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Original Article

Association of single nucleotide polymorphisms at *HSPB1* rs7459185 and *TGFB1* rs11466353 with radiation esophagitis in lung cancer

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ABSTRACT

Background and purpose: Radiochemotherapy (RCT) success in lung cancer (LC) can be limited due to the onset of adverse effects in the adjacent normal tissue such as radiation-induced esophageal toxicity (RIET). Therefore, specific biomarkers to customize the RCT dose administration and esophageal toxicity prediction are necessary to improve treatment effectiveness.

Materials and methods: 247 LC patients prospectively recruited between 2012 and 2016 from 3 institutions were genotyped for **7** SNPs along *TGFB1* and *HSPB1* genes seeking an association with RIET risk development. Kaplan–Meier cumulative probability and Cox proportional hazards analyses were used to evaluate the effect of *TGFB1* and *HSPB1* genotypes on such risk.

Results: Multivariate analyses showed that patients carrying the *HSPB1* rs7459185 CC genotype were associated with a significantly higher risk of acute grade 3 RIET than those carrying the GG/GC genotypes (HR = 17.73; 95% CI = 2.896–108.49; $p = 0.002$). LC patients who received higher (>median) volume of esophagus exposed to 30 Gy and harboring the rs7459185 GG/GC genotypes showed a significantly lower RIET incidence ($p < 0.001$). Additionally, LC patients carrying the *TGFB1* rs11466353 GG genotype were found to be associated with a lower risk of late grade 2 RIET compared with those with the TT/TG genotypes (HR = 0.29; 95% CI = 0.103–0.830; $p = 0.021$). Patients receiving a high (>60 Gy) radiation dose who presented the rs11466353 GG genotype had a significantly lower RIET incidence ($p = 0.025$).

Conclusion: The presence of different rs7459185/rs11466353 genotypes in LC patients associated with RIET risk and may be useful biomarkers along with other risk factors for guiding therapy intensity in an individualized therapy.

Lung cancer (LC) ranks among the leading causes of death associated with cancer worldwide due to its high incidence and mortality rates. This poses a significant challenge to the health system and a dire economic problem [1]. For advanced (non-metastatic) LC and inoperable cases, radiation therapy (RT) given with chemotherapy is the first-line treatment. RT with curative intention aims to accomplish a beneficial therapeutic index, leading malignant cells to lose their clonogenicity by cell death induction through DNA damage, with the goal of achieving uncomplicated local regional control [2,3]. However, in many cases, the normal adjacent tissue's radiation tolerance limits the administered radiation dose, thus RT treatment is often accompanied by adverse reactions such as radiation-induced esophageal toxicity (RIET) and radio-induced pneumonitis, affecting treatment efficacy and negatively impairing patient's quality of life [4–6].

RIET can be classified as acute, which usually occurs within 90 days after the completion of treatment and is normally addressed by conservative supportive care, or late, which is more persistent and troublesome with a median onset time of 6 months [7]. The development of different techniques such as three-dimensional conformal radiation therapy, Intensity-Modulated Radiation Therapy (IMRT), Volumetric Modulated Arc Therapy (VMAT), or image-guided radiation therapy (IGRT) has improved the aspects commented above [8,9]. Nevertheless, most patients present acute esophageal toxicity as a response to esophageal

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mucosa irradiation and dosimetric parameters are not enough to predict this event [10]. Therefore, it is necessary to find specific biomarkers to customize the RT dose administration and predict esophageal toxicity, leading to a personalized therapy.

Different molecular events have been described as potentially responsible for radiation-induced normal tissue damage and inter-individual genetic variations appear to be promising biomarkers to discriminate patients with high risks of treatment-related toxicities in different types of cancer [11–14]. Genes within oxidative stress and inflammation signaling networks have being widely studied for their influence to RT response [15,16]. Among them, the Transforming growth factor beta-1 (TGFB-1) plays important roles in inflammation and cell proliferation, which appear to be linked to radiation-induced fibrosis progression [17]. TGFB1 is activated as a feedback anti-inflammatory signal facing the release of cytokines, chemokines and growth factors during the early immune inflammatory response to radiation [18]. Previous studies attempted correlations of different candidate single nucleotide polymorphisms (SNPs) along *TGFB1* sequence and increased risk of RIET with the potential of being prognostic and/or predictive biomarkers for clinical practice [19–21]. Additionally, SNPs along Heat shock protein beta-1 (*HSPB1*) sequence, a chaperone responsible for remediating damage to proteins in response to high levels of stress, inhibition of apoptosis, regulation of cell development, and cell differentiation, have been associated with cellular radiosensitivity in LC patients [22–24].

To explore new planning and treatment technology in RT, SNPs along *TGFB1* and *HSPB1* might be valuable genetic biomarkers for the identification of patients' individual RIET susceptibility. Consequently, we have performed a multicenter prospective study in 247 LC patients for a better understanding of contributions of different SNPs to predict esophageal radio-sensitivity in LC patients.

Materials and methods

Ethics statement

The study was approved by the Ethics Committee for clinical research and complies with the tenets of the declaration of Helsinki and the Institutional Review Board of the participating centers. Written informed consent for molecular genetic studies was obtained from all participants.

Patient population

Subjects for this prospective analysis were recruited at the radiation oncology department of 3 different institutions between January 2012 and December 2016. Patient selection criteria were as follows: Patients $\geq$ 18 years old, newly diagnosed stage I-IV patients (undergoing thoracic RT with radical or palliative intent) with small cell, or non-small cell lung cancer were included. Patients presenting lung cancer recurrence were also included if no previous radiation therapy was administered in the first treatment course. The LC cohort consisted of 247 patients, 213 men (86%) and 34 (14%) women (Table 1).

Treatment planning

Patient immobilization and treatment planning were performed with the patient in the supine position. A vacuum sealed cradle for immobilization was made when necessary. All patients were scanned (contrast enhanced computed tomography [CT] scan in 0.5-cm thickness) from the atlas (C1) level to the second lumbar vertebra (L2) level, approximately, to include the whole neck and lungs. In brief, the gross tumor volume (GTV-primary and GTV-node) consisted of the lesion diagnosed by biopsy and therefore visible in the subsequent CT scan. The regions of tumor visible by endoscopy but not seen on CT images were also included in the GTV-primary. The GTV was expanded by 6–8 mm around the primary tumor and selected lymph node region to obtain a clinical target volume (CTV) which was further expanded laterally and vertically by 10 mm to obtain a planning target volume (PTV). The esophagus was contoured beginning at the level of cricoid cartilage on every CT image, until and including the gastroesophageal junction.

Patient evaluation and follow-up

During the course of radiotherapy, patients were seen at least weekly and more often if they needed clinical evaluation and disease management. They were evaluated at approximately 1–3 months after completion of therapy and then every 3 months. The follow-up evaluations consisted of a history and physical examination. Computerized axial tomography scans were obtained at intervals of 3–6 months. Double-contrast esophagography was performed if clinically indicated.

Genotyping method and SNP selection

Genomic DNA was isolated from peripheral blood sample using the DNeasy® Blood and Tissue kit (Qiagen, Hilden, Germany). DNA concentrations and purity were determined using the NanoDrop 2000 UV–Vis spectrophotometer (Nano Drop Technologies, Wilmington, DE, USA).

Four potentially functional variants of *TGFB1* and *HSPB1* genes were selected from the gene SNP database of the National Institute of Environmental Health Sciences Genome Program and related literature using the *LD TAG SNP Selection* tool (https://snpinfo.niehs.nih.gov/snpinfo/snptag.html). The selected SNPs met at least two of the following criteria: (1) a minor allele frequency of at least 5%, (2) to be located in the promoter, regulatory or untranslated region of the gene, (3) TaqMan® SNP Genotyping Assay available. Additionally, three previously described SNPs, rs1800469 rs2868371 and rs2868370, associated with RIET development have been selected for validation.

All the genotypes were obtained by real-time polymerase chain reaction on the Viia7 Real Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA) using TaqMan® SNP Genotyping Assays (Supplementary Table 1). The general PCR conditions used for the experiments were 60 °C × 30″, [95 °C × 10′, 95 °C × 15″, 60 °C × 1′, 60 °C × 30″] × 40.

Statistical analysis

Statistical analyses were performed using SPSS (version 19.0). The end points of analysis (development of RIET acute and late) were estimated and scored using Common Terminology Criteria for Adverse Events version 4.0. The RT starting point was considered to calculate the time to different RIET development; patients who did not experience the end points were censored at 3 months since RT started or at the time of death or last contact. Kaplan–Meier analysis was performed to estimate the cumulative RIET incidence. Cox proportional hazards analysis was performed to calculate hazard ratio (HR) and confidence interval (CI). Multivariate Cox regression analysis with a stepwise backward elimination procedure was used to adjust for factors significant on univariate analysis, as well as any other factors that might have misled genotypes univariate analysis. A p-value $\leq$ 0.05 was considered significant. In addition, the pointwise P value was adjusted for multiple testing using a Bonferroni approach by multiplying the pointwise P value by the number of tests.

Table 1
Patient's Characteristics.

Characteristic	No. of Patients (%) $n = 247$
Gender	
Female	34 (14)
Male	213 (86)
Age (years)	
Median (range)	65 (35–88)
Treatment	
Primary Tumor	195 (79)
Relapse	18 (7.3)
Palliative	34 (13.7)
Smoking status	
Current	126 (51)
Former	111 (45)
Never	10 (4)
N° of cigarettes per day	
Median (range)	30 (3–100)
No. of pack years*	
Median (range)	63 (1.5–275.15)
History of dysphagia	
No	234 (94.7)
Yes	13 (5.3)
Loss Weight history	
No	196 (79.3)
Yes	51 (20.7)
KPS	
≥80	187 (75.7)
<80	60 (24.3)
Weight before RT (Kg),	
Median (range)	75 (50–93.3)
Histology	
Squamous cell	102 (41.4)
Adenocarcinoma	69 (28)
Large-cell carcinoma	8 (3.3)
NSCLC, NOS	9 (3.6)
Small-cell carcinoma	57 (23.2)
Sarcoma	1 (0.5)
Primary clinical stage	
I	13 (5.3)
II	12 (4.8)
IIIA	92 (37.2)
IIIB	107 (43.3)
IV	23(9.4)
Surgery	
No	208 (84.2)
Yes	39 (15.8)
Radiation treatment (days)	
Median (range)	45 (1–127)
Radiation total dose (Gy)	
Median (range)	60 (2–70)
Radiation fractionation	
Once daily	201(81.4)
Twice daily	19 (18.6)
Concurrent chemoradiation	
No	140 (56.7)
Yes	107 (43.3)
PTV (cc)	
Median (range)	357,22 (23.63–1468.5)
CTV (cc)	
Median (range)	176.22 (7.5–1100)
GTV (cc)	
Median (range)	74.54 (0.98–595.8)
Volume of esophagus (cc)	
Median (range)	28.58 (7–94.43)
D_{min} of esophagus	
Median (range)	0.7 (0.1–15.44)
D_{max} of esophagus, Gy	
Median (range)	61.58 (7.2–75.7)
MED, Gy	
Median (range)	21.9(0.91–60.14)
Median dose of esophagus	
Median (range)	12.25 (0.29–65.2)
V_5	
Median (range)	56.625(1.47–100)
V_{10}	
Median (range)	51(0.07–100)
V_{15}	
Median (range)	47(0.09–100)
V_{20}	
Median (range)	43(0.04–98.4)
V_{25}	
Median (range)	40(0.02–97.41)
V_{30}	
Median (range)	37 (0.02–93.83)
V_{35}	
Median (range)	32.92 (0.09–92.04)
V_{40}	
Median (range)	28.07 (0.24–89.23)
V_{45}	
Median (range)	21.635 (0.09–80)
V_{50}	
Median (range)	15.37(0.78–79)
V_{55}	
Median (range)	9 (0.07–76)
V_{60}	
Median (range)	4 (0.15–74)
V_{65}	
Median (range)	2 (0.01–56)
V_{70}	
Median (range)	0 (0.01–54)

Abbreviations: KPS, Karnofsky Performance Status; NSCLC, NOS, non-small-cell lung cancer, not otherwise specified; D_{min}, minimum dose; D_{max}, maximum dose; MED, mean esophagus dose; V_x, volume of normal esophagus receiving × Gy or more radiation.

* Number of pack years = (packs smoked per day) × (years as a smoker).

SNPs in silico analysis

In order to identify functional effects, they were analyzed by RegulomeDB (www.regulomedb.org/), and SNPinfo (FuncPred). Additionally, differential expression analysis was performed using the data from the Genotype-Tissue Expression project (https://www.gtexportal.org/home/).

Results

Patient characteristics

The clinical–pathological characteristics of the 247 patients enrolled in the study are listed in Table 1. According to the classification of malignant tumors (TNM) staging system (7th ed), 25 patients (10.1%) were stage I-II, 92 (37.2%) were stage IIIA, 107 (43.4%) were stage IIIB and 23 (9.3 %) were stage IV. According to the histopathological classification, there were 69 (28%) adenocarcinoma, 102 (41.3%) squamous-cell carcinoma, 8 (3.2%) large-cell carcinoma, 9 (3.6%) not otherwise specified and 57 (23.4%) small cell carcinoma. Median follow-up time for all patients was 14 months (range, 1 to 88 months). Patients who died or progressed before 3 months from the start of radiation therapy ($n = 31$) were not included for the late toxicity assessment. At the time of analysis, 141 patients (57%) experienced acute RIET and 28 (13%) late RIET. Irrespective of the treatment modality, the acute grade 2 and 3 rate for all patients was 47% ($n = 116$), 4.5% ($n = 11$), respectively, and 8.3% ($n = 18$) and 1.4% ($n = 3$) for late RIET, respectively. There was no acute or late grade 4/5 toxicity. Median time between the first day of radiotherapy and the maximum acute RIET of (grade 2 and 3) was 0.7 (range, 0.17–2.90) and 1.6 (range, 0.5–2.03) months, respectively.

Table 2
Associations between patient-, tumor-, and therapy-related characteristics and radiation esophagitis toxicity.

Parameter	Acute Esophagitis (n = 247)						Late Esophagitis (n = 216)					
	Grade 2			Grade 3			Grade 1			Grade 2		
	HR	95% CI	p value	HR	95% CI	p value	HR	95% CI	p value	HR	95% CI	p value
Gender												
Female (ref)	1.00	0.48–1.29	0.342	1.00	0.22–13.62	0.596	1.00	0.15–3.24	0.635	1.00	0.40–	0.286
Male	0.79			1.74			0.69			3.00	22.52	
Age, years												
≤65 (ref)	1.00	0.32–0.70	**<0.001**	1.00	0.19–2.25	0.507	1.00	0.35–4.21	0.756	1.00	0.30–1.98	0.586
>65	0.47			0.66			1.22			0.77		
Treatment												
Primary Tumor (ref)	1.00	0.29–1.34	0.223	1.00	0–845.13	0.533	1.00	0–2254.28	0.569	1.00	0.06–3.67	0.485
Relapse	0.62			0.04			0.04			0.49		
Smoking status												
Former/Never (ref)	1.00	1.08–2.26	**0.018**	1.00	0.68–9.58	0.168	1.00	0.27–3.26	0.928	1.00	0.37–2.32	0.858
Current	1.56			2.54			0.94			0.92		
No. of cigarettes per day												
≤30 (ref)	1.00	0.75–1.58	0.638	1.00	0.19–2.38	0.537	1.00	0.12–1.98	0.319	1.00	0.57–4.03	0.409
>30	1.09			0.67			0.49			1.51		
No. of packs years[a]												
≤63 (ref)	1.00	0.69–1.46	0.994	1.00	0.30–3.81	0.909	1.00	0.16–2.18	0.426	1.00	0.75–3.26	0.234
>63	1.001			1.08			0.59			1.56		
History of alcoholism												
No (ref)	1.00	0.80–1.82	0.369	1.00	0–20.86	0.277	1.00	0.17–6.67	0.906	1.00	0.61–5.37	0.29
Yes	1.21			0.02			1.11			1.80		
History of dysphagia												
No (ref)	1.00	0.70–3.22	0.301	1.00	0.29–17.76	0.442	1.00	0.31–19.43	0.394	1.00	0.21–	0.669
Yes	1.50			2.25			2.46			1.56	11.81	
History of weight loss												
No (ref)	1.00	0.36–0.99	**0.045**	1.00	0.99–10.63	0.052	1.00	0.22–4.88	0.967	1.00	0.60–4.74	0.326
Yes	0.60			3.24			1.03			1.68		
KPS												
>80 (ref)	1.00	0.50–1.04	0.084	1.00	0.25–2.73	0.764	1.00	0.74–11	0.13	1.00	0.29–1.91	0.531
≤80	0.73			0.83			2.84			0.74		
Weight before RT, Kg												
≤75 (ref)	1.00	0.60–1.32	0.566	1.00	0.36–8.06	0.497	1.00	0.16–2.00	0.375	1.00	0.84–	0.074
>75	0.89			1.71			0.56			6.32	47.71	
Histology												
SCLC (ref)	1.00	0.44–1.01	0.054	1.00	0.23–3.21	0.813	1.00	0.24–5.51	0.866	1.00	0.39–4.80	0.626
NSCLC	0.67			0.85			1.14			1.37		
Clinical Stage												
I-II (ref)	1.00	0.97–4.50	0.058	1.00	0.007–86197	0.446	1.00	0.17–10.45	0.791	1.00	0.35–	0.346
III-IV	2.09			24.12			1.32			2.64	19.83	
Surgery												
No (ref)	1.00	0.42–1.25	0.25	1.00	0–35.33	0.347	1.00	0.06–3.64	0.462	1.00	0.11–2.11	0.335
Yes	0.73			0.04			0.46			0.48		
Radiation treatment, days												
≤45 (ref)	1.00	0.60–1.25	0.434	1.00	0.14–1.58	0.217	1.00	0.09–1.29	0.111	1.00	0.77–6.03	0.147
>45	0.87			0.46			0.33			2.15		
Radiation total dose, Gy												
≤60 (ref)	1.00	0.71–1.48	0.89	1.00	0.15–1.79	0.303	1.00	0.16–1.95	0.355	1.00	1.01–9.28	**0.049**
>60	1.03			0.52			0.55			3.05		
Radiation fractionation												
Once daily (ref)	1.00	2.14–6.25	**<0.001**	1.00	0–593.39	0.519	1.00	0.14–8.69	0.928	1.00	0.29–5.48	0.759
Twice daily	3.66			0.04			1.10			1.26		
Concurrent CRT												
No (ref)	1.00	1.21–2.53	**0.003**	1.00	0.87–12.39	0.079	1.00	0.44–5.58	0.482	1.00	0.53–3.42	0.528
Yes	1.75			3.29			1.58			1.35		
PTV, cc[b]	1.001	1,00001–1.001	**0.045**	1.001	0.99–1.003	0.201	1.00	0.998–1.003	0.816	1.001	0.99–1.003	0.202
CTV, cc[b]	1.001	1,0001–1.002	**0.025**	1.003	1.000008; 1.005	**0.049**	0.998	0.99–1.004	0.51	1.001	0.99–1.004	0.556
GTV, cc[b]	1.002	1,0003–1.004	**0.021**	1.003	0.99–1.01	0.32	1.003	0.997–1.01	0.331	1.00	0.99–1.01	0.957
Volume of esophagus[b]	0.98	0.97–1	0.056	1.026	0.98–1.07	0.274	1.01	0.97–1.06	0.595	1.01	0.97–1.05	0.724
D_{min} of esophagus[b]	1.05	0.98–1.12	0.155	1.057	0.83–1.34	0.65	0.84	0.41–1.73	0.634	1.11	0.97–1.27	0.119
D_{max} of esophagus[b]	1.02	1.004–1.03	**0.012**	1.022	0.97–1.08	0.397	0.99	0.96–1.03	0.642	1.01	0.98–1.05	0.424
MED[b]	1.03	1.01–1.05	**<0.001**	1.048	0.99–1.11	0.079	1.003	0.95–1.06	0.925	1.04	0.997–1.08	0.068
Median Dose of esophagus[b]	1.02	1.01–1.03	**0.002**	1.026	0.99–1.06	0.131	1.02	0.98–1.05	0.35	1.01	0.98–1.04	0.516
V_5[b]	1.02	1.01–1.03	**0.001**	1.02	0.98–1.06	0.292	1.01	0.97–1.05	0.611	1.03	0.999–1.06	0.056
V_{10}[b]	1.02	1.01–1.03	**0.001**	1.02	0.99–1.06	0.218	1.01	0.98–1.05	0.541	1.02	0.99–1.05	0.128
V_{15}[b]	1.02	1.01–1.03	**<0.001**	1.02	0.99–1.06	0.164	1.01	0.98–1.04	0.641	1.01	0.99–1.04	0.286
V_{20}[b]	1.02	1.01–1.03	**<0.001**	1.03	0.997–1.07	0.077	1.003	0.97–1.04	0.822	1.02	0.995–1.05	0.111

Table 2 (*continued*)

Parameter	Acute Esophagitis (n = 247)						Late Esophagitis (n = 216)					
	Grade 2			Grade 3			Grade 1			Grade 2		
	HR	95% CI	p value	HR	95% CI	p value	HR	95% CI	p value	HR	95% CI	p value
V_{25}[b]	1.02	1.01–1.03	**<0.001**	1.03	0.996–1.07	0.084	1.003	0.97–1.04	0.853	1.02	0.99–1.04	0.179
V_{30}[b]	1.02	1.01–1.03	**<0.001**	1.03	0.998–1.07	0.062	1.003	0.97–1.04	0.849	1.02	0.99–1.04	0.162
V_{35}[b]	1.02	1.01–1.03	**<0.001**	1.03	0.994–1.07	0.101	1.004	0.97–1.04	0.788	1.02	0.995–1.05	0.114
V_{40}[b]	1.02	1.01–1.03	**<0.001**	1.03	0.99–1.06	0.133	1.004	0.97–1.04	0.79	1.02	0.99–1.04	0.132
V_{45}[b]	1.02	1.01–1.02	**0.003**	1.03	0.99–1.06	0.132	1.004	0.97–1.04	0.819	1.01	0.99–1.04	0.284
V_{50}[b]	1.01	1.002–1.02	**0.019**	1.03	0.995–1.06	0.103	1.003	0.97–1.04	0.857	1.02	0.99–1.04	0.236
V_{55}[b]	1.01	1.0002–1.02	**0.045**	1.03	0.999–1.06	0.056	0.994	0.96–1.04	0.782	1.02	0.995–1.05	0.144
V_{60}[b]	1.01	0.996–1.02	0.177	1.03	0.996–1.06	0.081	0.995	0.95–1.04	0.812	1.02	0.997–1.05	0.086
V_{65}[b]	1.02	0.99–1.03	0.314	1.00	0.94–1.06	0.991	0.96	0.87–1.05	0.361	1.02	0.98–1.05	0.392
V_{70}[b]	0.99	0.97–1.02	0.599	1.03	0.97–1.08	0.364	0.971	0.86–1.01	0.644	0.99	0.92–1.06	0.779
rs11466353												
TT/TG (ref)	1.00	0.56–1.94	0.892	1.00	0.101–2.17	0.331	1.00	0.103–2.28	0.360	1.00	0.12–0.91	**0.033**
GG	1.04			0.47			0.49			0.33		
rs7459185												
GG/GC (ref)	1.00	0.71–2.61	0.346	1.00	1.35–19.15	**0.016**	1.00	0.15–9.44	0.866	1.00	0.08–4.62	0.635
CC	1.37			5.08			1.19			0.61		
rs2868371												
CC (ref)	1.00	0.73–1.54	0.758	1.00	0.49–4.37	0.489	1.00	0.56–5.29	0.338	1.00	0.23–1.75	0.383
CG/GG	1.06			1.47			1.73			0.64		
rs1800469												
AA (ref)	1.00	0.61–1.43	0.739	1.00	0.27–2.92	0.850	1.00	0.43–11.43	0.345	1.00	0.18–2.51	0.556
AG/GG	0.93			0.89			2.21			0.67		
rs1800468												
CC (ref)	1.00	0.61–1.74	0.902	1.00	0.34–7.33	0.557	1.00	0.59–15.72	0.183	1.00	0.58–5.1	0.332
CT/TT	1.03			1.58			3.05			1.72		
rs11466343												
CC (ref)	1.00	0.44–2.29	0.982	1.00	0.29–18.30	0.435	1.00	0.65–60.41	0.112	1.00	0–293.97	0.490
CT/TT	1.01			2.29			6.28			0.05		
rs2868370												
GG (ref)	1.00	0.54–1.69	0.874	1.00	0.27–13.40	0.525	1.00	0.09–24.18	0.775	1.00	0.57–17.02	0.191
GA/AA	0.96			1.89			1.50			3.11		

Abbreviations: KPS, Karnofsky performance status; NSCLC, non-small-cell lung carcinoma; SCLC, small-cell lung carcinoma; RT, radiation therapy; CRT, chemoradiation; GTV, gross tumor volume; CTV, clinical target volume; PTV, planning target volume; MED, mean esophagus dose; D_{min} of esophagus, the minimum dose; D_{max} of esophagus, the maximum dose; V(x), volume of normal esophagus receiving X Gy or more radiation.

[a] Number of pack years = (packs smoked per day) × (years as a smoker).

[b] Dosimetric parameters were analyzed as continuos variables.

Correlation between esophageal toxicity, clinical–pathological characteristics and the selected SNPs

The genotype distributions of *TGFB1* and *HSPB1* SNPs among LC patients are displayed in Supplementary Table 1. Univariate analyses in order to identify associations between patient-, tumor-, and therapy-related characteristics and acute and late RIET are shown in Table 2. Concerning patient characteristics, age, smoking status and weight loss history were found to be associated with grade 2 acute RIET in univariate analysis. The PTV and the GTV were also significantly associated with acute grade 2 esophagitis, whereas CTV was associated with both acute grade 2 and 3 esophagitis. Mean and median esophagus dose (MED) and dosimetric parameters from volume of esophagus (in percent) exposed to 5 Gy (V_5) to V_{55}, were likewise associated with acute RIET grade 2 risk in the univariate analysis. Regarding the type of treatment, radiation fractionation and concurrent radiochemotherapy, were associated with acute grade 2 esophagitis. Concerning late esophageal damage, median time for development after completion of RT was 9 months (3–18 months). Irradiation dose and fractionation were both correlated with grade 2 late RIET. Across the SNP selected for validation, there were no statistically significant connections between the rs2868371 CC genotype and higher risk of acute or late esophagitis (acute grade 2 esophagitis: p value = 0.758; acute grade 3 esophagitis: p value = 0.489; late grade 1 esophagitis: p value = 0.338; late grade 2 esophagitis: p value = 0.383). Identically, rs1800469 AG/AA genotypes previously associated with higher risk of RIET were not statistically significant (acute grade 2 esophagitis: p value = 0.739; acute grade 3 esophagitis: p value = 0.850; late grade 1 esophagitis: p value = 0.345; late grade 2 esophagitis: p value = 0.556). Concordant results were obtained related to the rs2868370 previously studied but not associated with RIET (acute grade 2 esophagitis: p value = 0.874; acute grade 3 esophagitis: p value = 0.525; late grade 1 esophagitis: p value = 0.775; late grade 2 esophagitis: p value = 0.191).

For the rest of SNPs, patients carrying the rs7459185 CC genotype were associated with a higher risk of acute grade 3 RIET (HR = 5.08; 95% CI = 1.35–19.15; p = 0.016). This effect was virtually unchanged when logistic regression model with a stepwise backward elimination procedure of factors associated with acute esophagitis was used (HR = 17.73; 95% CI = 2.896–108.49; p = 0.002), suggesting that the association between acute grade 3 esophagitis incidence and rs7459185 CC genotype is an independent factor (Table 3; Fig. 1). This association was significant after adjustment for multiple testing (p = 0.02), assuming a Bonferroni correction. Patients exhibiting rs7459185CC genotype had acute grade 3 RIET rate of 17% (3/18) compared with 4% (8/229) in patients having rs7459185 GG/GC. Fig. 2A shows the RIET as a function of time according to the rs7459185 SNP and V_{30}. Patients with high (>median) V_{30} and the rs7459185 GG/GC genotypes had

Table 3
Multivariate analysis using a Cox regression model with a stepwise backward elimination procedure of factors associated with acute and late esophagitis.

Parameter	*Acute esophagitis*						*Late esophagitis*		
	Grade 2			*Grade 3*			*Grade 2*		
	HR	95% CI	*p* value	HR	95% CI	*p* value	HR	95% CI	*p* value
Age, years									
≤65 (ref)	1.00	0.394–1.007	0.054						
>65	0.63								
KPS									
≤80 (ref)				1.00	1.606–286.12	0.020			
>80				21.43					
No. of cigarettes per day									
≤30 (ref)									
>30									
No. of packs years[a]									
≤63 (ref)									
>63									
rs11466353									
TT/TG (ref)							1.00	0.103–0.830	0.021
GG							0.29		
rs7459185									
GG/GC (ref)				1.00	2.896–108.49	0.002			
CC				17.73					
Radiation total dose, Gy									
≤60 (ref)							1.00	0.945–8.920	0.063
>60							2.90		
Radiation fractionation									
Once daily (ref)	1.00	1.604–5.067	<0.001						
Twice daily	2.85								
V_{20}	1.02	1.019–1.006	0.003						
V_{30}				1.04	0.993–1.087	0.097			

Abbreviations: KPS, Karnofsky Performance Status; V(x), volume of normal esophagus receiving × Gy or more radiation.
Bold value indicates a statistically significant difference with a p-value less than 0.05
[a] Number of pack years = (packs smoked per day) × (years as a smoker).

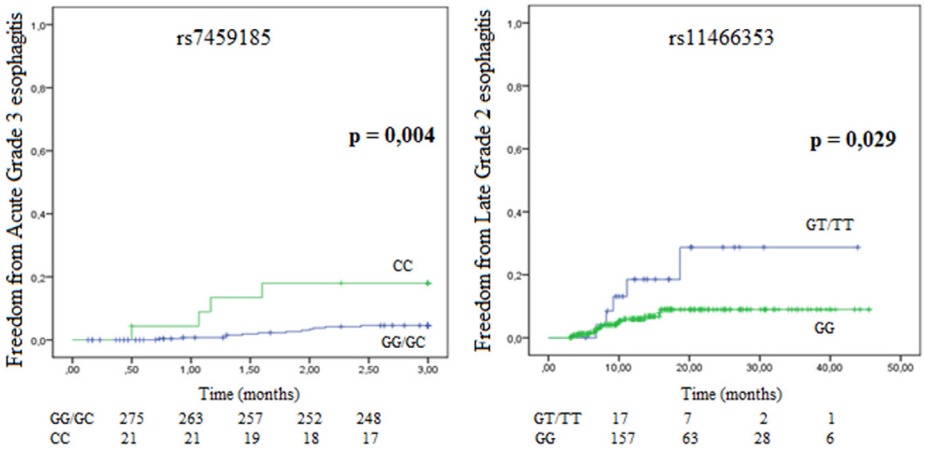

Fig. 1. Kaplan–Meier curve for acute grade 3 and late grade 2 radiation-induced esophagitis in lung cancer patients carrying different rs7459185 and rs11466353 genotypes.

a significantly lower RIET incidence ($p < 0.001$) compared with patients carrying the CC genotype.

Additionally, LC patients with the rs11466353 GG genotype showed an association with a lower risk of late grade 2 RIET development (HR = 0.33; 95% CI = 0.12–0.91; $p = 0.033$) which was further unchanged in multivariate analyses (HR = 0.29; 95% CI = 0.103–0.830; $p = 0.021$) (Table 3; Fig. 1). This association was not significant after adjustment for multiple testing ($p = 0.105$), assuming a Bonferroni correction. Patients harboring the rs11466353 TT/TG genotypes exhibit a late grade 2 RIET rate of 23% (5/21) compared with 7% (13/196) in patients with rs11466353 GG genotype. Fig. 2B shows the RIET as a function of time according to the rs11466353 SNP and the delivered radiation dose. Patients receiving high (>60 Gy) radiation doses and the rs11466353 GG genotype had a significantly lower RIET incidence ($p = 0.025$) compared with patients carrying the TT/TG genotypes. Eventually, no statistical significant association was found for the rest of SNPs analyzed linked to RIET risk development.

The SNPs were also analyzed according lung cancer subtypes. For patients with adenocarcinoma ($N = 69$), only the rs7459185 SNP showed a significant association with acute grade 2 radiation esophagitis (HR: 3.34; 95% CI 1.16–9.59; $p = 0.025$). Patients exhibiting rs7459185CC genotype had acute grade 2 RIET rate of 100% (4/4) compared with 51% (33/65) in patients having rs7459185 GG/GC. For patients with squamous cell carcinoma ($N = 102$), only the rs11466353 SNP showed an association with acute grade 3 (HR: 0.12; 95% CI 0.02–0.99; $p = 0.049$) and late grade 1 (HR: 0.13; 95% CI 0.10–0.86; $p = 0.025$) RIET. Patients harboring the rs11466353 TT/TG genotypes exhibit an acute grade 3 and late grade 1 RIET rate of 18% (2/11) and 20% (2/10) compared with 2%

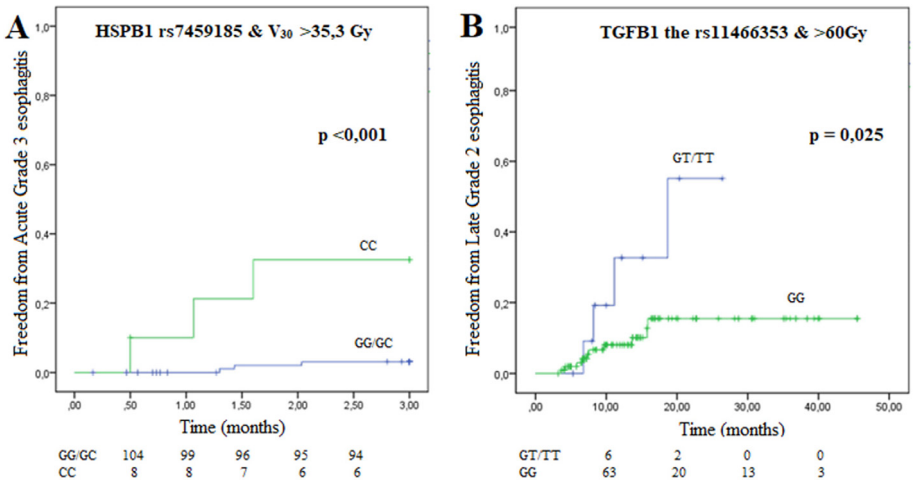

Fig. 2. Kaplan–Meier curve for: (A) the effect of the rs7459185 genotypes in lung cancer patients receiving high (>median) V_{30} on the cumulative incidence of acute grade 3 radiation-induced esophagitis; and (B) the effect of the rs11466353 genotypes in lung cancer patients receiving high (>median) radiation doses on the cumulative incidence of late grade 2 radiation-induced esophagitis.

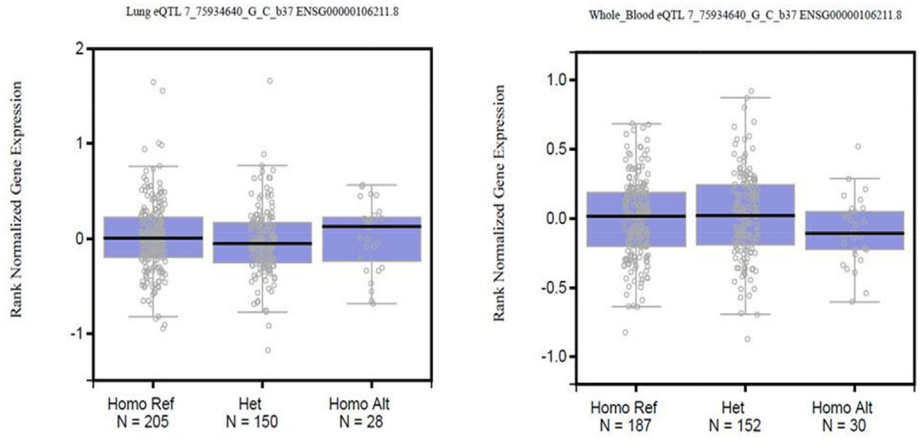

Fig. 3. Expression quantitative trait loci analysis of HSPB1 in the presence of the three different rs7459185 genotypes using data from the Genotype-Tissue Expression project.

(2/91) and 3% (2/78), respectively, in patients with rs11466353 GG genotype. For those cases with small-cell carcinoma ($N = 57$), there was not any statistical significant association.

The SNP rs7459185 is a downstream gene variant located in the chromosomal position chr7:76305323 (Supplementary Fig. 1). To provide biologically plausible support to the association and the prediction obtained, rs7459185 different genotypes and HSPB1 mRNA expression patterns were evaluated by expression quantitative trait loci analysis using lung tissue and blood cells expression data from the Genotype-Tissue Expression Database. The results showed a decrease in the HSPB1 mRNA expression levels in the presence of the rs7459185 CC genotype; while there were no evident changes between HSPB1 mRNA expression levels between different genotypes in case of lung tissue (Fig. 3).

Discussion

Over half of all LC patients are currently treated with RT; however the treatment is sometimes severely limited by the need to constrain the dose to the surrounding normal tissues in order to preserve their function. Esophageal toxicity caused by RT is a real and potentially debilitating toxicity which is estimated to be mainly caused by patient-related factors.

In the present study, half of patients suffered from acute esophagitis grade 2 or 3, which implies a complication that may require invasive treatments and delay the systemic therapies, despite a relatively homogeneous treatment. This common event has been previously explained as a result of the general concomitant platinum-based chemotherapy used in LC treatment due to radio-sensitizing nature of these agents [25–27]. Concurrent chemoradiation was associated with grade 2 acute esophagitis in univariate analysis but not for grade 3, much more severe and life-threatening in some cases, requiring enteral or parenteral nutrition. Therefore, concurrent chemoradiotherapy might not answer the more severe cases of toxicity nor explain the variability of the normal tissue response.

Alternatively, dosimetric factors such as the volume of the esophagus in percentage that receives a certain irradiation dose (Gy) as well as esophagus delineation might be suitable predictive dosimetric factors for esophagitis. Escalating the radiation therapy dose seems to increase toxicity risk development, especially when chemotherapy is concomitantly administered. Higher radiation therapy doses with concurrent chemotherapy result in poorer survival, partially due to the high levels of toxicity, which suggests that the optimal radiation dose has yet to be reached [28].

As a non-invasive alternative for molecular diagnosis and treatment monitoring we propose the association study between 7

SNPs, 2 previously described to be linked to RIET risk development (rs1800469 and rs2868371). Firstly, we did not find evidence of associations between the rs1800469, and rs2868371 in our cohort. The connection between the rs1800469 GA/AA genotypes was tested in early-grade RIET in a Chinese population [19], and next confirmed in an American study which also associated the presence of those genotypes with grade 3 toxicity [20]. Marked differences in cancer occurrence and treatment response may be markers of differences in genetic susceptibility. The SNP distribution is well known to differ among ethnicities and somehow polymorphic alleles can be under negative or positive selection in a population. The rs1800469 genotyping distribution is substantially different compared to the one found in our population. Relating to rs2868371, a previous American study linked a higher risk of grade 3 RIET with homozygosis for the most common allele C [24], with a very similar genotyping distribution compared to ours. Nevertheless, ethnic background should be carefully considered and some bias might affect those previous results owing to the small size and single-institution population analyzed.

Regarding the non-previously described SNP analyzed, the results obtained showed that patients carrying the rs7459185 CC genotype located in *HSPB1* sequence present a significantly higher risk of acute RIET while LC patient's harboring the rs11466353 TT/TG genotypes are associated with a higher susceptibility for late RIET development. The association between these genotypes and higher esophagitis risk resulted independent of other clinical–pathological and treatment factors although the SNP rs11466353 association did not retained statistical significance after Bonferroni correction. Interestingly, only the rs7459185 SNP showed a significant association with RIET when analyzing adenocarcinoma cases and only the rs11466353 SNP showed an association with RIET for patients with squamous cell carcinoma. We acknowledge that the distribution of cases across histological subtypes in our series is not representative in the clinical setting, where there is usually much more adenocarcinoma than squamous cell carcinomas. These SNPs are within the *TGFB1* and *HSPB1* sequences, which are widely-known for their role in RT clinical response and have been previously subjected as radiation tolerance biomarkers [29–31].

On one hand, TGFB1 has been well-established to be associated with radiation induced inflammation as a master switch for fibrosis development and persistence [17,32,33]. Moreover, ionizing radiation induces TGFB1 release, and overall the literature suggests plasma TGFB1 levels for its potential value to predict toxicities in LC [34,35]. However, TGFB1 plasma measurement is challenging because of its reproducibility, whereas DNA is more stable and easily testable, giving the opportunity to individualize treatment. Thus the use of associated *TGFB1* SNPs as biomarkers is notably convenient. The significant statistical association between the rs11466353 TT genotype and late esophagitis merely might be a prognostic biomarker and play an important role in the clinical outcomes of LC patients. Nonetheless, to test the rs11466353 intronic variant regulatory potential role *in silico* resulted in a struggle due to the little data available, thus the molecular mechanisms for the observed connection should be further investigated.

On the other hand, the rise of the rs7459185 CC genotype within *HSPB1* gene was linked to the risk of acute grade 3 esophagitis in LC patients. Bioinformatics' analysis showed a decrease in HSPB1 mRNA expression levels in the presence of the rs7459185 CC risk genotype. HSPB1 is also well-known for its function in stress-induced cellular damage prevention and protein stability, being strongly induced by different stressors, including ionizing radiation [36–38]. In this regard, high HSPB1 expression levels enhance the antioxidant capacity of ionizing radiation. Therefore, a HSPB1 down-regulation triggered by the rs7459185 C risk allele may increase cell sensitivity to RT, promoting RIET, which may partly explain the underlying biological mechanisms of the observed associations, although this hypothesis needs to be confirmed.

In conclusion, we were able to identify genetic variants associated with the development of short- and long-term side effects of RT. To our knowledge, this is the first evidence that the presence of different genotypes of both SNPs (rs7459185/rs11466353) associate with acute or late RIET risk, respectively. Together with radiation dosimetric and other risk factors, the genetic test performed in the current analysis might be useful as biomarker in the prescription of personalized RT.

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Conflicts of interest

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.radonc.2019.03.005.

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