Altered Wnt5a expression affects radiosensitivity of non-small cell lung cancer via the Wnt/β-catenin pathway

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Abstract. It has been reported that upregulation of wingless-type protein 5a (Wnt5a) is associated with poor prognosis in patients with non-small cell lung cancer (NSCLC). Wnt5a expression is often upregulated in radiation-resistant NSCLC cells. However, the biological functions or molecular mechanisms of radiosensitivity in NSCLC remain unknown. In the present study, MTT assay and flow cytometric analysis were performed to assess the effect of overexpression or knockdown of Wnt5a and/or radiation on the proliferation and apoptosis of NSCLC cells. Furthermore, western blot analysis was performed to detect canonical Wnt signaling (β-catenin) in H1650 and A549 cells. The results demonstrated that Wnt5a knockdown combined with irradiation inhibited proliferation and induced apoptosis in NSCLC cells compared with Wnt5a knockdown or radiotherapy alone. In addition, the combination of Wnt5a knockdown and irradiation decreased nuclear and increased cytoplasmic β-catenin expression in H1650 and A549 cells, the effects of which were reversed following overexpression of Wnt5a. The combination of overexpressing Wnt5a and irradiation resulted in significant tumor regression, while β-catenin knockdown reversed Wnt5a overexpression-induced NSCLC cell proliferation. Taken together, these results suggest that Wnt5a may be involved in the activation of β-catenin-dependent canonical Wnt signaling, and thus may influence the effectiveness of radiation therapy in NSCLC.

Introduction

Lung cancer is the leading cause of cancer-associated mortality worldwide for 36 cancers (18.0% of the total cancer deaths) in 185 countries in year 2020 (1), and non-small cell lung cancer (NSCLC) accounts for up to 80% of total pulmonary malignancies (2). Radiotherapy is the most common treatment method used for localized lung cancer; it is non-invasive and well-tolerated (3,4). In patients with NSCLC, radiotherapy plays a key role in local treatment by inducing DNA damage, triggering cell cycle arrest and apoptosis of tumor cells (5,6). However, radioresistance remains an obstacle in achieving successful treatment. Thus, novel therapeutic strategies are required to improve the effectiveness of radiotherapy for patients with NSCLC.

A previous study reported that the wingless-type (Wnt) pathway is associated with radioresistance in NSCLC (7,8). It has been reported that Wnt5a expression is upregulated in different types of cancer, including gastric, pancreatic and prostate cancer (9-11). A previous study demonstrated that silencing Wnt5a expression decreases migration, invasiveness and epithelial-to-mesenchymal transition (EMT) of NSCLC cells; these effects are reversed following overexpression of Wnt5a (12). Furthermore, preclinical and clinical studies have reported that the combination of gene therapy and conventional anticancer therapy can improve the therapeutic benefits (13-16). Although Wnt5a expression is upregulated in radioresistant NSCLC cells (17), whether Wnt5a promotes radioresistance in NSCLC cells remains unclear.

The present study aimed to investigate the efficacy of overexpression or knockdown of Wnt5a combined with radiotherapy in NSCLC cells. In addition, it has been reported that Wnt5a overexpression promotes the EMT and metastasis of pancreatic cancer cells through the β-catenin-dependent canonical signaling (9). Thus, the study also investigated whether the Wnt/β-catenin pathway was relevant in mediating radioresistance in NSCLC cells.

Materials and methods

Cell culture. The human NSCLC cell lines, H1650 (cat. no. CRL-5883) and A549 (cat. no. CCL-185) were purchased from the American Type Culture Collection. Cells
were maintained in RPMI-1640 medium (Thermo Fisher Scientific, Inc.) supplemented with 10% (w/v) fetal calf serum (Gibco; Thermo Fisher Scientific, Inc.) and 1% (w/v) penicillin/streptomycin in culture dishes, at 37°C with 5% CO2. Cells were seeded into six-well culture plates at a density of 5x10^4 cells/well.

**Cell transfection.** For the knockdown of endogenous Wnt5a expression in NSCLC cells, small interfering (si)RNAs were used. For transfection, 1x10^4 A549 and H1650 parental cells were seeded into six-well plates and cultured overnight at 37°C with 5% CO2 until they reached 80% confluence. The Wnt5a siRNA expression cassette was subcloned into the pcDNA6 expression vector (Invitrogen; Thermo Fisher Scientific, Inc.). The target sequence was 5'-GTGGTGCCACTGACTGA-3'. For overexpression of Wnt5a, sequences were amplified by PCR and inserted into pcDNA6.2 vector to generate fusion plasmids, namely Wnt5a and pcDNA empty vector as the control. The ratio of the plasmid to the transfection reagent was 1 µg:3 µl. Transfection was performed at room temperature using EzWay™ Transfection Reagent according to the manufacturer's instructions (Invitrogen; Thermo Fisher Scientific, Inc.). To assess the role of β-catenin, H1650 and A549 cells were transfected for 24 h with si-β-catenin (20 nM; Shanghai GenePharma Co., Ltd.; forward, 5'-CATGUGUTGGAAGCUCUA-3' and reverse, 5'-GCCACGTTGCAAGAGGGU-3'). A non-specific scramble siRNA was used as a negative control (20 nM; Shanghai GenePharma Co., Ltd.; forward, 5'-AUGCUATCGUGUGCATU-3' and reverse, 5'-CAGAGACGTGGUGAGatta-3'). Transfection efficiency was determined via western blotting and reverse transcription-quantitative PCR (RT-qPCR). Subsequent experiments were performed 48 h post-transfection.

**Radiation treatment.** Cell irradiation was performed using a Varian 21EX (Varian Medical Systems) linear accelerator with a coverage field of 10x10 cm. H1650 and A549 cells were cultured in 12-well culture plates (1x10^4 cells/well) and incubated for 5 days at 37°C with 5% CO2, then 100 µl isopropanol with 40 mM HCl was added to each well to dissolve formazan crystals. Optical density (OD) was measured at wavelengths of 560 and 620 nm, using a measurement parameter editor (Tecan Group, Ltd.). Cell viability was expressed as OD value of the transected cell/OD value of background control (untransfected cells).

**Colony formation assay.** H1650 and A549 cells were irradiated (0, 2, 4, 6 or 8 Gy in a single fraction) 48 h post-transfection and subsequently seeded into 6-well plates at a density of 1x10^3 cells/well. The RPMI-1640 medium (Thermo Fisher Scientific, Inc.) was replaced every day and cells were incubated for 14 days at 37°C with 5% CO2. After 14 days, cells were fixed with 4% paraformaldehyde in PBS for 30 min at room temperature and stained with crystal violet (0.4 g/l; Sigma-Aldrich; Merck KGaA) at room temperature for 5 min. The number of colonies (determined as containing >50 cells) was counted manually under a light microscope (magnification, x10). The surviving fraction (%) was calculated as follows: Colony forming efficiency = number of colonies formed following irradiation treatment/number of cells seeded x100. All experiments were performed in triplicates and repeated three times.

**Cell apoptosis assay.** Cell apoptosis was determined via Annexin V-FITC and PI staining. Following 24 h irradiation, H1650 and A549 cells were seeded into 24-well plates at a density of 5x10^4 cells/well and resuspended in 100 µl binding buffer (10.0 HEPES, 140.0 NaCl and 2.5 mM CaCl2; pH 7.4). The cells were subsequently stained with 5 µl Annexin V-FITC and 5 µl PI using a FITC Annexin V Detection kit (BD Biosciences) in the dark at room temperature for 15 min, according to the manufacturer's protocol. Cell apoptosis was analyzed via flow cytometry (BD FACSCanto™; BD Biosciences) and expressed as the percentage of cells in each population (viable, Annexin V⁻/PI⁻; early apoptotic, Annexin V⁻/PI⁺; late apoptotic, Annexin V⁺/PI⁻ and necrotic, Annexin V⁺/PI⁺). These data were analyzed by FlowJo v10.0.7 software (FlowJo LLC).

**Western blotting.** H1650 and A549 cells were harvested, and cytoplasmic and nuclear proteins were isolated using the Proteo JET™ Cytoplasmic and Nuclear Protein Extraction kit according to the manufacturer's instructions (Fermentas; Thermo Fisher Scientific, Inc.). The Bradford assay was used for protein quantification. Equal amounts of protein (20 µg/lane) were separated by SDS-PAGE, transferred onto polyvinylidene difluoride membranes (Cytiva) and blocked with blocking buffer containing 5% skimmed milk in TBS-Tween-20 (0.1% Tween-20 in 1X TBS) for 1 h at room temperature. The membranes were incubated with primary antibodies against β-catenin (1:1,000; cat. no. 9582; Cell Signaling Technology, Inc.), lamin A (1:2,000; cat. no. 86846s; Cell Signaling Technology, Inc.), Wnt5a (1:800; cat. no. sc-365370; Santa Cruz Biotechnology, Inc.) and GAPDH (1:2,000; cat. no. sc-47724; Santa Cruz Biotechnology, Inc.) overnight at 4°C. Following primary incubation, membranes were incubated with HRP-conjugated secondary antibodies [anti-rabbit (1:5,000; cat. no. 211-035-109; Jackson ImmunoResearch Laboratories Inc.) and mouse IgG (1:5,000; cat. no. 315-035-048; Jackson ImmunoResearch Laboratories Inc.)] for 1 h at room temperature. Protein bands were detected using an Enhanced Chemiluminescence System (Pierce: Thermo Fisher Scientific, Inc.). Immunoreactive bands were quantified with the TINA v2.10G software (Raytest Isotopenmegerifte GmbH).

**RT-qPCR.** Total RNA was isolated from H1650 and A549 cells using TRIzol® according to the manufacturer's protocol (Invitrogen; Thermo Fisher Scientific, Inc.). Total RNA (2 µg) was reverse transcribed into complementary DNA (cDNA)
using the First-Strand RT-PCR kit (Promega Corporation). cDNA was subsequently amplified using PCR specific primers for the target genes and GAPDH was amplified as the internal control. The amplification mixture contained 0.5 U of Taq polymerase (Takara Bio, Inc.). The thermocycling conditions were as follows: 95°C for 3 min; 30 cycles at 95°C for 40 sec, 58°C for 40 sec and 72°C for 90 sec; final elongation at 72°C for 10 min. The primer sequences were as follows: Wnt5a forward, 5'-CGA AGA CAG GCA TCA AAG AA-3' and reverse, 5'-GCA AAG CGG TAG CCA TAG TC-3'; and GAPDH forward, 5'-ACC ACA GTC CAT GCC ATC AC-3' and reverse, 5'-TCC ACC ACC CTG TTG CTG TA-3'. RT-qPCR products were electrophoresed via a 1.5% agarose gel with ethidium bromide. Signals were quantified by densitometric analysis using Labworks Image Acquisition 4.0 software (Analytik Jena US LLC). Statistical analysis was subsequently performed to calculate the gel intensity using Microsoft Excel software 2010 (Microsoft Corporation).

Statistical analysis. Statistical analysis was performed using SPSS v21.0 software (IBM Corp.). All experiments were performed in triplicates and data are presented as the mean ± SD. Statistical differences were analyzed using one-way ANOVA followed by Tukey's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Wnt5a knockdown enhances irradiation-induced inhibition of NSCLC cell proliferation and colony formation. To determine the effects of Wnt5a knockdown on antitumor radiotherapy in H1650 and A549 cells, MTT assay was performed to assess cell proliferation following radiation alone or combined with transfection with si-Wnt5a or empty vector control. Western blotting and RT-qPCR were performed to detect Wnt5a expression levels (Fig. 1A). Treatment with ionizing radiation (2-8 Gy) inhibited proliferation of H1650 and A549 cells in...
a dose-dependent manner. Furthermore, Wnt5a knockdown decreased the proliferation of H1650 (Fig. 1B) and A549 (Fig. 1C) cells compared with the control (empty vector). The present study investigated whether Wnt5a affects colony formation of H1650 and A549 cells following radiotherapy. The results demonstrated that cell colony formation was significantly inhibited by radiotherapy and Wnt5a knockdown significantly enhanced this inhibitory effect (Fig. 1D-G). Taken together, these results suggest that combined Wnt5a knockdown and irradiation may improve the inhibitory effect on NSCLC cell proliferation.

Overexpression of Wnt5a reverses irradiation-induced inhibition of NSCLC cell proliferation and colony formation. The effect of overexpressing Wnt5a and irradiation on proliferation of H1650 and A549 cells was investigated. Equal amounts of H1650 and A549 cells transfected with Wnt5a or empty vector control were analyzed via western blotting and RT-qPCR (Fig. 2A). The results demonstrated that overexpression of Wnt5a increased cell proliferation following irradiation at 2 or 4 Gy compared with the empty vector control (Fig. 2B and C). However, no significant differences were observed between the Wnt5a overexpression and empty vector control groups following irradiation at 6 or 8 Gy, suggesting that 6 and 8 Gy doses may be lethal. H1650 (Fig. 2D and E) and A549 (Fig. 2F and G) cells overexpressing Wnt5a were treated with radiotherapy; radiation significantly inhibited colony formation, while overexpression of Wnt5a significantly decreased this inhibitory effect. Collectively, these results suggest that overexpression of Wnt5a attenuated the radiotherapeutic effect on NSCLC cells.

Wnt5a knockdown increases irradiation-induced apoptosis in NSCLC cells. To determine whether Wnt5a knockdown sensitizes H1650 and A549 cells to irradiation-induced apoptosis, cells were transfected with si-Wnt5a and subsequently irradiated with either 0 or 4 Gy. After 24 h, the percentage of apoptotic cells was determined via Annexin V/PI staining...
The apoptosis of H1650 and A549 cells following Wnt5a knockdown or irradiation alone significantly increased compared with control cells. In addition, combination of Wnt5a knockdown and irradiation further increased apoptosis. Taken together, these results suggest that Wnt5a knockdown sensitized NSCLC cells to irradiation-induced apoptosis.

Overexpression of Wnt5a decreases irradiation-induced apoptosis in NSCLC cells. To determine whether overexpression of Wnt5a affects irradiation-induced apoptosis, H1650 and A549 were transfected with Wnt5a or empty vector control following irradiation at 0 or 4 Gy (Fig. 4). The results demonstrated that overexpression of Wnt5a significantly decreased the apoptosis of H1650 and A549 cells compared with the control group. In addition, irradiation (4 Gy) increased the apoptosis of both H1650 and A549 cells; this effect was reversed following overexpression of Wnt5a. Collectively, these results suggest that overexpression of Wnt5a attenuated irradiation-induced apoptosis in NSCLC cells.

β-catenin expression following Wnt5a knockdown and/or irradiation in NSCLC cells. To determine whether Wnt5a knockdown and irradiation inhibit proliferation and induce apoptosis of NSCLC cells via the β-catenin pathways, the
cytoplasm and nucleus were separated and β-catenin expression was detected via western blotting (Figs. 5 and S1). Cytoplasmic β-catenin expression was higher following Wnt5a knockdown or irradiation in H1650 and A549 cells compared with the control cells. Combined Wnt5a knockdown and irradiation was further enhanced the expression of β-catenin. Conversely, nuclear β-catenin expression was reduced by the combination of Wnt5a knockdown and irradiation in H1650 and A549 cells. β-catenin expression following overexpression of Wnt5a and/or irradiation in NSCLC cells. The effects of overexpressing Wnt5a and irradiation on cytoplasmic and nuclear expression of β-catenin in NSCLC cells were investigated (Figs. 6 and S2). Western blot analysis revealed that overexpression of Wnt5a decreased cytoplasmic but increased nuclear β-catenin expression in H1650 and A549 cells. In addition, irradiation treatment increased cytoplasmic and decreased nuclear β-catenin expression in both H1650 and A549 cells. Notably, overexpression of Wnt5a reversed the irradiation-induced alterations in cytoplasmic and nuclear β-catenin expression in H1650 and A549 cells. Taken together, these results suggested that overexpression of Wnt5a may cause translocation of β-catenin from the cytoplasm to the nucleus in NSCLC cells.

si-β-catenin reverses activation of NSCLC cell proliferation caused by overexpression of Wnt5a. To determine the role of the β-catenin pathway in NSCLC cell proliferation induced by overexpression of Wnt5a and irradiation, H1650 and
A549 cells were treated with siRNA for β-catenin knockdown. Knockdown of β-catenin was confirmed by western blotting and RT-qPCR (Fig. S3). Overexpression of Wnt5a blocked the irradiation-induced decrease in NSCLC cell proliferation (Figs. 7 and S4). In addition, si-β-catenin reversed the promotion of NSCLC cell proliferation due to combined Wnt5a overexpression and irradiation. Collectively, these results suggest that the β-catenin pathway may be a mediator of Wnt5a overexpression- and irradiation-induced increases in proliferation and decreases in apoptosis of NSCLC cells.

**Discussion**

Wnt5a expression is upregulated in NSCLC cells (18) and regulates several biological events associated with tumor growth, EMT and metastasis of NSCLC cells (9). It has also been reported that overexpression of Wnt5a increases colony formation, migration and invasion (12,19). Thus, to assess whether alterations of Wnt5a expression affected the response of NSCLC cell lines to radiotherapy, the present study knocked down or overexpressed Wnt5a in H1650 and A549 cells. The results demonstrated that Wnt5a knockdown combined with irradiation decreased proliferation and induced apoptosis of NSCLC cells more than irradiation or Wnt5a knockdown alone. Conversely, overexpression of Wnt5a blocked irradiation-induced apoptosis. These findings suggest that Wnt5a expression served a valuable role in the radiotherapeutic treatment of NSCLC.

Wnt5a signaling comprises non-canonical (β-catenin-independent) and canonical (β-catenin-dependent) pathways (20). β-catenin signaling serves an important role in regulating the transcription of several oncogenes, such as cyclin D1 and c-Myc (21,22); thus, different types of cancer exhibit aberrant activation of this signaling pathway (23,24).
Activated Wnt/β-catenin signaling pathway has been shown to induce translocation of β-catenin from the cytoplasm to the nucleus (25,26), thus promoting the initiation of EMT, tumor invasion, and metastasis (14,27-29). The present study demonstrated that the combination of Wnt5a knockdown and irradiation decreased nuclear but increased cytoplasmic β-catenin expression in NSCLC cells. These findings support the hypothesis that the combination of Wnt5a knockdown and irradiation decreases translocation of β-catenin from the cytoplasm to the nucleus, thus inhibiting NSCLC cell proliferation and enhancing apoptosis.

A previous study reported that Wnt5a plays a key role in regulating NSCLC cell migration and invasion by activating β-catenin-dependent canonical Wnt signaling (12). Consistent with this finding, the results of the present study demonstrated that Wnt5a knockdown increased NSCLC cell apoptosis. In addition, overexpression of Wnt5a attenuated the killing effect of radiation therapy on NSCLC cells, whereas si-β-catenin antagonized Wnt5a overexpression-induced proliferation of NSCLC cells. Increasing evidence suggest that the Wnt/β-catenin pathway is associated with radioresistance of cancer cells (7,30). The enhanced nuclear translocation of β-catenin was more evident in radioresistance cells (31-33). Consistent with these findings, the present study demonstrated that Wnt5a knockdown decreased nuclear β-catenin expression, whereas overexpression of Wnt5a enhanced nuclear β-catenin expression. Taken together, these results suggest that the Wnt5a/β-catenin pathway may exert a radiosensitizing effect in NSCLC.

In conclusion, the present study demonstrated that Wnt5a knockdown in combination with irradiation inhibited...
proliferation and induced apoptosis of NSCLC cells; these effects were reversed following overexpression of Wnt5a. In addition, Wnt5a influenced the susceptibility of NSCLC cells to radiotherapy via activation of β-catenin-dependent canonical Wnt signaling. Thus, Wnt5a gene therapy may enhance the therapeutic effect of radiation for the treatment of NSCLC.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors’ contributions

JL, SX and XX conceptualized and designed the study. JL, SX, XW, HD and LHW acquired the data and drafted the manuscript. JL, SX, XW, HD and LHW performed data analysis. SX and XX wrote the manuscript. JL, SX, XW, HD and XX revised the manuscript. JL, SX and XX confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.
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