Original Article

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

Jian-Ming Ding a, Wen-Rong Lin b, Zhao-Dong Fei a, Chuan-Ben Chen a*

a Department of Radiation Oncology, Fujian Medical University Cancer Hospital, Fujian Cancer Hospital, Fuzhou, Fujian, China
b Department of Ultrasound, Fujian Medical University Cancer Hospital, Fujian Cancer Hospital, Fuzhou, Fujian, China

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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.

* Corresponding author. Department of Radiation Oncology, Fujian Medical University Cancer Hospital, Fujian Cancer Hospital, No. 420 Fuma Road, Jinan District of Fuzhou, 350014, Fujian, China.
E-mail address: ccbben@126.com (C.-B. Chen).

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Introduction

The incidence rate of oral cavity and pharynx continues to increase in recent years. 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). 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.

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. 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. 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. 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. Previous studies suggested the inhibitory effect of PYHIN1 on cell invasion and cell growth in breast cancer. 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. 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, 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. 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 χ² 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 ssGSEA (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).
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 cells infiltration in oral cancer samples.
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. 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 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.
PYHIN1 was also regarded as an interferon pathway gene affecting mycobacterium tuberculosis infection and contributed to the induction of interferon response. The dysregulation of PYHIN1 in pediatric inflammatory bowel disease also suggested the involvement of PYHIN1 in immune responses. 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.

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)      |             |                          |         |
| ≤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%)  |         |

Univariate analyses

- PYHIN1: p = 0.004, Hazard ratio: 0.972 (0.512–0.881)
- age: p = 0.173, Hazard ratio: 1.206 (0.921–1.581)
- gender: p = 0.033, Hazard ratio: 1.368 (1.025–1.827)
- T: p < 0.001, Hazard ratio: 1.947 (1.418–2.673)
- N: p < 0.001, Hazard ratio: 1.890 (1.357–2.632)

Multivariate analyses

- PYHIN1: p = 0.017, Hazard ratio: 0.570 (0.360–0.903)
- gender: p = 0.037, Hazard ratio: 1.436 (1.022–2.018)
- T: p < 0.001, Hazard ratio: 2.007 (1.383–2.913)
- N: p < 0.001, Hazard ratio: 1.818 (1.300–2.543)

Correlation analyses

- CD8+ T cells infiltration: r = -0.699, P < 0.001
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).
A tumor suppressor in oral cancer. PYHIN1 expression levels indicated CD8+ T cells infiltration and predicted the good prognosis of oral cancer patients. Moreover, detection of PYHIN1 expression might be an alternative manner for predicting the efficiency of immunotherapy in oral cancer.

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

| Parameters          | Univariate analyses | Multivariate analyses |
|---------------------|---------------------|-----------------------|
|                     | CD8+ T cells infiltration |                     |
|                     | HR  95% CI  P value | HR  95% CI  P value |
| PYHIN1 expression   | 0.302 (0.114–0.899)  | 0.240 (0.012–0.408)  |
| Low vs. High        | 0.928                | 0.016                 |
| Age (years) Low vs. >Median | 1.218 (0.995–1.727)  | 1.094 (0.968–1.314)  |
| Gender Male vs. Female | 1.314 (0.893–1.933)  | 0.615 (0.464–2.094)  |
| T classification 1.135 (0.978–1.394) | 0.726 (0.434–2.110)  |
| T1–T2 vs. T3–T4     |                      |                       |
| N classification 1.176 (0.800–1.728)  | 0.702 (0.532–1.529)  |

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

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