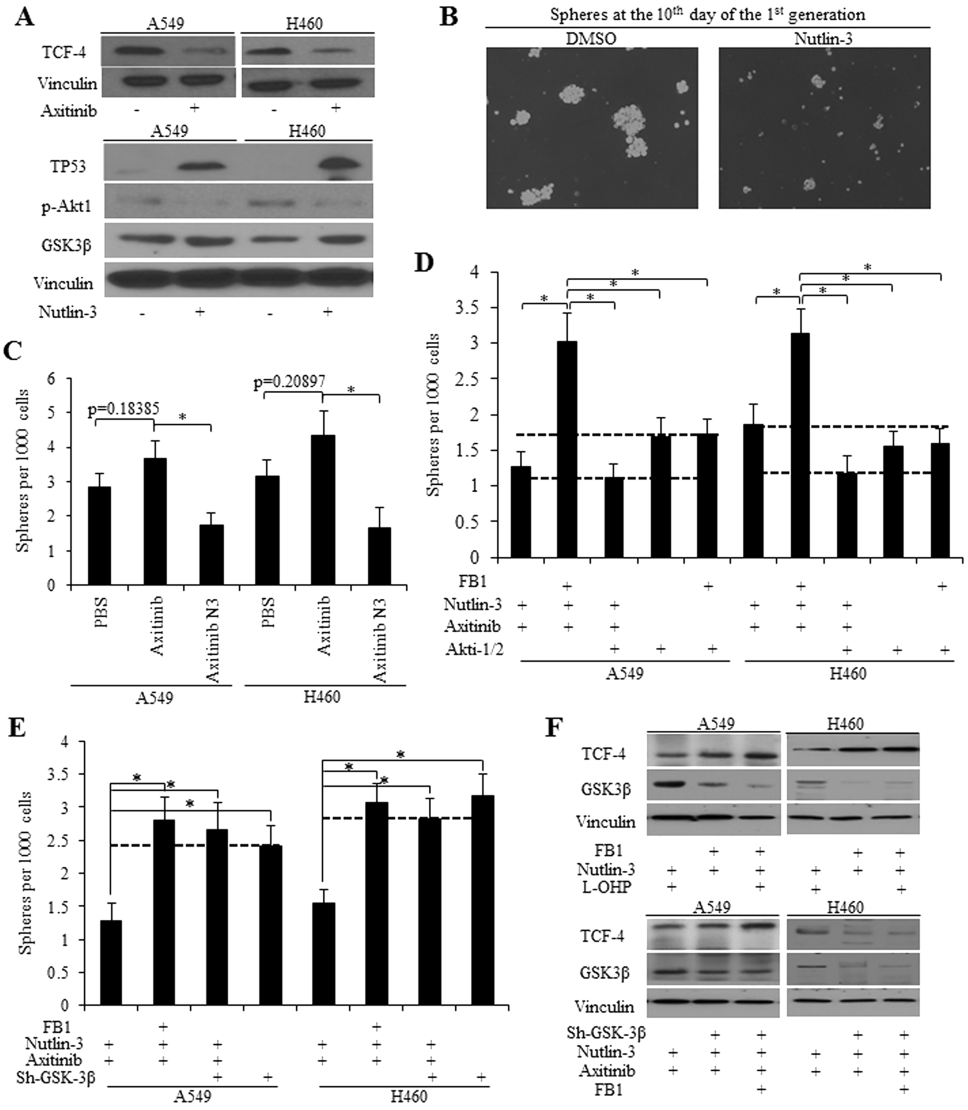

Figure 3. Nutlin-3 increased expression of GSK3β by inhibiting Akt1 phosphorylation and sensitized the CSCs to the axitinib-induced reduction of renewal ability in an Akt repression-dependent manner. (A) Axitinib reduced TCF-4 expression levels. p-Akt1 and GSK3β play a critical role in nutlin-3-induced sensitization to axitinib. (B, C) Nutlin-3 inhibited CSC sphere-forming ability in conjunction with axitinib. (D) Nutlin-3 sensitized CSCs to axitinib-induced reduction of cell number, but the inhibition of Akt1 activated by FB1 and GSK3β-shRNA offset the increase in axitinib's effect by nutlin-3. The data represent the means ± SD from three independent experiments. *$p < 0.01$. (E, F) Nutlin-3 inhibited the capacity of CSCs to renew themselves by inhibiting Akt phosphorylation and later rescued GSK3β. FB1 addition and GSK3β inhibition both abolished the sensitizing effect of nutlin-3 on axitinib-treated cells. The data represent the mean ± SD from three independent experiments. *$p < 0.01$.

important for developing more effective chemotherapy strategies and prolonging patient survival in the future[35,36]. CSCs are a special type of generalized stem cells, displaying a phenomenon called ACD. It is believed that one daughter cell of a CSC maintains its original features of a stem cell, while the other will proliferate by continuous rapid division to produce more cancer cells. Among all CSCs, most undergo symmetric division and produce two offspring stem cells that have such a strong proliferation ability that the tumor has an increased capacity for self-renewal. This, in turn, promotes the growth, maintenance, and recurrence of the tumor. Because of ACD, the tumor has different growth trends that can be changed by modulating cell polarity in the process of division, offering great hope for the treatment and control of the tumor at the source[37].

This study investigated the potential interaction between p53 and Akt1 in axitinib-induced apoptosis of NSCLC. We found that p53 has a promoting effect on lung cancer cell apoptosis induced by axitinib. Furthermore, nutlin-3

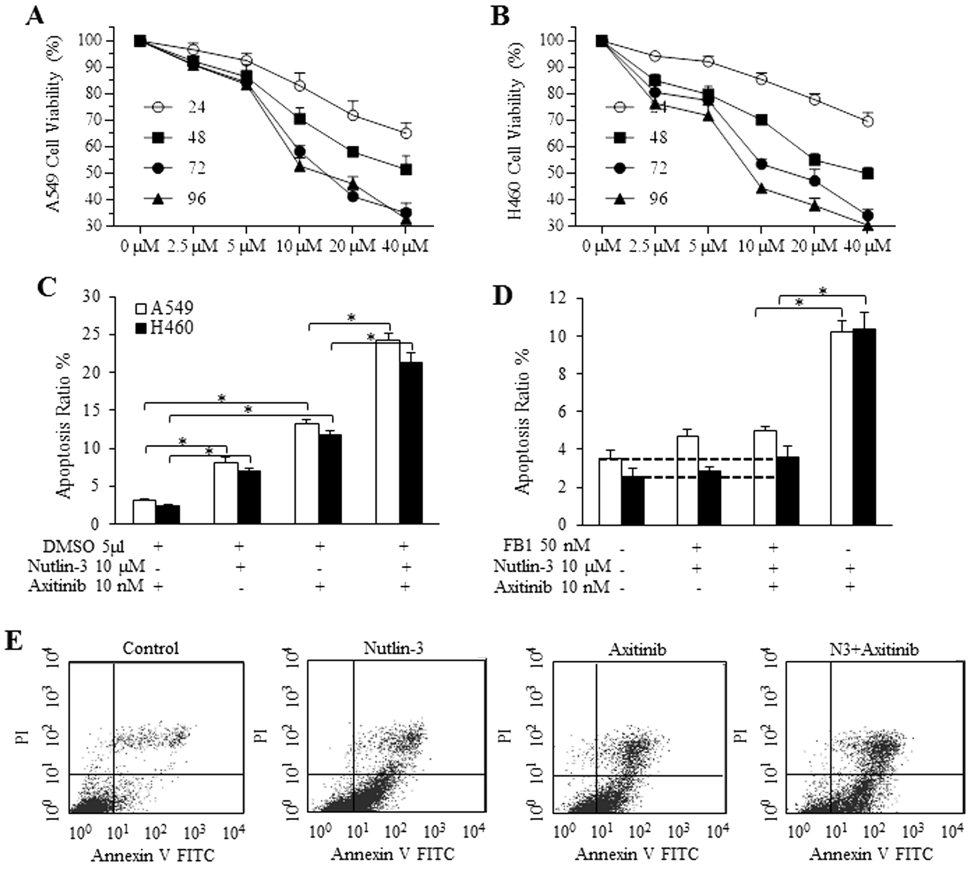

Figure 4. Immunohistochemistry and analysis of clinical tissue samples of NSCLC. (A) Low expression of GSK3β in NSCLC. (B) Akt was activated and overexpressed more often in NSCLC tissues. (C, D) The overall survival (OS) analysis using Kaplan–Meier plots. (E) Heat map revealing that Wnt signaling factors were often increased, while Akt1 was overexpressed or activated.

inhibited the symmetric division and the proportion of CSCs by regulating the activity of Akt1/GSK3β, which enhanced the effect of axitinib antitumor treatment. At the same time, these experiments provide further insights into the relationship between p53 and GSK3β in apoptosis of lung cancer cells. Therefore, p53 affects GSK3β by modulating Akt1 phosphorylation, and Akt1/GSK3β together play a decisive role in the regulation of the division mode of CSCs. Wnt signaling is a major controller of CSC division. Qu et al. have found that axitinib can direct ACD by blocking Wnt/β-catenin signaling, and this interaction was independent of the GSK3β/APC complex[28]. In our studies, we confirmed that nutlin-3, a small molecule regulator of p53, could sensitize the cancer cells to axitinib chemotherapy through repression of the Akt1/Wnt-mediated stem cell renewal. Interestingly, GSK3β may be involved in this regulation, which indicates that nutlin-3 might be a suitable adjuvant for axitinib therapy. Thus, the combination of nutlin-3 and axitinib may offer a potential novel strategy for cancer treatment. This novel approach has the potential to improve the effects of radiation and chemotherapy by regulating the division mode of NSCLC stem cells and the biological characteristics of NSCLC.

ACKNOWLEDGMENTS: This study was supported by the National Science Foundation for Young Scientists of China (Grant No. 81602597 to Xin Sun) and in part by the National Natural Science Foundation of China (Grant No. 81272418 to Hong Ren). The authors declare no conflicts of interest.

REFERENCES

1. Zhou B, Chen H, Wei D, Kuang Y, Zhao X, Li G, Xie J, Chen P. A novel miR-219-SMC4 JAK2/Stat3 regulatory pathway in human hepatocellular carcinoma. J Exp Clin Cancer Res. 2014;30:33:55.
2. Wang YH, Dong YY, Wang WM, Xie XY, Wang ZM, Chen RX, Chen J, Gao DM, Cui JF, Ren ZG. Vascular endothelial cells facilitated HCC invasion and metastasis through the Akt and NF-κB pathways induced by paracrine cytokines. J Exp Clin Cancer Res. 2013;32(1):51.
3. Iglesias-Bartolome R, Patel V, Cotrim A, Leelahavanichkul K, Molinolo AA, Mitchell JB, Gutkind JS. mTOR inhibition prevents epithelial stem cell senescence and protects from radiation-induced mucositis. Cell Stem Cell 2012; 11(3):401–14.
4. Parsons CM, Muilenburg D, Bowles TL, Virudachalam S, Bold RJ. The role of Akt activation in the response

to chemotherapy in pancreatic cancer. Anticancer Res. 2010;30(9):3279–89.
5. Phung TL, Du W, Xue Q, Ayyaswamy S, Gerald D, Antonello Z, Nhek S, Perruzzi CA, Acevedo I, Ramanna-Valmiki R, Rodriguez-Waitkus P, Enayati L, Hochman ML, Lev D, Geeganage S, Benjamin LE. Akt1 and akt3 exert opposing roles in the regulation of vascular tumor growth. Cancer Res. 2015;75(1):40–50.
6. Pestell RG. New roles of cyclin D1. Am J Pathol. 2013; 183(1):3–9.
7. Risso G, Blaustein M, Pozzi B, Mammi P, Srebrow A. Akt/PKB: One kinase, many modifications. Biochem J. 2015; 468(2):203–14.
8. Malak PN, Dannenmann B, Hirth A, Rothfuss OC, Schulze-Osthoff K. Novel AKT phosphorylation sites identified in the pluripotency factors OCT4, SOX2 and KLF4. Cell Cycle 2015;14(23):3748–54.
9. Padala RR, Karnawat R, Viswanathan SB, Thakkar AV, Das AB. Cancerous perturbations within the ERK, PI3K/Akt, and Wnt/β-catenin signaling network constitutively activate inter-pathway positive feedback loops. Mol Biosyst. 2017;13(5):830–40.
10. Secchiero P, Melloni E, di Iasio MG, Tiribelli M, Rimondi E, Corallini F, Gattei V, Zauli G. Nutlin-3 up-regulates the expression of Notch1 in both myeloid and lymphoid leukemic cells, as part of a negative feedback antiapoptotic mechanism. Blood 2009;113(18):4300–8.
11. Kaveri D, Kastner P, Dembélé D, Nerlov C, Chan S, Kirstetter P. β-Catenin activation synergizes with Pten loss and Myc overexpression in Notch-independent T-ALL. Blood 2013;122(5):694–704.
12. Cunningham LA, Candelario K, Li L. Roles for HIF-1α in neural stem cell function and the regenerative response to stroke. Behav Brain Res. 2012;227(2):410–7.
13. Sun X, Liu J, Xu C, Tang SC, Ren H. The insights of Let-7 miRNAs in oncogenesis and stem cell potency. J Cell Mol Med. 2016;20(9):1779–88.
14. Pang Y, Liu J, Li X, Zhang Y, Zhang B, Zhang J, Du N, Xu C, Liang R, Ren H, Tang SC, Sun X. Nano Let-7b sensitization of eliminating esophageal cancer stem-like cells is dependent on blockade of Wnt activation of symmetric division. Int J Oncol. 2017;51(4):1077–88.
15. Xu C, Xiao G, Zhang B, Wang M, Wang J, Liu D, Zhang J, Ren H, Sun X. CCAT1 stimulation of the symmetric division of NSCLC stem cells through activation of the Wnt signalling cascade. Gene Ther. 2018;25(1):4–12.
16. Sun X, Liu J, Xu C, Tang SC, Ren H. The insights of Let-7 miRNAs in oncogenesis and stem cell potency. J Cell Mol Med. 2016;20(9):1779–88.
17. Gómez-López S, Lerner RG, Petritsch C. Asymmetric cell division of stem and progenitor cells during homeostasis and cancer. Cell Mol Life Sci. 2014;71(4):575–97.
18. Sun X, Xu C, Tang SC, Wang J, Wang H, Wang P, Du N, Qin S, Li G, Xu S, Tao Z, Liu D, Ren H. Let-7c blocks estrogen-activated Wnt signaling in induction of self-renewal of breast cancer stem cells. Cancer Gene Ther. 2016;23(4):83–9.
19. Sun X, Tang SC, Xu C, Wang C, Qin S, Du N, Liu J, Zhang Y, Li X, Luo G, Zhou J, Xu F, Ren H. DICER1 regulated let-7 expression levels in p53-induced cancer repression requires cyclin D1. J Cell Mol Med. 2015;19(6): 1357–65.
20. Sun X, Fan C, Hu LJ, Du N, Xu CW, Ren H. Role of let-7 in maintaining characteristics of breast cancer stem cells. Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi. 2012;28(8): 789–92.
21. Sun X, Jiao X, Pestell TG, Fan C, Qin S, Mirabelli E, Ren H, Pestell RG. MicroRNAs and cancer stem cells: The sword and the shield. Oncogene 2014;33(42):4967–77.
22. Wang J, Zhang B, Meng J, Xiao G, Li X, Li G, Qin S, Du N, Zhang J, Zhang J, Xu C, Tang SC, Liang R, Ren H, Sun X. Analysis of risk factors for post-operative complications and prognostic predictors of disease recurrence following definitive treatment of patients with esophageal cancer from two medical centers in Northwest China. Exp Ther Med. 2017;14(3):2584–94.
23. Sakurai M, Miki Y, Masuda M, Hata S, Shibahara Y, Hirakawa H, Suzuki T, Sasano H. LIN28: A regulator of tumor-suppressing activity of let-7 microRNA in human breast cancer. J Steroid Biochem Mol Biol. 2012;131(3–5): 101–6.
24. Alamgeer M, Peacock CD, Matsui W, Ganju V, Watkins DN. Cancer stem cells in lung cancer: Evidence and controversies. Respirology 2013;18(5):757–64.
25. Barbieri E, Mehta P, Chen Z, Zhang L, Slack A, Berg S, Shohet JM. MDM2 inhibition sensitizes neuroblastoma to chemotherapy-induced apoptotic cell death. Mol Cancer Ther. 2006;5(9):2358–65.
26. Shimura T, Kakuda S, Ochiai Y, Kuwahara Y, Takai Y, Fukumoto M. Targeting the AKT/GSK3β/cyclin D1/Cdk4 survival signaling pathway for eradication of tumor radioresistance acquired by fractionated radiotherapy. Int J Radiat Oncol Biol Phys. 2011;80(2):540–8.
27. Dey-Guha I, Wolfer A, Yeh AC, Albeck JG, Darp R, Leon E, Wulfkuhle J, Petricoin EF 3rd, Wittner BS, Ramaswamy S. Asymmetric cancer cell division regulated by AKT. Proc Natl Acad Sci USA 2011;108(31):12845–50.
28. Qu Y, Gharbi N, Yuan X, Olsen JR, Blicher P, Dalhus B, Brokstad KA, Lin B, Øyan AM, Zhang W, Kalland KH, Ke X. Axitinib blocks Wnt/β-catenin signaling and directs asymmetric cell division in cancer. Proc Natl Acad Sci USA 2016;113(33):9339–44.
29. Hwang WL, Jiang JK, Yang SH, Huang TS, Lan HY, Teng HW, Yang CY, Tsai YP, Lin CH, Wang HW, Yang MH. MicroRNA-146a directs the symmetric division of Snail-dominant colorectal cancer stem cells. Nat Cell Biol. 2014;16(3):268–80.
30. Zhuang L, Wang X, Wang Z, Ma X, Han B, Zou H, Wu Z, Dong S, Qu Z, Zang Y, Wu L. MicroRNA-23b functions as an oncogene and activates AKT/GSK3β/β-catenin signaling by targeting ST7L in hepatocellular carcinoma. Cell Death Dis. 2017;8(5):e2804.
31. Jin Z, Cheng X, Feng H, Kuang J, Yang W, Peng C, Shen B, Qiu W. Apatinib inhibits angiogenesis via suppressing Akt/GSK3β/ANG signaling pathway in anaplastic thyroid cancer. Cell Physiol Biochem. 2017;44(4):1471–84.
32. Lau WM, Teng E, Huang KK, Tan JW, Das K, Zang Z, Chia T, Teh M, Kono K, Yong WP, Shabbir A, Tay A, Phua NS, Tan P, Chan SL, So JBY. Acquired resistance to FGFR inhibitor in diffuse-type gastric cancer through an AKT-independent PKC-mediated phosphorylation of GSK3β. Mol Cancer Ther. 2018;17(1):232–42.
33. Kumar MS, Armenteros-Monterroso E, East P, Chakravorty P, Matthews N, Winslow MM, Downward J. HMGA2 functions as a competing endogenous RNA to promote lung cancer progression. Nature 2014;505(7482):212–7.
34. Wang F, Mi YJ, Chen XG, Wu XP, Liu Z, Chen SP, Liang YJ, Cheng C, To KK, Fu LW. Axitinib targeted cancer

stemlike cells to enhance efficacy of chemotherapeutic drugs via inhibiting the drug transport function of ABCG2. Mol Med. 2012;18:887–98.
35. Sun X, Qin S, Fan C, Xu C, Du N, Ren H. Let-7: A regulator of the ERα signaling pathway in human breast tumors and breast cancer stem cells. Oncol Rep. 2013;29(5):2079–87.
36. Jilkine A, Gutenkunst RN. Effect of dedifferentiation on time to mutation acquisition in stem cell-driven cancers. PLoS Comput Biol. 2014;10(3):e1003481.
37. Ziauddin MF, Hua D, Tang SC. Emerging strategies to overcome resistance to endocrine therapy for breast cancer. Cancer Metastasis Rev. 2014;33(2–3):791–807.