RESEARCH ARTICLE

Radiation Induces Phosphorylation of STAT3 in a Dose- and Time-dependent Manner

Ling Gao[1&], Feng-Sheng Li[2&], Xiao-Hua Chen[3], Qiao-Wei Liu[3], Jiang-Bin Feng[1], Qing-Jie Liu[1]*, Xu Su[1]*

Abstract

Background: We have reported the radiation could activate STAT3, which subsequently promotes the invasion of A549 cells. We here explored the dose- and time-response of STAT3 to radiation and the effect of radiation on upstream signaling molecules. Materials and Methods: A549 cells were irradiated with different doses of γ-rays. The expression of and nucleus translocation of *p*-STAT3 in A549 cells were detected by immunoblotting and immunofluorescence, respectively. The level of phosphorylated EGFR was also assessed by immunoblotting, and IL-6 expression was detected by real time PCR and ELISA. Results: Radiation promoted the phosphorylation of STAT3 at Y705 in a dose- and time-dependent manner and nuclear translocation. The level of phosphorylated EGFR in A549 cells increased after radiation. In additional, the mRNA and protein levels of IL-6 in A549 cells were also up regulated by radiation. Conclusions: STAT3 is activated by radiation in a dose-and time-dependent manner, probably due to radiation-induced activation of EGFR or secretion of IL-6 in A549 cells.

Keywords: Radiation - STAT3 - phosphorylation - EGFR - IL-6

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Introduction

Radiotherapy is one of the common approaches for cancer therapy. It can be used alone or in combination with chemotherapy and/or surgery. Radiotherapy has shown therapeutic effect for most type of cancer including lung cancer, even has a curative potential in many solid human tumors (Krause et al., 2011), such as head and neck carcinoma and non-small cell lung cancer (Budach et al., 2005; Baumann et al., 2011). However, despite continuous progression of radiotherapy technology, a high proportion of patients will still die from tumor recurrence and metastasis due to the cancer cells with radioresistance (Rycaj et al., 2014). Radiation acts by inhibiting cell proliferation or inducing apoptotic cell death (Fu et al., 1991). However, radiaiton can also activate various radiation response genes. This leads to the production of various proteins, some of which might change the radiosensitivity of cancer cell and confer survival advantage to cancer cells. The survival cancer cells after radiation contributed to the recurrence and metastasis of cancer.

The transcription factor Signal Transducer and Activator of Transcription 3 (STAT3), a member of the STAT family, plays a key role in many physiological and pathological processes (Yu et al., 2007; Qi et al., 2014). STAT3 can translocate into the nucleus after the phosphorylation of STAT3 at Tyr705 and bind to many genes specific promoter sequences, where it regulates the transcription of targeting genes, including Survivin, Bcl2 and Bcl-xl which are all involved in the regulation of cell apoptosis (Bromberg, 2001; Ni et al., 2004). Recent study has also indicated that STAT3 can also translocate into mitochondria after the phosphorylation of STAT3 at Ser727, regulating the oxidative phosphorylation (Myers et al., 2009). More interestingly, our previous study showed that radiation could promote the phosphorylation of STAT3 at Tyr705, which resulted in the increase of invasion of A549 cells by up regulating the expression of MMP2 (Li et al., 2013). This result indicated that the activation of STAT3 by radiation play an important role in recurrence and metastasis of lung adenocarcinoma. Therefore, in the current study, we further investigate the relationship between the *p*-STAT3 and radiation, and the upstream molecular involved in radiation-induced activation of STAT3.

*[1]Key Laboratory of Radiological Protection and Nuclear Emergency, National Institute for Radiological Protection, Chinese Center for Disease Control and Prevention, [2]The Second Artillery General Hospital, [3]Department of Experimental Therapy of Acute Radiation Sickness, Beijing Institute of Radiation Medicine, Beijing, China [&]Equal contributors *For correspondence: suxu@nirp.cn, qjliu@nirp.cn*

Materials and Methods

Cell lines

The A549 cell line was obtained from the Beijing Xiehe Cell Culture Center and maintained in DMEM plus 10% fetal bovine serum (FBS).

Radiation

The A549 cells were irradiated using a Cobalt-60 unit at a source-skin distance of 4m. The dose rate was 2.17 Gy/min.

Immunoblotting

Cells were harvested 12 or 24 h after radiation, and were then directly lysed in a lysis buffer containing a mammalian protease cocktail (Sigma) to obtain total protein content. Protein concentrations were measured by the Bradford method. 50 μg of protein was separated by 12% SDS-polyacrylamide gel, and transferred to nitrocellulose filters. The filters were blocked with TBST buffer (10 mM Tris-HCl, pH 8.0, 0.15 M NaCl, 0.05% Tween 20) containing 5% skimmed milk. They were then incubated overnight with monoclonal antibodies to *p*-STAT3 $^{(Y705)}$ (Cell Signaling, USA), EGFR and *p*-EGFR (Santa Cruz, USA) and actin (Santa Cruz, USA). Finally, horseradish peroxidase-linked anti-mouse IgG (ZhongshanGoldenbridge Biotechnology Co., Beijing, China) was added to allow ECL visualization of the bands.

Immunofluorescence staining

A549 cells were placed on coverslips in 6-well plates and incubated in an environment with 5% CO_2 at 37 °C for 24 h. Then, these cells were irradiated at indicated dose; 24 h after radiation, cells were processed as follows: cells were washed in 1×PBS, and fixed for 20 min in 4% paraformaldehyde. Then, cells were treated with 0.2% Triton and 1% BSA for 1 h and with anti-pSTAT3 antibody (1:400) overnight at 4 °C. Following incubation with fluorescein isothiocyanate-conjugated rabbit anti-rat IgG (1:1000, Abcam) and DAPI (1:5000) for 30 min at room temperature, mounting was performed with antifade reagents (Prolong Gold), and photomicrographs were captured using an Olympus confocal laser scanning microscope.

Real-time PCR

Total RNA was extracted from the irradiated cells using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. The RNA (1μg) was reverse transcribed with reverse transcriptase using random hexamers as primers (Kaiji Sheng Wu, Nanjing, China). The resulting complementary DNAs (cDNAs) were diluted by 2.5-fold with DNase/RNase-free water and the levels of IL-6 and the housekeeping gene β-actin were determined by quantitative PCR on the LightCycler (BoriKeJi, Hangzhou, China) using the Master SYBR-Green I reagent kit under the following conditions: a step of denaturation at 95 °C for 2 min and 40 cycles at 95 °C for 5 sec, at 58 °C for 30 sec and at 72 °C for 30 sec. The endpoint used in PCR quantification (Ct) was defined as the PCR cycle number that crosses an arbitrarily placed signal threshold and is a function of the amount of target cDNA present in the starting material. The IL-6 levels were normalized to those of β-actin in the same samples. The primers used were 5'-ctttggagtttgaggtagtataccta-3' and 5'-gctgcgcagaatgagatgagttgtc-3'for IL-6 and 5'-tccgtggagaagagctacga-3' and 5'-gtacttgcgctcagaaggag-3' for β-actin.

Enzyme-linked immunosorbent assay (ELISA)

The supernatant of cell culture were collected. IL-6 level in the supernatant was determined by a human IL-6 enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocol.

Results

Radiation promoted the activation of STAT3 in a dose- and time-dependent manner

Our previous study had proved that 2 and 4 Gy of radiation could promote the phosphorylation of STAT3 at Tyr705 significantly (Li et al., 2013). Here, we extend the dose rang of radiation to further investigate the relationship of radiation and phosphorylation of STAT3. As shown in figure 1 (A), 24 h after radiation, the expression of phosphorylated STAT3 at Tyr705 in A549 cells increased with the increasing radiation dose. The immunofluorescence staining showed that exposure at 2 Gy could promote the phosphorylation and nuclear translocation of STAT3, and this effect was more significantly after 10 Gy of irradiation (Figure 1B). The time-effect of radiation-induced activation of STAT3 was

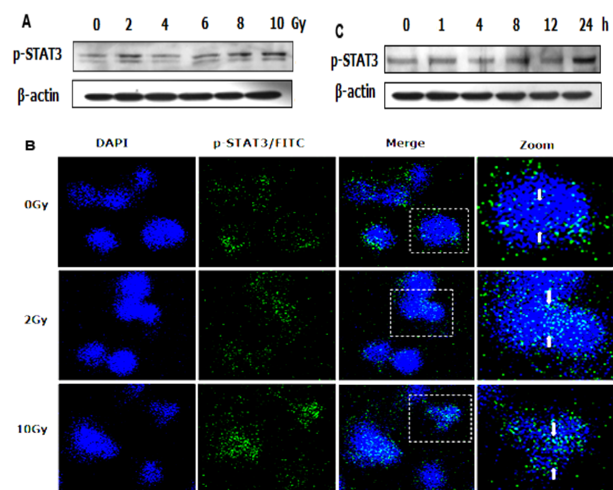

Figure 1. Radiation Promoted the Phosphorylation of Stat3 At Tyr705 in A Dose-and Time-dependent Manner. (A). The phosphorylated STAT3 (Y705) in A549 cells 24 h post different dose of irradiation were determined using specific antibodies and immunobloting. β-actin was used as a loading control. **(B)**. the A549 cells were exposed to dose of 2 or 10 Gy. 24 h after irradiation, the cells were processed as the protocol of immunofluorescence staining described in the section of methods and material. **(C)**. A549 cells irradiated with 10 Gy of γ raywere collected at different time point after radiation and the total protein was extracted. The phosphorylated STAT3 (Y705) was determined using specific antibodies and immunobloting. β-actin was used as a loading control

subsequently detected after the A549 cells were irradiated at dose of 10 Gy. As shown in figure 1 (C), the level of phosphorylated STAT3 at Tyr705 in A549 cells increased in a time dependent manner.

10 Gy of radiation caused the phosphorylation of EGFR

It has been reported that EGFR pathway can promote the phosphorylation of STAT3 (Ni et al., 2004), therefore we further investigated whether the EGFR-STAT3 pathway was responsible for radiation-induced activation of STAT3. As shown in figure 2, although the content of total EGFR in A549 cells remained unchanged at any examined time point after 10 Gy of radiation, the level of phosphorylated EGFR in A549 cells increased significantly from 1 to 12 h post radiation. 24 h after irradiation, the level of *p*-EGFR in cells went down to base line.

Radiation promoted the secretion of IL-6

Besides EGFR-STAT3 pathway, IL-6R-Jak2-STAT3 pathway is another classical pathway that regulates the activation of STAT3. Therefore, the IL-6 expression was further detected by real-time PCR and ELISA. As shown in figure 3 (A), the result of real-time PCR showed that the expression of IL-6 mRNA in A549 cells increased significantly 2 h after 2 Gy of radiation. 8 h after radiation, the expression of IL-6 mRNA reach the peak. However, after exposure to 4 Gy of γ ray, the level of IL-6 mRNA increased in a time dependent manner within 12h. The result of ELISA further confirmed that the protein expression of IL-6 was up regulated significantly 12 h after 2 and 4 Gy of irradiation, and exposure to 6 Gy of γ ray did not result in the significant increase of IL-6 secreted into the supernatant of cell culture (Figure 3B).

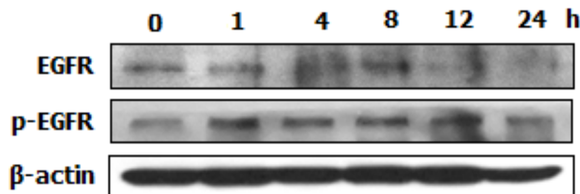

Figure 2. Radiation Resulted in the Phosphorylation of EgFR. A549 cells irradiated with 10 Gy of γ ray were collected at different time point after radiation and the total protein was extracted. The protein levels of EGFR and phosphorylated EGFR was determined using specific antibodies and immunobloting. β-actin was used as a loading control

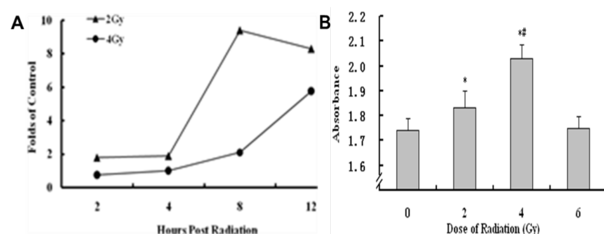

Figure 3. Radiaition Promoted the Expression of IL-6 in A549 Cells. (A). A549 cells were irradiated with 2 or 4 Gy of γ ray and collected at indicated time point after radiation. The total RNA were extracted and used as the templates of reverse transcription to obtain the cDNA. Then the real time PCR were performed to detected the level of IL-6 mRNA. **(B)**. The supernatant of cell culture were collected. IL-6 level in the supernatant was determined by ELISA. Compared with control, *$p<0.05$; Compared with 2 Gy group, #$p<0.05$

Discussion

Recent years, the incidence of lung cancer which already being the leading cause of cancer related death worldwide have been gradually rising because of escalating smoking rates (Jemal et al., 2011). Radiotherapy is the indispensable treatment regime, especially for inoperable lung cancer. Although radiotherapy can lead to increased survival time in lung cancer patients, the ultimate cause of death of most patients will still is regional recurrence and metastases of distant organs because of the radioresistance of lung cancer cells. It has been reported that cancer cells can adaptively response to radiation by regulating some signal pathway after they were irradiated, some of which will be contribute to the survival or metastasis of cells. Our previous study and other group had shown that radiation could promote the phosphorylation of STAT3 at Tyr705, which resulted in the increase of invasion of A549 cells by up regulating the expression of MMP2 (Yu et al., 2007; Li et al., 2013). In the current study, we found that radiation promoted the phosphorylation of STAT3 at Tyr705 in a dose-and time-dependent manner in A549 cells. Furthermore, the level of phosphorylated EGFR and IL-6, both of which were upstream signaling molecular of STAT3, in A549 cells was found to be up regulated after radiation.

STAT3, which was phosphorylated in several clinical cancer cells and considered as the therapeutic target of several cancers, such as colitis-associated cancer (Pandurangan et al., 2014), could be activated by numerous cytokines, growth factors and oncogenic proteins (Bromberg et al., 2001). Alterations in Stat3 phosphorylation status followed with change of cancer cell proliferation, cellular apoptosis and chemo-and radio-therapeutic sensitivity in colorectal cancer, small cell lung cancer, skin cancer cell, ovarian cancer cell and non-small cell lung adenocarcinoma cell line (Abdulghani et al., 2008; Liu et al., 2011; Zhao et al., 2012; Hao et al., 2013; Sun et al., 2013; Zhu et al., 2013).

In the current study, we showed that STAT3 could also be activated by radiation in a time-and dose-dependent manner. Similarly, radiation could activate the phosphorylation of STAT3 in prostate cancer cells (Singh-Gupta et al., 2009). In addition, it had been reported that the activation of STAT3 could activate the expression of downstream genes that regulate cell migration, invasion and angiogenesis at the transcriptional level (Zhang et al., 2010; Wu et al., 2014). These results indicated that the activation of STAT3 might play an important role in the radioresistance of lung adenocarcinoma.

Considering the key role of STAT3 in the resistant response of lung adenocarcinoma to radiotherapy, it will be very significant to investigate the mechanism resulting in radiation-induced activation, especially the upstream molecular which lies the cell membrane and is easy to be targeted, because the STAT3 lies in the cytoplasm or

nucleus and is difficult to be blocked. In the current study, we found that the two main upstream molecular of STAT3, *p*-EGFR and IL-6 were both up regulated, indicating they might be involved in radiation-induced activation of STAT3. However, it is still unclear whether these proteins should be responsible for this phenomenon. Further study using RNA interference or specific inhibitors of these pathways is needed to entirely elucidate the mechanism by which radiation activates STAT3.

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