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Original Article

PYHIN1 correlates with CD8+ T cells infiltration and confers good patient survival in oral cancer

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Abstract *Background/purpose:* Immunotherapy has become a research hotspot and is used for head and neck cancer treatment. This research aims to explore the prognostic value of PYHIN1 in oral cancer and the relationship between PYHIN1 and cancer immunity.
Materials and methods: The expression of PYHIN1 in clinical specimens was evaluated by bioinformatics analyses and immunohistochemistry.
Results: Gene ontology term enrichment analyses and gene set enrichment analyses showed the involvement of PYHIN1 in the modulation of adaptive immunity-associated signaling according to The Cancer Genome Atlas database and Gene Expression Omnibus dataset. Interestingly, the correlation analyses in The Cancer Genome Atlas database revealed a positive correlation between PYHIN1 expression and activated CD8+ T cells infiltration and a negative correlation between PYHIN1 expression and tumor purity. Moreover, activated CD8+ T cells infiltration predicted good patient survival and was negatively correlated with tumor purity. Importantly, PYHIN1 expression was negatively correlated with the pathological stage and was positively associated with a good prognosis in patients with oral cancer. The data obtained from the Gene Expression Omnibus dataset and immunohistochemistry confirmed the positive association between PYHIN1 and CD8+ T cells infiltration in oral cancer tissues.
Conclusion: We conclude that PYHIN1 is an indicator of cancer immunity, and is an independent prognostic factor that may be an alternative target for oral cancer treatment.

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Introduction

The incidence rate of oral cavity and pharynx continues to increase in recent years.[1] Immunotherapy has become a research hotspot, and more and more studies have been conducted to evaluate the effect of immune checkpoint inhibitors on head and neck cancer (HNSC).[2] Combination of PD-1/PD-L1 expression, tumor mutation burden, and microsatellite instability testing that are recommended to be used for predicting immune responses with other indicators of cancer immunity in oral cancer contributes to developing an individualized and precision oncology approach.[3–5]

Several genes were reported to be correlated with antitumor immunity in human cancers. CXCL14 from cancer cells exerted antitumor immunity through restoring MHC-I expression, thus facilitating antigen-specific CD8+ T cells responses in HPV-positive HNSC.[6] FBP1 from cancer cells was an inhibitor of PD-L1 expression and was correlated with cancer immunity, indicating its predictive role in immune responses to anti-PD-1 therapies.[7] The silence of CMTM6 downregulated PD-L1 expression and induced CD8+ and CD4+ T cell infiltration. Therefore, CMTM6 might be a promising therapeutic target for HNSC treatment.[8] According to these reports, cancer immunity is associated with specific gene expression of cancer cells.

PYHIN1 belongs to the HIN-200 family of interferon-inducible proteins that share a 200-amino acid motif at the C-termini. PYHIN1 has been reported to be involved in controlling adaptive immunity and innate immunity through modulating the production of cytokine, the function of macrophages and T cells, and the transcription of a specific target gene.[9,10] Previous studies suggested the inhibitory effect of PYHIN1 on cell invasion and cell growth in breast cancer.[11,12] However, the role of PYHIN1 in oral cancer has not been studied, and the association between PYHIN1 and cancer immunity in human cancers is yet to be determined.

Here, we demonstrated that PYHIN1 was positively correlated with CD8+ T cells infiltration of oral cancer and exhibited differential expression levels during cancer progression. Moreover, PYHIN1 expression negatively correlated with tumor purity of oral cancer and functioned as a favorable prognostic indicator for the overall survival of oral cancer patients.

Materials and methods

Bioinformatics analysis

The mRNA-Seq data of oral cancer in The Cancer Genome Atlas (TCGA) database and the mRNA-Seq data of oral cancer in the Gene Expression Omnibus (GEO) dataset (GSE41613) were extracted for performing bioinformatics analyses. The expression profiles of oral cancer were applied for differential analyses, gene set enrichment analyses (GSEA), Gene Ontology (GO) term enrichment analyses, correlation analyses, and survival analyses. The median values were regarded as the cut-offs, and the value higher than the median was assigned as a high group, the others were assigned as a low group. The R package ggplot2 was applied for drawing bubble charts. The R package ESTIMATE was applied for calculating the stromal score, immune score, and tumor purity. Tumor purity was calculated based on ESTIMATE score as previously described.[13] Single sample GSEA (ssGSEA) was applied for calculating the infiltration of specific immune cells.

Clinical data collection

Clinical data of the 335 patients with oral cancer were obtained from the TCGA dataset. Age, gender, T classification, N classification, M classification, and overall survival were extracted from the medical records of these patients. Some of the clinical data and PYHIN1 expression of oral cancer patients are not available in the TCGA database. Since only one patient had distant metastasis, M classification was not used for further analyses. Clinical data of the 97 patients were obtained from the GSE41613.

Collection of clinical tissues

A total of 55 paraffin-embedded oral cancer samples (HOraC060PG01) were purchased from Shanghai Outdo Biotech (Shanghai, China). The samples were collected during surgery, and diagnoses were confirmed by pathology reports. Age, gender, T classification, N classification were extracted from the medical records of these patients. The approved protocols were obtained from the Ethics Committee of Shanghai Outdo Biotech Company, and prior consent was collected from the patients.

Immunohistochemistry (IHC) and evaluation of IHC staining

Immunohistochemical detection of PYHIN1 and CD8 expression was performed on 55 paraffin-embedded oral cancer tissues collected from the hospital. Briefly, the sections of oral cancer tissues were dewaxed with xylene and rehydrated with graded ethanol. The slides were incubated with 3% hydrogen peroxide to eradicate the endogenous peroxidase activity after antigen retrieval with HIER antigen retrieval reagent (pH 6) (Abcam, MA, USA) for PYHIN1 and with Tris buffer (pH 9) for CD8 using microwave irradiation. Then, the slides were treated with normal goat serum and were subjected to incubation with primary

antibody anti-PYHIN1 (1:200, HPA051224, Atlas Antibodies, Bromma, Sweden) and anti-CD8 (1:200, ab17147, Abcam), followed by the treatment with the secondary antibody. Human tonsil tissue was used as a positive control. PBS was used as a negative control by substituting for primary antibodies. Finally, the antigen–antibody complexes were visualized using DAB chromogen and hematoxylin. Two independent pathologists who were blind to the data of patients independently evaluated the IHC staining of sections. The disagreement between these two independent pathologists was resolved with discussion, or by a third pathologist. The intensity scores were calculated as previously described.[14,15] For PYHIN1 staining, the score 0–4 was assigned as low expression, and the score 5–9 was assigned as high expression. For CD8 staining, the percent of infiltrating immune cells 0–5% was assigned as low expression, and the percent of infiltrating immune cells higher than 5% was assigned as high expression.

Statistical analysis

All data analyses were conducted by SPSS 21.0. Nonparametric tests were adopted for evaluating the differential expression levels of PYHIN1 in oral cancer. The $\chi 2$ test was conducted to explore the relationship between PYHIN1 expression and parameters of oral cancer. The correlation analyses were applied to elucidate the association between PYHIN1 and activated CD8+ T cells infiltration in oral cancer. The logistic regression analyses were conducted to clarify the relationship between parameters and CD8+ T cells infiltration. Survival analyses were performed by plotting Kaplan–Meier survival curves. The univariate and multivariate Cox proportional hazards models were used to evaluate the effect of various variables on oral cancer patient survival. $P < 0.05$ were considered statistically significant.

Results

PYHIN1 correlates with activated CD8+ T cells infiltration in the TCGA dataset

Firstly, bioinformatics analyses were conducted to investigate the role of PYHIN1 in oral cancer. Interestingly, GO term enrichment analyses and GSEA of the TCGA dataset indicated that PYHIN1 was positively participated in modulating adaptive immunity-associated signaling (in KEGG, Reactome, and Hallmark) (Fig. 1). Then, the infiltration scores of immune cells were calculated by the ESTIMATE and suggested that PYHIN1 mRNA levels were positively correlated with stromal score and immune score. However, PYHIN1 mRNA levels were negatively correlated with tumor purity (Fig. 2A). To explore the relationship between PYHIN1 expression and a specific type of cell, differential analyses were then performed and elucidated that PYHIN1 mRNA levels were correlated with the infiltration of immune cells and stromal cells determined by ssGESA (Fig. 2B). Interestingly, the most prominent correlation was identified between PYHIN1 expression and activated CD8+ T cells was (Fig. 2C).

The relationship between PYHIN1 expression and clinicopathological parameters of oral cancer patients

Further exploring the clinical value of PYHIN1 in the TCGA dataset, we found that PYHIN1 was downregulated in stage III-IV and T3-T4 oral cancer in comparison to those in stage I-II and T1-T2 oral cancer, respectively (Fig. 3A). However, the differential analyses revealed no difference of PYHIN1 expression in other clinicopathological parameters. Table 1 showed the clinicopathological parameters of 335 oral cancer patients. The median age of these patients was 60 years old, and 232 patients were male. The correlation analyses showed that PYHIN1 expression was associated with the gender of the patients. However, no correlation was elucidated between PYHIN1 expression and other clinicopathological features, such as age, T classification, N classification, and M classification in oral cancer.

High PYHIN1 expression predicts a good prognosis of oral cancer patients

Survival analyses were applied to investigate the correlation between PYHIN1 expression and the overall survival of oral cancer patients in the TCGA dataset. Univariate analyses and multivariate analyses were performed to probe into the relationship between PYHIN1 expression and the clinicopathological parameters and overall survival of oral cancer patients. Importantly, survival analysis suggested that high PYHIN1 expression predicted a good patient prognosis. Male patients had better overall survival than female patients. T1-T2, without lymph node metastasis, and high activated CD8+ T cells infiltration predicted a good patient prognosis (Fig. 3B). Multivariate Cox proportional hazard analyses identified high PYHIN1 expression as independent and favorable prognostic indicators for oral cancer patients. Moreover, female, T3-T4, and lymph node metastasis were identified as independent and unfavorable prognostic indicators for oral cancer patients (Fig. 3C). Although activated CD8+ T cells infiltration was not an independent indicator for the overall survival of oral cancer patients, activated CD8+ T cells infiltration was negatively correlated with tumor purity (Fig. 3D).

PYHIN1 is associated with activated CD8+ T cells infiltration in oral cancer

To verify the relationship between PYHIN1 and activated CD8+ T cells infiltration in oral cancer, we performed bioinformatics analyses based on the GEO dataset. GSEA and GO term enrichment analyses based on the GSE41613 dataset confirmed that PYHIN1 was positively participated in modulating adaptive immunity-associated signaling in oral cancer (Fig. 4A and B). The correlation analyses were further performed and verified that PYHIN1 mRNA levels were positively correlated with activated CD8+ T cells infiltration (Fig. 4C). Importantly, survival analysis showed that PYHIN1 mRNA levels were positively correlated with the overall survival of oral cancer patients based on the GSE41613 dataset (Fig. 4D).

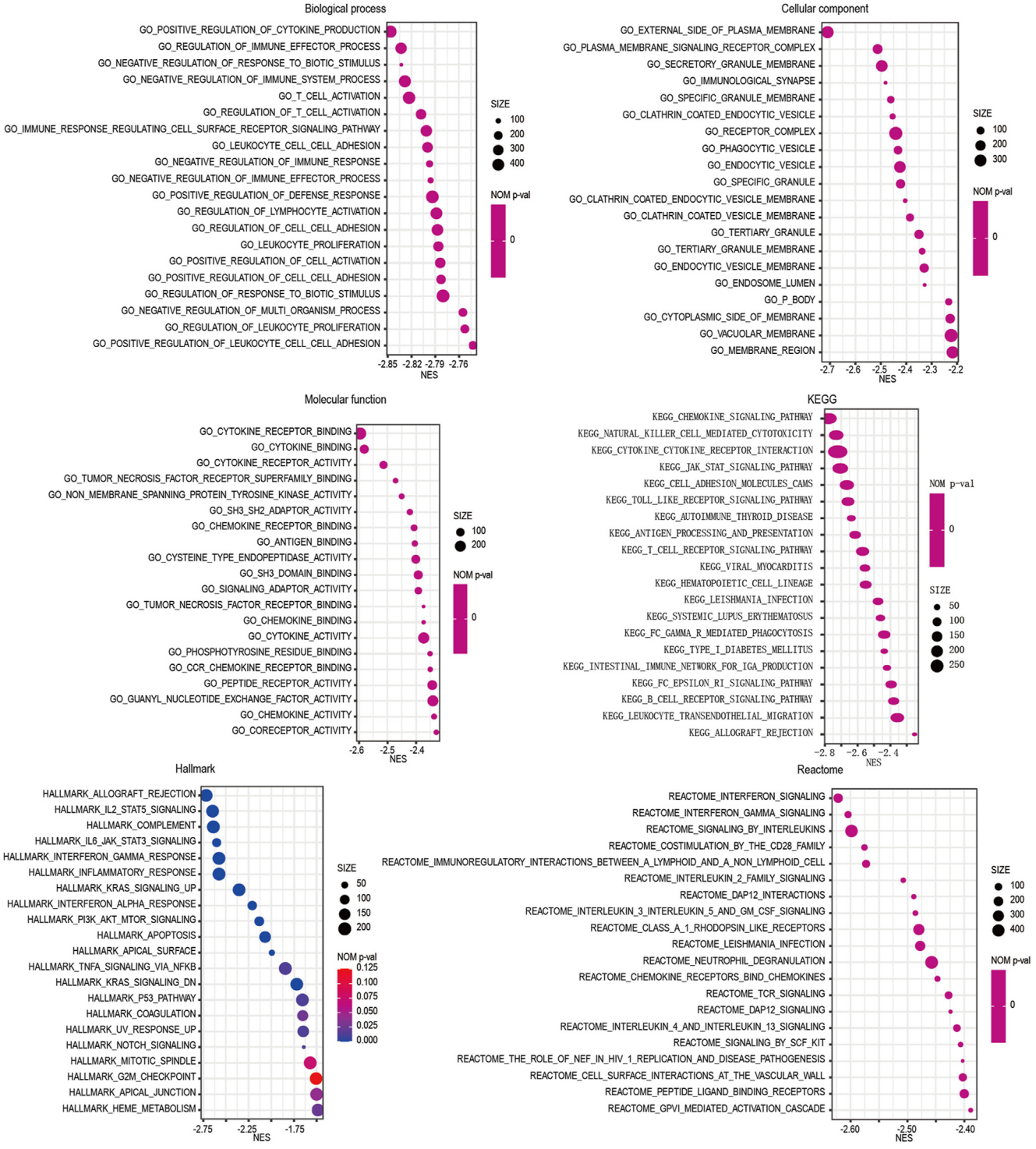

Figure 1 PYHIN1 participates in regulating adaptive immunity-associated signaling in oral cancer. The top list of gene ontology term enrichment analyses showing the impact of PYHIN1 on biological process, cellular component, and molecular function relying on the TCGA dataset, and the top list of gene set enrichment analyses showing the impact of PYHIN1 on signaling relying on the TCGA dataset (in KEGG, Reactome, and Hallmark).

Then, the expression of PYHIN1 and CD8 were measured by immunohistochemistry in collected oral cancer samples. Consistently, high PYHIN1 protein levels were positively correlated with CD8+ T cells infiltration (Fig. 4E). Then, the univariate and multivariate logistic regression analyses were conducted to clarify the relationship between observed parameters and CD8+ T cells infiltration. Intriguingly, PYHIN1 expression was an independent indicator of CD8+ T

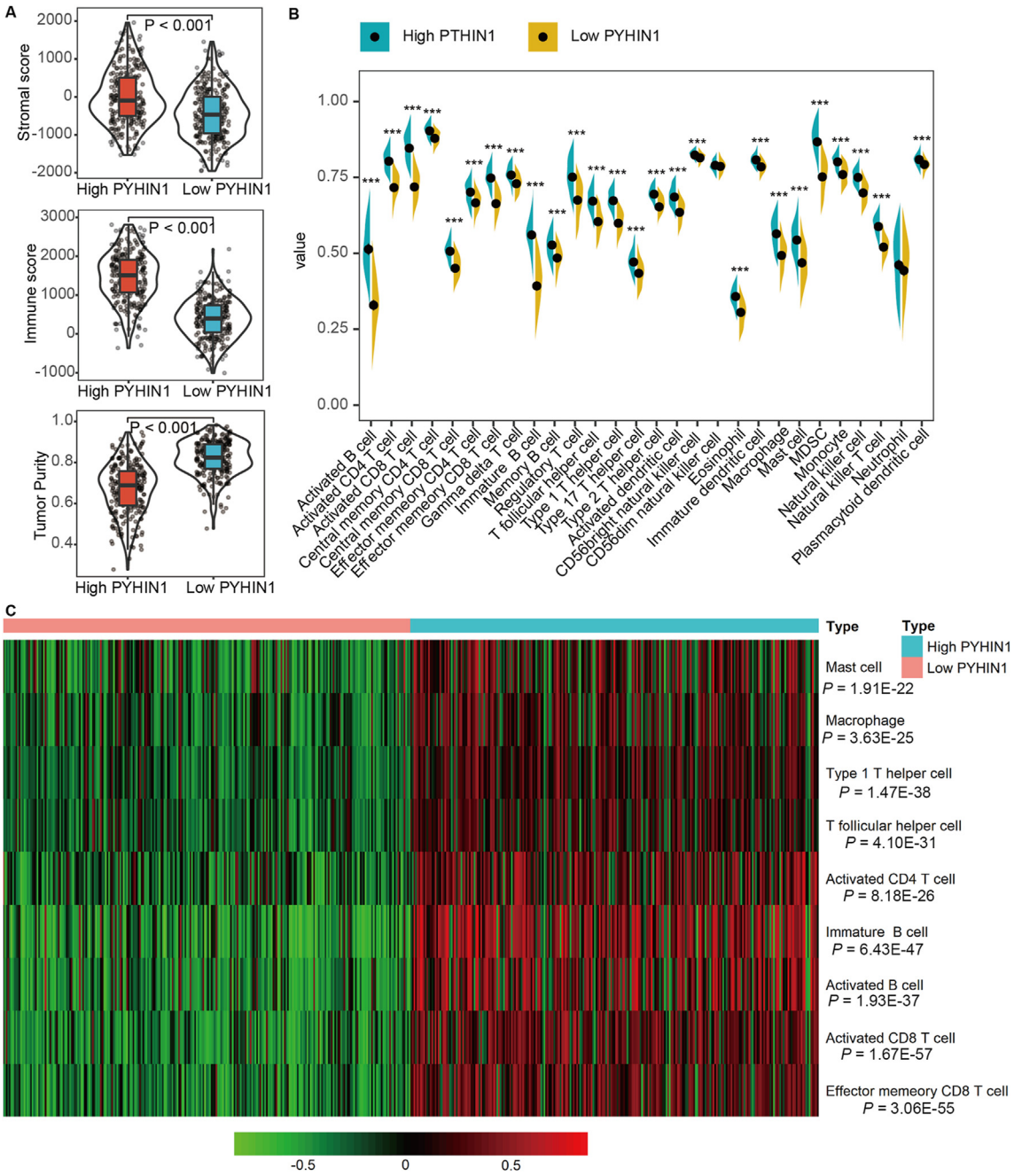

Figure 2 PYHIN1 expression is positively correlated with activated CD8+ T cells infiltration (A) The distribution of stromal score, immune score, and tumor purity in oral cancers with high and low PYHIN1 expression (B) The distribution of 28 types of immune cells in oral cancers with high and low PYHIN1 expression (C) The top list of single-sample gene set enrichment analyses showing the relationship between PYHIN1 expression and specific types of immune cells. ***$P < 0.001$.

cells infiltration. However, there were no correlation between age, gender, T classification, N classification, and CD8+ T cells infiltration (Table 2).

Discussion

Previous studies suggested that PYHIN1 was a candidate tumor suppressor gene in prostate cancer and breast cancer.[16,17] However, the role of PYHIN1 in oral cancer has not been reported. In this study, we investigated the role of PYHIN1 in oral cancer, and bioinformatics analyses and immunohistochemistry in clinical samples demonstrated that PYHIN1 might function as a tumor suppressor in oral cancer. Importantly, we identified PYHIN1 as a novel immune-associated gene in regulating cancer immunity.

The existing evidence demonstrated the regulation of PYHIN1 on immune responses. In bipolar disorder, biological pathways in relation to PYHIN1 were mainly associated with immune function, especially cytokine–cytokine receptor

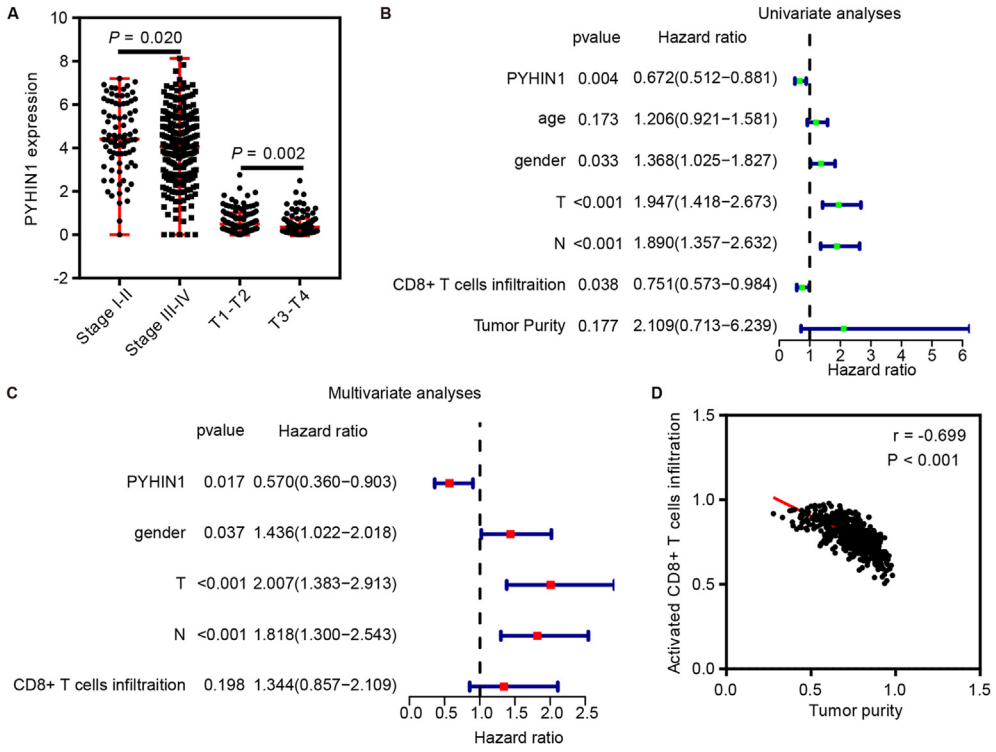

Figure 3 PYHIN1 is negatively correlated with pathological stage and tumor purity and predicts a good prognosis of oral cancer patients (A) PYHIN1 mRNA levels were downregulated in stage III-IV oral cancer compared to those in stage I-II oral cancer (B) Univariate survival analyses were performed to investigate the relationship between clinicopathological characteristics and the overall survival of oral cancer patients (C) Multivariate survival analyses were performed to investigate the relationship between clinicopathological characteristics and the overall survival of oral cancer patients (D) The correlation analyses were performed to elucidate the relationship between activated CD8+ T cells infiltration and tumor purity.

Table 1 Relationships between PYHIN1 expression levels and the clinicopathological parameters of oral cancer patients.

Parameters	Total (n)	PYHIN1 expression levels		*P* value
		Low, n (%)	High, n (%)	
Age (years)		168	167	
≤Median	161	82 (50.9%)	79 (49.1%)	0.783
>Median	174	86 (49.4%)	88 (50.6%)	
Gender				
Male	232	125 (53.5%)	107 (46.5%)	0.048
Female	103	43 (41.7%)	60 (58.3%)	
T classification				
T1-T2	128	63 (49.2%)	65 (50.8%)	0.753
T3-T4	200	102 (51.0%)	98 (49.0%)	
N classification				
N0	170	84 (49.4%)	86 (50.6%)	0.824
N+	154	78 (50.6%)	76 (49.5%)	
M classification				
No	320	160 (50.0%)	160 (50.0%)	1.000
Yes	2	1 (50.0%)	1 (50.0%)	

interaction.[18] PYHIN1 was also regarded as an interferon pathway gene affecting mycobacterium tuberculosis infection and contributed to the induction of interferon response.[10,19] The dysregulation of PYHIN1 in pediatric inflammatory bowel disease also suggested the involvement of PYHIN1 in immune responses.[20] In addition, the PYHIN protein family was a mediator of the immune responses in mammalian host defenses.[21] However, PYHIN1 has not been identified as an immune-associated gene in human cancers. Therefore, we conducted a further study to elucidate the relationship between PYHIN1 and cancer immunity. Here, we showed that PYHIN1 was positively correlated with activated CD8+ T cells infiltration in oral cancer, suggesting the participation of PYHIN1 in the regulation of cancer immunity. Besides, the obtained results were consistent with the data of GO term enrichment analyses and GSEA that PYHIN1 was involved in modulating adaptive immunity-associated signaling based on TCGA and GEO datasets.

Low tumor purity was associated with poor patient prognosis and was affected by different genomic change patterns, and tumors with low purity exhibit a strong immunophenotype.[22] The number of CD8 positive T cells was closely related to the patient prognosis in many cancers.[23] In our work, we also elucidated that PYHIN1 expression affected tumor purity, and the tumor purity of oral cancer negatively correlated with the infiltration of activated CD8+ T cells. Moreover, PYHIN1 expression was negatively associated with pathological stage, suggesting the tumor suppressor role of PYHIN1 in oral cancer. In subsequent survival analyses, we confirmed that high PYHIN1 expression predicted good patient survival. A previous study showed that the most common cell type

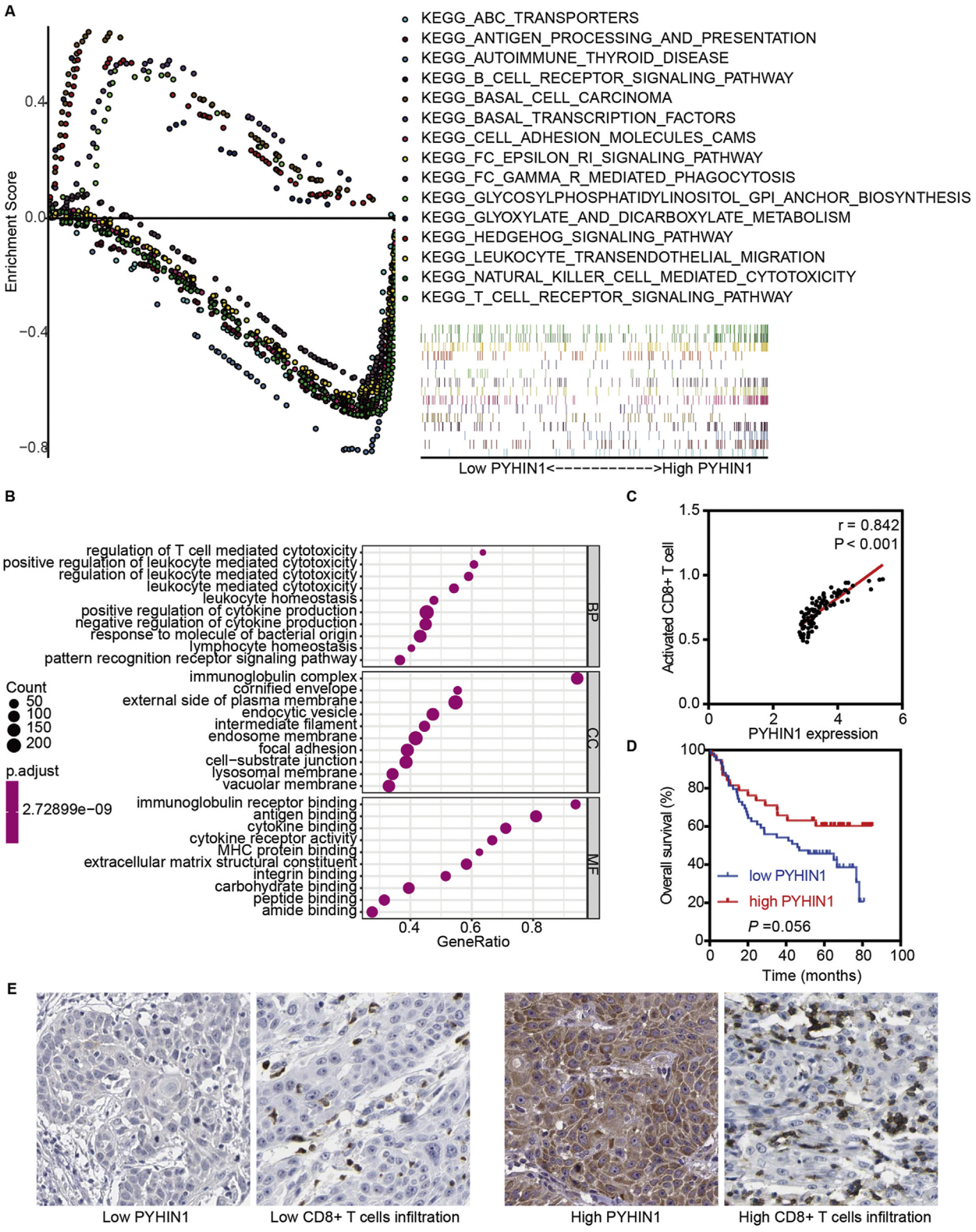

Figure 4 PYHIN1 participates in regulating cancer immunity in oral cancer relying on the GEO dataset (GSE41613) and oral cancer tissues (A) Gene set enrichment analyses were applied to elucidate the impact of PYHIN1 on KEGG signaling pathways (B) Gene ontology term enrichment analyses were applied to elucidate the impact of PYHIN1 on biological process, cellular component, and molecular function (C) The correlation analyses were performed to explore the relationship between PYHIN1 expression and activated CD8+ T cells infiltration (D) Survival analysis was performed to elucidate the relationship between PYHIN1 expression and the overall survival of oral cancer (E) Representative images showed the relationship between PYHIN1 expression and CD8+ T cells infiltration in oral cancer tissues (× 200).

Table 2 Logistics regression analyses of observed parameters and CD8+ T cells infiltration determined by immunohistochemistry.

Parameters	CD8+ T cells infiltration					
	Univariate analyses			Multivariate analyses		
	HR	95% CI	*P* value	HR	95% CI	*P* value
PYHIN1 expression Low *vs.* High	0.302	(0.114–0.899)	0.012	0.408	(0.240–0.928)	0.016
Age (years) ≤Median *vs.* >Median	1.218	(0.995–1.727)	0.059	1.104	(0.968–1.314)	0.144
Gender Male *vs.* Female	1.314	(0.893–1.933)	0.165	1.163	(0.646–2.094)	0.615
T classification T1 -T2 *vs.* T3-T4	1.135	(0.978–1.394)	0.101	1.238	(0.726–2.110)	0.434
N classification N0 *vs.* N+	1.176	(0.800–1.728)	0.409	0.903	(0.532–1.529)	0.702

significantly associated with good prognosis was Tem CD8+ cells, activated CD8+ cells, and Tem CD4+ cells.[24] Here, we confirmed the positive association between activated CD8+ T cells and the good prognosis of oral cancer patients.

Recently, immunotherapy has been well developed and has been widely used for cancer therapy. The immune checkpoint inhibitors that block the PD-L1/PD-1 and/or CTLA-4 signaling pathway and induce the activation of T cells were applied for HNSC treatment.[25–27] In this study, we showed a positive correlation between PYHIN1 and activated CD8+ T cells infiltration, indicating the application of PYHIN1 as a marker for predicting the efficiency of immunotherapy. Importantly, we confirmed the positive association between PYHIN1 protein levels and CD8+ T cells infiltration in oral cancer tissues determined by immunohistochemistry, suggesting the expression of PYHIN1 in oral cancer cells affecting the behavior of immune cells. Age, sex, and pathological stage were shown to affect cancer immunity.[15,24,28] Although we did not elucidate the correlation between age, sex, pathological stage, and CD8+ T cells infiltration, we indeed suggested PYHIN1 expression was an independent indicator of CD8+ T cells infiltration in oral cancer determined by immunohistochemistry. Age tended to be correlated with CD8+ T cells infiltration in oral cancer, we assumed that expanding the sample size might obtain significant results. To this end, we demonstrated that the abnormal expression of PYHIN1 was an independent indicator of CD8+ T cells infiltration and predicted the good prognosis of oral cancer patients.

Taken together, we demonstrated that PYHIN1 played as a tumor suppressor in oral cancer. PYHIN1 expression levels were positively associated with CD8+ T cells infiltration and overall survival of patients with oral cancer. We propose that PYHIN1 is a significant prognostic factor in oral cancer and may be applied as a potential target for oral cancer treatment. Moreover, detection of PYHIN1 expression might be an alternative manner for predicting the efficiency of immunotherapy in oral cancer.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

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