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HSPB1 Gene Polymorphisms Predict Risk of Mortality for U.S. Patients after Radio(chemo)therapy for Non-Small-Cell Lung Cancer

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Abstract

Purpose—We investigated potential associations between single-nucleotide polymorphisms (SNPs) in the heat shock protein beta-1 (*HSPB1*) gene and overall survival in U.S. patients with non-small-cell lung cancer (NSCLC).

Materials and Methods—Using available genomic DNA samples from 224 patients with NSCLC treated with definitive radio(chemo)therapy, we genotyped two SNPs of *HSPB1* (rs2868370 and rs2868371). We used both Kaplan-Meier cumulative probability and Cox proportional hazards analyses to evaluate the effect of *HSPB1* genotypes on survival.

Results—Our cohort comprised 117 men and 107 women, mostly white (79.5%), with a median age of 70 years. The median radiation dose was 66 Gy (range 63–87.5 Gy), and 183 patients (82%) received concurrent platinum-based chemotherapy. The most common genotype of the rs2868371 SNP was CC (61%). Univariate and multivariate analyses showed that this genotype was associated with poorer survival than carriers of CG/GG genotypes (univariate hazard ratio [HR]=1.39, 95% confidence interval [CI] 1.02–1.90, $P=0.037$; multivariate HR=1.39; 95% CI 1.01–1.92; $P=0.045$).

Conclusion—Our results show that the CC genotype of *HSPB1* rs2868371 was associated with poorer overall survival in patients with NSCLC after radio(chemo)therapy, findings that contradict those of a previous study of Chinese patients. Validation of our findings with larger numbers of

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similar patients is needed, as are mechanical and clinical studies to determine the mechanism underlying these associations.

Keywords

Non-small cell lung cancer; radiation therapy; overall survival; single-nucleotide polymorphisms; heat shock protein beta-1

Introduction

Lung cancer is the leading cause of cancer-related death in the United States [1]. Definitive radiotherapy with concurrent chemotherapy has become the standard treatment for locally advanced, unresectable non-small cell lung cancer (NSCLC) [2]. Unfortunately even with this aggressive treatment, the prognosis is still very poor, with 5-year survival rates of about 10%–16% [3]. Abundant research has been undertaken in search of biomarkers, including genetic factors, with which to predict the response to therapy and prognosis for patients with NSCLC before treatment begins.

Heat shock protein (HSP) 27 is encoded by the heat shock protein beta-1 gene (*HSPB1*) and is about 27 kDa in size. HSP27 is an adenosine triphosphate-independent molecular chaperone whose main function is protection against protein aggregation by facilitating the repair or degradation of damaged proteins potentially produced in stressed cells [4, 5]. HSP27 also increases the antioxidant defense capacity of cells by increasing their glutathione content and limits the toxicity of oxidized proteins by its chaperone activity [6, 7]. This latter characteristic may be of particular importance in the cellular response to radiation because reactive oxygen species are important in the induction of apoptosis [8]. HSP27 is abundantly expressed in cancer cells and strongly induced after stresses such as anticancer drugs and oxidative stress such as ionizing irradiation [9, 10]. Therefore HSP27 has been considered to be an important prognostic factor in malignant diseases [11]. A recent study from China [12] showed that the C allele of the rs2868371 functional promoter polymorphism of *HSPB1* was associated with an increased risk of lung cancer in the Chinese population but better survival among patients with advanced NSCLC compared with the G allele, possibly through reducing the expression levels of Hsp27 protein, which could impair DNA repair capacity. However, these findings resulted from analysis of only Chinese patients and have not been validated in separate groups of different races.

The primary aim of this study was to investigate the relationship between the genotypes produced by polymorphisms of the *HSPB1* gene and the risk of death in U.S. patients with NSCLC after radio(chemo)therapy. We further sought to determine whether the association between the *HSPB1* rs2868371 and survival observed by Guo et al. [12] in a Chinese population could be replicated in a U.S. population.

Methods and Materials

This study was approved by the appropriate institutional review board and was conducted in compliance with Health Insurance Portability and Accountability Act regulations.

Patients

The study cohort comprised 224 patients with NSCLC for whom DNA samples were available and who had been treated with radiation, with or without chemotherapy, at a single institution between 1998 and 2010. Patient, tumor, and treatment characteristics are described in Table 1.

Patient Evaluation and Follow-up

During the course of radiotherapy, patients were seen at least weekly and more often if needed for clinical evaluation and disease management. They were evaluated at approximately 1–3 months after completion of therapy and then 3 to 6 months afterward for the first 2 years and annually thereafter. Follow-up evaluations consisted of an interval history and physical examination. Follow-up imaging typically involved chest x-ray or computed tomography (CT). Further images, including magnetic resonance imaging, bone scans, and positron emission tomography, were obtained at the discretion of the treating physician, generally when patients presented with symptoms or abnormalities on the chest x-ray or CT.

Genotyping Methods

We searched the gene SNP database of the National Institute of Environmental Health Sciences Genome Program and related literature to identify all functional SNPs of the *HSPB1* gene with a minor allele frequency greater than 0.05 in a white population [13]. We selected two SNPs of the *HSPB1* gene that met at least two of the following three criteria: (1) a minor allele frequency of at least 5%, (2) location in the promoter untranslated region or coding region of the gene, and (3) previous reported association with lung or other cancers.

Genotypes were obtained as follows. From each blood sample, DNA was extracted from a leukocyte cell pellet obtained from buffy coat by centrifugation of 1 mL of whole blood by using the Qiagen DNA Blood Mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. DNA purity and concentration were determined by spectrophotometric measurement of absorbance at 260 and 280 nm. Genotyping of *HSPB1* (rs2868370 and rs2868371) was performed with standard TaqMan assays in 384-well plates, which were read with Sequence Detection Software on an ABI-Prism 7900 instrument according to the manufacturer's instructions (Applied Biosystems, Foster City, CA). Primers and probes were supplied by Applied Biosystems. Each plate included 4 negative controls (no DNA), duplicated positive controls, and 8 repeat samples. Amplification was done under the following conditions: 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. For all genotypes, the assay success rate was >99% and the results for the repeated samples were 100% concordant. TaqMan genotyping failed in 12 cases because the amount of DNA in the sample was too small ($n = 6$ for rs2868370; $n = 6$ for rs2868371).

Statistical Analysis

Data were analyzed with Stata/SE 10 software (Stata Corp LP, College Station, Texas). Patients were grouped according to genotype. The chi-square test was used to compare genotype distribution differences between cohorts. Cox proportional hazards analysis was used to calculate hazard ratios (HRs) and confidence intervals (CIs) to evaluate the influence of genotypes on survival. Multivariate Cox regression was performed to adjust for patient factors (age, sex, Karnofsky performance status [KPS], and smoking habits), disease factors (histology, stage), and treatment factors (type of chemotherapy). Survival time was measured from the date of diagnosis to the date of death or last contact. Kaplan-Meier analysis was used to estimate the cumulative probability of mortality. A logistic model was used to calculate the sensitivity and specificity and to generate receiver operating characteristic (ROC) curves and areas under the curve (AUCs), which estimate each model's ability to predict survival status. A P value of 0.05 or less was considered statistically significant in two-sided t tests.

Results

Characteristics of the 224 patients are shown in Table 1. The study cohort comprised 117 men (52.2%) and 107 women (47.8%) with a median age of 70 years (range 39–93 years). Most patients (79.5%) were white, had good performance status (KPS = 80, 79.5%), had stage III disease (78.2%), and received concurrent chemotherapy (81.7%). The median total radiation dose for all patients was 66 Gy (range 63–87.5 Gy) at 1.2 to 2 Gy/fraction (57 patients [25%] received 69.6 Gy/58 fractions at 1.2 Gy/fraction twice a day); 20% of patients received a dose greater than 70 Gy. Most patients (81.7%) also received concurrent platinum- and taxane-based chemotherapy. Therapies received included induction chemotherapy followed by radiation (n=15), induction chemotherapy followed by concurrent chemotherapy and radiation (n=74), and concurrent chemotherapy and radiation without induction treatment (n=109). Twenty-six patients were treated with radiation alone, 3 of whom subsequently received adjuvant chemotherapy. The median follow-up time was 18.6 months (range 5 days to 148 months), and the median overall survival time was 23 months (range 3–150 months).

The distribution of the *HSPB1* genotype for the rs2868370 SNP was 72.0% GG, 25.2% AG, and 2.8% AA; the distribution for the rs2868371 SNP was 60.5% CC, 29.4% CG, and 10.1% GG. No significant differences ($P>0.05$) were found in the frequency or distribution of either *HSPB1* SNP by sex, age, race, KPS, tumor histology, disease stage (I/II vs. III/IV), smoking status, chemotherapy, or tumor radiation dose.

Table 2 shows the associations between patient-, tumor-, and therapy-related characteristics and overall survival by univariate and multivariate analysis. Disease stage and KPS were associated with overall survival in both univariate and multivariate analysis. Figure 1 illustrates the Kaplan-Meier curves for mortality as a function of time among patients with different *HSPB1* genotypes. Kaplan-Meier estimates at 2 years indicated mortality rates of 57% for patients with the rs2868371 CC genotype and 43% for patients with the rs2868371 CG or GG genotype; corresponding rates were 48% for patients with the rs2868370 AG or AA genotype and 53% for patients with rs2868370 GG. No difference in overall survival time was found for patients with rs2868370 AG/AA or GG genotypes (median survival time, 25.1 months vs. 23.2 months, $P=0.1752$). However, patients with the rs2868371 CG/GG genotype had a longer overall survival time than did patients with CC genotype (median survival time, 29.8 months vs. 22.0 months, $P=0.0356$).

Univariate Cox proportional hazard analyses showed that the rs2868371 CC genotype was associated with poorer survival compared with the rs2868371 CG/GG genotypes (HR 1.39, 95% CI 1.02–1.90; $P=0.037$). This effect was virtually unchanged after adjustment for age, race, sex, KPS, tumor histology, disease stage, smoking status, receipt of concurrent chemotherapy, and radiotherapy fractionation in multivariate analysis (HR 1.39, 95% CI 1.01–1.92; $P=0.045$) (Table 3), suggesting that the rs2868371 SNP is an independent predictor of survival. To investigate a possible allele dosage effect, we also performed multivariate analyses with an additive model. No differences were found in overall survival for any of the three rs2868370 genotypes. As for rs2868371, survival was better for the CG genotype than for the CC genotype, but no difference was found between the GG and CC genotypes (Table 3). Thus we found no allele dosage effect for either SNP.

We then investigated the effect of combined unfavorable genotypes (rs2868370 GG or rs2868371 CC) on survival, and we found even poorer survival for those patients carrying both unfavorable genotypes (rs2868370 GG and rs2868371 CC) (HR=1.59, 95% CI 1.16–2.18; $P=0.004$) compared with those carrying 0 or 1 of these genotypes (Table 3). We further performed an ROC curve analysis (Table 4) to determine the strength of the variables

identified as being significant in the analyses described above for predicting overall survival. Disease stage was the most important predictor, with an AUC of 0.59. The addition of KPS or rs2868371 CC or both did not significantly improve the AUC (for KPS, AUC 0.60, $P=0.25$; for KPS+rs2868371, AUC 0.63, $P=0.12$). Finally, adding the genetic variants of both rs2868370 and rs2868371 significantly improved the predictive power of the survival model, enhancing the AUC by 0.06 (AUC 0.65, $P=0.047$; Table 4, Figure 2). Further analysis using Harrell's C statistics, a method that takes into consideration of incomplete follow-up, showed similar results (data not shown).

Discussion

In our cohort of U.S. patients, most of whom were white, we found that the CC genotype in rs2868371 of the *HSPB1* gene was associated with poorer survival after radiotherapy for NSCLC than other genotypes. The association between the rs2868371 CC genotype and poorer survival was independent of age, KPS, smoking status, tumor histology, tumor stage, receipt of concurrent chemoradiation, or radiotherapy fractionation. The ROC analysis also showed that the sensitivity and specificity of predicting overall survival was significantly improved by the addition of rs2868370 and rs2868371 to the model. Collectively, these results suggest that these two SNPs had some effect on survival and could enhance the power of predictions of survival. Our group recently reported [14] that the CC genotype of rs2868371 was associated with a higher risk of radiation-induced esophagitis in patients with NSCLC. This finding suggests that HSP SNPs could be valuable as a biomarker for predicting disease outcome and toxicity after radiotherapy for lung cancer. This information, if validated in larger groups with similar demographic and disease characteristics, could be used to design personalized treatment for patients with lung cancer. However, our results do differ from those of a recent analysis of a Chinese population by Guo et al. [12] in which the allele C of the *HSPB1* rs2868371 (compared with the allele G) was associated with increased risk of developing lung cancer but also with favorable survival for patients with advanced lung cancer, possibly by reducing the expression levels of Hsp27 protein, which could negatively affect DNA repair.

Several clinical and therapeutic factors may explain the difference in findings between our study and that of Guo et al.. First, the distribution of SNPs is well known to vary among different ethnic or racial groups, which may also account for differences between individuals in susceptibility to disease. This would seem to be confirmed by the apparent differences in the distribution of allele frequencies and genotypes in the two patient cohorts. The most common genotype of the rs2868371 SNP in our cohort was CC at 60.5%, a frequency similar to the 54.1% indicated in HapMap (<http://hapmap.ncbi.nlm.nih.gov/>) for residents of Utah of Northern and Western European ancestry. However, this genotype was much less common in the Chinese group at 16.9% (similar to the 15.3% found in HapMap for Han Chinese in Beijing, China). Indeed, the most common genotype of the rs2868371 SNP in the Chinese cohort reported by Guo et al. was CG. According to that report, the distribution of rs2868371 genotypes in the Chinese cohort was 16.9% CC, 46.8% CG, and 36.3% GG, which was significantly different from the distribution in our cohort ($P<0.001$). Similar ethnic differences have also been reported for SNPs in *TGF-β1*. For example, the *TGF-β1* T869C polymorphism has been linked with development of chronic obstructive pulmonary disease in whites [15], but no such associations have been observed for Asians [16]. This *TGF-β1* T869C polymorphism has also been linked with development of radiation pneumonitis in U.S. populations [17] but not in Chinese populations [18]. Therefore, differences in the genetic backgrounds of U.S and Chinese populations may explain, at least in part, these different results. Although separating the effects of ethnicity from those of environment is difficult at best, our results suggest that the frequency patterns of polymorphisms in the *HSPB1* gene vary greatly among different ethnic groups. Given the

different genotype frequencies and opposite associations with survival between the Chinese and U.S. cohorts, we suspect that rs2868371 may be simply a marker for another SNP that is inherited in linkage disequilibrium differently in these two ethnic groups.

Another possible explanation for the differences in results may reflect the differences in the type of treatment delivered to U.S. versus Chinese patients. For example, in the Chinese study [12] approximately 30% of patients underwent surgery and 50% had radiotherapy, whereas in the current study all patients received radiotherapy and none had surgery. Differences between groups in disease stage may also have affected survival; in our study only 5% of patients had stage IV disease at diagnosis, whereas almost 50% of patients in the Chinese study were diagnosed with advanced disease. However, when we evaluated only patients with advanced disease in our study, we did not find GG or CC genotype of rs2868371 was associated with poorer survival (results not shown).

Guo et al. [12] also reported findings from an assay of 30 patients showing that patients carrying the rs2868371 CC/CG genotypes had slightly lower expression of HSP27 protein in both normal bronchial epithelial and malignant cancer cells compared with patients carrying the GG genotype. However, this apparent difference was not statistically significant ($P>0.05$) and therefore does not seem to explain completely the mechanism by which the CC/CG genotype reduced mortality risk in that study. In another study, Guisasola et al. [19] reported that radiotherapy caused HSP27 downregulation, to the greatest extent in patients with the largest irradiated volumes, and led to decreased intracellular HSP levels. Thus, we hypothesize that patients carrying the rs2868371 CC genotype may have less HSP27 downregulation than those carrying other genotypes after radiotherapy and therefore may overexpress HSP27.

This study had several limitations, among them its single-institution nature, the relatively small number of patients, and the fact that the clinical data were collected and analyzed retrospectively. Differences in type of treatment and disease stage between the U.S and Chinese studies may have confounded the comparison of SNPs as prognostic factors for survival, although the magnitude of this effect is unclear. In addition, although the AUC was improved by adding the unfavorable genotypes (GG of rs2868370 and CC of rs2868371), an AUC of 0.65 may not be clinically relevant. Prospective studies with larger numbers of patients with similar baseline characteristics are needed to better understand the effect of the *HSPB1* SNPs on survival in different ethnic groups as well as the magnitude of the clinical impact. Therefore, it will be important to validate our findings in a prospective study of a larger number of patients treated at several institutions. The recently formed Radiogenomics Consortium would be an ideal venue for such studies [20]. Finally, although it was not the intent of this study, future studies are needed to investigate the mechanism by which the CC genotype increases mortality risk after radio(chemo)therapy.

Conclusion

Our results demonstrated significant associations between overall survival and SNPs in *HSPB1* rs2868371 in U.S. patients with NSCLC after radio(chemo)therapy, findings that are opposite to those of a previous Chinese study. Our results also suggest an ethnic difference in the distribution of these genotypes. Therefore, to discover the mechanisms underlying these differences, validation of genotype distributions and associations in similar populations with larger numbers of patients will be an important consideration in future studies. Mechanistic laboratory and clinical studies should also be included in the design of these future studies.

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Summary

HSP27, encoded by *HSPB1*, is important in radioresistance. SNPs in *HSPB1* have been associated with survival after lung cancer treatment, but these findings, obtained from Chinese patients, have not been replicated. We assessed SNPs in *HSPB1* in 224 U.S. patients with NSCLC treated with radio(chemo)therapy and found that the CC genotype of rs2868371 was associated with poorer overall survival, findings that contradict those of the previous Chinese study.

Figure 1. Kaplan-Meier survival curves for U.S. patients with non-small-cell-lung cancer by *HSPB1* rs2868370 (A) and rs2868371 (B) genotypes.

Figure 2. Receiver operating characteristic curves and the associated areas under the curves (AUCs) for disease stage (dotted line), for disease stage and Karnofsky performance status (KPS) (triangles), and for disease stage, KPS, and *HSPB1* polymorphisms (circles) as predictors of overall survival.

Table 1

Patient characteristics

Characteristic	No. of Patients (%)
Sex	
Male	117 (52.2)
Female	107 (47.8)
Age, years	
60	47 (21.0)
>60	177 (79.0)
Race	
White	178 (79.5)
Black	45 (20.1)
Asian	1 (0.4)
Disease stage	
I	26 (11.6)
II	11 (4.9)
III	175 (78.1)
IV	12 (5.4)
Tumor histology	
Squamous cell	79 (35.2)
Adenocarcinoma	80 (35.7)
NSCLC, NOS	65 (29.1)
Karnofsky performance status	
<80	46 (20.5)
80	178 (79.5)
Smoking status	
Current	53 (23.7)
Former/Never	144 (64.3)/27 (12.0)
No. of pack-years of smoking [*]	
49	123 (55.0)
>49	101 (45.1)
Surgery	
No	224 (100)
Yes	0
Chemotherapy	
No	23 (10.3)
Yes	201 (89.7)
Radiotherapy	
No	0
Yes	224 (100)
Radiotherapy fractionation	
Once a day	162 (72.3)

Characteristic	No. of Patients (%)
Twice a day	62 (27.7)
Radiation technique	
3D CRT	137 (61.2)
IMRT	69 (30.8)
PT	18 (8.0)

Abbreviations: NSCLC, NOS: non-small cell lung cancer, not otherwise specified; 3D: three-dimensional conformal radiotherapy; IMRT: intensity-modulated radiotherapy; PT: proton therapy.

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

Association analysis between clinical characteristics and overall survival of NSCLC patients after radiotherapy

Table 2

Variable	Univariate analysis			Multivariate analysis*		
	HR	95% CI	P	HR	95% CI	P
Sex						
Male	ref					
Female	0.78	0.58–1.04	0.09	0.89	0.65–1.23	0.49
Age, years						
60	ref					
>60	0.87	0.61–1.24	0.44	1.00	0.67–1.47	0.98
Race						
White	ref					
Other	1.17	0.82–1.68	0.38	1.16	0.79–1.71	0.46
Disease stage						
I	ref					
II	1.81	0.81–4.02	0.15	2.20	0.94–5.12	0.07
III	1.94	1.13–3.30	0.02	2.43	1.30–4.54	0.005
IV	2.40	1.08–5.37	0.03	3.72	1.48–9.37	0.005
Histology						
Adenocarcinoma	ref					
Squamous cell carcinoma	1.44	1.02–2.05	0.04	1.44	0.98–2.12	0.06
NOS	1.26	0.86–1.83	0.24	1.28	0.86–1.92	0.23
KPS						
80	0.70	0.49–1.0	0.05	0.66	0.45–0.96	0.03
<80	ref					
Smoking status						
Current	ref					
Former/never	0.83	0.59–1.18	0.30	0.80	0.55–1.16	0.23
No. of pack years [†]						
44	ref					
>44	1.07	0.80–1.44	0.64	1.02	0.73–1.42	0.90

Variable	Univariate analysis			Multivariate analysis*		
	HR	95% CI	P	HR	95% CI	P
Radiotherapy fractionation						
Once a day	ref					
Twice a day	1.23	0.90–1.70	0.20	1.16	0.82–1.65	0.40
Radiation technique						
3D	ref					
IMRT	1.04	0.75–1.46	0.81	0.85	0.59–1.25	0.42
PT	0.74	0.41–1.35	0.32	0.71	0.38–1.33	0.28
Concurrent chemotherapy						
Yes	0.99	0.68–1.44	0.95	0.70	0.44–1.11	0.13
No	ref					

Abbreviations: HR, hazard ratio; NSCLC, non-small-cell lung carcinoma, not otherwise specified; KPS, Karnofsky Performance Status; CRT, chemoradiation; 3D, 3-dimensional conformal radiotherapy; IMRT, intensity-modulated radiation therapy; PT, proton therapy.

* Multivariate analyses in this table were adjusted for age, race, sex, stage, histology, KPS, smoking status, concurrent chemotherapy and radiotherapy fractionation as covariates.

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

Table 3

Associations between *HSPB1* genotypes and overall survival

Genotype	No. (%)	No. of Deaths (%)	Univariate Analysis			Multivariate Analysis*		
			HR	95% CI	P	HR	95% CI	P
rs2868370								
GG	157 (72)	129 (82)	ref			ref		
AG/AA	61 (28)	44 (72)	0.79	0.56–1.11	0.18	0.84	0.58–1.21	0.36
AG	55 (25)	39 (71)	0.95	0.39–2.33	0.92	0.83	0.56–1.21	0.32
AA	6(3)	5 (83)	0.77	0.54–1.11	0.16	0.99	0.39–2.55	0.99
rs2868371								
CG/GG	86 (39)	63 (73)	ref			ref		
CC	132 (61)	109 (83)	1.39	1.02–1.90	0.037	1.39	1.01–1.92	0.045
CC	132 (61)	109 (83)	Ref			Ref		
CG	64 (29)	45 (70)	0.66	0.46–0.93	0.019	0.66	0.46–0.94	0.023
GG	22 (10)	18 (82)	0.93	0.56–1.53	0.76	0.95	0.55–1.64	0.86
HSPB1								
GG/CC [†]	85 (40)	75 (88)	1.60	1.17–2.17	0.003	1.59	1.16–2.18	0.004
Others	127 (60)	93 (73)	ref			ref		

Abbreviations: HR, hazard ratio; CI, confidence interval.

* Multivariate analyses in this table were adjusted for age, race, sex, stage, histology, Karnofsky performance status, smoking status, receipt of concurrent chemotherapy, and radiotherapy fractionation as covariates.

[†] Pooled patients with the unfavorable genotypes rs2868370 GG and rs2868371 CC.

Table 4

Comparison of predictive models for survival of the US cohort

Models	Δ Likelihood Ratio			AUC
	χ^2	DF	P*	
Stage (ref)	--	--	--	0.59
Stage + KPS	1.32	1	0.25	0.60
Stage + KPS + rs2868371	4.19	2	0.12	0.63
Stage + KPS + rs2868371+ rs2868370 [†]	7.93	3	0.047	0.65

Abbreviations: DF, degrees of freedom; AUC, area under the curve; KPS, Karnofsky performance status.

[†]Stage (I, II vs. IIIA vs. IIIB vs. IV); KPS (<80 vs. 80); rs2868370 (GG vs. AG/AA); rs2868371 (CC vs. CG/GG).

* P values for likelihood ratio test comparing alternative models to the reference model.