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Original Article

Serum toll-like receptors are potential biomarkers of radiation pneumonia in locally advanced NSCLC

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Abstract: Objective: Toll-like receptors (TLRs) are highly or lowly expressed in a wide variety of tumors and exhibit either pro-tumor or anti-tumor activities. In the present study, we investigate whether there are relationships between the expressions of TLRs and the occurrence of radiation pneumonia in advanced NSCLC patients treated with radiotherapy. Materials and methods: 76 patients diagnosed with NSCLC and 50 healthy controls were recruited from Oct 2012 to Jan 2014. The expressions of serum TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, and TLR9 were detected by ELISA techniques. Fisher exact test, x^2 test, ROC working curve and Cox regression model were applied to analyze all data. Results: serum TLR1, TLR2, TLR4, and TLR9 exhibited a relative high expression level in NSCLC patients compared with healthy controls. Importantly, pre-neutrophil granulocyte ratio was associated with the expression of TLR1, TLR2, and TLR4. Moreover, the patients with high ratio of neutrophil granulocyte significantly increased the occurrence of fever in comparison to normal neutrophil ratio in NSCLC patients during the course of radiotherapy. We further evaluated the containing of TLRs when patients had temperatures and found serum TLR1, TLR2 and TLR4 were over-expressed. Finally, 26 of 76 patients were diagnosed with different stages of radiation induced pneumonia; as a result, the contents of TLR1 and TLR4 before radiotherapy were identified as independent significances with pneumonia occurrence. Conclusions: The pretreatment levels of TLR1 and TLR4 have the predictive value to be clinically potential biomarkers of pneumonia risk in locally advanced NSCLC.

Keywords: NSCLC, clinical biomarkers, serum toll-like receptors, radiation pneumonia risk

Introduction

Toll-like receptors are the main intra/extra-cellular immune cell receptors which play an important role in the induction of immune response via activating innate and adaptive immunity [1]. These receptors express in various kinds of immune cells such as B-lymphocytes, monocytes, plasmacytoid dendritic cells and at low levels in human respiratory cells, as well as epithelial cells [2]. TLRs can recognize pathogen associated molecular patterns (PAMPs) of infectious agents, and then activate the immune response including nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activation, migration, phagocytosis, and inflammatory cytokine expression. Until now this family of ten related molecules has been identified in humans [3, 4]. Structurally, TLRs are characterized by extracellular leucine-rich repeats and intracellular Toll/IL-1R (TIR) domains. The functional TLRs signal lead to the activation of transcription factors including activator protein-1 (AP-1) and nuclear factor-κB (NF-κB) via myeloid differentiation factor 88- or cytoplasmic Toll/interleukin-1 receptor domain (TIR) dependent pathways [5, 6]. In this way, TLRs mediate the production of proinflammatory cytokines and chemokine resulting in inflammation.

Chronic or recurrent inflammation plays an essential role in the development of many human tumors including urinary bladder, stomach, esophagus, large intestine and liver cancers, which process may be partly mediated through recognizing various stimuli like TLRs [7]. As reported, Toll-like receptors are expressed in sorts of cancer cells, what's more, function as modulators of tumor development

and progression [8]. On one side, activated TLRs signaling pathways stimulate tumorigenesis by promoting cell proliferation, invasion, and migration, as well as by inducing an immunosuppressive microenvironment [9]. On the other side, TLRs can also act as anti-tumor regulators by activating the immune system, inducing apoptosis, and enhancing chemo-sensitivity [10]. In brief, the seemingly two opposite effects are accounted by the different TLR types and specific tumor context.

Current standard strategies for un-resectable patients are combined treatment of chemotherapy, targeted-therapy and radiotherapy [11]. Radiation therapy using advanced technology has been identified as an excellent local treatment for NSCLC patients. Currently, numerous studies in recent years have reported the attempt to increase doses to improve local control rate and prolong survival time, nevertheless, the tolerance doses of surrounding normal tissues inhibited the implication on the targeted prescription doses, at the same time, the occurrence of radiation induced pneumonia (RP) was an independent risk factor and increased the risk of deaths [12, 13].

Recently, few researches were focused on developing novel diagnostic strategies for radiation induced pneumonia and no paper clarified the association of serum TLR-levels with the occurrence of RP. As a result, in this paper we firstly demonstrate that TLRs may serve as potential biomarkers to predict the occurrence of radiation induced pneumonia.

Materials and methods

Clinical data

This study was approved by the Airforce General Hospital, Beijing Chao-Yang Hospital and Tianjin Huanhu Hospital. All participants signed informed consent for the use of their blood samples prior to recruitment. All anonymous specimens were handled according to the ethical and legal standards.

A total of 76 eligible patients with NSCLC who had no previous treatment and 50 normal persons comprised control group were recruited into the study from Oct 2012 to Jan 2014. A total of 126 participants were investigated and the serum samples from these people were obtained during study. The recruited patients must attain the following eligibility criteria: age older than 18 years old; pathologically confirmed NSCLC; PET-CT or CT confirmed stage (UICC TNM classification 5th and 6th editions); Karnofsky score (KPS) ≥70; the diseases treated in the three designated hospitals and the complete clinical data obtained thoroughly with pathology reports. Patients who previously received chemotherapy or surgery as part of regiment treatment of NSCLC, who had a history of another type of invasive cancers, recent symptomatic pneumonia, prior RT to the pulmonary, prior chemotherapy therapy or the presence of any serious medical conditions were completely excluded from the study group. Volunteers who were not diagnosed previously with any type of cancer and didn't have a history of infection agents within 3 months were chosen and matched to the patients by age, gender and ethnicity.

Clinical evaluation and treatment description

ALL patients were immobilized in the supine position by plastic molds with arms up for CT simulation and treatment. CT scanning rage including the entire lung volume was performed at 5 mm scan thickness. The gross tumor volume (GTV) was determined by chest CT and defined as the visible tumor from the lung-window plus the visible lymph nodes from the mediastinal-window. The clinical target volume (CTV) was defined by extending the GTV with additional uniform 0.5-1 cm expansion. The supraclavicular nodal regions and uninvolved mediastinal were routinely excluded in the CTV, but high risk nodal stations near GTV nodes were included occasionally. The planning target volume (PTV) was obtained by a 0.7~1.0 cm margin 3-D expansion from the CTV. The organs at risk, such as, lung, heart, esophagus, spinal cord, and trachea were contoured layer by layer in the CT images. CT images with delineated targets and organs at risk were transferred to the TomoPlan 2.2.4.15 treatment planning systems via DICOM 3.0. The dose prescriptions were 66-70 Gy/20-25f for GTV, and 60 Gy/20-25f for CTV, 1 fractions/day, 5 fractions/week. The dose prescriptions were designed to cover 100% of the target volume. The dose limits for organs at risk were according to the RTOG criteria: lung V20 <30%, heart V50 <50%, a maximum dose to spinal cord ≤45 Gy and esopha-

gus V55 <50%. After making correction for the tissue inhomogeneity, the mean lung dose (MLD), V5, V10, V20 and V30 were calculated from the lung Dose Volume Histogram (DVH). Tomo-therapy platform (Accuary Inc., Sunnyvale, CA) was performed to deliver radiation treatments for lung cancers.

Follow-up and the definition of RP

All patients paid a visit to the hospital for follow-up at 0.5, 1, 3 and 6 months after radiotherapy. Follow-up evaluations included an interval history, physical examination, routine blood tests, and pulmonary functional tests. Chest CT was performed at 1, 3 and 6 months after RT or any time the doctors believing it was necessary. RP was evaluated by the symptoms of patients and reviewing all available radiographic images up to at least 6 months after treatment. Grading of the RP was recorded using the scale defined by Common Terminology Criteria for Adverse Events, version 3.0, which is based on the severity of clinical symptoms of patients with radiographic changes. It is outlined as follows: Grade 1 pneumonitis is for asymptomatic patients or who have mild symptoms (dry coughing or dyspnea on exertion) with radiographic findings; Grade 2 pneumonitis is defined as symptomatic, requiring medical management but not interfering with activities of daily living. Grade 3 pneumonitis is severely symptomatic RP that interfered with daily activities or required the administration of oxygen; Grade 4 pneumonitis is defined as severe respiratory insufficiency and continuous oxygen or assisted ventilation needed.

Serum TLRs extraction

Preoperative serum samples were collected from 76 patients with histologically proven NSCLC. As a control, serum was also collected from healthy volunteers (n=50). After collection, samples were centrifuged at 1,500 g for 20 min at 4°C. The supernatant fluids were then transferred into fresh collection tubes and stored at -80°C until further processing.

ELISA techniques

Human TLRs ELISA Kits were detected by the GBD ELISA system (GBD Systems, USA) following the manufacturer's instructions. In brief, 96-wellmicrotiter plates were coated for overnight at 4°C followed by washing with PBS and blocking with 6% skimmed milk in PBS for 1 h at 37°C. Serum samples were diluted in dilution buffer and 50 μL of diluted serum and standards was added in the appropriate well of the antibody pre-coated microtiter plates. All standards and samples were added in duplicate to the plates. 10 μL of biological was added to sample wells. And then 50 μL of enzyme conjugate was added to each well. The plates were incubated for 1 h at 37°C and washed five times with PBS. Following washing steps, 50 μL Substrate A&B was added in each well and plate was kept incubating for 15 minutes at 20-25°C in dark. Add to each well. Finally, the reaction was stopped by addition of 50 μL of stop solution and the absorbance was read at 450 nm in a microtiter plate reader within 30 minutes.

Statistical analysis

SPSS 13.0 statistical software was applied to analyze all data. X^2 test and Fisher exact test were used to classify datum. ROC working curve was used to analyze the value of TLRs function prognostic evaluation in the patients; the risk factors of radiation pneumonia in patients were analyzed by Cox regression model. Comparisons between the two groups were using t test, and the Kruskal-Wallis test was used to compare more than two groups. *P* value of <0.05 was considered statistically significant for each analysis.

Results

Clinical characteristics of patients

In total, 76 NSCLC patients and 50 healthy volunteers participated in the present study. All patients were of Chinese ethnicity with a male predominance (63.2%), and included 28 females. The median age at diagnosis of NSCLC was 58-year old (ranging from 35 to 78). The median Karnofsky Performance Status was 80 (range 70-100). There was no patient performing systematic medications or other therapies before consulting to our departments. 36 of 76 patients were identified with squamous cell carcinoma, and the other 40 patients were confirmed as adenocarcinoma; 42 patients were Stage III A, and 34 patients were Stage III B according to UICC TNM classification. 86.7% received a combination of chemotherapy and

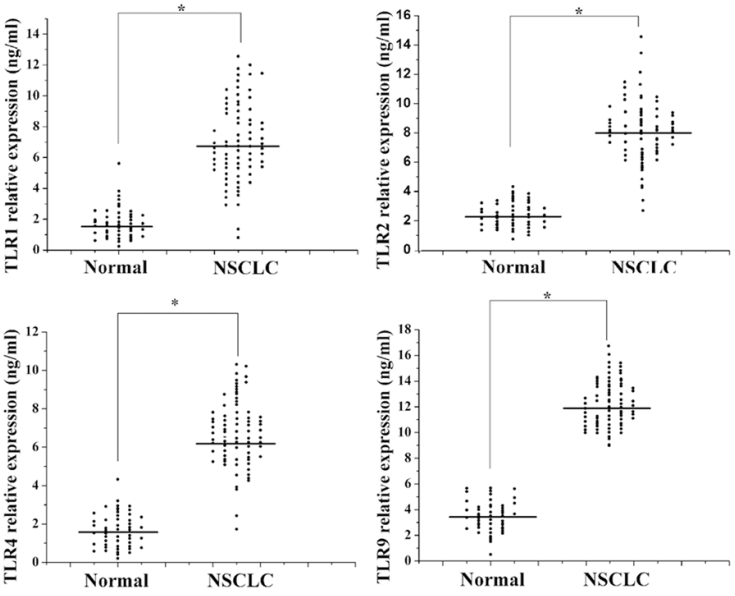

Figure 1. Serum TLR1, TLR2, TLR4, and TLR9 exhibited a relative high expression level in NSCLC patients compared with healthy controls (P < 0.01).

radiation therapy. The results of the pulmonary function test for all the patients before RT were as follows: the median FEV1 was 1.85 L, the median FEV1/FVC 69% and the median DLCO 80%. The median radiation dose received by patients was 66 Gy (range, 66-70 Gy) with a median PTV volume of 42.6 cm^3, (range, 4.67-142.5 cm^3). The median $V20_{Lung}$ was 18% (range, 9%-26%) and the median mean lung dose (MLD) was 18.7 Gy (range, 3.5-28.9 Gy). There was no significant difference between the NSCLC patients and the control individuals in the distribution of age, gender, smoking status, alcohol consumption, and family history of lung tumors.

Values of serum TLRs expression levels in early diagnosis of NSCLC

Based on our prior observations, we found that the similar volume tumor and same dose of radiation may lead to different risks of radiation pneumonia. Therefore, we are committed to search the biomarkers which can exactly indicate the occurrence of pneumonia after radiation. Here, we identified that serum TLRs can serve as potential biomarkers for radiation induced pneumonia. The expression level of serum TLRs in local advanced NSCLC patients and healthy controls were analyzed by ELISA techniques. After normalization, serum TLR1, TLR2, TLR4, and TLR9 exhibited a relative high expression level in NSCLC patients (mean ± SD: 6.79± 1.84, 8.03±1.69, 5.83±1.59 and 11.98±2.32), whereas, its expression was significantly down-regulated in healthy controls (P <0.01, **Figure 1**). But the mean serum levels of TLR3, TLR5, TLR6, TLR7 and TLR8 in 50 healthy volunteers were no statistically significant difference in the patients diagnosed with NSCLC (P > 0.05). Therefore, the TLR1, TLR2, TLR4, and TLR9 may be as a biomarker in early diagnosis of NSCLC.

Association of serum TLRs levels with fever expression

Importantly, we discovered that the expression of TLR1, TLR2, and TLR4 was associated with neutrophil granulocyte ratio before radiotherapy. The expressions of serum TLR1, TLR2, and TLR4 were enriched with ascending neutrophil containing (P < 0.001, **Table 1**) in 39 patients who did not have infectious sign. The following, we observed that the patients with high ratio of neutrophil granulocyte who are also high-expressed in TLR1, TLR2, and TLR4 significantly increased the occurrence of fever in comparison to normal neutrophil ratio in NSCLC patients during the course of radiotherapy. 28 of 39 patients with high ratio of neutrophil granulocyte and 6 of 37 patients with normal neutrophil ratio occurred in the fever symptom, and the difference was statistically significant. All the patients were detected the containing of TLRs when they had temperatures and found serum TLR1, TLR2 and TLR4 were over-expressed and more than ever before radiotherapy (6.79±1.84 vs. 15.83±2.31, P < 0.01; 8.03±1.69 vs. 19.43±3.31, P < 0.01; 5.83± 1.59 vs. 18.21±2.15, P < 0.01 **Table 2**).

Clinical characteristics of patients diagnosed with radiation pneumonia

The median follow-up time was 9.5 months (range, 3.0-17.5 months). 26 of 76 patients

Table 1. Association of TLR expression with neutrophil granulocyte ratio before RT

Mean ± SD (ng/ml)	Normal NGR (37)	High NGR (39)	*P* value
TLR1	5.63±0.86	8.94±1.29	<0.01
TLR2	6.25±0.84	9.58±1.76	<0.01
TLR3	9.66±1.53	9.04±1.24	0.78
TLR4	4.21±0.95	8.59±1.37	<0.01
TLR5	5.88±0.71	6.03±0.76	0.83
TLR6	4.35±0.63	3.99±0.87	0.54
TLR7	6.91±0.81	6.43±0.76	0.75
TLR8	3.72±0.86	3.59±0.99	0.84
TLR9	11.45±1.82	12.56±1.88	0.79

Table 2. Changes of mean TLRs levels in the time of fever occurrence

Mean ± SD (ng/ml)	Before RT	Fever occurring	*P* value
TLR1	6.79±1.84	15.83±2.31	<0.01
TLR2	8.03±1.69	19.43±3.31	<0.01
TLR3	9.35±1.21	10.55±1.32	0.45
TLR4	5.83±1.59	18.21±2.15	<0.01
TLR5	5.92±0.82	6.25±0.86	0.52
TLR6	4.20±0.93	4.36±0.82	0.38
TLR7	6.61±1.32	7.31±1.15	0.77
TLR8	3.65±1.21	4.52±1.36	0.26
TLR9	11.98±2.32	12.03±2.55	0.89

were diagnosed with different grades of radiation induced pneumonia during or after the radiotherapy, including 4 patients in grade 1, 12 patients in grade 2, 9 patients in grade 3, and 1 patient in grade 4. The median time of RP occurrence was 2.5 months (1.0-4.8 months), and the overall incidence of severe RP was 13.2%. The patient who developed grade 4 pneumonitis died of aggravation of RP. He was 68 year-old ex-smoker and diagnosed for squamous cell carcinoma stage IIIB. Ratio of neutrophil granulocyte was above normal and the serum TLR1 was exceptionally high (12.56 ng/ml) compared with the mean containing in NSCLC patients before RT ($P < 0.001$), but other TLRs were no significance to the mean level. He was relatively well tolerable but occurring fever during treatment, the detection of serum TLR1, and TLR4 (12.56 vs. 17.83 ng/ml & 6.75 vs. 20.56 ng/ml) were more than ever before radiotherapy ($P < 0.001$). After 1.0 months completion of RT he had coughing, fever and progressive dyspnea. CT scan showed diffuse patchy shadow corresponding to radiation field. He received intravenous steroid therapy, continuous oxygen inhalation and ventilator care, but died after 4 weeks of intensive care.

Serum TLRs expression levels as a potential diagnostic biological marker for radiation pneumonia

26 of 76 patients were diagnosed with different grades of radiation induced pneumonia after the radiotherapy. To find out the factors related to the occurrence and severity of pneumonia, we evaluated the association between clinic-pathologic characteristics such as age, gender, CCRT, tumor volume, the mean dose of bilateral pulmonary, $V20_{Lung}$ and contents of TLRs and RP risk in **Table 3.** As demonstrated, the levels of TLR1 and TLR4 before radiotherapy were confirmed as statistically significant increased risk of RP in both univariate and multivariate Cox proportional hazard analyses. In addition, patient age, CCRT, $V20_{Lung}$, and MLD were all significantly associated with RP risk in the univariate analysis, but all these factors that showed a p value of ≤0.05 were analyzed in multivariate analyses, only the TLR1 and TLR4 serum level proved to be significant (hazard ratio [HR] for TLR1 (ng/ml) (≥6.79, <6.79), 5.4 95% CI, 2.5-20.7, $P < 0.001$; hazard ratio [HR] for TLR4 (ng/ml) (≥5.83, <5.83), 95% CI, 7.3 (3.6-26.7), $P < 0.001$).

Furthermore, we performed an ANOVA model for analysis the association of the pretreatment TLR1 and TLR4 expression with the risk of ≥ RP 2. The results showed TLR1 and TLR4 level of the patient before RT could predict the severe damage of lung function induced by radiation, and the difference was statistically significant (**Table 4**).

Next, we generated ROC curves to assess the potential usefulness of serum TLR1 and TLR4 as a noninvasive biomarker for the diagnosis of radiation induced pneumonia in local advanced NSCLC patients. Our ROC analyses revealed that serum TLR1 and TLR4 levels were robust in discriminating patients diagnosed with pneumonia from control subjects, with an AUC value of 0.957 (95% CI = 0.887 to 0.976) and 0.923 (95% CI = 0.865 to 0.953) ($P < 0.001$). Therefore, the levels of TLR1 and TLR4 before RT are significant predictors to the occurrence of PR.

Table 3. Univariate and multivariate analysis of factors associated with the occurrence of radiation pneumonitis

	Univariate analysis		Multivariate analysis	
	P value	Hazardratio, 95% CI	*P* value	Hazard ratio, 95% CI
Age (≥60, <60)	0.01	7.6 (1.5-25.3)	0.63	3.4 (1.6-7.4)
Sex (F, M)	0.70	1.5 (0.4-5.6)		
CCRT (+, -)	0.01	8.4 (1.9-16.7)	0.51	2.5 (1.2-5.7)
PTV volume (cm^3) (≥42.6, <42.6)	0.43	1.8 (0.5-3.9)		
Total dose (≥68, <68)	0.35	1.9 (0.6-4.5)		
$V20_{Lung}$ (%) (≥18, <18)	0.01	6.7 (1.8-19.3)	0.81	3.9 (1.1-5.9)
Mean lung dose (Gy) (≥18.7, <18.7)	0.007	8.6 (1.7-36.5)	0.23	3.2 (1.9-6.7)
TLR1 (ng/ml) (≥6.79, <6.79)	<0.001	9.6 (1.3-56.7)	<0.001	5.4 (2.5-20.7)
TLR2 (ng/ml) (≥8.03, <8.03)	0.01	5.0 (0.4-23.5)	0.56	2.6 (0.9-4.2)
TLR3 (ng/ml) (≥9.35, <9.35)	0.53	1.5 (0.2-5.7)		
TLR4 (ng/ml) (≥5.83, <5.83)	<0.001	9.4 (1.2-46.5)	<0.001	7.3 (3.6-26.7)
TLR5 (ng/ml) (≥5.92, <5.92)	0.68	1.4 (0.8-4.3)		
TLR6 (ng/ml) (≥4. 20, <4.20)	0.58	1.3 (0.8-3.5)		
TLR7 (ng/ml) (≥6.61, <6.61)	0.21	2.3 (1.4-9.8)		
TLR8 (ng/ml) (≥3.65, <3.65)	0.69	1.6 (0.7-6.9)		
TLR9 (ng/ml) (≥11.98, <11.98)	0.05	5.7 (2.9-20.8)	0.45	4.3 (5.2-15.6)

Table 4. Analysis of TLRs expression and ≥ Grade 2 radiation pneumonitis in patientsbefore RT

	Mean ± SD (ng/ml)	Non-RP	RP	*P* value
RP 2 (n=12)	TLR1	4.23±0.51	9.65±0.72	<0.01
	TLR2	7.54±0.64	8.03±0.69	0.66
	TLR3	9.35±0.83	9.44±0.84	0.81
	TLR4	4.57±0.55	7.93±0.77	<0.01
	TLR5	5.93±0.62	6.05±0.63	0.78
	TLR6	4.21±0.49	4.15±0.42	0.74
	TLR7	6.57±0.61	7.03±0.63	0.36
	TLR8	3.21±0.35	3.78±0.42	0.28
	TLR9	11.07±0.82	12.79±0.85	0.26
≥ RP 3 (n=10)	TLR1	4.23±0.51	11.57±0.84	<0.01
	TLR2	7.54±0.64	7.79±0.78	0.86
	TLR3	9.35±0.83	9.75±0.88	0.75
	TLR4	4.57±0.55	10.54±0.96	<0.01
	TLR5	5.93±0.62	6.21±0.77	0.71
	TLR6	4.21±0.49	4.34±0.52	0.73
	TLR7	6.57±0.61	6.98±0.67	0.54
	TLR8	3.21±0.35	3.93±0.41	0.36
	TLR9	11.07±0.82	12.01±0.92	0.24

Discussion

Recent reports demonstrated that a great variety of tumor cells over-expressed TLRs, such as TLR4 and TLR9, which indicated that the activation of TLRs may be an important event in most cancer cells [14-17]. Recent studies suggested TLRs are widely expressed on not only infiltrating cells of myeloid and lymphoid origin which can activate the innate and adaptive immunity polarization, but also resident normal tissue cells which are associated with tumor occurrence [18]. In the literature, TLRs including TLR1, TLR2, TLR3, TLR4, TLR5, TLR6 and TLR9 can be detected on the surface of airway epithelial cells [19]. Interestingly, TLR1, TLR2, TLR4, TLR7/8 and TLR9 are overexpressed in lung carcinoma compared with the normal lung tissues [20]. Furthermore, the development and progression of malignant tumors have largely been associated with chronic inflammatory reactions. Based on above explanations, biological researches for expression of TLRs in malignant cells remain elusive. Thus, what we can conclude is that TLR system may be a double-edge sword, which needs to be carefully wielded in the treatment of cancer disease.

In the previous study, all the researches were engaged in demonstrating TLRs either on the

activation of the immune system to fight cancerous cells or promoting carcinogenesis by the enhancement of chronic inflammatory responses. Nevertheless, the present study was focused on the revelation of biomarker values in TLRs to predict radiation pneumonia risk in local advanced NSCLC patients.

Currently, a combination of chemotherapy and radiotherapy is recognized with standard treatment approach which may lead to good performance status in the advanced stage of NSCLC. With the development of modern planning and delivery, dose escalation and hypofractionation become technically feasible and safe, which is thought to be the clinical benefit of overall survival directly [21]. However, the new problem often accompanied to dose escalation is the high morbidity of radiation pneumonitis, radiation fibrosis, chronic oxygen dependence and even death.

As reported in the literature, 109 patients treated with a higher dose, grade 2 to 3 pneumonitis was occurred and associated with lung-dosimetric parameters such as the mean lung dose (MLD), volume of lung that received at least 20 Gy (V20), and the normal-tissue complication probability (NTCP) of the lung [22]. Moreover, there are increasing evidences that the irradiation of comparatively highly functional lungs leads to more pulmonary damage [23, 24]. Yet, it was reported that factors other than these physical parameters could also influence the incidence and severity of RP. So the search for predictive biochemical markers to identify the individuals at risk for symptomatic pneumonitis is highly desirable. It was reported that KL-6 levels before and after SBRT would help to predict the occurrence of ≥ Grade 2 radiation pneumonitis, at the same time, showed the highest sensitivity and specificity in the evaluation of the pulmonary injury [25]. Early changes of circulating IL-6 and IL-10 levels during radiotherapy may serve as independent predictive factors for the risk of radiation pneumonitis [25]. It has been demonstrated that the expression of nuclear factor kappa B (NF-κB), which is a key responsive transcription factor under oxidative stress, plays a crucial role in inducing various inflammatory cytokines to mediate and amplify the RP [26].

It is widely recognized that signaling via TLRs by their ligands can activate NF-κB pathway and regulate a variety of pro-inflammatory genes, such as interleukin-8, IL-1 and IL-6, which factors not only constitute the tumor microenvironment in proliferation and survival of genetically altered cells and then lead to additional genetic changes associated with malignancy, but also induce inflammatory responses to tumor or normal tissues [26-28]. Furthermore, the expression of inflammatory cytokines, chemokines, adhesion molecules, the receptors and the resultant cellular interactions seem to trigger the expression of fibrotic cytokines, such as transforming growth factor β (TGF-β), basic fibro-blast growth factor (bFGF), platelet-derived growth factor, and vascular endothelial growth factor (VEGF) which was identified as the relationship with pulmonary fibrosis [28-30]. Based on the above theories, we speculated the exceptional expressions of TLRs in NSCLC patients are the radical reason in inducing RP. But which kind of TLR plays an important role in the complicated process that has not been reported until now. Therefore, we designed this clinical experiment to discover the association of differential TLRs with RIP.

In this paper, our pertinent findings are as follows: firstly, we found serum TLR1, TLR2, TLR4, and TLR9 exhibited a relative high expression level in NSCLC patients compared with healthy controls. Secondly, a robust association between higher pre-neutrophil granulocyte ratio and expression of TLR1, TLR2, and TLR4 were identified. Next, the patients with high ratio of neutrophil granulocyte significantly increased the occurrence of fever in comparison to normal neutrophil ratio in NSCLC patients during the course of radiotherapy. Finally, the severity of RP was reflected in incremental differences in expression of serum TLR1 and TLR4. Similarly, if TLR1 and TLR4 are the basic reasons of RP occurrence, the main question to answer is whether the exception of TLRs on immune cells or on tumor cells. Thus, further studies are needed to better elucidate the differently specific cell types of TLRs in lung carcinoma.

In conclusion, our group firstly identified an important role of serum TLR1 and TLR4 as potential clinically biomarkers of radiation pneumonia risk in local advanced NSCLC patients. Certainly, further supporting evidences from larger independent studies are needed before clinical application. In addition, Future studies will be focused on the sources (immune

cells or tumor cells) of TLRs which lead to radiation pneumonia in NSCLC patients.

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Disclosure of conflict of interest

None.

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