Radiotherapy and Oncology 136 (2019) 29–36

Contents lists available at ScienceDirect

Radiotherapy and Oncology

journal homepage: www.thegreenjournal.com

Original Article

The single nucleotide variant rs2868371 associates with the risk of mortality in non-small cell lung cancer patients: A multicenter prospective validation

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ARTICLE INFO

Article history:
Received 7 December 2018
Received in revised form 23 March 2019
Accepted 26 March 2019
Available online 6 April 2019

Keywords:
Non-small cell lung cancer
HSPB1
Functional SNP
Glucocorticoid receptor

ABSTRACT

Background and purpose: Definitive radiation therapy (RT) with or without chemotherapy has become the standard treatment for non-metastatic unresectable non-small cell lung cancer (NSCLC). However, treatment outcomes can differ substantially and patients' genetic background could play a crucial role. Potential associations between single-nucleotide polymorphisms (SNP) in Heat shock protein beta-1 (*HSPB1*) and survival have been reported in prior single-institution retrospective reports.

Materials and methods: The current assay aims to validate such connection in a prospective multicenter study in a European cohort including 181 NSCLC patients. Median follow-up time for all patients was 13 months (range, 3–57 months).

Results: The results obtained show an association between the rs2868371 GG genotype and better overall survival (HR: 0.35; 95%CI: 0.13–0.96; $p = 0.042$) in multivariate analysis. Two-year overall survival rate was 72% for patients carrying the rs2868371 GG genotype versus 36% for those patients harboring the rs2868371 CC/CG genotypes ($p = 0.013$). Additionally, the rs2868371 GG genotype was found to be associated with better disease-free survival in the multivariate analysis (HR: 0.36; 95%CI: 0.13–0.99; $p = 0.048$). *In silico* analysis of the potential functional SNP suggested significant difference in the affinity of the Glucocorticoid Receptor binding site between alternative allelic variants, confirmed by chromatin immunoprecipitation analysis displaying stronger affinity for the risk allele (C). Furthermore, our findings indicate that the rs2868371 influences (mRNA) *HSPB1* expression, offering insight into the regulation of *HSPB1* transcription.

Conclusion: The functional *HSPB1* rs2868371 promoter variant may affect lung cancer survival by regulation of HSPB1 expression levels through glucocorticoid receptor interaction.

Lung cancer is a leading cause of mortality and morbidity worldwide. Among all lung cancer cases, non-small-cell lung carcinoma (NSCLC) accounts for approximately 85%, with two major histological types: adeno- and squamous cell carcinoma. Over the past decade the management for lung cancer has greatly improved, based on prevention, early detection, and surveillance. Regardless, long-term survival is very limited, especially in locally advanced and metastatic lung cancer [1]. Early-stage NSCLC patients can benefit from surgical resection or stereotactic body radiation therapy (SBRT), whereas a multidisciplinary treatment strategy including the combination of definitive radiation therapy (RT) and chemotherapy is the principal treatment modality for unresectable advanced NSCLC [2,3]. However, NSCLC patients with similar clinical and pathological characteristics develop very different responses to treatment [4,5]. The individual variation in such clinical response is estimated to be caused by patient-related factors,

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https://doi.org/10.1016/j.radonc.2019.03.025
0167-8140/

likely due to the heterogeneity of different gene expression profiles. A plausible explanation for this fact may rely on distinct protein activities due to the presence of single nucleotide polymorphisms (SNPs) on coding regions or located within promoter regions able to alter the regulation of gene transcription [6,7]. Thus, novel molecular biomarkers and genetic variants are required within clinical practice to improve loco-regional control and survival.

To this end, earlier radiogenomic studies attempted correlations of candidate SNPs in different genes as predictive biomarkers for radiation-induced damage risk and survival in NSCLC patients' treatment [8–16]. Among them, HSPB1 that belongs to the heat-shock protein family (HSP) encoded by the *HSPB1* gene, is a master regulator of radio(chemo)therapy response [17–19]. HSPs are a diverse group of highly conserved proteins classified according to their molecular-weight which play important roles in the prevention of stress-induced cellular damage [20,21]. The small heat-shock protein of 27 kDa HSPB1 is overexpressed in lung cancer tissue and serum. HSPB1 plays important roles in cancer progression and prognosis arising from its anti-aggregation and anti-apoptotic properties and SNPs along its sequence have been associated with one or more late effects of RT and lung cancer survival in replication studies [19,22–25]. Two prior retrospective studies [22,25] have shown an association between genetic variants in *HSPB1* and survival in NSCLC patients. The first study [22] included a cohort which underwent RT in 50% of cases while the second study [25] focused only on patients receiving RT. In both studies, the vast majority of patients also received chemotherapy. Guo et al [22] showed that the relative luciferase activity driven by the *HSPB1* gene promoter containing 1271G allele was significantly higher than that driven by the promoter containing 1271C allele in four cell lines ($P < 0.05$ for all), which suggested that the 1271C allele may lead to a lower Hsp27 protein synthesis level than the 1271G allele. All of these findings suggest that HSPB1 is connected with the existing individual radiobiology in lung cancer patients and is associated with an increased risk of lung cancer as well. Thus, regulatory SNPs along the HSPB1 sequence are valuable biomarkers to assess disease outcome.

In this sense, we have performed a multicenter prospective study to validate whether the genetic polymorphism rs2868371 within the *HSPB1* sequence may contribute directly to disease outcome among NSCLC patients.

Materials and methods

Ethics statement

Prior to their participation, written informed consent for genetic studies was obtained from all participants. 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.

Patient population

NSCLC patients ($N = 181$) for this prospective analysis were recruited at three institutions between January 2012 and December 2016. Exclusion criteria included having either prior history of other cancer or previous oncologic treatments. All had whole-blood samples available. Our cohort (Table 1) consisted of 163 men and 18 women with a median age of 67 years. One hundred and forty-six patients (81%) underwent platinum-based chemotherapy. A total of 155 patients (86%) received RT with a median radiation dose of 60 Gy (concurrent with chemotherapy in 34% of cases). Twenty-seven patients (15%) underwent surgery. Seven patients did not receive any oncologic treatment due to poor performance status in 2 cases, one patient refused recommended treatment, and 2 patients died while waiting to be treated.

Table 1
Patient's characteristics.

Characteristic	No. of patients (%) $N = 181$
Gender	
Female	18 (10)
Male	163 (90)
Age (years)	
Median (range)	67 (37–88)
KPS	
100	39 (22)
90	43 (24)
80	46 (25)
≤70	53 (29)
Tumor histology	
Squamous cell	103 (57)
Adenocarcinoma	61 (34)
Large-cell lung carcinoma	6 (3)
NSCLC, NOS*	11 (6)
Disease stage	
I–II	10 (6)
IIIA	82 (45)
IIIB	64 (35)
IV	25 (14)
Smoking status	
Current	86 (48)
Former	88 (49)
Never	7 (4)
Surgery	
No	154 (85)
Yes	27 (15)
Thoracic radiation therapy	
No	26 (14)
Yes	155 (86)
Radiation dose to the primary tumor (Gy)	
Median (range)	60 (4–68)
Chemotherapy	
No	35 (19)
Yes	146 (81)
Concomitant chemotherapy	
No	120 (66)
Yes	61 (34)
Rs2868371	
CC	102 (56)
CG	65 (36)
GG	14 (8)

Genotyping method and SNP selection

A 6-ml peripheral blood sample was drawn into coded heparinized tubes and genomic DNA was extracted from whole blood samples using the DNeasy® Blood and Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. DNA concentrations and purity were determined by spectrophotometric measurement of absorbance at 260 and 280 nm by the NanoDrop 2000 UV–Vis spectrophotometer (Nano Drop Technologies, Wilmington, DE, USA). As far as we know, the rs2868371 is the only *HSPB1* genetic variant previously described by its implication in NSCLC survival and treatment outcome in different ethnic groups [22,25]. For the study, we selected this SNP for validation in our cohort. The genotyping was performed by real-time polymerase chain reaction (PCR) on the Viia7 Real Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA) using TaqMan® SNP Genotyping Assays (C__16146175_10). 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. For all genotypes, the assay success rate was 95%, and concordance of repeated sample testing was 100%. When the call rate was less, DNA was re-extracted and a new genotyping assay was performed.

DNA isolation from paraffin tissue sections, PCR amplification and direct sequencing

Homogenized tumor-normal formalin-fixed paraffin-embedded (FFPE) tissues samples from 37 NSCLC patients were cut into 10-μm-thick sections. Sample material included fine-needle aspirations, core biopsies, surgical samples, and cytology from malignant effusions. DNA was isolated using QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. PCR conditions were optimized for amplification of DNA sequence surrounding SNP rs2868371 (primer pair: 5′-TGTT AGGCGTGTGGACTTTG-3′/5′-TTTTCCAGATGGGGAAACTG-3′), using MyTaq DNA Polymerase (Bioline GmbH, Luckenwalde, Germany) according to the manufacturer recommendations. PCR product was purified with ExoSAP-IT PCR Clean-up Kit (GE Healthcare, Little Chalfont, UK) and immediately sequenced using Big-Dye Terminator version 3Æ1 cycle sequencing kit (Applied Biosystems, Waltham, MA, USA) in an ABI 3500 × L DNA sequencer (Applied Biosystems, Waltham, MA, USA).

Quantitative PCR-based mRNA analysis

RNA was extracted from 35 FFPE tissue samples in TRIsure™ (Bioline GmbH, Germany). cDNA was generated from total RNA (1 μg) by SensiFAST™ cDNA Synthesis Kit (Bioline GmbH, Luckenwalde, Germany). Primer pairs used for HSPB quantitative PCR were: HSPB1: 5′-GTGGAGATCACCGGCAAG-3′/5′-ACGGT CAGTGTGCCCTCA-3′; GAPDH: 5′-GCCAAAAGGGTCATCATCTC-3′/5 ′-TGTGGTCATGAGTCCTTCCA-3′. PCR conditions were optimized for each primer pair and results were normalized to the internal standard GAPDH. Quantitative PCR was performed using iTaq Universal SYBR Green Supermix (Bio-Rad, Irvine, CA, USA) and Viia7 Real Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA) under the following conditions**:** 50 °C × 2′, [95 °C × 10′, 95 °C × 15″, 60 °C × 1′, 60 °C] × 40. Every experiment was performed in triplicate. Analysis was based on the ΔΔCt method. The upper limit of the Ct was set at 35.

In silico analysis of transcription factor binding sites

In order to identify functional effects of the SNP, it was analyzed computationally using PERFECTOS-APE [26] with the following parameters: HOCOMOCO v11, 0.0005 as a threshold for motif *P*-value for any of two alternative alleles, and 5 as a threshold for the ratio between the motif *P*-values for two alternative alleles. Additionally, the analysis was performed using the software PROMO v3.0.2, (which uses TRANSFAC v6.4) [27,28]. The sequences carrying each allele were loaded as the query sequence. The prediction was carried out considering only human sites and transcription factors.

Chromatin immunoprecipitation–quantitative PCR (ChIP–qPCR)

Twenty-four frozen tissue specimen sections were washed in PBS, cross-linked with 1% formaldehyde for 15 min at room temperature and quenched with 2.5 M glycine for 5 min, at room temperature. The samples were then homogenized with Lysis buffer (50 mM Tris–HCL, pH8.0, 10 mM EDTA, 1% SDS) treated with a protease inactivation cocktail (2 μg/μl of the serine protease inhibitor AEBSF (AG Scientific, San Diego, CA, USA); cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail (Roche, Basel, Switzerland). The cross-linked material was subsequently sonicated for 15 min (Intensity level: 5; 30seg on/30seg off) on a Bioruptor (Diagenode, Liège, Belgium). Soluble chromatin was quantified fluorometrically using a Q-bit (Thermo Fisher Scientific, Waltham, MA, USA), and ChIP was performed using agarose bead columns (Active Motif, Carlsbad, CA, USA) with 10 μg of antibody against Glucocorticoid Receptor (D6H2L) (Cell signaling Technology, Danvers, MA, USA) or the same amount of mouse IgG isotype control (Normal Rabbit IgG #2729) (Cell signaling Technology, Danvers MA, USA). Immunoprecipitated DNA or Input chromatin was diluted in Elution Buffer (10% SDS, 1 M $NaHCO_3$) and cross-links were reversed adding 5 M NaCl overnight at 65 °C. Proteinase K treatment was performed with Proteinase K solution 20 mg/ml (Thermo Fisher Scientific, Waltham, MA, USA) and incubation for 2 h at 45 °C. DNA was extracted with Phenol:Chloroform:Isoamyl Alcohol 25:24:1 (Sigma, Saint Louis, MO, USA) and diluted in 50 μl of nuclease-free water. Immunoprecipitated DNA was analyzed by real-time PCR (the same primers used for PCR amplification and direct sequencing) using iTaq Universal SYBR Green Supermix (Bio-Rad, Irvine, CA, USA) and Viia7 Real Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). Relative DNA quantity was normalized by the Percent Input Method.

Statistical analysis

Patients were grouped according to their genotypes. Multiple models were explored for rs2868371 in our analyses. SPSS (version 19.0) statistical software was used for data analyses. The primary outcome was the overall survival (OS). The sample size required for a power of 80% at a 5% level significance, and assuming a probability of survival difference of 10% at 2 years based on prior studies [17,18], is 174. The Kaplan–Meier product-limit method provided estimates of the following endpoints: OS, disease-free survival (DFS; defined as any disease recurrence [loco-regional, or distant]), loco-regional recurrence (LR), and distant metastases (DM). OS was defined as the interval between the diagnosis date to the date of death from any cause or the last date of follow-up for censored patients. DFS was defined from the date of diagnosis until the date of first recurrence, loco-regional or systemic. LR and DM were defined as the interval between the diagnosis date and the date of loco-regional or distant progression, respectively, as determined by clinical, pathologic and radiologic evidence. Cox proportional hazards analysis was performed to calculate HRs and CIs to evaluate the influence of patient, tumor, and treatment characteristics on risk of mortality and recurrence. In addition, multivariate analyses including the statistically significant features in the univariate analysis were performed using a Cox's proportional hazards model. The results of the quantitative data are presented as the mean with standard deviation (mean ± SD) of three independent experiments. All tests were two-tailed. $p < 0.05$ was considered significant.

Results

Patient characteristics

Clinical and pathological characteristics of the patients enrolled in the study are summarized in Table 1. Median follow-up time for all patients was 13 months (range, 3 to 57 months). At the time of analysis, 73 (40%) were alive and 108 (60%) had died. According to the TNM (7th ed), 10 (5.5%) were stage I-II, 82 (45.4%) were stage IIIA, 64 (35.3%) were stage IIIB and 25 (13.8%) were stage IV. According to the histopathological classification, there were 61 (33.7%) adenocarcinoma, 103 (57%) squamous cell carcinoma, 6 (3.3%) large cell carcinoma, and 11 (6%) NSCLC not otherwise specified.

Association of rs2868371 and survival in NSCLC patients

The genotype distributions of *HSPB1* SNP among NSCLC patients are described in Table 1. In order to identify risk factors that corre-

lated with patients' outcome, univariate analysis was performed. Correlations between corresponding patients' clinic-pathological features and OS, DFS, local and distant relapse are shown in Table 2. KPS, disease clinical stage, surgery, chemotherapy and radiation therapy were all significantly associated with OS in univariate analysis ($P < 0.05$). Univariate Cox proportional hazards regression analyses showed that the rs2868371 CC/GC genotypes were associated with poorer survival compared with the rs2868371 GG genotype (univariate crude hazards ratio [HR] = 0.30; 95% confidence interval [CI] 0.11–0.82, $p = 0.019$). The results obtained were similar when analyzing only those who had received RT (HR: 0.29; 95% CI: 0.09–0.90; $p = 0.033$; Table 2). In addition, patients receiving either surgery or concomitant radiochemotherapy and having the rs2868371 GG genotype were all alive at the time of analysis. We also evaluated the interaction between rs2868371 and treatment modality. There was only a statistically significant difference according to the surgery. Patients receiving surgery had the rs2868371 GG genotype in 18% of cases while it was only 6% in those who were not operated ($P = 0.013$). Multivariate analysis adjusted for KPS, disease clinical stage, and treatment (surgery, radiation therapy, and chemotherapy) showed again a significant independent association between the rs2868371 SNP and OS (HR = 0.35; 95% CI = 0.13–0.96, $p = 0.042$; Table 3). The Kaplan–Meier curves for mortality, as a function of time among patients with different *HSPB1* SNPs genotypes, estimate at 24 months OS for 72% of patients carrying the rs2868371 GG genotype and 36% for patients with rs2868371 CC/CG genotypes ($p = 0.013$; Fig. 1A).

Multivariate Cox proportional hazards regression was also used to evaluate the SNP for its association with DFS. After adjustment for clinical stage and surgery, the *HSPB1* rs2868371 polymorphism

Table 2
Univariate analyses of the associations between patient characteristics and *HSPB1* genotypes and the outcome.

Parameter	Overall Survival			Disease Free Survival			Local Recurrence			Distant Metastases		
	HR	95% CI	*p* value	HR	95% CI	*p* value	HR	95% CI	*p* value	HR	95% CI	*p* value
Gender												
Male	2.111	0.98–4.548	0.056	1.094	0.548–2.188	0.798	1.377	0.547–3.466	0.497	1.023	0.488–2.146	0.952
Female (ref)												
Age, years												
≤67 (ref)												
>67	0.951	0.65–1.391	0.794	0.917	0.596–1.412	0.695	1.613	0.929–2.802	0.089	0.917	0.567–1.481	0.723
KPS												
≥80 (ref)												
<80	1.681	1.138–2.482	0.009	0.791	0.469–1.335	0.38	0.988	0.526–1.854	0.969	0.57	0.305–1.066	0.078
Tumor Histology Adeno (ref)												
Squamous cell	1.272	0.862–1.878	0.226	1.176	0.761–1.817	0.464	1.372	0.79–2.382	0.262	0.865	0.535–1.4	0.555
Large-cell	0.647	0.205–2.044	0.458	0.444	0.108–1.817	0.259	0.357	0.049–2.602	0.31	0.286	0.04–2.066	0.215
NSCLC, NOS	1.928	0.888–4.186	0.097	1.114	0.407–3.047	0.834	1.728	0.536–5.568	0.36	1.164	0.364–3.721	0.798
Clinical stage												
IV (ref)												
I, II, IIIA	0.515	0.35 –0.757	0.001	0.534	0.346–0.825	0.005	1.141	0.648–2.009	0.648	0.496	0.305–0.808	0.005
Smoking status												
Current	1.002	0.685–1.466	0.991	0.996	0.648–1.533	0.987	1.081	0.627–1.861	0.78	1.109	0.686–1.79	0.673
Former/Never (ref)												
Surgery												
No (ref)												
Yes	0.279	0.135–0.576	0.001	0.412	0.217–0.783	0.007	0.272	0.107–0.688	0.006	0.614	0.32–1.179	0.143
Chemotherapy												
No (ref)												
Yes	0.518	0.334–0.802	0.003	0.627	0.352–1.116	0.113	0.493	0.253–0.961	0.038	0.715	0.374–1.367	0.31
Thoracic radiation												
No (ref)												
Yes	0.46	0.277–0.765	0.003	2.964	0.93–9.442	0.066	2.527	0.61–10.468	0.201	2.174	0.679–6.957	0.191
Concurrent CT												
No (ref)												
Yes	0.891	0.592–1.339	0.578	1.097	0.703–1.713	0.683	0.573	0.306–1.074	0.082	1.086	0.657–1.793	0.748
rs2868371												
CC (ref)												
GC	1.202	0.814–1.77	0.355	1.161	0.74–1.822	0.516	1.912	0.084–3.371	0.025	0.761	0.453–1.27	0.302
GG	0.325	0.118–0.89	0.03	0.326	0.117–0.909	0.032	0.478	0.144–1.585	0.227	0.396	0.141–1.10	0.078
rs2868371												
CC/GC (ref)												
GG	0.301	0.111–0.82	0.019	0.308	0.112–0.846	0.022	0.372	0.115–1.197	0.097	0.439	0.159–1.21	0.111
rs2868371												
CC (ref)												
GC/GG	0.987	0.674–1.44	0.945	0.907	0.588–1.4	0.66	1.422	0.824–2.453	0.206	0.666	0.407–1.09	0.106
rs2868371*												
CC (ref)												
GC	1.135	0.737–1.747	0.565	1.223	0.774–1.932	0.388	2.081	0.165–3.719	0.013	0.786	0.464–1.331	0.37
GG	0.3	0.093–0.967	0.044	0.344	0.123–0.962	0.042	0.52	0.156–1.736	0.288	0.436	0.155–1.228	0116
rs2868371*												
CC/GC (ref)												
GG	0.285	0.09–0.903	0.033	0.318	0.116–0.875	0.026	0.39	0.121–1.257	0.115	0.479	0.173–1.325	0.156
rs2868371*												
CC (ref)												
GC/GG	0.939	0.616–1.432	0.771	0.956	0.616–1.485	0.841	1.54	0.885–2.7	0.126	0.7	0.424–1.154	0.162

Abbreviations: HR, hazards ratio; KPS, Karnofsky's performance status; NSCLC, non-small cell lung cancer; NOS, not otherwise specified; CT, chemotherapy.
* Patients receiving thoracic radiation therapy ($N = 155$).

Table 3
Multivariate analyses for overall survival and disease free survival.

Parameter	Overall survival			Disease free survival		
	HR	95% CI	*P*	HR	95% CI	*P*
Karnofsky's performance status						
≥80 (ref)						
<80	1.126	0.709–1.790	0.616			
Clinical stage						
I, II	0.406	0.166–0.995	0.049	0.867	0.367–2.046	0.744
III, IV (ref)						
Surgery						
No (ref)						
Yes	0.334	0.162–0.691	0.003	0.470	0.244–0.908	0.025
Chemotherapy						
No (ref)						
Yes	0.511	0.309–0.845	0.009			
Thoracic radiation						
No (ref)						
Yes	0.559	0.307–1.018	0.057			
rs2868371						
CC/CG (ref)						
GG	0.349	0.127–0.964	0.042	0.357	0.128–0.991	0.048

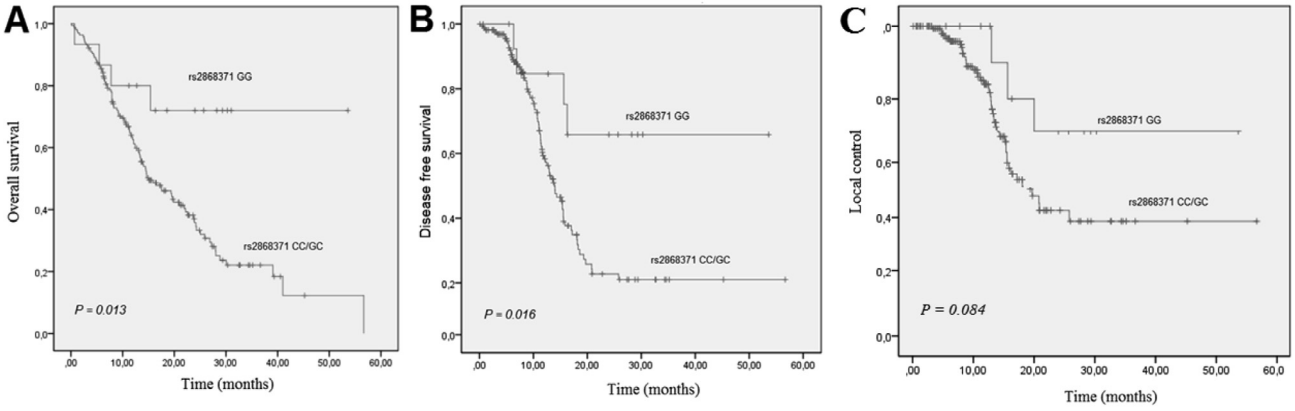

Fig. 1. The Kaplan–Meier curve for overall survival (A), disease-free survival (B), and local control (C) in patients with non-small-cell lung cancer by the presence of the single nucleotide variant rs2868371.

showed a statistically significant association with DFS ([HR] = 0.36; 95% confidence interval [CI] = 0.13–0.99; $p = 0.048$; Table 3). Two-year DFS rate for patients carrying the rs2868371 GG genotype was 66% vs 23% for those with rs2868371 CC/CG genotypes ($p = 0.016$; Fig. 1B). In addition, there was a tendency of lower risk for local recurrence in patients with rs2868371 GG genotype ($p = 0.084$; Fig. 1C).

In order to evaluate putative somatic changes in the tumor related with the rs2868371 genotype associated with lung cancer survival, we validated the genotypes obtained from 37 DNA samples isolated from whole blood with DNA isolated from their counterparts' FFPE tumor/normal samples. The genotyping distribution at FFPE tumor samples obtained was 54.3% CC, 20% GG and 25.7% CG with a 91.6% concordance. There were two patients' samples with insufficient tumor cell percentage (<20%). Since the proportion of tumor DNA was insufficient, the presence or absence of the polymorphism might have been overshadowed by an excess of normal DNA from non-tumor cells, therefore the corresponding genotypes were not considered.

Gene expression analysis shows differences between expression levels of (mRNA) HSPB1 and rs2868371 genotypes in lung tissue

Since the abundance of a gene transcript can be directly modified by polymorphism in regulatory elements, gene expression analysis was performed in 35 lung tumor tissue samples from NSCLC patients in order to study (mRNA) *HSPB1* expression in the presence of the different genotypes. Obtained results showed

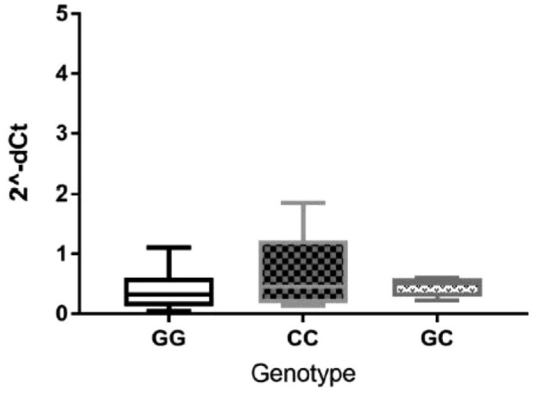

Fig. 2. (mRNA) *HSPB1* expression levels in lung cancer tissues in relation to rs2868371 genotypes. Box plots display relative (mRNA) *HSPB1* expression levels in patients carrying the three different rs2868371 genotypes (CC, CG, GG). All transcripts were quantified by quantitative polymerase chain reaction and normalized for Ct values of the housekeeping gene *GAPDH* using the dCt method. There was no significant difference between (mRNA) *HSPB1* expression levels in patients carrying different genotypes.

higher (mRNA) *HSPB1* expression (Fig. 2) in patients carrying the CC genotype compared with the GG genotype. However, these differences were not statistically significant among the three different genotypes (Anova test, p value = 0, 4891).

ChIP analysis demonstrates altered DNA-binding ability of the glucocorticoid receptor to the HSPB1 promoter region where the rs2868371 is located

Bioinformatic analyses for transcription factor binding sites affected by alternating rs2868371 allelic variants predicted several transcription factors with significantly different predicted affinity to the promoter region surrounding the rs2868371 (Supplementary Table 1). The glucocorticoid receptor showed significant differences in predicted affinity for the two alternative alleles. This receptor can bind DNA directly to regulate target gene expression through specific DNA sequences, being the C allele part of the binding site core sequence of the receptor (Fig. 3A). To test if the endogenous glucocorticoid receptor binds to the *HSPB1* intronic region containing rs2868371 in lung tissue, ChIP experiments were performed in frozen tumor samples from 24 patients carrying different genotypes. The differential binding affinity of the human glucocorticoid receptor to alternative rs2868371 alleles was confirmed by 150-bp genomic sequence embedding rs2868371 q-PCR amplification. The glucocorticoid receptor binding in the presence of the G allele was less efficient, and the differences between genotypes GG-CC, and GG-GC were statistically significant (p = 0.003/p = 0.009 respectively; Fig. 3B).

Discussion

Interindividual differences have been found in lung cancer patients' survival. Despite the fact that factors such as age, coexisting morbidities, type of treatment, etc. might be responsible for this phenomenon; biological differences also contribute to the variability of patients' disease outcome. Thus, radiosensitivity should be regarded as a complex phenotype which varies considerably among individuals depending on the combined effects of different genetic alterations. Here we present the validation of the association of rs2868371 SNP within *HSPB1* gene with lung cancer survival and recurrence in NSCLC patients.

The rs2868371 had been previously reported to be associated with cancer risk and survival. However, virtual differences in genotype distribution frequency and patients' outcome have been described among different ethnicities in retrospective analysis [22,25]. Our results support the association between rs2868371 GG genotype and better survival in patients with NSCLC in a prospective setting. Furthermore, we found that the differences in survivals rates according the genotypes were higher in our prospective study compared with prior retrospective reports [22,25]. In accordance with the American study [25], in our population the most common genotype was the rs2868371 CC and it was associated with poorer survival. However, the rs2868371 CC genotype was correlated with better survival in the Chinese study, being the less common genotype in this population [22]. Although these results seem opposite, it could be explained because the SNP is described in a different DNA strand orientation. According to the refSNP Clustering and refSNP Orientation, "the orientation of a refSNP, and hence its sequence and allele string, is set by the first submitted SNP (ss) used to create a refSNP (rs) cluster..." and "...dbSNP reports all variants and variant alleles on the + strand of the assembly...". However, the Chinese study [22] described the functional HSPB1 promoter as -1271G > C while in the American report [25] the polymorphism was described as c.-1271C > G.

The functional SNP rs2868371 represents an upstream variant placed in the promoter region of the *HSPB1* gene, therefore able

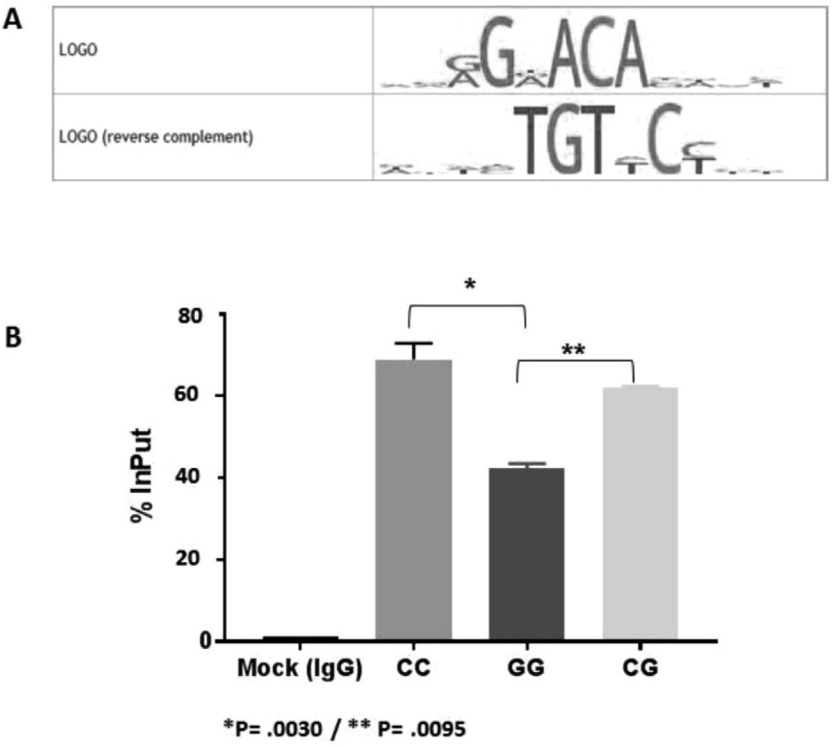

Fig. 3. Chromatin Immunoprecipitation analysis of Glucocorticoid Receptor binding to *HSPB1* gene promoter. Core sequence logos for Glucocorticoid Receptor were downloaded from *HOCOMOCO* database; the dominant nucleotides with the best scoring binding site are placed at the bottom (A). ChIP was performed using anti-GR specific antibody in tissue samples from lung cancer patients carrying different rs2868371 genotypes. Unspecific immunoprecipitation using IgG (Mock) was also used as a negative control. DNA in both the input and immunoprecipitated fractions was quantified by qRT-PCR using primers specific to a genomic sequence containing rs2868371. Data are shown as the enrichment of input DNA in the immunoprecipitated fractions (B).

to influence gene expression and have a putative role in the physiological and disease process. In our study, different (mRNA) *HSPB1* expression patterns were found related to the presence of different genotypes in NSCLC tumor samples. Unless these differences were not statistically significant, it is important to note that patients carrying rs2868371 GG genotype showed less expression compared with the other two genotypes. In agreement with us, Guo et al. [22] reported differences in the Hsp27 expression levels among three genotypes of the rs2868371 polymorphism in tumor tissues, but they did not reach a significant level. These differences were marginally significant in the normal tissues ($p = 0.057$). High HSPB1 expression levels are being associated with worse clinical outcome and resistance to radiation and/or chemotherapy in NSCLC patients [18]. In this regard, high HSPB1 expression levels in lung cancer cells play important roles in prevention of stress-induced cellular damage enhancing the antioxidant capacity against different inflammatory mediators or oxidative stress as ionizing radiation, counteracting the formation of misfolded proteins and allowing the correct protein refolding, reducing DNA damage and apoptosis [17,29–31]. Therefore, HSPB1 down-regulation triggered by the rs2868371 G allele may increase sensitivity of lung cancer cells to chemo (radio) therapy promoting a favorable survival. These expression differences might be explained by the presence of a highly conserved regulatory motif within the *HSPB1* promoter region key for transcription. *In silico* prediction analysis displayed a set of transcription factors whose ability to bind this region differs between rs2868371 alleles. The glucocorticoid receptor can bind DNA directly to regulate target gene expression through specific DNA sequences, being the rs2868371 C allele part of the binding site core sequence of the receptor. The glucocorticoid receptor showed significant differences in predicted affinity for the two alternative rs2868371 alleles. The glucocorticoid receptor binding in the presence of the G allele was less efficient, and the differences between genotypes GG-CC, and GG-GC were statistically significant. Specifically, the presence of the less frequent rs2868371 G allele disrupts a highly conserved sequence for the glucocorticoid receptor recognition [32,33]. This might be susceptible to altering the glucocorticoid receptor affinity for overlapping the nucleotide recognition element located at the *HSPB1* promoter as it was shown by chromatin immunoprecipitation analysis performed in patient tumor samples. Further studies are necessary to elucidate the complex mechanism of this association.

The glucocorticoid receptor, abundantly expressed in both normal and cancerous lung tissue, belongs to the nuclear receptor superfamily of ligand-activated transcription factors able to modulate different gene transcription by the association with specific DNA binding sequences with nearly invariant positions which are highly conserved across species [34,35]. In the cytoplasm the HSP70 and HSP90 are associated in a heterocomplex with the glucocorticoid receptor [36,37]. High levels of glucocorticoids induce glucocorticoid receptor dissociation from the HSP and translocation to the nucleus where it directly regulates gene transcription being *HSPB1* one of glucocorticoid receptor targets identified in different tissues [38–40]. Because of its profound anti-inflammatory property, glucocorticoids and their synthetic analogs are widely prescribed in lung cancer treatment. However, glucocorticoids sensitivity and actions vary considerably among individuals and tissues promoting or inhibiting tumor progression and regulating radio(chemo)therapy-induced cell apoptosis existing an incomplete understanding of the underlying mechanism in each case [41,42].

We validated the association between the rs2868371 SNP and survival in NSCLC patients in a multicenter prospective setting. The results obtained show an association between the rs2868371 GG genotype and better OS. Additionally, we propose that *HSPB1* represents a direct, functional target of the glucocorticoid receptor. Different affinity to the *HSPB1* promoter region where the rs2868371 is located may improve the tumor cells' tolerance to the stress produced by oncologic treatments such as radiation because of the cytoprotective role of HSPs. Although functional HSPB1 inhibition may be a good strategy for combination with radio(chemo)therapy, and different strategies under development offer a promising approach [43–45], the effect of glucocorticoids on the radio(chemo)resistance through HSPB1 activation requires further study as it may have important implications in clinical practice.

Conflicts of interest

None.

Acknowledgements

This work was supported by grants from ISCIII (Fis: PI13/01155, PI16/02104) and Consejeria de Salud of the Junta de Andalucia (PI-0096-2012).

The authors thank the donors and the Hospital Universitario Virgen del Rocío-Instituto de Biomedicina de Sevilla Biobank (Andalusian Public Health System Biobank and ISCIII-Red de Biobancos PT17/0015/0041) for the human specimens used in this study.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.radonc.2019.03.025.

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