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Original article

# Differential effect of soy isoflavones in enhancing high intensity radiotherapy and protecting lung tissue in a pre-clinical model of lung carcinoma

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ABSTRACT

*Background:* Radiotherapy of locally-advanced non-small cell lung cancer is limited by radiation-induced pneumonitis and fibrosis. We have further investigated the role of soy isoflavones to improve the effect of a high intensity radiation and reduce lung damage in a pre-clinical lung tumor model.
*Methods:* Human A549 NSCLC cells were injected i.v. in nude mice to generate a large tumor burden in the lungs. Mice were treated with lung irradiation at 10 Gy and with oral soy. The therapy effect on the tumor cells and surrounding lung tissue was analyzed on lung sections stained with H&E, Ki-67 and Masson's Trichrome. Pneumonitis and vascular damage were evaluated by measurements of alveolar septa and immunofluorescent staining of vessel walls.
*Results:* Combined soy and radiation caused a significantly stronger inhibition of tumor progression compared to each modality alone in contrast to large invasive tumor nodules seen in control mice. At the same time, soy reduced radiation injury in lung tissue by decreasing pneumonitis, fibrosis and protecting alveolar septa, bronchioles and vessels.
*Conclusions:* These studies demonstrate a differential effect of soy isoflavones on augmenting tumor destruction induced by radiation while radioprotecting the normal lung tissue and support using soy to alleviate radiotoxicity in lung cancer.
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Lung cancer is the second most common malignancy in both men and women in the USA and the leading cause of death. It is estimated that over 215,000 people per year will be diagnosed with lung cancer [1]. Approximately, 85% of lung cancers are classified as non-small cell lung cancer (NSCLC), which includes squamous cell carcinoma, adenocarcinoma and large cell carcinoma. A third of patients with newly diagnosed NSCLC present with unresectable stage III locally advanced disease with an overall 5-year survival rate of 20%, emphasizing the need to improve the therapeutic ratio [2]. Locally advanced disease is currently treated by concurrent chemo-radiotherapy [3,4]. High intensity radiotherapy could be more effective but is limited by lung tissue toxicity presenting as radiation pneumonitis, which is an interstitial pulmonary inflammation that develops in up to 30% of patients after thoracic radiation [5–7]. Radiation pneumonitis is caused by an early inflammatory process triggered by damage to lung parenchyma, epithelial cells, vascular endothelial cells and stroma that involves induction of pro-inflammatory cytokines and chemokines which recruit inflammatory immune cells in the lung tissue [8–11]. It is now believed that this acute early pneumonitis actually progresses to a chronic inflammation mediated by cyclical phases of cytokines, chemokines and growth factors released in the tissue microenvironment [9,12]. These complex events culminate in the later stage of lung fibrosis which is due to excessive accumulation of collagen and other extracellular (ECM) components [9,12,13]. These adverse events of radiotherapy affect patients' breathing and their quality of life [5,6,12,14]. It is axiomatic that biological-based strategies that enhance damage to malignant cells and reduce damage to normal cells, would be able to increase the volume that could be irradiated with higher doses without increasing toxicity.

Our previous studies indicate that soy isoflavones, which are plant estrogens extracted from soy beans and non-toxic dietary compounds, could be exploited as biological agents to sensitize cancer to radiation while simultaneously protect surrounding normal tissues. These studies demonstrated that soy isoflavones sensitized cancer cells to radiation both *in vivo* and *in vitro* in

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pre-clinical tumor models of lung cancer, prostate cancer and renal cell carcinoma [15–22]. Our mechanistic studies showed that soy isoflavones radiosensitized human NSCLC cells by inhibition of critical survival pathways which are constitutively activated in cancer cells and are further upregulated by radiation [20,22]. These include DNA repair processes and key transcription factors such as nuclear factor-kappaB (NF-κB) and hypoxia inducible factor (HIF-1α) that are responsible for transcription of proteins involved in cell-cycle progression, proteolysis, and angiogenesis and are implicated in cancer radioresistance [20–22]. In contrast, normal cells do not express such activated malignant survival pathways and thus, are not affected directly by soy isoflavones.

Furthermore, we found that soy isoflavones can have the dual capability of enhancing radiation damage in the malignancy and simultaneously protecting the normal lung from radiation injury using a xenograft pre-clinical lung model [15]. Soy isoflavones increased radiation-induced destruction of lung tumor nodules, and also mitigated the vascular damage, inflammation and fibrosis, caused by radiation injury to lung tissue [15]. Those initial studies were performed with hemithorax irradiation to the left lung to discriminate between radiation and soy effects in the same mouse by analyzing the left and right lungs separately. We have now expanded these observations by performing studies using full lung irradiation to quantitate the tumor response in both lungs and the radioprotective effects of soy on a larger area of lung tissues, as the left lung comprises one lobe and the right lung has four lobes in mice. We also tested the response of a greater tumor burden, which was generated by injecting a double dose of tumor cells to mimic a more advanced model of lung cancer. To further explore the effect of soy isoflavones on irradiated tumor nodules and irradiated lung tissues, new techniques were developed to monitor and quantify soy radioenhancement effect for lung tumors versus soy radioprotection effect for lung tissues. The anti-tumor effect mediated by soy and radiation was assessed by Ki-67 proliferative index of tumor cells and Masson's Trichrome (MT) staining of tumor nodules to evaluate tumor-associated stroma, which are important issues for translational purposes. New and original methodologies were developed to evaluate and quantitate the radioprotective effects of soy that were previously observed [15]. The thickness of alveolar septa was measured quantitatively as a criterion for pneumonitis. Vascular damage was further investigated by fluorescent staining of the basement membrane of vessels using collagen, endothelial and pericyte specific antibodies to detect vessel abnormalities including thickening and projections. These new studies now confirm and establish the differential effect of soy isoflavones on malignant versus normal tissue.

## Materials and methods

### *Establishment of NSCLC advanced lung tumor model*

The human non-small cell lung carcinoma (NSCLC) A549 (purchased from ATCC) was cultured in F-12K culture medium containing 7% heat-inactivated fetal bovine serum with supplements. A549 cells, at $4 \times 10^6$ in 200 μl HBSS, were injected i.v. in the tail vein of 5–6 week old female Hsd Athymic Nude-Foxn1$^{nu}$ *nu*/*nu* nude mice (Harlan, Indianapolis, IN) [15]. Mice were housed and handled under sterile conditions in facilities accredited by the American Association for the Accreditation of Laboratory Animal Care (AAALAC). The animal protocol was approved by Wayne State University Animal Investigation Committee (IACUC). To monitor tumor establishment in the lungs of mice, preliminary kinetics experiments were performed and mice were sacrificed at different time points after i.v. injection of A549 cells. The lungs were resected and processed for histological staining with hematoxylin–eosin (H&E). Established tumor nodules of about 600 μm in diameter were observed by day 16–18 in the midst of the lung tissue, therefore this time point was selected to initiate treatment with soy isoflavones.

### *Soy Isoflavones*

The G-4660 soy isoflavone mixture used is a pure extract of 98.16% isoflavones from soybeans consisting of 83.3% genistein, 14.6% daidzein and 0.26% glycitein (manufactured by Organic Technologies and obtained from NIH). The soy isoflavone mixture was dissolved in 0.1 mol/l $Na_2CO_3$ and mixed with sesame seed oil at a 2:1 ratio just prior to treatment to facilitate gavage and avoid irritation of the esophagus by $Na_2CO_3$ [15]. Mice were orally treated with soy isoflavones by gavage. Control mice received the vehicle alone.

### *Tumor-bearing lung irradiation*

Photon irradiation was performed at a dose of 10 Gy with a Siemens Stabilipan X-ray set (Siemens Medical Systems, Inc) operated at 250 kV, 15 mA with 1 mm copper filtration at a distance of 47.5 cm from the target. Three anesthetized mice, in jigs, were positioned under a 6.4 mm lead shield with 3 cut-outs in an aluminum frame mounted on the X-ray machine to permit selective irradiation of tumor-bearing lungs in the thoracic area, as described previously [15]. The radiation dose to the lung and the scattered dose to areas of the mouse outside of the radiation field were carefully monitored. To minimize backscattering of radiation, the bottom of the aluminum frame that holds the jigs was hollowed out and the backplate of the jig was thinned to 1.6 mm thickness. Under these conditions and with the lead shielding, the X ray dose to the shielded regions was reduced to 1% of the thoracic dose. The dose rate was 101 cGy/min and HVL was 2 mm Cu.

### *Experimental protocol*

Mice bearing established A549 lung tumor nodules were pretreated with soy isoflavones at a dose of 5 mg/day (equivalent to 250 mg/kg) for 3 days from days 16 to 18 after cell injection. On day 19, the full lung was selectively irradiated by delivering 10 Gy to the thorax while shielding the rest of the mouse body with lead. Soy treatment was then continued on a daily basis for 5 more days at 5 mg/day. Then mice were treated with a lower dose of 1 mg/day (equivalent to 50 mg/kg) for an additional 4 weeks administered 5 days a week. The rationale for giving a higher dose of soy isoflavones for pre-treatment and just after radiation is to optimize the effect of soy for radiosensitization, based on previous studies [15–19]. We found that either dose causes radioenhancement on the tumor. At a dose of 1 mg/day of soy the levels of isoflavones measured in the serum of mice treated with soy isoflavones reflected typical *in vivo* metabolism with significant levels of daidzein (1.6 μM) and genistein (1.7 μM) [18]. To assess the therapeutic response of lung tumors to soy and radiation, 9–10 mice per experimental group were treated. By day 50 after cell implant, the tumor nodules in untreated lungs were very large up to 1200 μm in diameter. Therefore this time point was selected for termination of the experiment so that the tumor nodules in treatment groups could be directly compared with those in control group prior to possible mouse death from tumor burden. Mice were killed and lungs were perfused with 10% buffered formalin prior to resection.

### *Lung tissue preparation for histology*

Formalin fixed lungs were embedded in paraffin and cut into 5 μm sections. Sections were stained with hematoxylin–eosin

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