

The Preliminary Study for Postoperative Radiotherapy Survival Associated with *RUNX3* and *TLR9* Expression in Lung Cancer

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Background: Many studies have reported that the inflammatory immune response related to *TLR9* signaling activation participates in tumor development and affects the treatment outcome. *RUNX3* functions as a tumor suppressor by regulating DNA methylation. *RUNX3* protein plays an important role in TGF- β signaling pathway that is involved in tumor growth inhibition and apoptosis. At present, radiotherapy is still an important treatment in lung cancer, which induces immune response and affects the therapeutic outcome. The role of *TLR9* signaling activation and *RUNX3* in this process is not clear.

Methods: In this study, we investigated the expression of *TLR9* in tumor and *RUNX3* in surrounding tissues by immunohistochemical methods and analyzed the relationship on postoperative survival in lung cancer.

Results: We found that the high expression of *TLR9* was the risk factor in postoperative survival of lung cancer with no difference in lifetime. The high expression of *RUNX3* in lung cancer with *TLR9* signaling activation was in favor of progression-free survival and overall survival in postoperative radiotherapy. It suggested that *RUNX3* played an important role in lung cancer radiotherapy. In order to determine the effect of *RUNX3* in lung cancer radiation with *TLR9* signaling activation, we introduced 5-Aza-2'-deoxycytidine (5-Aza-CdR) and exposed lung cancer A459 cells repeatedly. The high expression of *RUNX3* especially *RUNX3-B* in cells treated with 5-Aza-CdR was observed. We examined that 5-Aza-CdR induced more cell blocking in G2/M phase in combining irradiation.

Conclusion: The result implied that it was feasible to improve radiosensitivity of lung cancer with *TLR9* signaling activation by increasing *RUNX3* expression, and 5-Aza-CdR was an option in this process.

Keywords: *TLR9* signaling activation, *RUNX3* expression, 5-Aza-CdR, radiotherapy, lung cancer

Introduction

Although radiotherapy is one of the most effective treatments for non-small-cell lung carcinoma, local recurrence and metastasis are the main reasons for the failure of clinical treatment. There is an urgent need to explore the relationship of radiotherapy and tumor development for lung cancer treatment currently.

TLR9 is a member of toll-like receptor family, binding DNA in bacteria and viruses. *TLR9* is a pattern recognition receptor connecting innate and adaptive immune systems.¹ The researchers have demonstrated that *TLR9* is expressed in multiple cancer cells.² The signaling activation causing inflammatory cytokine response such as TNF α ,

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IL-1, IL-6, IL-8 promotes tumor proliferation and microenvironment development, contributing to a huge impact on clinical tumor treatment.^{3,4} Ren et al reported that the activation of *TLR9* signal in lung cancer cells upregulated inflammatory cytokines, MMP-2, chemokines, and immunosuppressive factor (IL-10) levels, leading to significant enhancement of tumor proliferation, invasion and metastasis.^{5,6} CpG-oligodeoxynucleotide induced *TLR9* activation increasing prostate cancer metastasis has been confirmed.⁷ And the correlation of *TLR9* high expression and poor tumor grading or later tumor staging had been reported in prostate cancer, glioma and esophagus tumor.^{8–10} *RUNX3* is a protein-coding gene that functions as a tumor suppressor and regulates cell development, apoptosis, signaling transduction, etc.^{11,12} Recently it was reported that *RUNX3* has a regulatory function on tumor immunity, whose upregulation can facilitate the expression of T-bet and promote Th1 immune response.^{13–15} Irradiation triggering signaling cascade and leading to upregulation of more than 60 genes involved in cell cycle regulation, DNA damage and repair, apoptosis, oncogenes and tumor suppressor genes, cell metabolism, etc have reported.¹⁶ And how *TLR9* signal activation and *RUNX3* expression influence radiotherapy outcome have rarely been reported. In this study, we surveyed the effect of *TLR9* in tumor tissues and *RUNX3* in adjacent carcinoma tissues on postoperative radiotherapy survival in lung cancer. 5-Aza-2'-deoxycytidine (5-Aza-CdR) is one of the inhibitors of DNA methyltransferase. Studies have shown that epigenetic changes of DNA methylation and histone modification affect tumor development and treatment.¹⁷ It is believed that DNA methyltransferase could inhibit transcription of target genes by forming transcription repressors with histone methyltransferase at promoter region.¹⁸ The application of 5-Aza-CdR and the analog 5-azacitidine in tumor chemoradiotherapy has been reviewed.^{19,20} We had determined the radiation resistance in lung cancer A549 cells exposed repeatedly in preliminary study, which *TLR9* expression upregulated.²¹ In the study we would investigate the role of *RUNX3* in 5-Aza-CdR combined radiotherapy for cells with *TLR9* signal activation and further elucidated that it was significant in raising *RUNX3* expression for lung cancer radiotherapy.

Materials and Methods

Clinical Information Related to the Cases

Sixty-three patients were enrolled in the study for tumor resection and postoperative chemoradiotherapy in JinShan

Hospital of Fudan University from March 1, 2010 to December 31, 2011. Fifty-two males and 11 females were selected. The oldest patient was 76 years old, and the youngest was 36 years old. The median age was 63 years. Forty-two cases had a smoking history. All patients received a preoperative pathological diagnosis. Sixty-one patients accepted lobectomy and lymph node dissection while two patients took unilateral pneumonectomy. Thirty-six squamous carcinomas apart from three sarcomatoid carcinomas and 27 adenocarcinomas, including three adeno-squamous carcinomas were diagnosed pathologically after tumor resection. There were 28 cases of mediastinal lymph node metastasis. The postoperative tumor tissues of 63 patients were selected as the effective specimens for this experiment. This was conducted in accordance with the Declaration of Helsinki. The study was approved by the Ethics Committee of Jinshan Hospital, Fudan University (Shanghai, China), together with the confirmation that all patients whose tissue was used provided written informed consent.

Thirty-six patients received radiotherapy within three months postoperatively. The conditions for postoperative radiotherapy were as follows: positive edge-cutting and/or mediastinal lymph node metastasis, KPS score ≥ 85 and FEV 1.0 of pulmonary function $\geq 80\%$. The signature confirmation for postoperative radiotherapy was signed. Intensity-modulated radiation therapy (IMRT) was adopted for postoperative radiotherapy. The target drawings referred to the NCCN non-small-cell lung cancer clinical practice guidelines. Clinical target volume (CTV) put on 5–8 mm externally as the planning target area (PTV). Ninety-five percent isodose line covering the 100% volume of PTV was approved. The dose limits of dangerous organs was as follows: the side lung V30 $\leq 25\%$, total lung V20 $\leq 25\%$, mean dose of the side lung ≤ 18 Gy, heart maximum dose ≤ 45 Gy, spinal cord ≤ 45 Gy. The plan was administrated by 6 MV X-ray for the total dose 50 Gy/25 Fx, adding up to 56–60 Gy/28–30 Fx for the patient's positive surgical margin. Thirty-six patients completed postoperative radiotherapy with no serious side effects.

The follow-up began three months after surgical treatment and/or chemoradiotherapy and ended in February 2015. It was once every three months for two years and every six months for two years. The median for follow-up was 38 months. The time from the first visit to tumor recurrence was determined as progressive-free survival (PFS). The time from the visit ending or death

in follow-up was defined as overall survival (OS). Forty-eight patients died of lung cancer and one non-neoplastic death. Twelve patients were alive except for four survival with tumor recurrence and two lost. The ratio of follow-up was 96.8%. The outpatient booking for follow-up was preferred, letter or telephone visit as a supplement.

Expression of *TLR9* and *RUNX3* in Cancer Tissues

The wax blocks of lung cancer tissues were cut into sections of 4 μm , fixed and labeled. After being deparaffinized, hydrated and antigen retrieval, the sections were treated with 3% hydrogen peroxide and 1% FBS. Diluted primary antibody (Rabbit Anti-*TLR9* polyclonal antibody 1:1000 and Rabbit Anti-*RUNX3* monoclonal antibody 1:1000, Abcam, US) was added and incubated at 4 $^{\circ}\text{C}$ overnight. HRP-Polymer Antimouse Immunohistochemistry (IHC) Kit (KIT-5930 Maixin Biology, Fuzhou, China) was incubated for 30 min at room temperature, followed by diaminobenzidine for color development. Finally the sections were counterstained with hematoxylin and mounted with Permount. The scores were determined by the proportion of stained cells and intensity. The cells binding *TLR9* protein in tumor tissues and *RUNX3* protein in paracarcinoma tissues was counted respectively. The proportion of stained cells was graded as follows: 0 (no stained cells), 1 (10% stained cells), 2 (10–50% stained cells) and 3 (50% stained cells). The intensity of the stain was recorded on a scale of 0 (no staining), 1 (weak staining, light yellow), 2 (moderate staining, yellowish brown) and 3 (strong staining, brown). The staining index was calculated as follows: staining index = staining intensity + score of stained cells. Tissue sections with staining index over 4 were defined as high expression and staining index less than 4 as low expression.

Preparation for Lung Cancer A549 Cell with *TLR9* Signal Activation and the Experimental Groups

Human lung cancer A549 cell line from Shanghai Tumor Hospital, Fudan University (Shanghai, People's Republic of China) was approved by the Ethics Committee of Jinshan Hospital, which was used for the preparation of the target cell line based on our previous study. A549 cell in logarithmic growth was exposed 6 MV X-ray, source to surface distance (SSD=100 cm) irradiation with dose rate

2 Gy/min and once 6.37 Gy for 10 exposures.²¹ The irradiation was performed once again until 63.7 Gy. And this cell line was designated as lung cancer A549 cell with high-*TLR9* expression (A549^{high-*TLR9*}). 5-Aza-CdR (Merck Drugs & Biotechnology, Sigma-Aldrich, Germany) was prepared for experimental cells in 0.5, 1.0, 2.0, 5.0 and 10 $\mu\text{mol/L}$, respectively. The absorbance was measured by microplate spectrophotometer at 450 nm. According the measurement, 5.0 $\mu\text{mol/L}$ 5-Aza-CdR and 10 $\mu\text{g/mL}$ chloroquine (CQ) were adopted for the treatment in experimental cells.

The cells were divided into six groups: A549 group, A549^{high-*TLR9*} group, A549^{high-*TLR9*}/IR (cell irradiation) group, A549^{high-*TLR9*}+CQ/IR (chloroquine combining irradiation) group, A549^{high-*TLR9*}+5-Aza-CdR/IR (5-Aza-CdR combining irradiation) group, A549^{high-*TLR9*}+5-Aza-CdR (5-Aza-CdR treatment) group. The cells were administrated in serum-free medium with 10 $\mu\text{g/mL}$ chloroquine and 5 $\mu\text{mol/L}$ 5-Aza-CdR respectively before IR 30 min.

Detection of *TLR9* and *RUNX3* Protein by Western blotting

After cell disruption and centrifugation, protein extracts concentration were determined by the BCA method and separated by 10% SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membranes (EMD Millipore, Billerica, MA, USA). The membrane was blocked with 10% nonfat dry milk in Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBS-T) for 2.5 h at room temperature and then incubated with the following primary antibodies at 4 $^{\circ}\text{C}$ overnight: Rabbit *TLR9* monoclonal antibody 1:5000 (Abcam, USA), Rabbit *RUNX3* monoclonal antibody 1:1000 (Abcam), Mouse GAPDH monoclonal antibody 1:3500 (Proteintech, USA). Membranes were washed with TBS-T three times and probed with secondary antibodies for two hours at room temperature. Protein bands were visualized after incubation with chemiluminescence HRP substrate (EMD Millipore).

Cycle Distribution of Cells in Experimental Groups

The cells were exposed with 6 MV X-ray, a single dose of 10 Gy. The cell culture was replaced once after irradiation 24 h. The cells irradiated 0 h, 12 h, 24 h, 36 h, 48 h were harvested and fixed with 75% ethanol at 4 $^{\circ}\text{C}$ overnight, then incubated with 500 μL propidium iodide/RNase staining buffer (BD Biosciences) at room temperature for 15 min. After

centrifugation and PBS washing, the cell cycle reagent PI (50 mg/mL) was added to suspended cells avoiding light at 4°C for 20 min. The cell cycle was determined by flow cytometer.

Statistical Analysis

SPSS 17.0 statistical software (SPSS, Inc., Chicago, IL, USA) was used in the study. The one-way variance analysis of *TLR9* and *RUNX3* and clinicopathological features was tested by chi-squared test. The risk assessment of factors on postoperative survival of lung cancer was measured by logistic regression. The prediction of factors associated on postoperative radiotherapy survival was examined by Cox regression. The life tables were used for calculating the survival time related to *TLR9* and *RUNX3*. The measurement data showed on $\bar{x} \pm s$ and analysis of variance was adopted in comparison of the groups. The statistical figures were made by GraphPad Prism 5.0 software. $P < 0.05$ was regarded as statistically significant.

Results

The Relationship of *TLR9* and *RUNX3* and Postoperative Radiotherapy Survival in Lung Cancer

The expression of *TLR9* in tumor tissues and *RUNX3* adjacent to cancer was shown in Figure 1. Forty-three and 28 of 63 patients presented positive expression of *TLR9* and *RUNX3* respectively. The relationship of *TLR9* and *RUNX3* and clinicopathological characteristics by one-

way variance analysis was presented in Table 1. The results displayed that the high expression of *TLR9* in cancer tissues emerged in later T stage significantly ($\chi^2=8.620$, $P=0.013$), which the high expression of *RUNX3* in paracarcinoma tissues was likely to in earlier T stage ($\chi^2=5.920$, $P=0.052$). *RUNX3* expression in non-squamous carcinoma showed statistic difference, the chi-squared value being 4.200 and $P=0.040$. The expression of *TLR9* and *RUNX3* in tumor differentiation, N staging, and postoperative radiotherapy had no distinction.

Then we conducted the risk assessment on *TLR9*, *RUNX3* and above clinical features for postoperative survival in lung cancer by logistic regression, shown in Table 2. The result revealed that the relative risk value associated with tumor T staging and *TLR9* expression in the model was 2.716 and 5.057, respectively. It suggested that the high expression of *TLR9* was harmful for postoperative survival in lung cancer with the P -value 0.019 significant. The median time of PFS and OS associated with *TLR9* expression was shown in Table 2, with no difference in postoperative or radiotherapy patients. The cumulative survival ratio on *TLR9* expression in postoperative or radiotherapy patients was exhibited in Figure 2. Subsequently the median survival time of PFS and OS in postoperative radiotherapy associated with *TLR9* and *RUNX3* expression was analyzed and shown in Table 3. The statistical value of PFS and OS in postoperative radiotherapy related *RUNX3* expression and negative *TLR9* was 1.905 and 4.224, respectively, $P=0.168$ and 0.040. With positive *TLR9* it was 6.097 and 6.521, respectively, $P=0.014$ and 0.011 significantly, as shown in

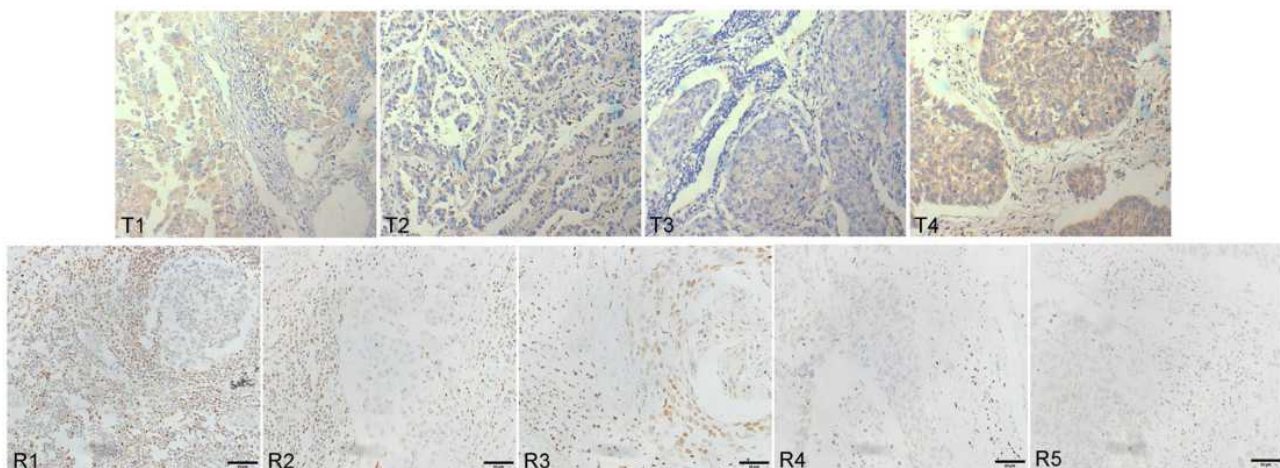


Figure 1 The expression of *TLR9* and *RUNX3* in lung cancer tissues (immunohistochemical SP method). Expression of *TLR9* in tumor tissues (T1-T4) and *RUNX3* in adjacent to cancer (R1-R5) was examined with DAB magnification 400X. Compared with normal bronchoalveolar cells, tumor cells became larger in volume and abnormally clumped together. *TLR9* protein stained yellow or brown distributed equality in cytoplasm of tumor cells with unusual karyomegaly, multileaf nuclear and nuclear membrane thickened, etc. *RUNX3* protein stained yellow or brown granular distributed in nucleus.

Table 1 The One-way Analysis of Variance on *TLR9* and *RUNX3* Expression in Lung Cancer Tissues

	n	<i>TLR9</i> Expression		χ^2	P	<i>RUNX3</i> Expression		χ^2	P	
		Low (%)	High (%)			Low (%)	High (%)			
Pathology	Squamous carcinoma	36	11 (17.5)	25 (39.7)	0.055	0.815	24 (38.1)	12 (19.0)	4.200	0.040
	Nonsquamous carcinoma	27	9 (14.3)	18 (28.6)			11 (17.5)	16 (25.4)		
Differentiation	Highly	12	5 (7.9)	7 (11.1)	0.685	0.710	10 (15.9)	2 (3.2)	5.181	0.075
	Moderately	30	9 (14.3)	21 (33.3)			16 (27.8)	14 (22.2)		
	Poorly	21	6 (9.5)	15 (23.8)			9 (14.3)	12 (19.0)		
Tumor (T)	T1 (≤ 3 cm)	17	10 (15.9)	7 (11.1)	8.620	0.013	6 (9.5)	11 (17.5)	5.920	0.052
	T2 (3 cm $<$ T ≤ 5 cm)	26	7 (11.1)	19 (30.2)			14 (22.2)	12 (19.0)		
	T3 ($>$ 5 cm)	20	3 (4.8)	17 (27.0)			15 (23.8)	5 (7.9)		
Lymph	No	36	14 (22.2)	22 (34.9)	1.978	0.160	22 (34.9)	14 (22.2)	1.050	0.306
	Yes	27	6 (9.5)	21 (33.3)			13 (20.6)	14 (22.2)		
Postoperative radiotherapy	No	27	8 (12.7)	19 (30.2)	0.098	0.755	17 (27.0)	10 (15.9)	1.050	0.306
	Yes	36	12 (19.0)	24 (38.1)			18 (28.6)	18 (28.6)		

Notes: Based on the principles of the AJCC (American Joint Committee on Cancer) and the UICC (International Union Against Cancer) in 1997, the criterion of newly revised TNM staging were adopted to clinical stage of lung cancer.

Table 2 The Correlation of *TLR9* Expression in Cancer Tissues and Postoperative Survival

(A) The risk evaluation of <i>TLR9</i>, <i>RUNX3</i> and cliopathological factors in lung cancer by Logistic Regression Model								
	B	SE	Wald	df	Sig.	Exp(B)	95%CI for EXP(B)	
							Lower	Upper
T stage	0.999	0.493	4.103	1	0.043	2.716	1.033	7.143
<i>TLR9</i>	1.621	0.693	5.465	1	0.019	5.057	1.299	19.678
Constant	-1.630	0.921	3.137	1	0.077	0.196		
(B) The postoperative survival analysis on <i>TLR9</i> expression in lung cancer								
		PFS (Med time: mon)		OS (Med time: mon)				
		Postoperation	Radiotherapy	Postoperation	Radiotherapy			
<i>TLR9</i>	Negative	18.00	16.50	36.00	27.00			
	Positive	19.50	16.88	34.12	29.57			
Wilcoxon statistic		0.014	0.010	1.241	0.030			
Sig.		0.906	0.921	0.265	0.958			

Figure 3. This result implied that the patients with *TLR9* signaling activation was a survival benefit from postoperative radiotherapy in *RUNX3* high expression. With Cox regression for PFS on lung cancer radiotherapy, the risk evaluation of tumor differentiation, T staging and *RUNX3*

in equation in **Table 3** was statistic significance, $P=0.026$, 0.055 and 0.001, respectively. The relative risk was 2.109, 1.861 and 0.178 with 95%CI for Exp(B) (1.093–4.071), (0.987–3.509) and (0.067–0.475). The result illuminated that the high expression of *RUNX3* was helpful for

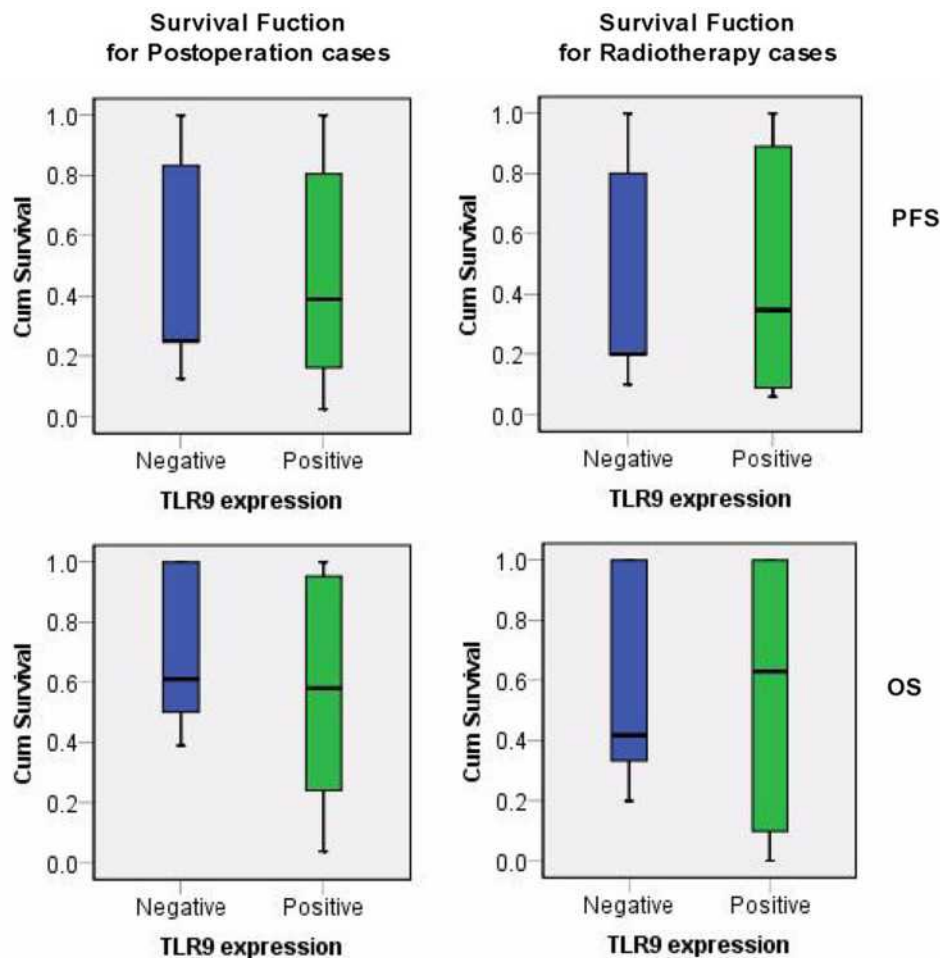


Figure 2 The survival function of lung cancer postoperation related *TLR9* expression. X axis was identified for *TLR9* status, Y axis indicated the cumulative survival of PFS and OS on lung cancer postoperation and radiotherapy respectively.

Note: PFS and OS refer to progression-free survival and overall survival respectively.

postoperative radiotherapy survival in lung cancer. It was meaningful for upregulating *RUNX3* expression to improve radiotherapy outcome in lung cancer.

RUNX3 Expression in Experimental Cells Before and After Radiotherapy

In order to demonstrate the effect of *RUNX3* in lung cancer radiotherapy, the levels of *TLR9* and *RUNX3* protein in experiment cells was detected by Western blotting (shown in Figure 4). Based on *RUNX3* antibody instructions and double-band pattern, *RUNX3-A* and *RUNX3-B* standing for the band with larger and smaller molecular weight, respectively were adopted to data analysis in the study. The results displayed the levels of *RUNX3* protein in 5-Aza-CdR treatment cells was increased especially

RUNX3-B before irradiation. Compared with *A549^{high-TLR9}* cells, *RUNX3-B* high expression in 5-Aza-CdR treatment cells was significance, with the *t* value 3.785, $P < 0.05$. The levels of *RUNX3* protein in cells post-irradiation were decreased with no difference. *TLR9* expression in *A549^{high-TLR9}* and *A549* cells was statistical significance, $t = 4.081$ and $P < 0.01$. It was the explanation for *TLR9* signal activation in *A549* cell exposure repeatedly.

Cycle Distribution Related *RUNX3* Upregulation in Lung Cancer Radiotherapy

The distribution of the cell cycle in experiment was tested, shown in Figure 5A. Compared with the cell irradiation

Table 3 The Effect of *TLR9* and *RUNX3* Expression on Postoperative Radiotherapy Survival of Lung Cancer

(A) The analysis of postoperative radiotherapy survival on <i>TLR9</i> and <i>RUNX3</i> expression									
		PFS (Med time: mon)		Wilcoxon Statistic	Sig.	OS (Med time: mon)		Wilcoxon Statistic	Sig.
		<i>RUNX3</i> expression				<i>RUNX3</i> expression			
		High	Low			High	Low		
<i>TLR9</i>	Negative	45.00	15.00	1.905	0.168	59.63	23.25	4.224	0.040
	Positive	21.00	12.00	6.097	0.014	34.72	24.00	6.521	0.011

(B) The Cox proportional hazard regression of <i>TLR9</i>, <i>RUNX3</i> and clinicopathological features for postoperative radiotherapy survival in lung cancer									
	B	S.E.	Wald	df	Sig.	Exp(B)	95%CI for EXP(B)		
							Lower	Upper	
Differentiation	0.746	0.336	4.945	1	0.026	2.109	1.093	4.071	
T stage	0.621	0.324	3.680	1	0.055	1.861	0.987	3.509	
<i>RUNX3</i>	-1.724	0.500	11.907	1	0.001	0.178	0.067	0.475	

only, the cell proportion in G2/M and G1/G0 in A549^{high-*TLR9*}+5-Aza-CdR/IR group was 44.8±1.76 and 45.73±1.67, respectively with the *t* values 14.15 and 13.52, *P*<0.001 equally. There was no change in cell distribution in 5-Aza-CdR treatment and the irradiation only group. The fewer G2/M cells and more G1/G0 cells in the A549^{high-*TLR9*}+CQ/IR group than that in A549^{high-*TLR9*}/IR group were examined. Furthermore we examined 5-Aza-CdR induced cells blocking in G2/M with maximization at irradiation 24 h and maintained for 36 h and 48 h after radiotherapy, as shown in Figure 5B. The distribution of S cells in chloroquine combining irradiation was more than in the A549^{high-*TLR9*}/IR group at 12 h with the *t* values 11.04 and *P*<0.001. Meanwhile we saw fewer G2/M cells and many more G1/G0 cells in A549^{high-*TLR9*}+CQ/IR group than the irradiated cells alone.

Discussion

Radiotherapy is a common clinical treatment for lung cancer patients. *TLR9* is a transmembrane protein. It is activated by binding unmethylated CpG sequences in DNA molecules to TIR domain.^{22,23} The researchers have demonstrated that irradiation leads to DNA damage and cells debris and proteins associated with hypoxia and necrosis activates *TLR9* signal cascades by endogenous DAMPs, which promotes

downstream pro-inflammatory cytokines synthesis. The result contributes to the alternative tumor micro-environment and host immunity and influences clinical treatment outcomes.²⁴⁻²⁶ *RUNX3* encodes a heterodimer comprised of a Runt domain containing α subunit and a DNA-binding β subunit.^{27,28} It is reported that the high expression of *RUNX3* in CD8+ T cells is essential to suppress transcription of CD4 silencer and regulates the expression of CD8+ T-cell specific genes.²⁹⁻³¹ The differentiation of Th1 cells is determined by transcription factor T-bet and the induction of *RUNX3* expression. The interaction between transcription factor T-bet and *RUNX3* causes large production of IFN- γ and the silence of *IL4* gene.^{15,32} However, the low expression of *RUNX3* due to DNA methylation at CpG site has also been demonstrated in various tumors.³³ For these reasons, the aim of this study was to investigate whether the high expression of *RUNX3* influences irradiation immune responses related *TLR9* signal activation and enhanced radiotherapy effects.

We characterized the expression of *TLR9* in tumor tissues and *RUNX3* in paracarcinoma tissues and explored the relationship with postoperative radiotherapy survival in lung cancer. The result present that *TLR9* expression and tumor T staging among clinicopathologic features were the variables of relative risk

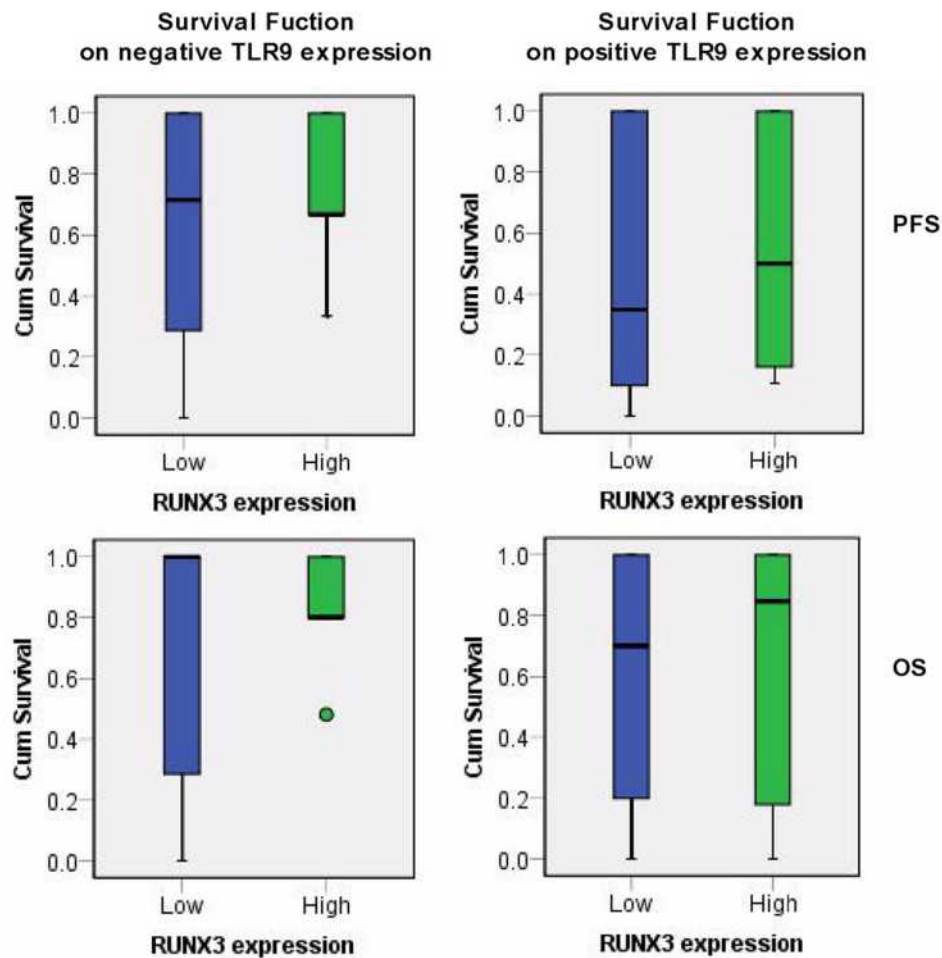


Figure 3 The survival function of lung cancer radiotherapy related *TLR9* and *RUNX3* expression. X axis was identified for *RUNX3* status, Y axis indicated the cumulative survival of PFS and OS on lung cancer radiotherapy with negative and positive *TLR9* expression.

Note: PFS and OS refer to progression-free survival and overall survival respectively.

in postoperative survival of lung cancer with statistic value 5.057 and 2.716, respectively. It suggested that *TLR9* signal activation was the disadvantage of postoperative survival in lung cancer. And we observe no difference in PFS and OS of postoperative or radiotherapy survival in lung cancer. However patients with *RUNX3* high expression had the better PFS and OS in postoperative radiotherapy, either positive or negative *TLR9* expression. This result confirmed that *RUNX3* expression in paracarcinoma tissues of lung cancer mediated irradiation effects and impact the survival on postoperative radiotherapy. The analysis of Cox regression on PFS in lung cancer radiotherapy revealed that the relative risk on tumor differentiation, tumor

T staging, and *RUNX3* was 2.109, 1.861, and 0.178, respectively. It was certain that the high expression of *RUNX3* was significant to improve radiotherapy outcomes associated with *TLR9* activation.

To test the effect of *RUNX3* on lung cancer radiotherapy related *TLR9* signal activation, we introduced 5-Aza-CdR and lung cancer A549 cell was exposed repeatedly and detected the expression of *TLR9* and *RUNX3* in experimental cells. The increasing levels of *TLR9* protein obviously in A549^{high-*TLR9*} cells was observed. We also examined the levels of *RUNX3* protein especially *RUNX3-B* in 5-Aza-CdR treatment cells was higher than in A549^{high-*TLR9*} cells. Furthermore we found that the G2/M cells in 5-Aza-CdR combining

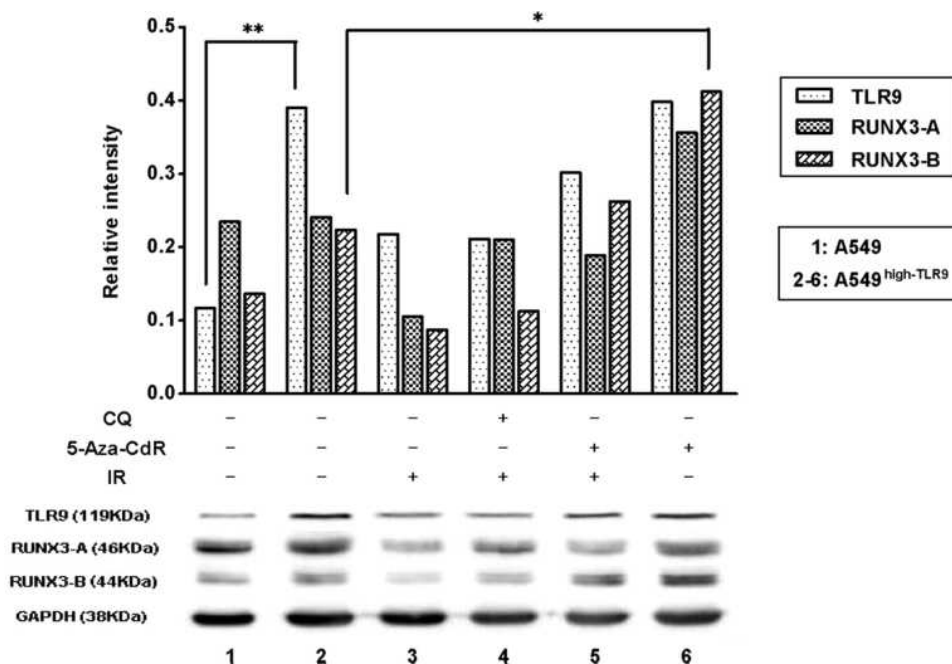


Figure 4 *RUNX3* and *TLR9* expression in cells before and after radiotherapy. The levels of *RUNX3* and *TLR9* protein in experimental cells were detected by Western blotting. *RUNX3-A* and *RUNX3-B* standard for the larger and smaller molecular weight band separately. The levels of *TLR9* protein in the A549^{high-TLR9} cell was significantly more than in the A549 cell, $t=4.081$ and $P<0.01$. It indicated that *TLR9* signaling activated in A549 cells exposure repeatedly. The result displayed that *RUNX3* protein especially *RUNX3-B* was increased in 5-Aza-CdR treatment cells. In comparison with the A549^{high-TLR9} cell, the increasing levels of *RUNX3-B* protein was significant, with the t value 3.785 and $P<0.05$. The decreased levels of *RUNX3* protein in cells after irradiation was observed with no difference.

Notes: $*t=3.785$, $P<0.05$; $**t=4.081$, $P<0.01$.

Abbreviations: IR, irradiation; A549^{high-TLR9} cell, A549 cell with *TLR9* signal activation; 5-Aza-CdR, 5-Aza-2'-deoxycytidine; CQ, chloroquine.

radiotherapy was sevenfold to the 5-Aza-CdR treatment group and twofold to the cell irradiated solely, respectively. It was maximized at irradiation 24 h and kept the irradiation advantage until 36 h and 48 h. It is known that the G2/M cells has the most sensitivity to irradiation. It was remarkable that 5-Aza-CdR treatment combined radiotherapy made more cells block in the G2/M phase and enhanced irradiation effect. In contrast, we observed the S cells prolonged in chloroquine combining radiotherapy, which was insensitive to X-rays. It suggested that only inhibiting *TLR9* signaling was unable to improve the radiation resistance related *TLR9* signaling activation. And this result also conformed to our first clinical investigation. Considering the higher proportion of G2/M cells in 5-Aza-CdR adjuvant radiotherapy we thought that

RUNX3 expression upregulation played a key role in this process. It showed the radiosensitization of 5-Aza-CdR in lung cancer with *TLR9* signaling activation by upregulating *RUNX3* expression. We concluded that it was necessary to raise *RUNX3* expression to promote the irradiation effect in lung cancer with *TLR9* signaling activation.

Conclusion

In summary, *TLR9* signaling activation involved in tumor progression and influenced the postoperative radiotherapy survival in lung cancer. It was notable that the increasing levels of *RUNX3* protein could contribute to reverse radioresistance in this process and improve the survival related lung cancer radiotherapy. Of course, 5-Aza-CdR was considerable.

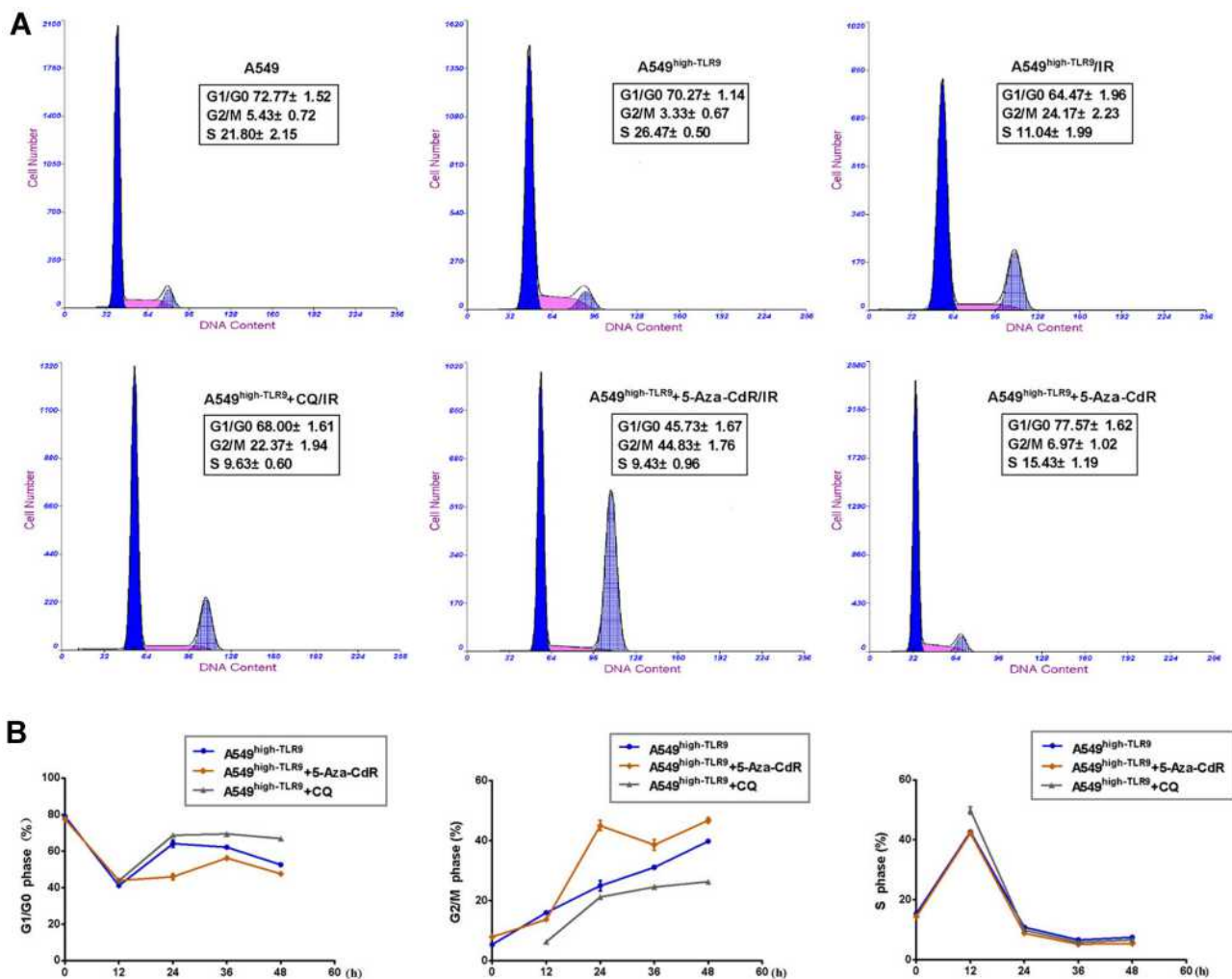


Figure 5 Comparison of cell distribution on experimental cells before and after radiotherapy. The cycle distribution in experimental cells before and after radiotherapy (**A**). The proportion of G2/M cells in 5-Aza-CdR treatment at irradiation 24 h was 44.83 ± 1.76 , twice that of solely irradiated cells significantly, $t=15.52$ and $P<0.001$. It maintained for irradiation 36 h and 48 h (**B**). The G1/G0 cells in 5-Aza-CdR adjuvant radiotherapy remained low for irradiation 48 h. The cells ratio in S phase in chloroquine combining radiotherapy at irradiation 12 h was 15.43 ± 1.19 , which was significantly more than the solely irradiated cells, $P<0.001$. The result was that after irradiation more cells kept in G2/M phase in 5-Aza-CdR treatment group as well more cells in S phase in chloroquine combining radiotherapy.

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Disclosure

The authors report no competing interests in this work.

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