Integrating Intrinsic Radiosensitivity and Immune Status for Predicting Benefits of Radiotherapy in Head and Neck Squamous Cell Carcinoma

Authors' Contribution:
Study Design A
Data Collection B
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Background: HNSCC (head and neck squamous cell carcinoma) is a heterogeneous disease for which radiotherapy is a main treatment. As intrinsic radiosensitivity and immune status affect the initial and effective stage of the radiation-induced cancer immunity cycle, respectively, it is important to consider both of them when we select patients who can benefit from radiotherapy.

Material/Methods: Our study included all HNSCC patients with complete survival and radiotherapy information in TCGA database. Patients were divided into RS (radiosensitive), RR (radioresistant), immune, and non-immune groups according to their RSI (radiosensitivity index) and immune score calculated by the ESTIMATE algorithm. Survival analysis was performed to compare OS (overall survival) between patients receiving and not receiving radiotherapy. GO and KEGG pathway enrichment analysis was performed for functional analysis. Univariate Cox and ridge regression analysis were performed to construct a predictive gene signature based on the combined stratification.

Results: Only patients in the RS-immune group could benefit from radiotherapy, and the survival analysis results remained consistent after we performed propensity score matching between patients receiving and not receiving radiotherapy. The differentially expressed genes between the RS-immune and non-RS-immune groups were mainly enriched in pathways related to immune process. The 3-gene signature we built exhibited predictive value in training and validation cohorts when treated as a binary or continuous variable.

Conclusions: The combined stratification of intrinsic radiosensitivity and immune status was superior to considering intrinsic radiosensitivity or immune status alone and could be used in preclinical evaluation to select patients or to decide whether radiotherapy sensitizers and immunotherapy should be used at the same time.

Keywords: Immunity, Active • Radiotherapy • Squamous Cell Carcinoma of Head and Neck

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Background

HNSCC (head and neck squamous cell carcinoma) is the sixth most common cancer in the world [1]. The incidence of HNSCC varies from region to region, and is generally associated with the exposure to tobacco-derived carcinogens or excessive alcohol consumption [2]. Over 600 000 new cases and 350 000 deaths occurred in a year. The 5-year survival rate of patients with locally advanced HNSCC is about 50% and 40-60% of patients relapse and do not respond to subsequent treatment. The median survival time of patients with recurrent/metastatic disease is only 10 months [3,4]. Radiotherapy is one of the main treatments for HNSCC; however, because of the complexity and heterogeneity of HNSCC, not all patients will benefit from radiotherapy. Some patients cannot tolerate the adverse reactions, resulting in treatment termination or even death [5-8]. Therefore, biomarkers for predicting radiosensitivity in HNSCC are necessary for radiotherapy selection and predicting therapeutic response.

In recent years, some studies have proposed use of gene expression signatures to predict survival in HNSCC patients receiving radiotherapy. For example, Eschrich et al [9] developed the intrinsic radiosensitivity index (RSI) through the SF2 of 48 cancer cell lines, and RSI has already been systematically validated as a predictor of clinical outcomes in multiple independent radiotherapy cohorts including head and neck cancer [10]. Ma et al identified a 4-gene methylation signature to predict survival in HNSCC patients [11]. You et al identified 4 key markers in 4 core functional pathways representing radiosensitivity of HNSCC [12]. However, although most of the existing signatures are prognostically relevant, few have been shown to be predictive in HNSCC.

Previous studies on improving radiotherapy effects mostly focused only on cancer cells, but there is now growing evidence that the therapeutic effect of radiotherapy is influenced and regulated by the tumor microenvironment, including the immune system [13]. In the radiation-induced cancer immunity cycle, intrinsic radiosensitivity affects the release of cancer cell antigen, and immune status affects antigen-specific T cell activation [14]. In this work, we explored the effect of combined stratification of intrinsic radiosensitivity and immune status on the benefit of radiotherapy in HNSCC patients by integrating 2 different biological processes. Based on this combined stratification, a predictive gene expression signature was established to select patients who could benefit from radiotherapy.

Material and Methods

Data Acquisition and Processing

HNSCC clinical and raw gene count expression data were downloaded from TCGA (http://cancergenome.nih.gov/, 2020/03/15).

Patients with both survival and radiotherapy information were retained (n=392). Genes with maximum expression values less than 10 and average expression values less than 1 were excluded because their expression values were too low. The DEseq2 package [15] was used to standardize raw gene expression data and change gene expression to log2 (count+1) form. All protein coding genes were extracted for subsequent analysis. Immune scores calculated by ESTIMATE algorithm was downloaded from the website (https://bioinformatics.mdanderson.org/estimate/, 2020/03/31).

Patient Stratification and Survival Analysis

Patients were divided into an immune group and a non-immune group based on the median immune score. RSI value of each patient was calculated based on the gene model established by Eschrich et al [9]. We selected patients who received radiotherapy (N=254) and determined the optimal cut-off value as the RSI value that could separate those patients into 2 groups with the most significant difference in OS using X-tile software (version 3.6.1, Yale University School of Medicine, New Haven, CT, USA). All patients were divided into an RS group (N=157), an RR group (N=235), an immune group (N=196), and a non-immune (N=196) group. We compared overall survival between patients receiving and not receiving radiotherapy in the 4 groups. After that, patients were divided into 4 subgroups: RS-immune (N=83), RS-non-immune (N=74), RR-immune (N=113), and RR-non-immune (N=122). The overall survival of patients receiving radiotherapy and not receiving radiotherapy was compared in the 4 subgroups.

Patient Matching and Survival Analysis

To minimize selection bias and confounding factors, we performed propensity score matching (PSM) between patients receiving and not receiving radiotherapy. We considered RS-immune as 1 group and the 3 other groups as another group, called the non-RS-immune group. Because of the lack of clinical information, we matched patients with a ratio of 1: 4 based on 7 clinical and therapeutic features including neoplasm histological grade (grade I, II, III), clinical stage (stage I, II, III, IV), lymphovascular invasion (no, yes), margin status (negative, positive\approach), presence of pathological nodal extra-capsular spread (no, micro-invasion\ gross invasion), surgery (no, yes), and chemotherapy (no, yes). After that, overall survival of patients receiving radiotherapy and not receiving radiotherapy was compared in 2 matching cohorts.

Gene Differential Expression Analysis and Pathway Enrichment Analysis

DEG (differentially expressed genes) between RS-immune and non-RS-immune group were identified with a cut-off value
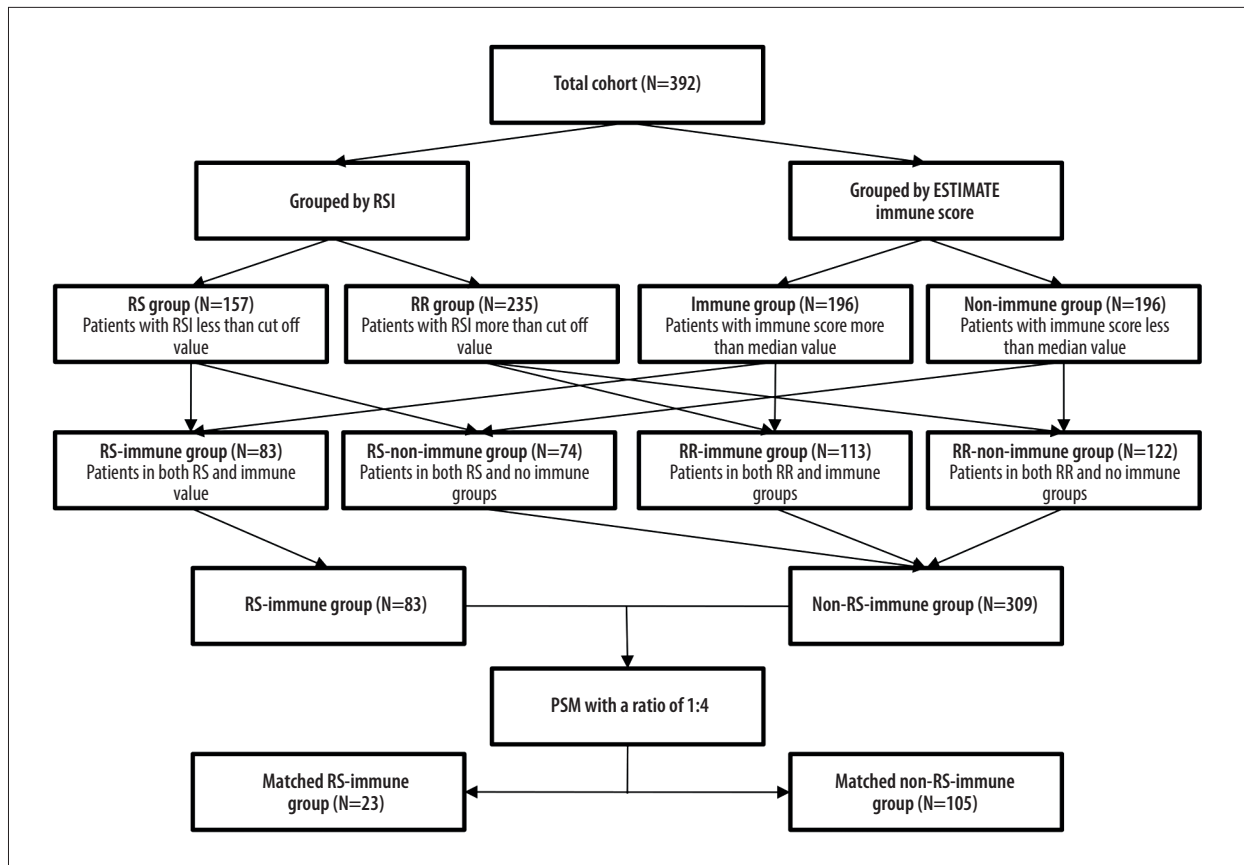
Figure 1. Schema of patient grouping. RSI – radiosensitivity index; RS – radiosensitive; RR – radioresistant; PSM – propensity score matching.

of adjust p value <0.05 and |log2 fold change (FC)| ≥1 using Limma package [16]. GO (Gene Ontology) biological process and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway enrichment analysis was performed using DAVID website (https://david.ncifcrf.gov/) with a selection criterion of FDR<0.05.

Gene Signature Establishment with Ridge-Regularized Cox Model

To establish a gene expression model that can predict the benefit of radiotherapy, we randomly divided the TCGA dataset into a training (N=236) and a validation cohort (N=156) with a ratio of 3: 2. We analyzed the DEG between the RS-immune and non-RS-immune group. Genes passed the selection criteria of |log2FC| ≥1.2, and adjusted P value <0.01 were selected for subsequent survival analysis. We use the method developed by Tian et al [13,17] to train this predictive gene model with the Penalized package [18]. First, we performed univariate Cox regression analyses with the formula below:

$$h(t|X, T)=h_0(t)\exp{(\alpha T + \beta XT)}$$

X represents the expression of DEGs. T represents radiotherapy status and was coded as {-1,1}. Genes having significant interaction with radiotherapy (P<0.05) in univariate Cox regression analyses were further included in a multivariate ridge-regularized Cox model. The formula was as follows:

$$h(t|X_1, ...X_k, T)=h_0(t)\exp\left(\alpha T+\sum_{k=1}^{K}\beta_k X_k T\right), s.t.\ |\alpha|^2+\sum_{k=1}^{K}|\beta|^2 <\lambda$$

$X_1....X_k$ represents the genes selected by univariate Cox regression analyses and lambda represents the regularization intensity determined by 10-fold cross-validation. The final gene signature for RSS was:

$$\sum_{k=1}^{K}\beta_k X_k$$

Statistical Analysis

X-tile software (version 3.6.1) was used to calculate RSI best cut-off value, and all other statistical analysis were performed by R Studio software (version 1.1.453). Kaplan-Meier survival analysis and Cox regression analyses were used for survival analysis. The chi-square test and Fisher exact test were used
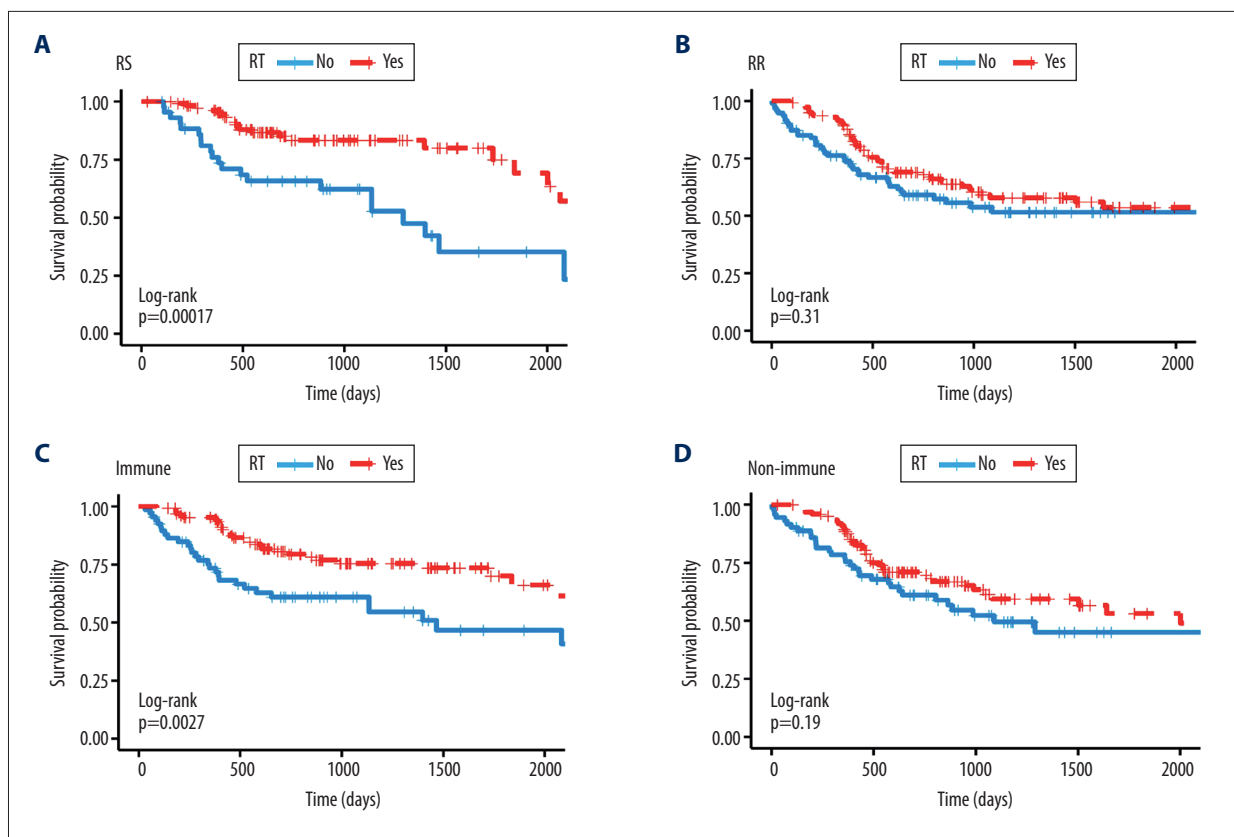
Figure 2. Kaplan-Meier survival analysis. Kaplan Meier plots were drawn to compare OS between patients receiving and not receiving RT in the group of (**A**) RS (**B**) RR (**C**) immune (**D**) non-immune. RS – radiosensitive; RR – radioresistant; RT – radiotherapy.

to compare clinical and pathological factors between corresponding groups. All tests were 2-sided. A *P* value <0.05 was considered statistically significant.

Results

Patient Stratification and Survival Analysis

The RSI cut-off value performed by X-tile software was 0.8. Schema of patient grouping is provided in **Figure 1**. Based on the cut-off value of RSI and the median value of immune score, we divided the cohort into the RS group and RR group, and the immune group and non-immune group. Kaplan-Meier survival analysis suggested that better overall survival in patients receiving radiotherapy were seen in the RS and immune groups (**Figure 2A**, P<0.001, HR=0.338, 95% CI=0.187-0.611; **Figure 2C**, P=0.003, HR=0.482, 95% CI=0.296-0.785). On the contrary, there were no significant difference in overall survival between patients receiving and not receiving radiotherapy in RR or in the non-immune group (**Figure 2B**, P=0.313, HR=0.814, 95% CI=0.545-1.214; **Figure 2D**, P=0.197, HR=0.742, 95% CI=0.472-1.167)

After that, we combined the 2 stratification methods to divide patients into 4 subgroups – the RS-immune, RS-non-immune, RR-immune, and RR-non-immune groups – showing that patients treated with radiotherapy had better overall survival compared with those without radiotherapy only in the RS-immune group (**Figure 3A**, P<0.001, HR=0.194, 95% CI=0.788-0.480). Among the rest groups, there were no significant differences in overall survival between patients receiving radiotherapy and not receiving radiotherapy (**Figure 3B**, P=0.276, HR=0.595, 95% CI=0.234-1.515; **Figure 3C**, P=0.571, HR=0.836, 95% CI=0.450-1.555; **Figure 3D**, P=0.492, HR=0.831, 95% CI=0.490-1.409).

Patients Matching and Survival Analysis

In order to exclude the influence of confounding factors, we performed PSM between patients receiving and not receiving radiotherapy. After PSM, RS-immune group remained 23 patients, non-RS-immune group remained 105 patients. There were no significant differences in the 7 matching variables between patients treated with and without radiotherapy in both groups (**Tables 1, 2**).
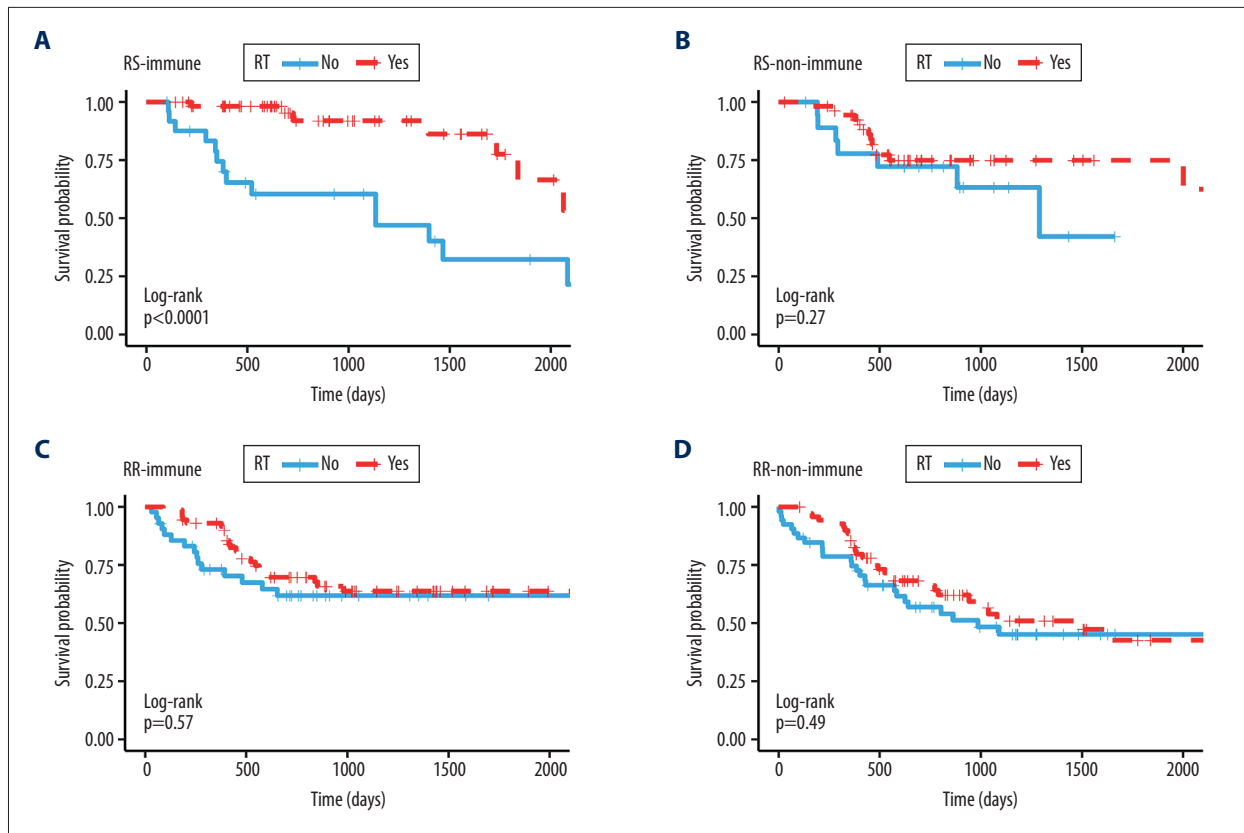
Figure 3. Kaplan Meier survival analysis. Kaplan Meier plots were drawn to compare OS between patients receiving and not receiving RT in the subgroup of (**A**) RS-immune (**B**) RS-non-immune (**C**) RR-immune (**D**) RR-non-immune. RS – radiosensitive; RR – radioresistant; RT – radiotherapy.

We performed survival analysis in the matching cohorts and found similar results to that before PSM. In the RS-immune group, the overall survival was better in patients receiving radiotherapy (**Figure 4A**, P=0.015, HR=0.102, 95% CI=0.011-0.933). In the non-RS-immune group, there was no significant difference in overall survival between patients receiving and not receiving radiotherapy (**Figure 4B**, P=0.112, HR=0.600, 95% CI=0.320-1.126).

Bioinformation Analysis of Differentially Expressed Genes.

To explore potential biological processes, we analyzed the differentially expressed genes between RS-immune group and non-RS-immune group. The results showed that there were 674 differentially expressed genes with 525 genes upregulated in RS-immune group and 149 downregulated. GO and KEGG pathway enrichment analysis were performed using differentially expressed genes. Top 10 GO biological processes and KEGG pathways were shown (**Figure 5A, 5B**). GO biological process enrichment analysis showed that the differentially expressed genes were mainly enriched in pathways related to immunity such as immune response, adaptive immune response, and T cell co-stimulation, suggesting an active immune

response in the RS-immune group. KEGG pathway enrichment results showed that the differentially expressed genes were mainly enriched in cytokine-cytokine receptor interaction, intestinal immune network for IgA production, and cell adhesion molecules.

Predictive Value of the Signature in HNSCC

To establish a gene expression model that can predict radiotherapy benefit, we divided the TCGA dataset into a training and a validation cohort. The information of the 2 cohorts is shown in **Table 3**. The differentially expressed genes analysis in the training cohort identified 382 genes, all of which were included in the univariate Cox regression analyses. The results showed that 3 genes had significant interactions with radiotherapy (P<0.05) and they were further included in the multivariate ridge-regularized Cox model. We successfully constructed a 3-gene signature: RSS=0.0352306203* CCNA1-0.0366707616* CLGN-0.0224280179* KRT15, lambda=674.5671(**Figure 6**).

In the training cohort, we used the median value of RSS to divide the cohort into 2groups; the RSS-L group was classified as the radiotherapy-effective group because patients receiving

Table 1. PSM result in RS-immune group.

Matching variables	No RT	RT	P value
Total number	7	16	
Neoplasm histologic grade			0.732
I	1	1	
II	5	11	
III	1	4	
Clinical stage			0.718
I	0	1	
III	2	3	
IV	5	12	
Lymphovascular invasion			0.657
No	4	11	
Yes	3	5	
Margin status			1.000
Negative	6	12	
Positive\close	1	4	
Pathological nodal extra-capsular spread			0.621
No	6	11	
Micro-invasion\gross invasion	1	5	
Surgery			0.371
No	5	7	
Yes	2	9	
Chemotherapy			–
No	7	16	
Yes	0	0	

PSM – propensity score matching; RS – radiosensitive.

Table 2. PSM result in non-RS-immune group.

Matching variables	No RT	RT	P value
Total number	34	71	
Neoplasm histologic grade			0.793
I	2	7	
II	25	50	
III	7	14	
Clinical stage			0.180
I	3	1	
II	1	4	
III	11	17	
IV	19	49	
Lymphovascular invasion			0.367
No	26	47	
Yes	8	24	
Margin status			0.769
Negative	30	60	
Positive\close	4	11	
Pathological nodal extra-capsular spread			0.504
No	25	46	
Micro-invasion\gross invasion	9	25	
Surgery			0.661
No	21	48	
Yes	13	23	
Chemotherapy			–
No	34	71	
Yes	0	0	

PSM – propensity score matching; RS – radiosensitive.

radiotherapy had better OS (**Figure 7A**, P=0.001, HR=0.377, 95% CI=0.202-0.703), while patients in the RSS-H group were classified as radiotherapy-defective as no significant survival difference could be seen between patients receiving and not receiving radiotherapy (**Figure 7B**, P=0.634, HR=1.156, 95% CI=0.636-2.103). The validation cohort was also split into 2 groups according to RSS median value. Consistent with the result of the training set, patients in RSS-L group benefited from radiotherapy (**Figure 7C**, P<0.001, HR=5.215, 95% CI=2.235-12.166) and there was no significant difference in overall survival between patients treated with or without radiotherapy in the RSS-H group (**Figure 7D**, P=0.915, HR=1.041, 95% CI=0.494-2.196). Finally, we considered RSS as a continuous variable and performed univariate and multivariate Cox regression analyses in the training and validation cohorts. RSS

showed significant interaction with radiotherapy in univariate and multivariate analyses in the training and validation cohorts (**Table 4**).

Discussion

In this study, we first demonstrated that the combined stratification of intrinsic radiosensitivity and immune status could predict radiotherapy benefit and was better than considering intrinsic radiosensitivity or immune status alone. In addition, we developed a gene signature based on the combined stratification for predicting radiotherapy benefit of HNSCC independently.
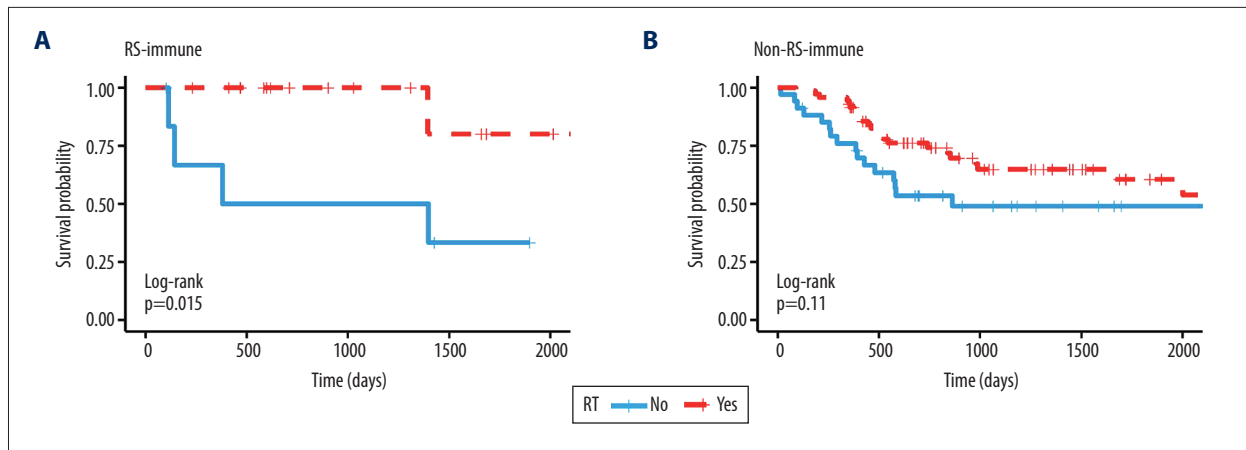
Figure 4. Kaplan Meier survival analysis. Kaplan Meier plots were drawn to compare OS between patients receiving and not receiving RT in the matching group of (**A**) RS-immune (**B**) non-RS-immune. RS – radiosensitive; RR – radioresistant; RT – radiotherapy.
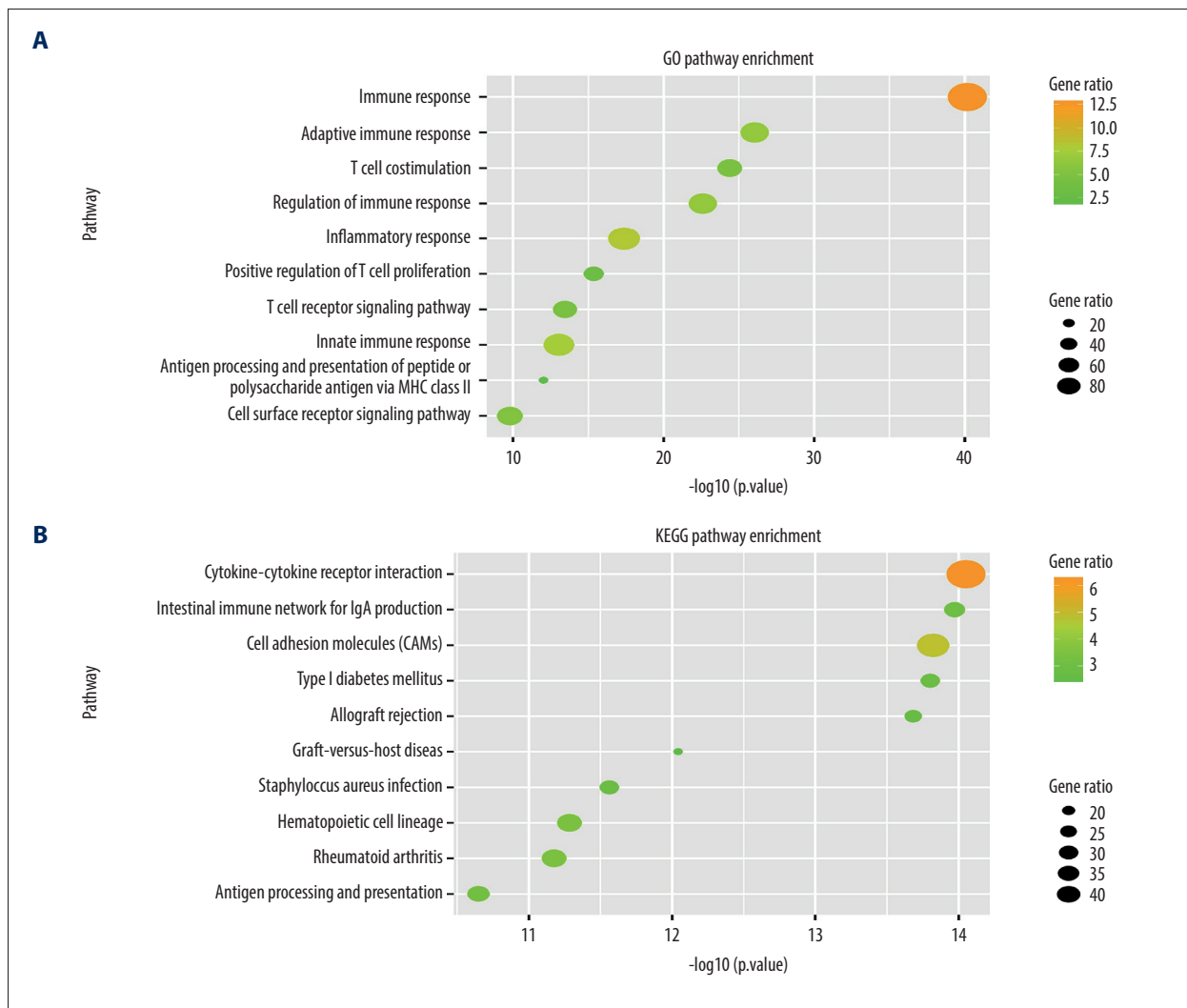

Figure 5. Gene Ontology and KEGG pathway enrichment analysis of differentially expressed genes between the RS-immune group and non-RS-immune group. The top 10 GO biological process enrichment results (**A**) and top 10 KEGG enrichment results (**B**) for differentially expressed genes.

Table 3. Comparison of patients clinical and pathological characteristics in training and validation cohorts.

Matching variables	Training	Validation	P value
Total number	236	156	
Neoplasm histologic grade			0.662
I	30	19	
II	140	97	
III	57	35	
IV	2	0	
Clinical stage			0.152
I	9	7	
II	50	20	
III	44	38	
IV	127	86	
Lymphovascular invasion			0.350
No	119	68	
Yes	49	36	
Margin status			
Negative	167	111	
Positive\close	44	30	
Pathological nodal extra-capsular spread			0.350
No	117	71	
Micro-invasion\gross invasion	47	37	

Given the heterogeneity of HNSCC, we need effective biomarkers to select dominant patients and guide the optimal use of radiotherapy. At present, clinical and pathological parameters such as TNM stage, lymph node extra-capsular invasion, lymph vascular invasion, surgical margin, and nerve invasion are the main factors affecting radiotherapy decision-making in HNSCC. However, none of these parameters fully explain the differences in treatment response for individual patients undergoing radiotherapy. Other patient characteristics, such as smoking status, age, and nutritional status [19,20] are also considered to be factors that influence radiotherapy response and prognosis, but it is still difficult to predict which patients will be completely relieved and which will not. This condition makes the treatment of a large number of patients inadequate, ineffective, or unnecessary.

In this paper, we first demonstrate that the intrinsic radiosensitivity affects radiation response, which is widely accepted. Then, we demonstrate that patients with high immune score are more likely to benefit from radiotherapy. Previous articles have proved that immune cells in the tumor microenvironment

are necessary for sufficient radiation response in preclinical mouse models [21-23]. Kawaguchi et al [24] put forward that high CD8+ TIL density is a favorable biomarker for patients with nasopharyngeal carcinoma or oropharyngeal carcinoma. Alternatively, Gurin et al [25] also suggested that patients with high density of stromal CD8+ TILs displayed significantly better overall survival (OS) and progression-free survival (PFS) in oropharyngeal squamous cell carcinoma patients receiving IMRT. However, there are limited articles combing these 2 factors together. Patel et al [26] demonstrated that the combination of radiation, G2/M cell cycle checkpoint regulator Wee1 kinase inhibitor ADZ1775 and immunomodulator PD-1 can induce rejection of established tumor in 73% of the oral cancer in a mouse model, which is better than radiation or ADZ1775 dual therapy with PD-1 and is also superior to PD-1 monoclonal antibody monotherapy. This is consistent with our results. Our results indicate that only patients who are intrinsically radiosensitive and have abundant immune cells infiltration can benefit from radiotherapy. The possible explanation is that the radiation-induced cancer immunity cycle kills tumor cells by inducing tumor cell death, triggering innate immune system, activating tumor specific T cells that can bind to tumor cells, killing cancer cells, releasing other tumor related antigens, and repeating and expanding the above process again [14]. Tumor antigen releasing is the first step to initiate anti-cancer immunity, and radiotherapy can accelerate this step by inducing immunogenic cell death [27]. Patients who are not sensitive to radiotherapy have weaker response; therefore, less tumor antigens are released and this may result in insufficient CTL infiltration. On the other hand, the presence of antigen-presenting defects, depletion of T cells, tumor insensitivity to T cells, immunosuppressive cells, and other immunosuppressive checkpoints in the tumor microenvironment may also weaken cytotoxic effect of CTL [14,28]. The complementary effect of the 2 biological processes makes intrinsic radiosensitivity and immune status become 2 essential conditions for patients to benefit from radiotherapy. Through our conclusion, we can identify patients who may not respond well to standard radiotherapy and may benefit from radiation sensitizers or immunotherapy.

Some gene expression-based signatures have been constructed to predict the overall survival of HNSCC patients treated with RT. Ma et al [11] established a 4-gene methylation signature to predict survival rate of HNSCC patients with RT, and You et al [12] identified 4 key molecular markers based on 4 core functional pathways. These markers are prognostic at the time of establishment. However, we established a predictive model, RSS, by calculating the interaction between gene expression and radiotherapy with the method of Tian et al [17]. As a result, our signature may be more suitable to help make treatment decisions in clinical practice.
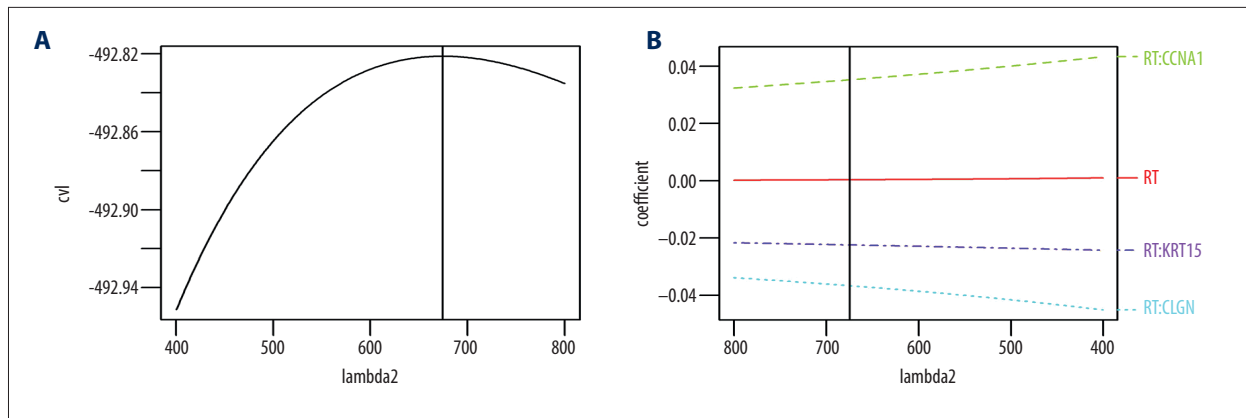
Figure 6. Signature established by L2 regularized (ridge) Cox regression model. (**A**) Parameter (Lambda2) selection by L2 regularized (ridge) Cox regression model adopted 10-fold cross-validation; (**B**) L2 regularized (ridge) Cox regression model coefficient profile plot of interaction between radiotherapy and genes selected by univariate Cox regression analyses. cvl – cross-validated likelihood; RT – radiotherapy.
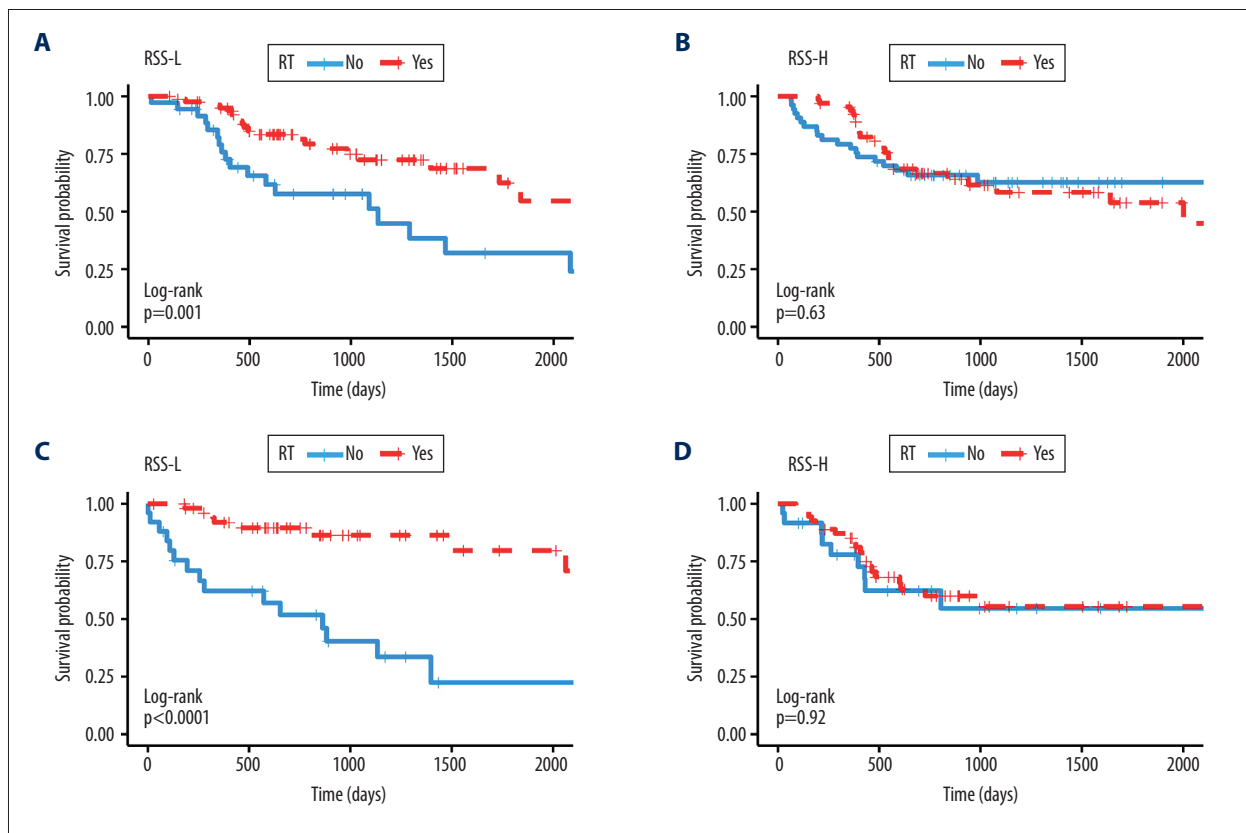

Figure 7. Kaplan Meier survival analysis. Kaplan Meier plots were drawn to compare OS between patients receiving and not receiving RT in the group of (**A**) RSS-L (**B**) RSS-H in the training cohort and in the group of (**C**) RSS-L (**D**) RSS-H in validation cohort. The median values of the RSS score were used as the cut offs to define the high and low RSS groups in both training and validation cohorts. RRS-L – RSS low expression; RSS-H – RSS high expression; RT – radiotherapy.

Table 4. Univariate and multivariate Cox regression analyses of interaction between RSS (as continues variable) and RT in training and validation cohort.

Variables	Univariate		Multivariate	
	HR (95% CI)	P value	HR (95% CI)	P value
Training				
RSI * RT	0.650 (0.393-1.074)	0.093		
Immune score * RT	0.999 (0.999-1.000)	0.004	0.999 (0.999-1.000)	0.249
RSS * RT	2.873 (1.431-5.769)	0.003	3.367 (1.323-8.565)	0.011
Clinical stage * RT	0.980 (0.899-1.068)	0.638		
Neoplasm histologic grade * RT	0.956 (0.832-1.099)	0.527		
Lymphovascular invasion * RT	1.233 (0.920-1.636)	0.146		
Margin status * RT	1.401 (1.055-1.861)	0.020	0.982 (0.679-1.420)	0.923
Pathological nodal extra-capsular * RT	1.720 (1.324-2.234)	4.83e-05	1.953 (1.466-2.603)	4.8e-06
Validation				
RSI * RT	0.588 (0.304-1.139)	0.166		
Immune score * RT	1.000 (0.999-1.000)	0.671		
RSS * RT	3.109 (1.216-7.952)	0.018	3.073 (1.004-9.406)	0.049
Clinical stage * RT	0.919 (0.829-1.019)	0.110		
Neoplasm histologic grade * RT	0.923 (0.775-1.099)	0.367		
Lymphovascular invasion * RT	0.936 (0.639-1.371)	0.735		
Margin status * RT	0.863 (0.568-1.312)	0.492		
Pathological nodal extra-capsular * RT	1.527 (1.104-2.111)	0.011	1.534 (1.110-2.119)	0.010

RT – radiotherapy; RSI – radiosensitivity index; RSS – radiosensitivity signature; HR – hazard ratio; 95% CI – 95% confidence interval.

The main limitation of our study was the combination of retrospective cohort and non-randomized radiotherapy. Ideally, predictive biomarkers should be tested in prospective randomized controlled trials. To reduce the potential selection bias in retrospective cohort studies, we used PSM to balance the relevant clinical and pathology characteristics between the corresponding groups [13]. Another limitation is that the constructed gene signature needs further biological and clinical verification as biomarkers of radiosensitivity in HNSCC.

Conclusions

In conclusion, we proved that the combined stratification of intrinsic radiosensitivity and immune status could serve as a preclinical evaluation to identify patients benefiting from radiotherapy and helped patients to choose the best treatment strategy. Based on this combined stratification, a gene signature was established to independently predict radiotherapy benefits in HNSCC.

Conflicts of Interest

None.

Declaration of Figures Authenticity

All figures submitted have been created by the authors, who confirm that the images are original with no duplication and have not been previously published in whole or in part.

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