

X-ray inhibits FUT4-mediated proliferation in A549 cells by downregulating SP1 expression

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Abstract

Objective: To identify the mechanism of down-regulation of Lewis Y antigen caused by X-ray irradiation.

Method: The present original research study was conducted at Zhejiang University City College, Hangzhou, Republic of China, from 2020 to 2022. Western blotting, Co-immunoprecipitation (CO-IP), electrophoretic mobility shift assay and Cell Counting Kit-8 (CCK8) were performed to confirm the effect of X-ray irradiation on A549 cell proliferation and its mechanism. Data was analysed using Statistical Package for Social Sciences (SPSS) 11.5.

Results: The expressions of fucosyltransferase IV and Lewis Y were decreased after X-ray irradiation, thus inhibiting the proliferation of A549 lung cancer cells. Deoxyribonucleic acid damage caused by the irradiation caused higher level of poly- adenosinediphosphate-ribosylated Specific Protein 1 (SP1), and translocation of SP1 from the nucleus, decreasing the expression of fucosyltransferase IV and Lewis Y.

Conclusion: There was a significant role of glycosylation in radiation therapy for lung cancer.

Keywords: Fucosyltransferase 4, SP1, X-ray, Lung cancer, Cell proliferation.

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Introduction

Lung cancer is one of the most life-threatening malignant tumours and it is the top cause of cancer deaths¹. Because of its occult onset, a large number of patients with lung cancer are missed out on surgery after being diagnosed with lung cancer, and some other patients cannot have the surgery because of physical reason. For such patients, radiotherapy is a good choice. Because of the heterogeneity of lung cancer, different patients have different effects on radiotherapy, and some lung cancer tissues are not sensitive to radiotherapy. X-ray is commonly used in radiotherapy, but its molecular biological mechanism on lung cancer has not been fully studied. Therefore, an in-depth study of the mechanism between X-ray and lung cancer may be helpful to improve the sensitivity of radiotherapy for lung cancer.

The glycans of cell membrane proteins participate in interactions as ligand binding, signal transduction and molecular adhesion, and are closely related to such vital processes as cell growth, apoptosis, motility and differentiation². Abnormal glycosylation is a characteristic change of malignant transformation of cells that is related to the cytotoxicity, radio sensitisation and chemo-

sensitisation of tumour cells³. However, the effect of radiation on the glycans of tumour cells and its mechanism are not clear.

The fucosyl residues at the terminus of the glycans on the cell surface are closely related to the malignant transformation of cells⁴. Fucosyltransferase can catalyse the synthesis of fucose oligosaccharides, wherein alpha-1 (α-1), 3 fucosyltransferase IV (FUT4) is a specific synthetase gene that mediates the synthesis of sialylated Lewis Y (LeY) on the cell surface. Several studies showed that FUT4 affected the proliferation⁵, apoptosis⁶ and metastasis⁷ of tumour cells by synthesising LeY, and increased the malignancy of tumours. Gao et al. demonstrated that FUT4 inhibited the chemosensitivity of lung cancer to cisplatin⁴. Liu et al. found that significant FUT4 correlation with immune response and programmed death-1 (PD-1) expression led to worse survival in lung adenocarcinoma⁸. These results indicated that FUT4 might be a potential target gene for lung cancer treatment, but the upstream regulatory mechanism was relatively rare. Therefore, exploring how to inhibit the expression of FUT4 may have certain significance for the treatment of lung cancer.

In a previous study, we revealed the relationship between FUT4 expression and chemosensitivity in lung cancer⁴. However, the correlation between FUT4 and radiotherapy has not been reported. X-rays may act on tumour cells, leading to the changes in the structure of glycans on the surface of cells, affecting the proliferation and apoptosis

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of tumours, killing the tumour cells. In the pre-experiment, we confirmed that the expression of LeY decreased after X-ray irradiation. The current study was planned to further identify the mechanism of down-regulation of LeY caused by X-ray irradiation.

Materials and Methods

The present original research study was conducted at Zhejiang University City College, Hangzhou, Republic of China, from 2020 to 2022. Western blotting, CO-IP, electrophoretic mobility shift assay and CCK8 were performed to confirm the effect of X-ray irradiation on A549 cell proliferation and its mechanism.

Reagents used were 3-aminobenzamide (3-AB) (Sigma-Aldrich Co), small interfering RNA (siRNA) targeting FUT4, SP1 siRNA and negative control siRNA, which were designed and synthesised (GenePharma), FUT4, proliferating cell nuclear antigen (PCNA), SP1, Polyadenosine diphosphate[ADP]-ribose polymerase-1 (PARP-1), Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β -actin, Histone H3 antibodies (Proteintech), antibodies against H2A histone family member X (γ H2AX), LeY and poly(ADP-ribose) (pADPr) (Abcam).

For cell culture and transfection, human lung adenocarcinoma cell A549 was used (Type Culture Collection, Chinese Academy of Sciences). It was cultured in Roswell Park Memorial Institute (RPMI-1640) supplemented with 10% Foetal Bovine Serum (FBS), 100IU/ml penicillin and 100 μ g/ml streptomycin at 37°C with 5% CO₂. Transient transfection of A549 cells was performed using Lipo3000 reagent, according to the manufacturer's instructions. The treated cells were irradiated with a 10Gy dose by 6MV X-ray (Varian Trilogy linear accelerator).

For western blotting, cells were lysed using radioimmunoprecipitation assay buffer (RIPA) buffer (25 mM Tris-HCl (pH 7.6), 150mM NaCl, 1% nonylphenoxy polyethoxyethanol-40 (NP-40), 1% sodium deoxycholate, 0.1% Sodium dodecyl-sulfate (SDS) with protease inhibitors. The supernatant was taken as the total cell lysate after sonication for 2min and centrifugation for 10min at 4°C. Protein concentration was quantified using the Bradford method⁹. Equal amounts of total protein were analysed by Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Following transfer to the nitrocellulose membrane, the blot was probed with primary antibodies and then incubated with horseradish peroxidase (HRP)-labelled secondary antibodies for visualisation using enhanced chemiluminescence reagents. The images were obtained by the Bio-Rad Imaging System⁵. Nuclear and Cytoplasmic Protein

Extraction Kit (Beyotime Biotechnology) was used to extract the nuclear and cytoplasmic proteins.

Cells were lysed using RIPA buffer with protease inhibitors. Protein 500 μ g was incubated with an anti-SP1 antibody for 1h at 4°C. Antibodies were precipitated using Protein G-coated Dynabeads (ThermoFisher Scientific) for overnight at 4°C, then immunoprecipitated proteins were detected by SDS-PAGE using pADPr antibody¹⁰.

For electrophoretic mobility shift assay (EMSA), biotin end-labelled probe (Beyotime Biotechnology). EMSA was measured by Light Shift Chemiluminescent EMSA kit (ThermoFisher Scientific), according to the manufacturer's instructions followed by deoxyribonucleic acid (DNA)-binding reactions contained with biotin-labelled oligonucleotides and nuclear extracts. Additional unlabelled oligonucleotides were added for competition. Reaction products were then separated by electrophoresis. Thereafter, protein-DNA complexes were transferred onto a positively charged nylon membrane and detected by chemiluminescence¹¹.

Cell proliferation was measured by the CCK-8 kit (Dojindo), according to the manufacturer's instructions. Treated cells were reacted with CCK-8 reagent at 37°C for 2h, followed by measuring the absorbance at 450nm on a microplate reader.

Data was analysed using SPSS 11.5⁵. Mean $\pm$ standard deviation (SD) values were calculated after experiments carried out three times or more. Groups were compared using student's t-test or one-way analysis of variance (ANOVA), and Newman-Keuls / Student-Newman-Keuls (SNK q) test analysis. $P < 0.05$ was considered statistically significant.

Results

X-ray inhibited A549 cells proliferation by down-regulating FUT4 expression. Human lung adenocarcinoma cell line A549 was used as experiment cells. A549 cells were irradiated with 10Gy X-ray as the experimental group, while the control group was not irradiated. After 24 hours of irradiation, the expression of FUT4 and PCNA proteins decreased in X-ray irradiated cells (Figure 1A). In CCK-8 assay, cell proliferation was inhibited after X-ray irradiation (Figure 1B). FUT4 siRNA was transfected into A549 cells and western blotting showed a significant decrease in FUT4 expression (Figure 1C). Besides, the expression of LeY and PCNA in FUT4 siRNA cells were decreased (Figures 1C-D).

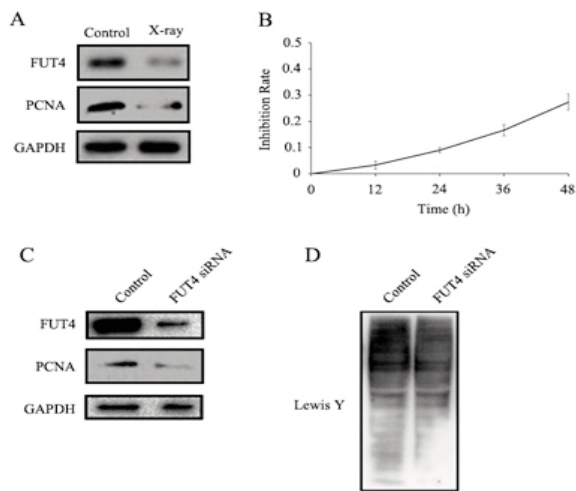


Figure-1: X-ray inhibited A549 cells proliferation by down-regulating fucosyltransferase IV (FUT4) expression. (A) Western blot analysis was used to assess the FUT4 and PCNA protein level in A549 cells after 10Gy X-ray irradiation. (B) The inhibition rate of A549 cells after irradiation was tested by CCK8. (C) The expression of FUT4 and PCNA after FUT4 siRNA detected by western blot. (D) Lewis Y (LeY) expression after FUT4 siRNA detected by western blot.

X-ray inhibited FUT4 expression by down-regulating SP1 in the nucleus. The molecular biological mechanism of X-ray induced FUT4 reduction. The expression of FUT4 and LeY in X-ray irradiated A549 cells treated with PARP inhibitor 3-AB was higher than the cells without 3-AB, and the decrease of FUT4 and LeY after 3-AB treatment was significantly reduced (Figures 2A-B). The expression of γ H2AX was significantly increased after X-ray irradiation (Figure 2C). DAPI staining showed that the staining was deepened after X-ray irradiation, and apoptotic bodies could be observed (Figure 2D). The expressions of cleaved PARP-1 and pADPr increased after X-ray irradiation, but decreased after the addition of 3-AB, while the expression trend of SP1 was the opposite, but not significant (Figure 2E). In the EMSA assay, the affinity of SP1 for DNA decreased after irradiation, but it was significantly increased after the addition of 3-AB (Figure 2F). The immunoprecipitation assay showed that the pADPr bound to SP1 protein increased after irradiation, but decreased after the addition of 3-AB (Figure 2G). After irradiation, the expression of SP1 in nucleoproteins significantly decreased, while SP1 in

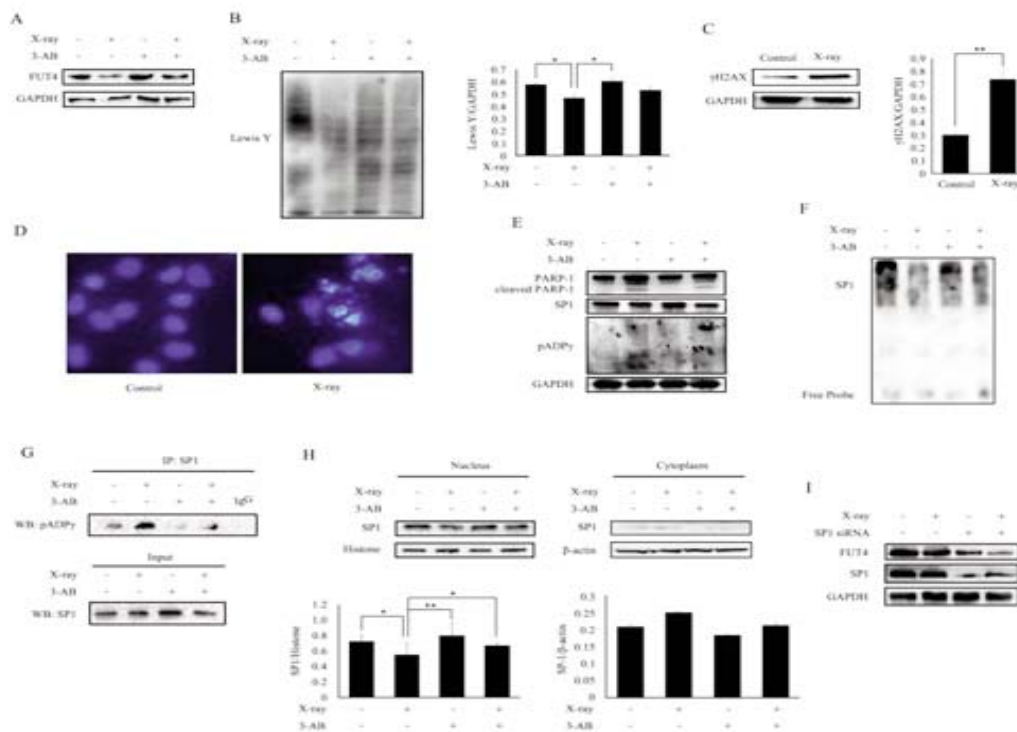


Figure-2: X-ray inhibited fucosyltransferase IV (FUT4) expression by down-regulating SP1 in the nucleus. FUT4 (A) and Lewis Y (LeY) (B) expression after 10Gy X-ray irradiation or treated with Poly-adenosinediphosphate[ADP]-ribose polymerase (PARP) inhibitor 3-aminobenzamide (3-AB) by western blot. (C) γ H2AX expression after irradiation by western blot. (D) DAPI staining after irradiation. (E) PARP-1, SP-1 and pADPr expression after irradiation or treated with 3-AB by western blot. (F) The affinity of SP1 for deoxyribonucleic acid (DNA) was detected by electrophoretic mobility shift assay (EMSA) assay. (G) The interaction between SP1 and pADPr after irradiation or treated with 3-AB by immunoprecipitation. (H) The expression of SP1 in nucleus or cytoplasm by western blot. (I) FUT4 and SP1 expression after SP1 siRNA by western blot. *: $p < 0.05$, **: $p < 0.01$.

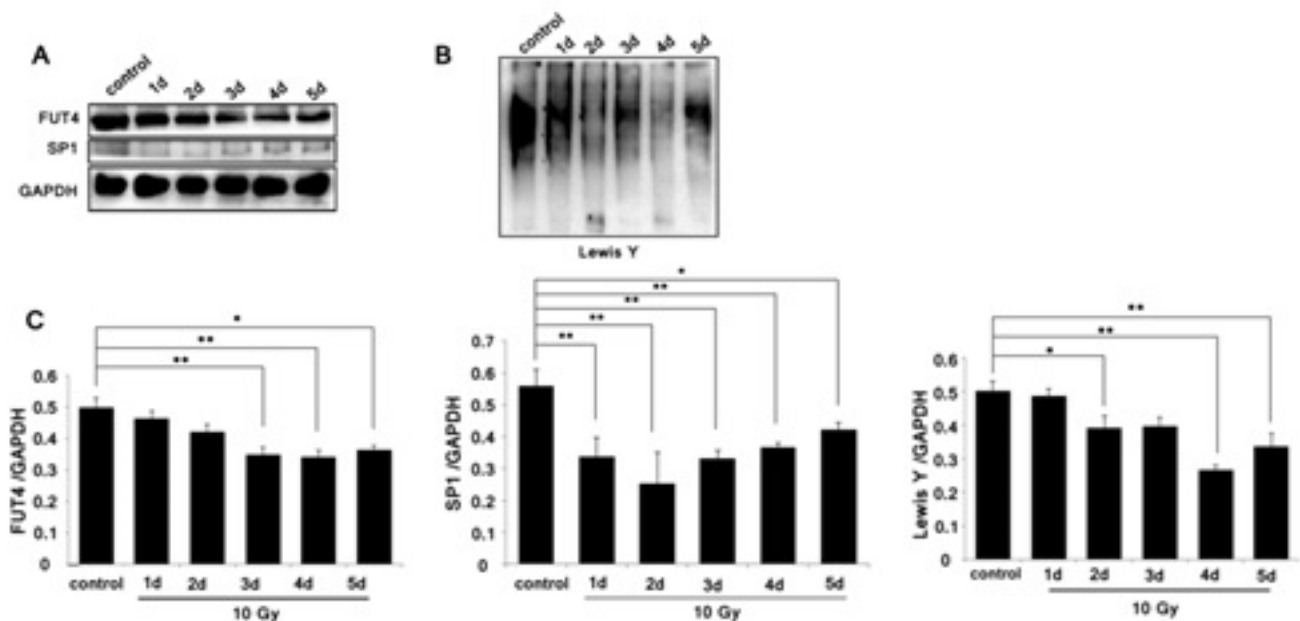


Figure-3: X-ray decreased fucosyltransferase IV (FUT4) expression and had time limitation. Changes of FUT4, SP1 (A) and Lewis Y (LeY) (B) expression after 10Gy X-ray irradiation by Western blot in 5 days. (C) *: $p < 0.05$, **: $p < 0.01$.

cytoplasmic proteins increased, and 3-AB could inhibit the change (Figure 2H). A549 cells were transfected with SP1 siRNA and the expression of FUT4 decreased after the decrease of SP1 expression (Figure 2I).

X-ray decreased FUT4 expression and had time limitation. The expressions of SP1, FUT4 and LeY protein decreased after X-ray irradiation (Figures 3A-B). SP1 decreased the most on the 2nd day after irradiation; the expression of SP1 increased gradually on the 3rd day after irradiation; the expression of FUT4 was the lowest on the 3rd and 4th days after irradiation, and increased on the 5th day (Figure 3C). The expression of LeY was the lowest on the 4th day after irradiation, and increased on the 5th day after irradiation (Figure 3C).

Discussion

Radiotherapy is one of the main treatments for lung cancer, but in some patients the effect of radiotherapy is not significant, which also leads to the failure of lung cancer treatment¹². Multiple studies have shown that radiation-resistance is associated with multiple signalling pathways in lung cancer cells¹²⁻¹⁴. However, the mechanism of radiotherapy in lung cancer needs further investigation. Therefore, the current study explored the mechanism of X-ray commonly used in radiotherapy for lung cancer.

Glycosylation, as a common post-translational

modification, participates in ligand binding, signal transduction, and molecular adhesion, and is closely related to cell growth, apoptosis, motility and differentiation¹⁵. Abnormal glycosylation is a characteristic change of malignant transformation of cells and involved in tumour progression, immune modulation and metastasis¹⁵. Toth et al. reported that glycosylation of plasma proteins does change in response to partial body irradiation (~60 Gy), and the effects last during follow-up¹⁶. However, there are relatively few studies on the effect of radiotherapy on the glycosylation of tumour cells, and the mechanism of radiotherapy on the surface glycans of tumour cells is still unclear. The current study found that the expression of LeY decreased after X-ray irradiation in lung cancer A549 cell line, which also confirmed that X-ray had certain effects on the glycans of lung cancer cells.

The change of glycosylation in tumour cells is caused by the change of glycosyltransferase expression in Golgi apparatus. Fucosyltransferase is a key enzyme in the biosynthesis of glycan complexes and plays an important role in the proliferation, invasion and metastasis of cancer cells as well as tumour immune monitoring¹⁷. FUT4, a member of the fucosyltransferase family, can synthesise LeY oligosaccharides¹⁸. Studies have shown that FUT4 regulates multiple signal pathways in tumours, which is related to tumour proliferation, apoptosis and metastasis^{5,6,18-20}. The present study found that the

expression of FUT4 and cell proliferation in A549 cells decreased after X-ray irradiation. However, after interfering with FUT4 expression with siRNA, LeY expression and cell proliferation decreased, indicating that X-ray reduced LeY synthesis and led to decreased cell proliferation by down-regulating the expression of FUT4.

The current study also explored how X-ray affects FUT4 expression. X-ray irradiation mainly kills cancer cells through DNA damage to achieve the purpose of cancer treatment²¹. DNA damage caused by X-ray was confirmed by detecting the expression of protein γ H2AX and DAPI staining in the current study. PARP, a multifunctional protein posttranslational modification enzyme, is present in most eukaryotic cells and activated by recognising DNA fragments with structural damage, playing an important role in DNA damage repair and apoptosis²². Liu et al. reported that PARP inhibitor increased the sensitisation to radiotherapy in FaDu cells²³. The current found an increase in PARP-1 and cleaved PARP-1 expression in A549 cells after X-ray irradiation. It indicated that apoptosis and DNA repair occurred after radiation, and the tumour cells initiated self-repair. However, the study found that the expressions of FUT4 and LeY were increased after the addition of PARP inhibitor 3-AB to A549 cells. Besides, the expressions of FUT4 and LeY in 3-AB treatment group after X-ray irradiation were significantly higher than those in group without 3-AB treatment. The results suggested that PARP inhibitors might decrease the sensitivity to X-ray irradiation, which is in contrast to earlier results²³, which might be related to differences in the cells studied. SP1 is a basal transcription factor that is closely related to cell proliferation, differentiation, DNA damage response, apoptosis, aging and angiogenesis, which is highly expressed in many cancers and is associated with poor prognosis²⁴. The current study found that the expression of pADPr increased significantly after X-ray irradiation, but the change of SP1 was not significant. The affinity between SP1 and DNA decreased after irradiation. However, the current study interestingly found that there was an interaction between pADPr and SP1 by immunoprecipitation, the SP1-bound pADPr significantly increased after X-ray irradiation. It was also found that the expression of SP1 in the nucleus decreased and cytoplasm increased after irradiation. These results indicated that the expression of PARP increased after X-ray irradiation and resulted in the increase of pADPr expression and its binding to SP1, causing SP1 to move out of the nucleus and into the cytoplasm. Yang et al. reported that SP1 regulated the expression of FUT4⁵. The current study used siRNA to inhibit the expression of SP1, and found that the expression of FUT4 and LeY decreased

significantly after SP1 interference, which was consistent with earlier results⁵.

The killing effect of X-ray irradiation on tumour cells is not lasting, and as the tumour cells repair themselves, they return to their original traits. Therefore, fractionated radiotherapy is given to tumour patients clinically. The current study explored the time of influence of X-ray on A549 cells, and found that the expression of SP1 was the lowest on the 2nd day, while FUT4 and LeY had it on the 4th day after irradiation, after which the expression gradually increased and recovered to the pre-irradiation level. These results indicated that the effect of X-ray on lung cancer cells was only temporary, and it needed to be irradiated several times in order to obtain a better therapeutic effect.

The current study has its limitations. The impact of X-ray irradiation on non-cancerous cell line was not evaluated, and no in vivo test was done.

Conclusion

DNA damage caused by X-ray increased pADPr bound to transcription factor SP1, leading to SP1 removal from the nucleus, and thus decreased FUT4 and LeY expressions, leading to decreased A549 cell proliferation. This effect could only last 2-4 days, and cell proliferation began to gradually recover. Findings suggest X-ray can affect glycan on the surface of tumour cells and reduce the proliferation of tumour cells. SP1 may become a new target treatment site for lung cancer. PARP inhibitor 3-AB could reverse this change and reduce the effect of X-ray on cell proliferation, indicating that PARP inhibitor combined with radiotherapy might not be a good recommendation for the treatment of lung cancer.

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Conflict of Interest: None.

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