Radiotherapy and Oncology 141 (2019) 86–94

Contents lists available at ScienceDirect

Radiotherapy and Oncology

journal homepage: www.thegreenjournal.com

Original Article

Validation study of the association between genetic variant of IL4 and severe radiation pneumonitis in lung cancer patients treated with radiation therapy

Yang Tang [a], Li Yang [b], Wan Qin [a], Min'xiao Yi [a], Bo Liu [a], Xiang'lin Yuan [a,b,*]

[a] Department of Oncology; and [b] Department of Hematology, Tongji Hospital, Huazhong University of Science and Technology, Wuhan, China

ARTICLE INFO

Article history:
Received 20 April 2019
Received in revised form 26 August 2019
Accepted 2 September 2019
Available online 17 September 2019

Keywords:
Radiation pneumonitis
Lung cancer
IL4
Inflammation
SNP

ABSTRACT

Background and purpose: Recent researches demonstrated that single nucleotide polymorphisms (SNPs) of genes involving inflammation, DNA repair, etc. were associated with risk of radiation pneumonitis (RP). However, these studies were single-centered, from single ethnic origin, without validation from independent cohort studies from other populations. In order to identify clinical valuable SNPs for RP, in this study we selected 19 RP-related SNPs candidates previously published before 2016 for validation in our cohort.

Material and methods: 359 lung cancer patients with radiotherapy were included in our prospective study (NCT02490319). Peripheral blood samples from these patients were genotyped by MassArray and Sanger Sequence method. Multivariate Cox hazard and other analyses were applied to estimate the hazard ratio (HR) and 95% confidence intervals (CIs) of all factors possibly related to the risk of RP.

Results: Patients with elder age, MLD ≥15 Gy, V_{20} ≥24% had higher risk of RP ≥grade 3 compared with their counterparts (HR = 2.020, 95% CI: 1.045–3.906, $P = 0.037$; HR = 2.502, 95% CI: 1.346–4.652, $P = 0.004$; HR = 2.256, 95% CI: 1.191–4.272, $P = 0.013$, respectively). Moreover, patients receiving IMRT were associated with decreased incidence of RP (HR = 0.520, 95% CI: 0.280–0.963, $P = 0.037$). Importantly, CT + TT genotype of *IL4:* rs2243250 was strongly related to decreased risk of RP ≥grade 3 (HR = 0.195, 95% CI: 0.090–0.424, $P = 0.000037$, $P_c = 0.0006$).

Conclusion: IL4: rs2243250 was validated to be significantly related to RP of grade ≥3 in our cohort. Our results further emphasized the prevalence and clinical value of *IL4:* rs2243250 on RP, and may thus be one of the important predictors of severe RP before radiotherapy.

Up till now, lung cancer remains as the leading cause of cancer-related mortality worldwide. According to the latest statistical report, there are 2.1 million new lung cancer cases and 1.8 million deaths predicted globally in 2018, nearly close to 1 in 5 (18.4%) of all cancer deaths [1]. China is among the countries with the highest lung cancer rates (above 40 per 100,000). For lung cancer patients, radiotherapy (RT) with or without chemotherapy remains to be the fundamental treatment. However, the efficacy of radiotherapy is restrained due to a series of RT-related complications that result in patients' intolerance.

As the most common complication and the major dose-limiting toxicity associated with radiotherapy, radiation pneumonitis (RP) is characterized by tissue inflammation and subsequent fibrosis which occurs after irradiation. RP hinders the tumor-controlling effects of radiotherapy by limiting the radiation dose that can be applied and the size of the irradiated volume [2,3]. Further, RP can cause poor quality of life or life-threatening symptoms in 15–40% of all lung cancer patients receiving radiotherapy [4]. Therefore, establishing reliable predictors for RP occurrence is of great significance such that the therapeutic effects of RT can be maximized, and its adverse effects can be minimized. In addition to the patient and treatment-related factors reported by previous studies [5], including Karnofsky performance status (KPS), chronic

Abbreviations: RP, radiation pneumonitis; KPS, Kamofsky performance status; CRT, concurrent chemoradiation; IMRT, intensity-modulated radiation therapy; MLD, mean lung dose; V_{20}, volume of normal lung receiving 20 Gy or more radiation; COPD, chronic obstructive pulmonary disease; CBLB, B-lineage lymphoma b protein; HSPB1, heat shock protein family B member 1; ATM, ataxia telangiectasia mutated; VEGF, vascular endothelial growth factor; LIG4, DNA ligase IV; XRCC1, X-ray repair cross complementing 1; APEX1, apyrimidinic endodeoxyribonuclease 1; IL1A, interleukin 1A; IL4, interleukin 4; BMP4, bone morphogenetic protein 4; BRCA1, breast cancer 1; FGF5, fibroblast growth factor 5.

* Corresponding author at: Department of Oncology, Tongji Hospital, Huazhong University of Science and Technology, Wuhan, Hubei Province, China.
E-mail address: xlyuan1020@163.com (X. Yuan).

https://doi.org/10.1016/j.radonc.2019.09.002
0167-8140/

lung disease [6], smoking status, chemotherapy [7,8], dosimetric parameters and plasma values of TGFβ [9,10], some genetic variants recently have been found to be associated with the occurrence and development of RP [11–16].

Recently, multiple studies demonstrated that SNPs of genes which function in various fundamental cellular processes are associated with increased risk and severity of radiation pneumonitis. Specifically, SNPs of genes participating in DNA repairing response including *ATM* [17], *LIG4* [18], *XRCC1* [19], *APEX1* [19], *BRCA1* [20] and those participating in inflammatory response including *IL1A*, *IL4*, *TNFRSF*, *MIF*, *NOS3*, *IL13* [21], *FGF5* [22] have been previously reported to be related with RP occurrence risk. In addition, genes functioning in immune regulation (*CBLB*) [23], oxidative stress pathway (*HSPB1*) [24], angiogenesis (*VEGF*) [25] and TGFβ pathway (*BMP-4*) [26] also have been demonstrated to possess intriguing links with RP occurrence and severity. As a large-scale Chinese Han population prospective cohort, our center has also discovered a number of SNPs associated with RP risk and severity [11,12,14]. However, all of these studies are single-centered from single ethnic origin. And they lacked independent cohort study validation from other populations. In order to identify SNPs which are clinical valuable in RP occurrence and severity prediction, in this study we selected those RP-related candidates previously published before 2016 in other cohorts to validate their association with RP in our cohort of Chinese Han population.

Material and methods

Patient population

For our prospective study (NCT02490319), 422 lung cancer patients were initially enrolled. All patients were treated with radiation therapy at Tongji Hospital, Huazhong University of Science and Technology (Wuhan, Hubei Province, China) between 2009 and 2016. We included the patients with radiation dose at least 45 Gy, age >18 years old, KPS >60 and life expectancy of at least 6 months. Patients with previous thoracic irradiation or severe cardiopulmonary diseases were excluded from our study. Of the 422

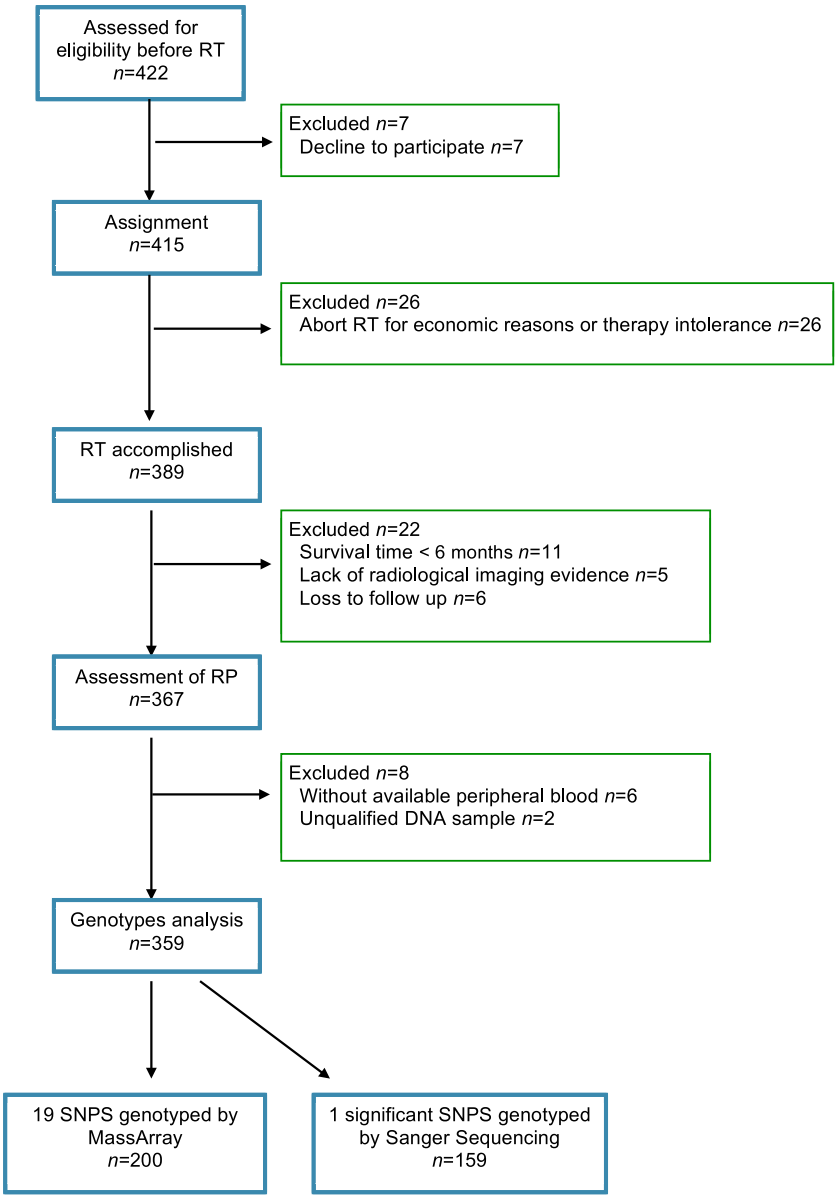

Fig. 1. Patient flow diagram.

patients, 359 patients (256 with non-small cell lung cancer and 103 with small-cell lung cancer) were eventually included for the final genotyping analysis (Fig. 1). In order to identify clinically significant RP-related SNPs by the quickest and most financially efficient way, we conducted two-step method for sample enrollment. According to their enrollment time, group of samples from 200 patients were firstly genotyped by MassArray to screen for the RP-susceptibility variants of the 19 candidate SNPs. And the second group of 159 patient samples were genotyped by Sanger sequencing for the significant SNPs that have been identified. This study was carried out in accordance with Declaration of Helsinki and approved by the Review Board of Tongji Hospital. Written informed consents were obtained from all patients for the use of their clinical information and for obtaining their blood and DNA.

Treatment and follow-up

All patients received radiotherapy with 6-MV X-rays from a linear accelerator (Elekta Synergy, Elekta, Sweden). The median total radiation dose was 54 Gy (range: 45–74 Gy), with 1.5–2 Gy administered per radiation treatment. IMRT (intensity-modulated radiation therapy) was administered to 63.3% of patients (n = 227). Computed tomography simulation (CT/e, GE, Fairfield, Connecticut, USA) was performed before the RT treatment was planned. The target volumes and critical normal organs were delineated by the three-dimensional planning system (Pinnacle Version 9.2). The baseline clinical characteristics and treatment details of the patients are shown in Table 1.

All patients enrolled in this study were examined during and one month after radiotherapy. Then, the patients were followed every three months for the first year and every six months thereafter. Before initiation of radiotherapy, we evaluated patients' baseline lung function through gathering information on patients' clinical symptoms, such as coughing, wheezing, dyspnea on exertion or at rest, etc., history of chronic lung disease, including COPD, and clinical imaging including chest X-ray or CT. At each follow-up visit, all patients were asked to undergo chest X-ray or CT scan and related clinical information, including symptoms, was collected. RP grade assessment is based on the patients' clinical imaging and symptoms comparison before and after radiotherapy. Two radiation oncologists assessed RP grading according to the Common Terminology Criteria for Adverse Events 4.0 as follows: Grade 0, no change; Grade 1, asymptomatic and diagnosed by radiographic findings only; Grade 2, symptomatic, not interfering with daily activities; Grade 3, symptomatic, interfering with daily activities or oxygen required; Grade 4, assisted ventilation required; Grade 5, fatal. The median follow-up time for RP of any grade was 21 months (ranging from 12 months to 80 months).

Table 1
Patient characteristics (N = 359).

Characteristic	No. of patients	%
Sex		
Male	271	75.5
Female	88	24.5
Age, years		
Median	62	
Range	28–83	
Histology		
SCLC	103	28.7
NSCLC	256	71.3
Stage		
I–II	46	12.8
III–IV	313	87.2
KPS		
80–100	280	78.0
<80	79	22.0
Smoking		
Non-smoker	142	39.6
Smoker	217	60.4
Induction Chemotherapy		
Yes	343	95.5
No	16	4.5
CRT		
Yes	93	25.9
No	266	74.1
Surgery		
Yes	196	54.6
No	163	45.4
IMRT		
Yes	227	63.3
No	132	36.7
Radiation dose (cGy)		
Median	5400	
Range	4500–7400	
MLD (cGy)		
Median	1310	
Range	178–2017	
V_{20}		
Median	24.00	
Range	0–42.00	
COPD		
Yes	27	7.5
No	332	92.5

Abbreviations: KPS, Kamofsky performance status; CRT, concurrent chemoradiation; IMRT, intensity-modulated radiation therapy; MLD, mean lung dose; V_{20}, volume of normal lung receiving 20 Gy or more radiation; COPD, chronic obstructive pulmonary disease.

Genotyping methods

Genomic DNA was extracted with a PureLink Genomic DNA Mini Kit (Invitrogen, K1820-01) from peripheral blood. All of the SNPs were selected from those RP-related SNPs that were published before 2016. All of the SNPs had minor allele frequencies greater than 5% in the Chinese population based on the HapMap HCB data, and all correlated alleles were captured at LD coefficient r^2 >0.8. Meanwhile all of the SNPs should locate at functional region (5 prime UTR region, 3 prime UTR region, exonic region, splice region, and regulatory region) base on functional prediction from Ensemble Gene Browser. 11 SNPs located in intronic region which were presumed less significant were excluded in this study (S. Table 3). Nineteen single-nucleotide polymorphisms (SNPs) of 16 genes were selected eventually (Table 3). For all 19 SNPs, genotypes were firstly determined using the MassArray system (Sequenom iPLEX assay, San Diego, United States) in 200 patients. The sample DNA was amplified by a multiplex PCR reaction, and the PCR products were then used for a locus-specific single-base extension reaction. Finally, the resulting products were desalted and transferred to a 384-element SpectroCHIP array. The alleles were discriminated by mass spectrometry (Sequenom, San Diego, United States). After analysis, the RP-susceptibility SNPs, *IL4:* rs2243250 were then genotyped by Sanger Sequencing method in the remaining 159 patients. The primer pairs for rs2243250 were F: 5′-GGTGA CAGAGTGAGACCCTG-3′; R: 5′-GGGCTCCTTCTCTGCATAGA-3′. The PCR products were then subjected to DNA sequencing to detect mutations.

Statistical analysis

The end point for this study was the development of RP ≥grade 3. The time to the end point was calculated from the start of radiotherapy. Patients who did not experience RP ≥grade 3 within 12 months of RT were censored. SPSS 21.0 statistical software (SPSS, Inc., Chicago, IL, USA) was used for the statistically analysis. Patients were divided into groups according to their genotypes, and Cox proportional hazard analysis was applied to estimate the

Table 2
Association between patient-, tumor-, and therapy-related characteristics and Grade ≥3 radiation pneumonitis ($N = 359$).

Parameter	Univariate Analysis			Multivariate Analysis		
	HR	95%CI	P	HR	95%CI	P
Sex						
Male	1			1		
Female	0.731	0.338–1.582	0.426	0.681	0.314–1.480	0.333
Age, years						
<62	1			1		
≥62	2.069	1.072–3.994	0.030	2.020	1.045–3.906	0.037
Histology						
SCLC	1			1		
NSCLC	0.775	0.406–1.478	0.439	0.882	0.456–1.707	0.710
Stage						
I–II	1			1		
III–IV	1.076	0.422–2.743	0.878	1.030	0.394–2.694	0.951
KPS						
<80	1			1		
80–100	0.864	0.424–1.763	0.689	0.853	0.416–1.749	0.664
Smoking						
Nonsmoker	1			1		
Smoker	1.294	0.679–2.468	0.434	1.210	0.632–2.317	0.565
Surgery						
No	1			1		
Yes	0.630	0.340–1.167	0.142	0.709	0.362–1.392	0.318
Chemotherapy						
No	1	1		1		
Yes	0.543	0.168–1.760	0.309	0.771	0.232–2.536	0.669
CRT						
No	1			1		
Yes	1.523	0.799–2.905	0.201	1.601	0.796–3.218	0.187
IMRT						
No	1			1		
Yes	0.536	0.291–0.989	0.046	0.520	0.280–0.963	0.037
Radiation dose, cGy						
<5400	1			1		
≥5400	1.521	0.806–2.872	0.196	1.223	0.636–2.354	0.546
MLD, cGy						
<1500	1			1		
≥1500	2.310	1.246–4.280	0.008	2.502	1.346–4.652	0.004
V_{20}						
<24%	1			1		
≥24%	1.889	1.009–3.539	0.047	2.256	1.191–4.272	0.013
COPD						
No	1			1		
Yes	1.730	0.679–4.409	0.251	1.240	0.467–3.289	0.666

Note: Multivariate analyses were adjusted for age, MLD and IMRT.
*Either MLD or V_{20} was used in the multivariate analyses, but not both.

hazard ratio (HR) and 95% confidence intervals (CIs) of all factors possibly related to the risk of RP. Moreover, multivariate Cox regression analysis was used for the adjustment of covariates. Those covariates were proved to meet the proportionality assumption of the Cox model by Schoenfeld Residual Tests. Tied events were handled by Breslow method. The influences of the genotypes on RP risk were assessed by Kaplan-Meier analysis and compared with log-rank tests. For genotype analysis, *P*-values were corrected by the Benjamini and Hochberg False Discovery Rate correction.

Results

359 patients were included in this study with 271 males and 88 females. Their characteristics are listed in Table 1. The median age of the population was 62 years (range from 28 to 83 years); 256 patients had NSCLC, and 103 had SCLC. In the study cohort, 87.2% of patients had stage III-IV disease, 54.6% underwent surgery before RT, almost all patients (95.5%) received induction chemotherapy followed by radiotherapy and 25.9% had concurrent chemoradiation. The median radiation dose was 54 Gy (range from 45 to 74 Gy), the median MLD was 13.10 Gy (range from 1.78 to 20.17 Gy), and the median V_{20} was 24% (range from 0 to 42.00%). The characteristics of 200 patients, who were firstly genotyped by MassArray to screen for RP-susceptibility variants, are listed in S. Table 1.

Within 12 months of radiotherapy, 41 patients (11.4%) suffered RP ≥grade 3. The associations between patient-, tumor- and therapy-related characteristics and RP ≥grade 3 are listed in Tables 2. The univariate and multivariate analysis by Cox regression model revealed that MLD, V_{20}, age, IMRT was significantly related to RP ≥grade 3. Patients with elder age, MLD ≥15 Gy, V_{20} ≥24% had higher risk of RP ≥grade 3 compared with those counterparts (HR = 2.020, 95% CI: 1.045–3.906, $P = 0.037$; HR = 2.502, 95% CI: 1.346–4.652, $P = 0.004$; HR = 2.256, 95% CI: 1.191–4.272, $P = 0.013$, respectively). Moreover, patients receiving IMRT were associated with decreased incidence of RP (HR = 0.520, 95% CI: 0.280–0.963, $P = 0.037$) (Table 2), which were consistent with the results of other publications.

A total of nineteen candidate SNPs were screened by MassArray in 200 patients for RP-susceptibility variants (Table 3). Among them, *IL4:* rs2243250 was found to be significantly associated with the occurrence of RP ≥grade 3 (Table 4). Then it was genotyped by Sanger Sequencing method in the remaining 159 patients, bringing the total population of 359 patients genotyped for *IL4:* rs2243250. Fig. 2 is a plot of the RP-free survival percentage for RP ≥grade 3 for each genotype of *IL4:* rs2243250 determined by the Kaplan-Meier

Table 3
Genes and single nucleotide polymorphisms selected for analysis.

Genes and SNP	Gene function	Predicted functional Consequence*	MAF*		Reference
			European	Chinese	
CBLB	Immune system				
rs2305035		Synonymous variant	23.8% (A)	19.9% (A)	Li et al. [23]
HSPB1	Oxidative stress pathway				
rs2868371		Regulatory region variant	15.5% (C)	12.6% (C)	Pang et al. [24]
ATM	DNA repair				
rs1801516		Missense variant	16.2% (A)	5.15% (A)	Xiong et al. [17]
rs228590		Non coding transcript exon variant	42.4% (A)	59.4% (A)	
VEGF	Angiogenesis				
rs2010963		5 Prime UTR variant	42.7% (C)	61.6% (C)	Yin et al. [25]
rs3025039		3 Prime UTR variant	14.8% (T)	17.6% (T)	
LIG4	DNA repair				
rs1805388		Missense variant	19.1% (A)	13.6% (A)	Yin et al. [18]
XRCC1	DNA repair				
rs25487		Missense variant	34.8% (T)	31.8% (T)	Kelsey et al. [20]
APEX1	DNA repair				
rs1130409		Missense variant	48.6% (G)	29.4% (G)	Yin et al. [19]
IL1A	Inflammation				
rs1800587		5 Prime UTR variant	29.9% (A)	7.2% (A)	Hildebrandt et al. [21]
rs17561		Missense variant	30.1% (A)	22.3% (A)	
IL4	Inflammation				
rs2243250		Regulatory region variant	21.1% (T)	8.2% (T)	Hildebrandt et al. [21]
TNFRSF18	Inflammation				
rs1061622		Missense variant	23.5% (G)	22.3% (G)	Hildebrandt et al. [21]
MIF	Inflammation				
rs755622		5 Prime UTR variant	19.3% (C)	19.8% (C)	Hildebrandt et al. [21]
NOS3	Inflammation				
rs1799983		Missense variant	32.1% (T)	15.3% (T)	Hildebrandt et al. [21]
IL13	Inflammation				
rs1800925		Non coding transcript exon variant	22.4% (T)	16.0% (T)	Hildebrandt et al. [21]
BMP4	TGFβ pathway				
rs762642		Splice region variant	43.2% (C)	40.7% (C)	Yang et al. [26]
BRCA1	DNA repair				
rs16942		Missense variant	33.7% (C)	45.9% (C)	Kelsey [20]
FGF5	Inflammation				
rs3733336		3 Prime UTR variant	65.3% (A)	75.3% (A)	Pu et al. [22]

Abbreviations: CBLB, B-lineage lymphoma b protein; HSPB1, heat shock protein family B member 1; ATM, ataxia telangiectasia mutated; VEGF, vascular endothelial growth factor; LIG4, DNA ligase IV; XRCC1, X-ray repair cross complementing 1; APEX1, apyrimidinic endodeoxyribonuclease 1; IL1A, interleukin 1A; IL4, interleukin 4; TNFRSF18, TNF receptor superfamily member 18; MIF, macrophage migration inhibitory factor; NOS3, nitric oxide synthase 3; IL13, interleukin 13; BMP4, bone morphogenetic protein 4; BRCA1, breast cancer 1; FGF5, fibroblast growth factor 5.
*The possible function of SNPs was predicted from Ensemble Gene Browser.

Table 4
Association between genotypes and Grade ≥3 RP ($N = 200$).

Polymorphism and Genotype	No. of event	No. of total	Univariate analysis			Multivariate analysis		
			HR	95% CL	P	HR	95% CL	P
CBLB:rs2305035								
GG	14	113	1			1		
GA	11	82	1.12	0.508–2.466	0.779	1.204	0.545–2.662	0.647
AA	1	5	1.805	0.237–13.733	0.568	1.496	0.194–11.505	0.699
HSPB1:rs2868371								
CC	12	78	1			1		
CG	13	108	1.095	0.507–2.367	0.817	0.722	0.327–1.593	0.419
GG	1	14	0.633	0.167–2.399	0.501	0.421	0.055–3.241	0.406
ATM:rs1801516								
GG	6	43	1			1		
AG	15	122	0.877	0.340–2.260	0.786	0.679	0.259–1.776	0.43
AA	5	35	1.044	0.319–3.421	0.943	0.842	0.250–2.832	0.781
ATM:rs228590								
GG	5	25	1			1		
GA	21	163	0.581	0.219–1.541	0.275	0.705	0.261–1.905	0.49
AA	0	25	0	0.000–	0.98	0	0	0.98
VEGF:rs2010963								
CC	4	40	1			1		
CG	20	146	1.077	0.570–2.036	0.82	1.179	0.398–3.490	0.766
GG	2	14	1.206	0.448–3.248	0.711	1.402	0.255–7.700	0.697
VEGF: rs3025039								
CC	14	132	1			1		

Table 4 (*continued*)

Polymorphism and Genotype	No. of event	No. of total	Univariate analysis			Multivariate analysis		
			HR	95% CL	P	HR	95% CL	P
CT	12	65	1.818	0.841–3.931	0.129	1.842	0.851–3.986	0.121
TT	0	3	0	0.000–	0.978	0	0	0.979
LIG4:rs1805388								
GG	17	139	1			1		
GA	9	60	1.233	0.550–2.766	0.612	1.117	0.486–2.566	0.794
AA	0	1	0	0.000–	0.98	0	0.000-	0.982
XRCC1:rs25487								
CC	16	91	1			1		
CT	9	103	0.469	0.207–1.061	0.069	0.554	0.236–1.298	0.174
TT	1	6	0.896	0.119–6.757	0.915	0.847	0.112–6.408	0.872
APEX1:rs1130409								
TT	9	56	1			1		
GT	14	122	0.693	0.300–1.601	0.391	0.733	0.315–1.703	0.469
GG	3	22	0.79	0.214–2.919	0.724	0.747	0.202–2.763	0.662
IL1A: rs1800587								
GG	21	162	1			1		
AG	5	38	1.052	0.397–2.790	0.919	1.38	0.502–3.795	0.533
IL1A: rs17561								
CC	22	168	1			1		
AC	4	32	1.005	0.346–2.916	0.993	1.037	0.357–3.015	0.947
IL4: rs2243250								
CC	4	7	1			1		
CT	5	57	0.106	0.028–0.396	0.001	0.107	0.029–0.401	0.001
TT	17	136	0.158	0.053–0.473	0.001	0.18	0.060–0.541	0.002
TNFRSF18: rs1061622								
TT	18	147	1					
TG	7	46	0.884	0.155–8.704	0.608	1.155	0.481–2.772	0.747
GG	1	7	1.257	0.525–3.008	0.884	1.3	0.172–9.806	0.799
MIF: rs755622								
GG	19	154				1		
CG	6	43	1.192	0.476–2.986	0.707	1.202	0.471–3.070	0.7
CC	1	3	3.101	0.415–23.185	0.27	3.222	0.407–25.486	0.268
NOS3: rs1799983								
GG	25	191	1			1		
GT	1	9	0.843	0.114–6.224	0.867	0.947	0.128–7.019	0.957
IL13: rs1800925								
CC	16	129	1			1		
CT	9	68	1.042	0.460–2.358	0.921	1.022	0.452–2.314	0.957
TT	1	3	3.024	0.401–22.828	0.283	3.946	0.507–30.707	0.19
BMP4: rs762642								
AA	13	81	1			1		
AC	11	92	0.738	0.331–1.648	0.459	0.701	0.314–1.566	0.387
CC	2	27	0.455	0.103–2.018	0.3	0.333	0.074–1.505	0.153
BRCA1: rs16942								
TT	7	74	1			1		
CT	14	94	1.64	0.662–4.063	0.285	1.764	0.709–4.393	0.222
CC	5	32	1.714	0.544–5.400	0.358	1.67	0.529–5.269	0.382
FGF5: rs3733336								
AA	12	107	1			1		
GA	14	82	1.531	0.708–3.310	0.279	1.588	0.729–3.461	0.244
GG	0	11	0	0.000–	0.981	0	0.000–	0.982

Note: Multiple analyses in this table were adjusted for all of the factors listed in S. Table 1.
Abbreviations: HR, hazard ratio; CI: confidence interval.

method. Patients with the CC genotype of *IL4:* rs2243250 had significantly higher risks of RP ≥grade 3 ($P < 0.0001$). Furthermore, multiple Cox proportional hazard analyses with adjustments for all of the characteristics listed in Table 1 revealed that the CT + TT genotype of *IL4:* rs2243250 were strongly related to the decreased risk of RP ≥grade 3 (HR = 0.195, 95% CI: 0.090–0.424, $P = 0.000037$, $P_c = 0.0006$) (Table 5). Additionally, multivariate analysis including only IL4: rs2243250 SNP and MLD or V_{20} were performed. The results indicated that IL4: rs2243250 SNP was associated with RP independent of MLD and V_{20} (S. Table 4).

Patients were divided to four groups based on the dosimetric factors-V_{20} or MLD and *IL4:* rs2243250 genotypes in order to evaluate the impact of the *IL4:* rs2243250 genotypes on RP in different dosimetric groups. Interestingly, the patients with CT/TT genotype of *IL4:* rs2243250 rely on the dosimetric factors. For the patients with CT/TT genotype of *IL4:* rs2243250, groups with MLD ≥15 Gy or V_{20} ≥24% had the higher incidence of RP ≥grade 3 with the patients who received MLD less than 15 Gy or V_{20} less than 24% ($P < 0.05$ and $P < 0.05$, respectively, Fig. 3A, B, S. Fig. 1A and B). However, patients with CC genotype of *IL4:* rs2243250 had much higher risk of RP grade ≥3 compared with other groups ($P < 0.0001$ and $P < 0.0001$, respectively, Fig. 3A, B), regardless of MLD or V_{20} level. KM curve comparison and log-rank test showed that for patients within CC genotype of *IL4*:rs2243250 group, differences in MLD and V_{20} status seemed had no significant impact on the risk of RP grade ≥3 ($P = 0.858$ and $P = 0.921$ respectively, S. Fig. 1B and D).

Discussion

In this study, we evaluated the variants of several genes involving in the pathogenesis of radiation pneumonitis, including

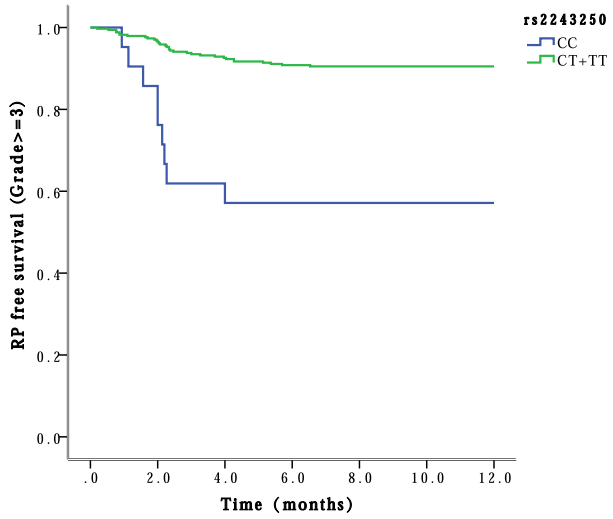

Fig. 2. Kaplan–Meier estimates RP-free survival (RP ≥grade 3) for each genotype. Patients with CC genotype of *IL4: rs2243250* had significantly higher risks of RP ≥grade 3 ($P < 0.0001$).

inflammation, immune response, DNA repairing and angiogenesis. Through this study we aimed to discover the potential associations between the SNPs and the occurrence of RP in lung cancer patients with radiotherapy. Among 19 SNPs tested, *IL4*: rs2243250 was found to be significantly associated with the risk of RP ≥grade 3. Patients with the CT/TT genotype of *IL4:* rs2243250 had significantly decreased risk of RP after radiotherapy for lung cancer comparing with CC genotype counterparts. We also discovered that the association between *IL4*: rs2243250 and RP grade ≥3 was independent of MLD and V_{20}. Comparing with previous report by Hildebrandt et al. that demonstrated *IL4*: rs2243250 significantly associated with RP risk in 173 NSCLC patients of western population [21], our result confirmed the above finding for the first time in an independent Chinese cohort.

The occurrences of RP ≥grade 3 were 11.4%, which were similar to those reported previously. Due to the prospective nature of our study, the incidence rate of RP was relatively higher than in some retrospective studies. We also confirmed that age, MLD and V_{20} was closely related to the risk of RP. In our cohort, patients with age ≥58, MLD ≥15 Gy and V_{20} ≥24% and without receiving IMRT had greater risk of developing RP grade ≥3, which verified the associations between the patient- and radiation dosimetric-related factors and the occurrence of severe RP [27].

As is well known that pro-inflammatory and fibrogenic cytokines induced by irradiation are involved in the pathogenesis of RP [28–30]. However, the exact roles that anti-inflammatory cytokines such as IL4, etc. play in the pathogenesis of RP still remain elusive. IL4 is multifunctional cytokine produced in various cell types, including macrophage, lymphocyte and fibroblast, etc. It generally promotes the humoral response, and inhibits the inflammatory chemokines such as TNF and IL1β [31]. IL4 also facilitates the differentiation of Th2 helper T cells [32]. Up till now, several experiments demonstrated that IL4, IL13 and TGFβ signaling pathways were involved in RP occurrence. Animal model study showed local *IL4* expression correlated with development of radiation induced pulmonary injury [33]. Another study demonstrated that IL4 amplified the fibrotic process through stimulation of *IL13* and *TGFβ* expression [34]. In addition, IL4 and IL13 can promote free radical generation that results in chronic oxidative stress [35]. Moreover, research indicated IL4 was vital for macrophage accumulation and maintenance in lung tissue after radiation, and macrophage infiltration was one of the main pathological process in RP occurrence [36]. As for the *IL4* SNP functional impact, studies demonstrated that several genetic variants of *IL4* were associated with 3-fold increased risk of pneumonitis [37]. Studies also showed increased IgE production for asthma patients carrying *IL4*: rs2070874 and rs2243250 [38]. Generally speaking, the above evidence suggests that *IL4* pathway is a vital regulator in inflammation and fibrosis, indicating the biological plausibility of the relevance of *IL4* SNP to RP risk validated in our research.

Our results further emphasized the prevalence and clinical value of *IL4:* rs2243250 on RP in another independent cohort, and may thus be one of the important predictors of severe RP before radiotherapy in addition to the radiation dosimetric factors. Those patients with RP susceptibility genotypes will greatly benefit from early prediction and prevention of RP by genotyping before the initiation of RT. And this study will help us to individualize radiation dose appropriately for better control of tumor according to their RP risk SNPs genotypes. Especially for the patients with favorable genotypes, elevated MLD and V_{20} will not increase their incidence of severe RP, which could assist the oncologist to adjust the radiation dose personally. Moreover, our findings suggest the

Table 5
Association between genotypes and Grade ≥3 RP ($N = 359$).

Polymorphism and Genotype	No. of event	No. of total	Univariate analysis			Multivariate analysis			P_c
			HR	95% CL	*P*	HR	95% CL	*P*	
IL4: rs2243250									
CC	8	21	1			1			
CT + TT	33	338	0.173	0.082–0.363	6.6E-5	0.195	0.090–0.424	3.7E-5	0.0006
Age, years									
<62	13	173	1			1			
≥62	28	186	2.069	1.072–3.994	0.030	2.240	1.152–4.358	0.018	
IMRT									
No	21	132	1			1			
Yes	20	227	0.536	0.291–0.989	0.046	0.575	0.310–1.066	0.079	
MLD, cGy									
<1500	23	264	1			1			
≥1500	18	95	2.310	1.246–4.280	0.008	2.021	1.068–3.825	0.031	
V_{20}									
<24%	16	194	1			1			
≥24%	25	165	1.889	1.009–3.539	0.047	2.014	1.062–3.817	0.032	

Note: Multiple analyses in this table were adjusted for age, MLD, IMRT and rs2243250.
Abbreviations: HR, hazard ratio; CI, confidence interval.
Pc: P-value corrected by Benjamini and Hochberg False Discovery Rate correction.
*Either MLD or V_{20} was used in the multivariate analyses, but not both.

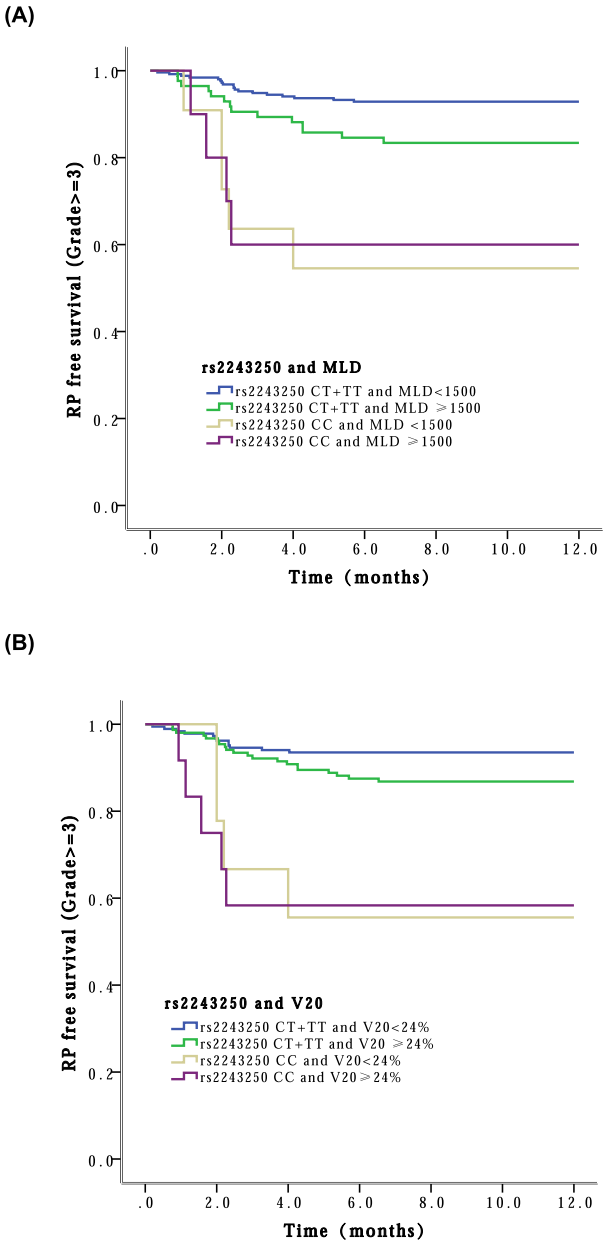

Fig. 3. Kaplan–Meier estimates effect of genotype in IL4: rs2243250 and dosimetric parameters on RP-free survival (RP ≥grade 3). (A) IL4: rs2243250 and MLD; (B) IL4: rs2243250 and V_{20}.

possible role of IL4 in the pathogenesis of RP, which will aid in the discovery of targeted therapy for RP in future research.

On the other hand, other SNPs tested in this study failed to exhibit significant association with RP in our cohort. This could be partially explained by the ethnic variation of SNP frequencies. However, despite the fact that *IL4:* rs2243250 and RP association has been demonstrated in Han Chinese population in our study and western Caucasian population in previous reports, it still requires further validation in expanded cohorts from different races, since the substantial ethnic variation exists in SNP frequencies. Moreover, *IL4:* rs2243250 warrant further investigation to identify the causative SNPs and their molecular mechanisms. Furthermore, we need to explore the potential role of IL4 pathway in the pathogenesis of RP, which would provide novel insight into the treatment of RP.

In summary, it is the first study to confirm the association between RP risk and *IL4:* rs2243250 in an independent Chinese cohort. Our results further emphasized the prevalence and clinical value of *IL4:* rs2243250 on RP. Our study indicated that in addition to the radiation dosimetric factors, *IL4* SNP can be used as useful predictive biomarker of RP risk before RT. Thus, patients will greatly benefit from early prediction and prevention of RP by rs2243250 genotyping before the initiation of RT. And this study will benefit lung cancer patients receiving radiotherapy since appropriately tailored radiation dose might result in better control of their diseases and lower occurrence and severity of RP.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

None.

Funding

This study was supported by grants from the National Natural Science Foundation of China (grants 81773360 and 81700145).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.radonc.2019.09.002.

References

[1] Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries Available from. CA Cancer J Clin [Internet] 2018;68:394–424. http://www.ncbi.nlm.nih.gov/pubmed/30207593.
[2] Jain V, Berman AT. Radiation pneumonitis: Old problem, new tricks. Cancers (Basel) 2018;10:1–16.
[3] Tsujino K, Hashimoto T, Shimada T, Yoden E, Fujii O, Ota Y, et al. Combined analysis of V20, VS5, pulmonary fibrosis score on baseline computed tomography, and patient age improves prediction of severe radiation pneumonitis after concurrent chemoradiotherapy for locally advanced non-small-cell lung cancer. J Thorac Oncol 2014;9:983–90.
[4] Rodrigues G, Lock M, D'Souza D, Yu E, Van Dyk J. Prediction of radiation pneumonitis by dose – volume histogram parameters in lung cancer–a systematic review. Radiother Oncol Ireland 2004;71:127–38.
[5] Vogelius IR, Bentzen SM. A literature-based meta-analysis of clinical risk factors for development of radiation induced pneumonitis. Acta Oncol 2012;51:975–83.
[6] Takeda A, Kunieda E, Ohashi T, Aoki Y, Oku Y, Enomoto T, et al. Severe COPD is correlated with mild radiation pneumonitis following stereotactic body radiotherapy. Chest 2012;141:858–66.
[7] Onishi H, Kuriyama K, Yamaguchi M, Komiyama T, Tanaka S, Araki T, et al. Concurrent two-dimensional radiotherapy and weekly docetaxel in the treatment of stage III non-small cell lung cancer: a good local response but no good survival due to radiation pneumonitis. Lung Cancer Ireland 2003;40:79–84.
[8] Parashar B, Edwards A, Mehta R, Pasmantier M, Wernicke AG, Sabbas A, et al. Chemotherapy significantly increases the risk of radiation pneumonitis in radiation therapy of advanced lung cancer. Am J Clin Oncol 2011;34:160–4.
[9] Zhao L, Wang L, Ji W, Wang X, Zhu X, Hayman JA, et al. Elevation of plasma TGF-beta1 during radiation therapy predicts radiation-induced lung toxicity in patients with non-small-cell lung cancer: a combined analysis from Beijing and. Michigan Int J Radiat Oncol Biol Phys 2009;74:1385–90.
[10] Shi S, Zeng Z, Ye L, Huang Y, Risk He J. Factors associated with symptomatic radiation pneumonitis after stereotactic body radiation therapy for stage I non-small cell lung cancer. Technol Cancer Res Treat 2017;16:316–20.
[11] Tang Y, Liu B, Li J, Wu H, Yang J, Zhou X, et al. Genetic variants in PI3K/AKT pathway are associated with severe radiation pneumonitis in lung cancer patients treated with radiation therapy. Cancer Med 2016;5:24–32.
[12] Yi M, Tang Y, Liu B, Li Q, Zhou X, Yu S, et al. Genetic variants in the ITGB6 gene is associated with the risk of radiation pneumonitis in lung cancer patients treated with thoracic radiation therapy. Tumour Biol 2016;37:3469–77.
[13] Liu B, Tang Y, Yi M, Liu Q, Xiong H, Hu G, et al. Genetic variants in the plasminogen activator inhibitor-1 gene are associated with an increased risk of radiation pneumonitis in lung cancer patients. Cancer Med 2017;6:681–8.

[14] Liu B, Yi M, Tang Y, Liu Q, Qiu H, Zou Y, et al. MMP-1 promoter polymorphism is associated with risk of radiation-induced lung injury in lung cancer patients treated with radiotherapy. Oncotarget 2016;7:70175–84.
[15] Xiao Y, Yuan X, Qiu H, Li Q. Single-nucleotide polymorphisms of TGFbeta1 and ATM associated with radiation-induced pneumonitis: a prospective cohort study of thoracic cancer patients in China. Int J Clin Exp Med 2015;8:16403–13.
[16] Wen J, Liu H, Wang L, Wang X, Gu N, Liu Z, et al. Potentially functional variants of ATG16L2 predict radiation pneumonitis and outcomes in patients with non-small cell lung cancer after definitive. Radiother J Thorac Oncol 2018;13:660–75.
[17] Xiong H, Liao Z, Liu Z, Xu T, Wang Q, Liu H, et al. ATM polymorphisms predict severe radiation pneumonitis in patients with non-small cell lung cancer treated with definitive radiation therapy. Int J Radiat Oncol Biol Phys 2013;85:1066–73.
[18] Yin M, Liao Z, Liu Z, Wang L-E, O'Reilly M, Gomez D, et al. Genetic variants of the nonhomologous end joining gene LIG4 and severe radiation pneumonitis in nonsmall cell lung cancer patients treated with definitive radiotherapy. Cancer 2012;118:528–35.
[19] Yin M, Liao Z, Liu Z, Wang L-E, Gomez D, Komaki R, et al. Functional polymorphisms of base excision repair genes XRCC1 and APEX1 predict risk of radiation pneumonitis in patients with non-small cell lung cancer treated with definitive radiation therapy. Int J Radiat Oncol Biol Phys 2011;81:e67–73.
[20] Kelsey CR, Jackson IL, Langdon S, Owzar K, Hubbs J, Vujaskovic Z, et al. Analysis of single nucleotide polymorphisms and radiation sensitivity of the lung assessed with an objective radiologic endpoin. Clin Lung Cancer 2013;14:267–74.
[21] Hildebrandt MAT, Komaki R, Liao Z, Gu J, Chang JY, Ye Y, et al. Genetic variants in inflammation-related genes are associated with radiation-induced toxicity following treatment for non-small cell lung cancer. PLoS One 2010;5:e12402.
[22] Pu X, Wang L, Chang JY, Hildebrandt MAT, Ye Y, Lu C, et al. Inflammation-related genetic variants predict toxicity following definitive radiotherapy for lung cancer. Clin Pharmacol Ther 2014;96:609–15.
[23] Li P, Wang X, Liu Z, Liu H, Xu T, Wang H, et al. Nucleotide polymorphisms in CBLB, a regulator of T-cell response, predict radiation pneumonitis and outcomes after definitive radiotherapy for non-small-cell lung. Cancer Clin Lung Cancer 2016;17:253–262.e5.
[24] Pang Q, Wei Q, Xu T, Yuan X, Lopez Guerra JL, Levy LB, et al. Functional promoter variant rs2868371 of HSPB1 is associated with risk of radiation pneumonitis after chemoradiation for non-small cell lung cancer. Int J Radiat Oncol Biol Phys 2013;85:1332–9.
[25] Yin M, Liao Z, Yuan X, Guan X, O'Reilly MS, Welsh J, et al. Polymorphisms of the vascular endothelial growth factor gene and severe radiation pneumonitis in non-small cell lung cancer patients treated with definitive radiotherapy. Cancer Sci 2012;103:945–50.
[26] Yang J, Xu T, Gomez DR, Yuan X, Nguyen Q-N, Jeter M, et al. Polymorphisms in BMP2/BMP4, with estimates of mean lung dose, predict radiation pneumonitis among patients receiving definitive radiotherapy for non-small cell lung cancer. Oncotarget 2017;8:43080–90.
[27] Tang X, Li Y, Tian X, Zhou X, Wang Y, Huang M, et al. Predicting severe acute radiation pneumonitis in patients with non-small cell lung cancer receiving postoperative radiotherapy: Development and internal validation of a nomogram based on the clinical and dose-volume histogram parameters. Radiother Oncol Ireland 2019;132:197–203.
[28] Lierova A, Jelicova M, Nemcova M, Proksova M, Pejchal J, Zarybnicka L, et al. Cytokines and radiation-induced pulmonary injuries. J Radiat Res 2018;59:709–53.
[29] Cappuccini F, Eldh T, Bruder D, Gereke M, Jastrow H, Schulze-Osthoff K, et al. New insights into the molecular pathology of radiation-induced pneumopathy. Radiother Oncol 2011;101:86–92.
[30] Hill RP, Zaidi A, Mahmood J, Jelveh S. Investigations into the role of inflammation in normal tissue response to irradiation. Radiother Oncol 2011;101:73–9.
[31] Abramson SL, Gallin JI. IL-4 inhibits superoxide production by human mononuclear phagocytes. J Immunol 1990;144:625–30.
[32] Chomarat P, Banchereau J. Interleukin-4 and interleukin-13: their similarities and discrepancies. Int Rev Immunol 1998;17:1–52.
[33] Buttner C, Skupin A, Reimann T, Rieber EP, Unteregger G, Geyer P, et al. Local production of interleukin-4 during radiation-induced pneumonitis and pulmonary fibrosis in rats: macrophages as a prominent source of interleukin-4. Am J Respir Cell Mol Biol 1997;17:315–25.
[34] Azmoonfar R, Amini P, Saffar H, Rezapoor S, Motevaseli E, Cheki M, et al. Metformin protects against radiation-induced pneumonitis and fibrosis and attenuates upregulation of dual oxidase genes expression.. Adv Pharm Bull 2018;8:697–704.
[35] Ameziane-El-Hassani R, Talbot M, de Souza Dos Santos MC, Al Ghuzlan A, Hartl D, Bidart J-M, et al. NADPH oxidase DUOX1 promotes long-term persistence of oxidative stress after an exposure to irradiation. Proc Natl Acad Sci U S A 2015;112:5051–6.
[36] Groves AM, Johnston CJ, Misra RS, Williams JP, Finkelstein JN. Effects of IL-4 on pulmonary fibrosis and the accumulation and phenotype of macrophage subpopulations following thoracic irradiation. Int J Radiat Biol 2016;92:754–65.
[37] Vasakova M, Striz I, Slavcev A, Jandova S, Dutka J, Terl M, et al. Correlation of IL-1alpha and IL-4 gene polymorphisms and clinical parameters in idiopathic pulmonary fibrosis. Scand J Immunol 2007;65:265–70.
[38] Kabesch M, Tzotcheva I, Carr D, Hofler C, Weiland SK, Fritzsch C, et al. A complete screening of the IL4 gene: novel polymorphisms and their association with asthma and IgE in childhood. J Allergy Clin Immunol 2003;112:893–8.