

Aberrant Expression Of PDCD4/eIF4A1 Signal Predicts Postoperative Recurrence For Early-Stage Oral Squamous Cell Carcinoma

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Background: Programmed cell death 4 (*PDCD4*) as a tumor suppressor gene inhibits growth and metastasis of cancer cells, which involved with eIF4A1, the inhibitor of translation initiation. Although the prognosis of early-stage oral squamous cell carcinoma (OSCC) is generally better, but many patients occur recurrence after surgery. Understanding the clinical expression pattern of PDCD4/eIF4A1 signal would provide diagnostic biomarker and target therapy premise for early-stage OSCC patients.

Methods: Immunohistochemical analysis was performed on 69 early-stage (T1/2N0M0) OSCC samples to evaluate temporal expression and prognostic value of eIF4A1 and PDCD4 in early-stage OSCC according to cell types and microlocalization. The correlations between PDCD4/eIF4A1 signal and Ki-67, postoperative recurrence and metastasis were determined.

Results: We found that PDCD4 was presented in tumor cells (TCs) and tumor-infiltrating lymphocytes (TILs) but absent in fibroblast-like cells (FLCs). eIF4A1 was only presented in TCs. PDCD4^{TCs} was negative associated with eIF4A1^{TCs} in tumor center, and patients with low PDCD4^{TCs} or high eIF4A1^{TCs} had poorer differentiation. Moreover, aberrant PDCD4/eIF4A1 signal led to higher Ki-67 level. Interestingly, patients with low expressed PDCD4^{TILs} had better prognosis, indicating the function heterogeneity of PDCD4 in different cell types. Furthermore, low PDCD4^{TCs} and high eIF4A1^{TCs} predicted higher postoperative recurrence rate and are significant independent risk factors for early-stage OSCC.

Conclusion: Patients with low PDCD4^{TCs} and high eIF4A1^{TCs} have higher recurrence rate and poor clinical outcome. Of note, PDCD4^{TILs} exerts contradictory function. Thus, PDCD4/eIF4A1 targeting therapeutics should consider the function heterogeneity of PDCD4.

Keywords: PDCD4, eIF4A1, early-stage OSCC, prognosis, diagnosis

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Introduction

Oral squamous cell carcinoma (OSCC) is malignant oral tumor which accounts for 24% of head and neck cancers. Postoperative local recurrence is a main reason affecting 5-year survival rate of OSCC in early stage,^{1,2} therefore, discovery of effective biomarkers and their effects on therapeutic responses are awaited to improve the early-stage OSCC patient prognosis.

PDCD4 is a tumor suppressor gene that located at human chromosome 10q24. Compared with normal tissues, PDCD4 has a lower expression in many cancers, such as colorectal cancer, esophageal squamous cell carcinoma and medullary thyroid carcinoma.³⁻⁵ The deficiency of PDCD4 in colorectal cancer cells ultimately promoted cell survival, proliferation and metastasis.³ On the other hand,

overexpression of PDCD4 in human prostate cancer cells induced a significant reduction in cell growth.⁶ The present research of PDCD4 are mainly conducted on cancer cells, but tumor is a heterogeneous cell population, it is necessary to study the expression pattern of PDCD4, including location and cell types.

The DEAD-box helicase eIF4A1 is needed to unwind structured RNA elements within the 5' untranslated region (5'UTR) to enable ribosome binding and scanning. A number of known oncogenes such as CBC25B, SMAD2, c-myc, c-myb and TGF β 1 were confirmed as requiring eIF4A1 for their efficient translation.⁷ PDCD4 binds with eIF4A1 to inhibit its enzymatic activity, thereby leaving the mRNA methylated decapping process unfinished and inhibiting the proliferation of tumor cells. PDCD4/eIF4A1 signal influences breast cancer cell proliferation and cell cycle, decreased eIF4A1 activity slowed down cellular proliferation significantly.⁸ Degraded PDCD4 greatly enhanced eIF4A activity, then eIF4A-mediated enhancement of oncogene translation may be a critical component for lymphoma progression.⁹ However, the clinical significance of PDCD4/eIF4A1 signal axis is still unclear in OSCC, which limits its efficacy of targeting therapy.

In the present study, we focused on the expression pattern of PDCD4/eIF4A1 signal in OSCC, we analysed the temporal distribution of PDCD4/eIF4A1 signal in early-stage OSCC by IHC according to distinct cell components in tumor micro-environment, including tumor cells and tumor-infiltrating lymphocytes (TILs). Further, we determined correlations between the expression of PDCD4/eIF4A1 signal and clinic pathological parameters and postoperative local recurrence in this study. Our findings reveal this signal may promote OSCC progression with diagnostic and prognostic value, of which early-stage OSCC patients may have a worse prognosis.

Materials And Methods

Patients And Samples

The experimental study group randomly included 69 patients diagnosed from 2007 to 2014 with early-stage OSCC (T1N0M0-T2N0M0). The 5-year survival rate was 69.6% in the 69 samples. All the 69 cases of OSCC included 8 cases of gingival cancer, 8 cases of buccal cancer, 9 cases of palate cancer and 44 cases of tongue cancer. The patients with primary tumors were diagnosed by haematoxylin and eosin (H&E) staining by experienced pathologists, and this study was approved by the Research Ethics Committee of Nanjing

Stomatology Hospital, Nanjing University. Written informed consent was obtained from all the patients. All these retrospective specimens were handled and anonymized according to ethical and legal standards. There were 21 patients died from OSCC (n=69) in our study until January 2019. None of the patients had received chemotherapy or radiotherapy prior to surgery and all 69 patients were followed-up until January 2019.

Immunohistochemistry

IHC was employed on 3 μ m formalin-fixed paraffin-embedded sections using anti-PDCD4 (1:200; ab80590; Abcam, Cambridge, MA, USA), anti-Ki-67 (1:100; ab16667; Abcam) and anti-eIF4A1 (1:200; ab31217; Abcam). All sections were subsequently incubated with secondary antibody (Vector Laboratories, Burlingame, CA, USA) and developed in diaminobenzidine (DAB). All sections were then washed in PBS. Appropriate positive and negative controls were included for each relevant stain.

Quantification Of Immunohistochemistry

To evaluate the immune expression of PDCD4, eIF4A1 and Ki-67 in tumor cells, tumor-infiltrating lymphocytes (TILs) and stroma fibroblast-like cells (FLCs), slides were visualized by two senior pathologists who evaluated each expression quantitatively. The patterns of PDCD4 and eIF4A1 expression locations in OSCC specimens were defined and divided into two regions: tumor center and invasive front. Ki-67 staining was only scored in the tumor cells. We distinguished stroma FLCs from tumor cells according to the typical fibroblast shape, long spindle-shaped cells with small cytoplasmic protrusions located in the tumor stroma.

IHC staining was scored according to the percentage of positive cells and staining intensity.¹⁰ Staining intensity was graded as negative (=0), weak (=1), moderate (=2) or strong (=3), and reactivity was assessed by the percentage of positively staining cells (0–5% [=0], 5–25% [=1], 26–50% [=2], 51–75% [=3] or 76–100% [=4]). The IHC score was calculated by multiplying the grade of the staining intensity by that of the staining percentage, giving a minimum score of 0 and a maximum of 12. High and low expressions of PDCD4 and eIF4A1 were defined by the median of IHC scores.

Statistical Analysis

The SPSS version 16.0 (SPSS Inc., Chicago, IL, USA) and Prism statistical software packages (version 8.0, GraphPad Software Inc., San Diego, CA, USA) were used for

statistical analyses. Subgroups of each immunostaining parameter were divided by their median values. Pearson's chi-square test and Fisher's exact test were used to compare clinicopathological parameters of the patients. Kolmogorov–Smirnov and Shapiro–Wilk tests showed that the expression scores of PDCD4, eIF4A1 and Ki-67 did not follow a normal distribution. The Mann–Whitney *U*-test was used to compare the two groups (e.g. PDCD4 in TCs with recurrence, with recurrence versus without recurrence). OS and relapse-free survival (RFS) comparing high expression groups and low expression groups were estimated using Kaplan–Meier curves. Survival time was defined as the interval between the date of surgery and the last date when the patient was known to be relapse-free or alive (censored). The Cox regression model was used to examine interactions between different prognostic factors in a multivariate analysis. Differences were considered statistically significant with $P < 0.05$.

Results

PDCD4 Is Expressed In Both TCs And TILs, But eIF4A1 Is Only Expressed In TCs

We assessed the PDCD4/eIF4A1 signal in different cell types including TCs, TILs and FLCs from tumor center to invasive front. A wide distribution of PDCD4 can be observed in TCs as well as in TILs, but it was absent in FLCs (Figure 1A and D). The expression of eIF4A1 was observed in TCs but absent in TILs and FLCs (Figure 1B and E). Considering the compartment heterogeneity of the tumor, we also assessed the expression of PDCD4/eIF4A1 signal in tumor center and invasive front, respectively. Interestingly, the expression of PDCD4 in IF is significantly lower than tumor center. Since the interaction between PDCD4 and eIF4A1 has been proved, we analysed the correlation of them and found that patients with high expression of PDCD4 attend to have lower

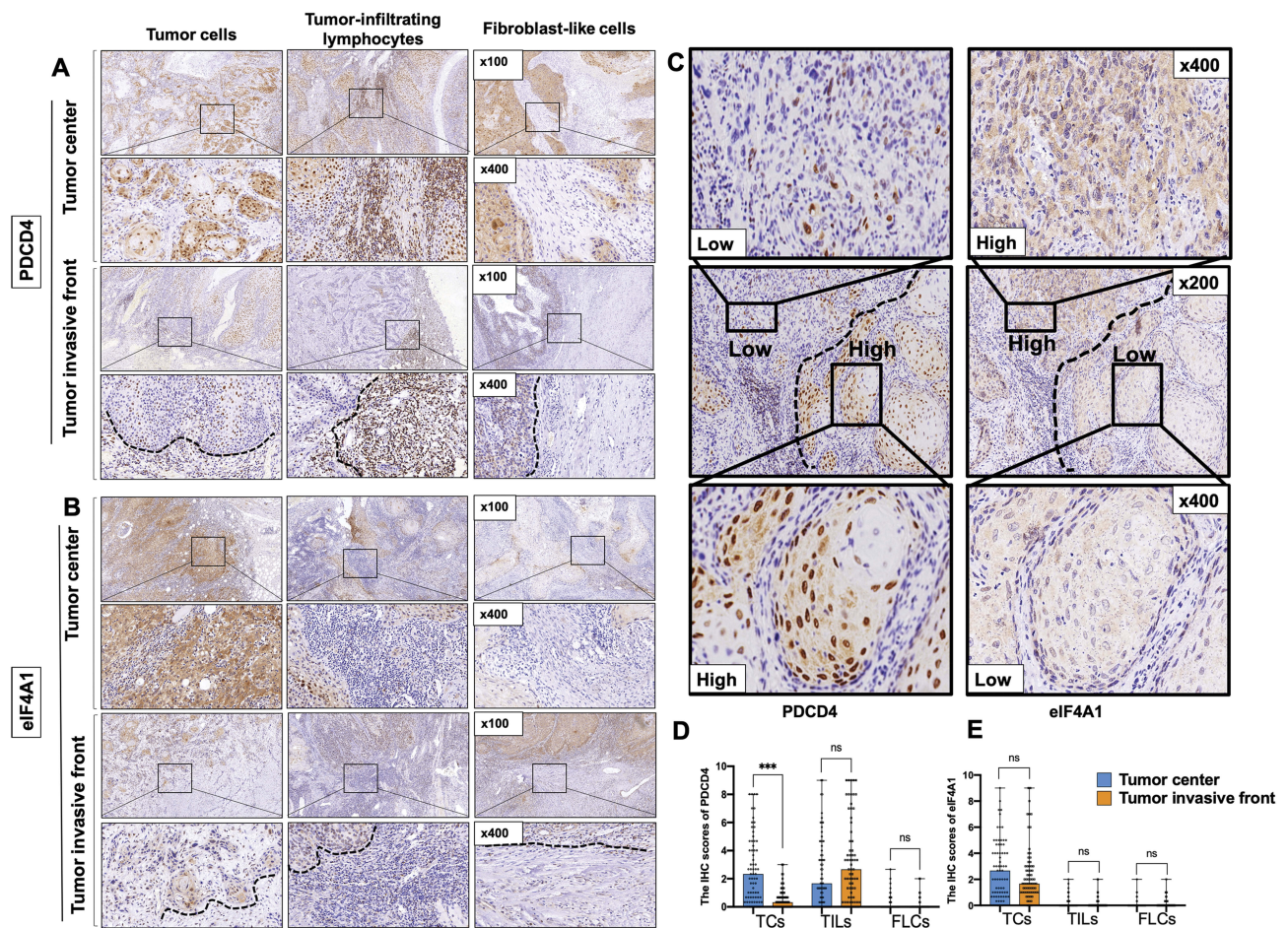


Figure 1 Representative images showing immunohistochemical staining of PDCD4 (A and D) and eIF4A1 (B and E) in TCs, TILs and FLCs in tumor center or invasive front of OSCC tissues. The staining of PDCD4 and eIF4A1 showed an opposite trend in different differentiated regions of the same part of the sample (C). *** and ns represent that differences are considered statistically significant with $P < 0.05$ and no significance, respectively.

expression of eIF4A1 in TCs (Tables 1 and 2). The staining results of 69 consecutive sections showed that the intensity of PDCD4 and eIF4A1 was conversed in the same region of the same specimen (Figure 1C).

Associations Of PDCD4/Eif4a1 Signal With Clinicopathological Characteristics

Considering that PDCD4/eIF4A1 signal was correlated with OSCC progression, we further analysed correlations between the distinct signal expression patterns and the clinicopathological characteristics of early-stage OSCC patients (Table 3 and S1). Here, we focus on PDCD4 in TCs and TILs and eIF4A1 in TCs. In tumor center, both PDCD4 and eIF4A1 expression had no association with age, smoking habits and T-stage, but we found that low expressed PDCD4 in TCs had poorer differentiation (Figure 2A) and a worse WPOI (Figure 2C). On the contrary, low expressed PDCD4 in TILs and eIF4A1 in TCs were found to have a better differentiation (Figure 2B and E). High expressed eIF4A1 in TCs was found to have a worse WPOI (Figure 2D). In invasive front, only the low expressed PDCD4 in TCs was detected to associate with a poorer differentiation (Figure 2F). Based on the conversed correlation between PDCD4 expression pattern in TCs and TILs with differentiation, the expression of

PDCD4 seemed to exert distinct functions between TCs and TILs. According to these results, tumor cell-derived PDCD4 may negatively regulate the function of eIF4A1 to promote OSCC malignant phenotype.

Low PDCD4 And High eIF4A1 Were Accompanied With Higher Ki-67 Staining

Since PDCD4 was found to inhibit cell proliferation in intra-ductal papillary mucinous neoplasm,¹¹ we proposed the hypothesis that cell proliferation is attributed to the aberrant PDCD4/eIF4A1 signal expression. Herein, we assessed the expression of proliferation index Ki-67 in OSCC according to the PDCD4/eIF4A1 level. Results indicated that patients with low PDCD4 expression or high eIF4A1 expression were accompanied with higher Ki-67 staining (Figure 3A and B), suggesting PDCD4 and eIF4A1 may regulate OSCC cell proliferation.

Recurrent Patients Have A Low PDCD4^{TCs} But A High eIF4A1^{TCs} And PDCD4^{TILs} Expression

In this study, all the 69 patients underwent complete surgical excision with negative margin, but there are still 14 patients occurred postoperative recurrence. Patients with recurrence have a lower expression of PDCD4 and a higher eIF4A1 expression in TCs (Figure 3C). PDCD4 overexpressed in TILs correlated with high recurrence rate, which also indicated the conversed function of PDCD4 in TILs and TCs. We also evaluated the correlation between metastasis and the signal expression, but the results demonstrated no significance (Figure 3D). Thus, we could speculate that the recurrence potential of early-stage OSCC is susceptible to low expressed PDCD4^{TCs}, high expressed eIF4A1^{TCs} and PDCD4^{TILs}.

Low Expressed PDCD4^{TCs} And High Expressed eIF4A1^{TCs} Correlated With Shorter Survival Time Both In OS And RFS

The potential relationship between PDCD4/eIF4A1 signal expression and OS and RFS were evaluated in the 69 patients included in this study. All the 69 OSCC patients were divided into PDCD4^{high/low} and eIF4A1^{high/low} in TCs or TILs groups. The results indicated that high expression of PDCD4 in TCs correlated with shorter overall survival time (P=0.0004) (Figure 4A), but high expression of eIF4A1 in TCs correlated with longer overall survival time than the

Table 1 Correlations Between PDCD4 And eIF4A1 Expression On Tumor Cell In Tumor Center

	eIF4A1 in TCs			χ^2	P
	Low	High			
PDCD4 inTCs					
Low	13	21	34	5.219	0.022*
High	23	12	35		
Total	36	33	69		

Note: *Represents that differences are considered statistically significant with P<0.05. Abbreviations: TCs, tumour cells; PDCD4, programmed cell death 4.

Table 2 Correlations Between PDCD4 And eIF4A1 Expression On Tumor Cell In Tumor Invasive Front

	eIF4A1 in TCs			χ^2	P
	Low	High			
PDCD4 inTCs					
Low	15	7	34	3.636	0.057
High	20	26	35		
Total	36	33	69		

Abbreviations: TCs, tumour cells; PDCD4, programmed cell death 4.

Table 3 Relationships Between PDCD4 With eIF4A1 Expression On Different Cell Types In Tumor Center And Clinicopathological Characteristics In OSCC Patients

Characteristics	n=(69)	PDCD4 Expression in TCs		χ^2	P	PDCD4 Expression in TILs		χ^2	P	eIF4A1 Expression in TCs		χ^2	P
		Low	High			Low	High			Low	High		
Sex													
Male	37	21	16	1.787	0.181	18	19	0.794	0.313	16	21	2.550	0.110
Female	32	13	19			19	13			20	12		
Age (years)													
<60	30	16	14	0.350	0.544	20	10	3.631	0.057	17	13	0.429	0.512
≥60	39	18	21			17	22			19	20		
Smoking													
No	49	24	25	0.006	0.939	26	23	0.021	0.884	28	21	1.673	0.196
Yes	20	10	10			11	9			8	12		
T-stage													
I	25	13	12	0.116	0.733	13	12	0.042	0.839	16	9	2.197	0.138
II	44	21	23			24	20			20	24		
Differentiation													
Well	29	5	24	20.538	<0.001*	20	9	4.735	0.030*	24	5	18.753	<0.001*
Moderate to poor	40	29	11			17	23			12	28		
WPOI													
I-3	32	11	21	5.301	0.021*	20	12	1.891	0.169	22	10	6.571	0.010*
4-5	37	23	14			17	20			14	23		
Total	69	34	35			37	32			36	33		

Note: *Represents that differences are considered statistically significant with $P < 0.05$.

Abbreviations: PDCD4, programmed cell death 4; OSCC, oral squamous cell carcinoma; TC, tumour cell; TILs, tumor-infiltrating lymphocytes.

low expression group ($P=0.0119$) (Figure 4B). The contrasting correlation between PDCD4 and eIF4A1 expression and clinical outcome suggested that their impact on prognosis is completely opposite. Thus, our further analysis found that the patients with PDCD4^{low} eIF4A1^{high} expression in TCs had a poorer prognosis than those who with low expression of PDCD4 or high expression of eIF4A1 in TCs (Figure 4C). The same result also presented in the analysis of RFS in tumor center (Figure 4G–L). However, in invasive front, we found no significant difference between OS or RFS and PDCD4/eIF4A1 signal expression in this study (Figure 4D–F).

Low Expressed PDCD4^{TCs} And High Expressed PDCD4^{TILs} In Tumor Center Are Significant Independent Risk Factors For Early-Stage OSCC

The prognostic significance of clinicopathologic features were analyzed by univariate and multivariate analysis

(Table 4 and S2). Results revealed that age, smoking, T-stage, differentiation, WPOI and eIF4A1 expression in TCs were not significant prognostic indicators for OS or RFS ($P > 0.05$). However, low expression of PDCD4 in TCs or high expression in TILs were significant independent risk factors for OSCC with conversed function. However, eIF4A1 is not an independent risk factor for early-stage OSCC.

Discussion

Although one study has investigated the role of PDCD4 in OSCC,¹² little is known about the co-expression pattern and prognostic value of PDCD4 and eIF4A1 in the same tumor. Ovarian cancer patients with lower PDCD4 expression were found to have shorter disease-free survival.¹³ Patricia et al found that loss of PDCD4 expression in OSCC is correlated with poorer survival and poorer disease-free survival of OSCC patients.¹⁰ eIF4A1 was also found to predict a poor prognosis in breast cancer.⁸ In our

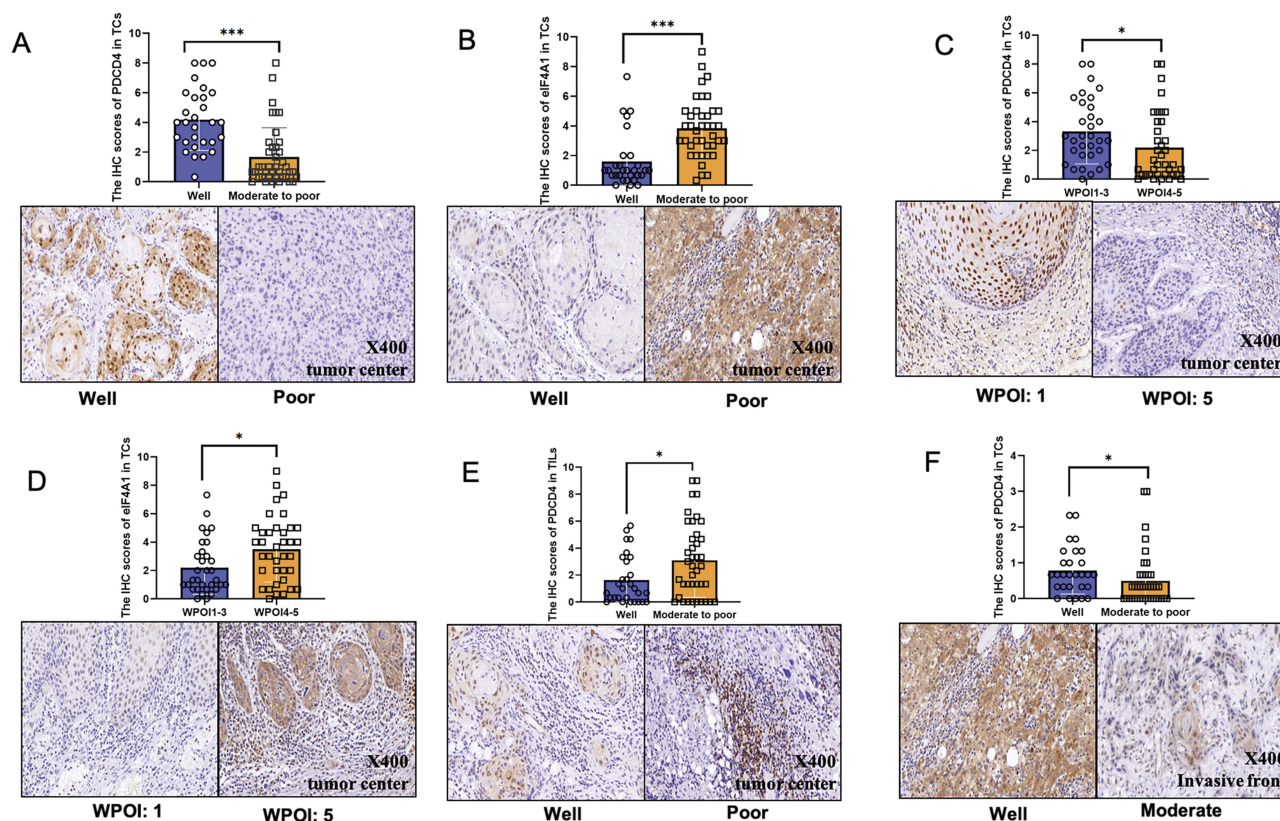


Figure 2 PDCD4 expression in tumor center with (A) different grades of tumor differentiation and (C) WPOI in TCs and (E) different grades of tumor differentiation in TILs. eIF4A1 expression in tumor center with (B) different grades of tumor differentiation and (D) WPOI. PDCD4 expression in invasive front with (E) different grades of tumor differentiation. * and *** represent that differences are considered statistically significant with $P < 0.05$ and $P < 0.001$, respectively.

survival analysis, patients with low expression of PDCD4 and high expression of eIF4A1 in tumor cells had a greater risk of OSCC recurrence. These findings suggest that the PDCD4/eIF4A1 signal could be a potential indicator with an increased risk of early-stage OSCC recurrence.

PDCD4 is downregulated or lost in certain aggressive human carcinomas including the breast cancer, lung and colon cancer,^{14–16} and it is speculated to shuttle between nucleus and cytoplasm of tumor cells.¹⁷ eIF4A1 has not been widely investigated in tumors, while its upregulation in cytoplasm of ER-negative breast cancer cells was associated with a poor prognosis.⁸ We found that the expression pattern of PDCD4/eIF4A1 signal in TCs of OSCC is consistent with the results of previous studies on other cancer cells. However, due to the limited expression of eIF4A1 in TILs, the effect by PDCD4 on tumor progression may through other factors in TILs. Studies on PDCD4 mainly focused on tumor center, but was deficient on invasive front. Interestingly, we detected a significant decrease expression of PDCD4 in the invasive front. Thus, PDCD4 expression exhibited a highly heterogeneous

pattern within the tumors depending on the different area of tumor, which could certainly have a contribution on the functional heterogeneity of these tumor regions.

Previous studies revealed that PDCD4 inhibits eIF4A1 to suppress translation of mRNAs with highly structured 5' UTR.¹⁷ However, the widespread clinical significance of this signal is impeded by the current lack of studies on the PDCD4/eIF4A1 in tumors. Limited evidence showed that the expression of PDCD4 and eIF4A1 was negatively related in breast cancer,⁸ similarly, we found a conversed expression pattern of these two proteins in OSCC, which was consistent with the known inhibitory effect on eIF4A1 by PDCD4.

This study demonstrated that the PDCD4/eIF4A1 signal expression is associated with the differentiation and WPOI of tumors. It is well known that epithelial differentiation is a determining factor in the prognoses of cancers, poorly differentiated cancers are highly proliferative compared with highly differentiated counterparts. In addition, differentiation is important in maintaining the tumorigenic and treatment-resistant of cancer stem cell in head and neck cancers.¹⁸ Valproic acid and all-trans retinoic

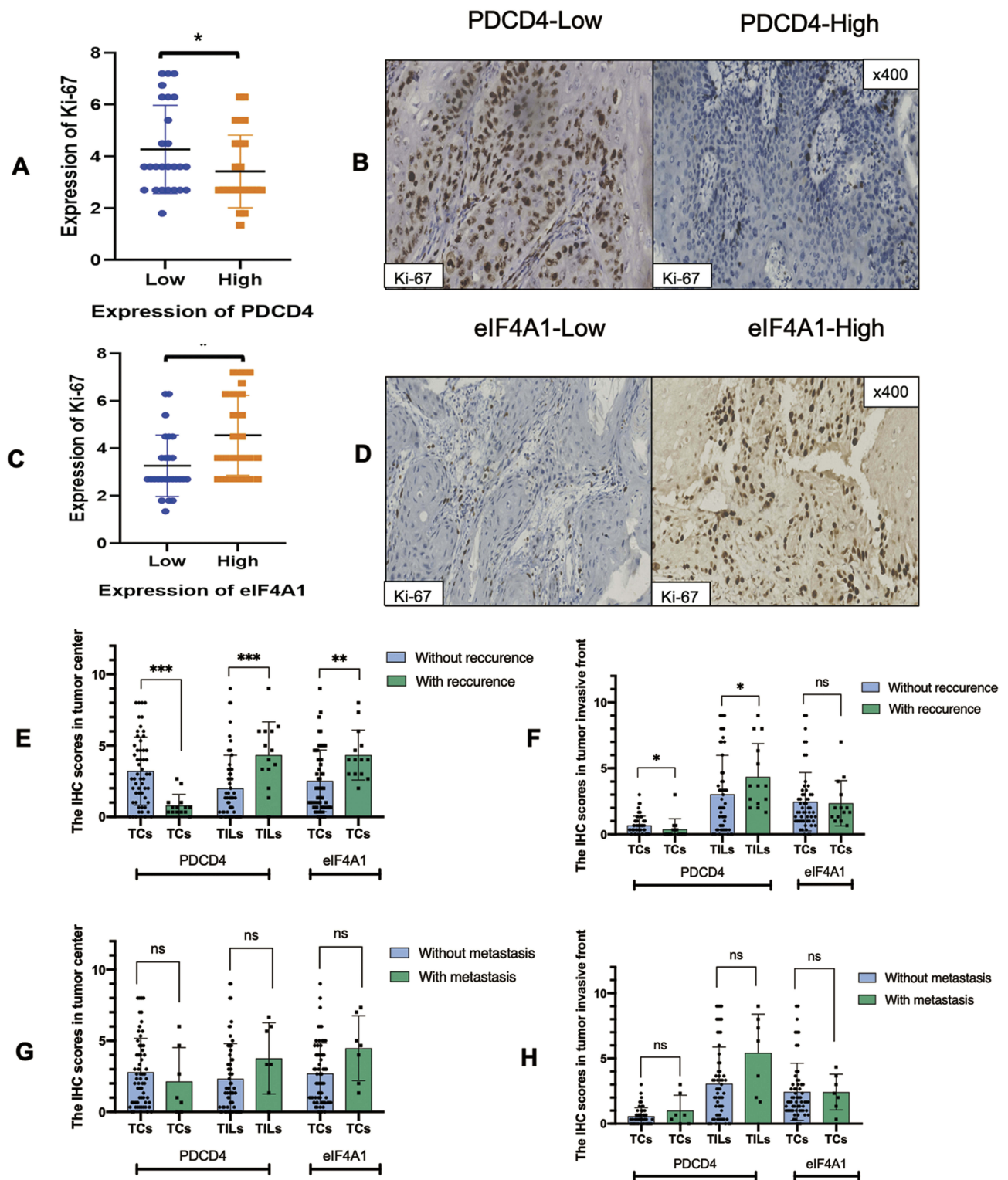


Figure 3 Correlation between Ki-67 staining in PDCD4 high/low groups (**A** and **B**) and elf4A1 high/low groups (**C** and **D**) in tumor cells. Correlation between PDCD4/elf4A1 expression in tumor center and invasive front with recurrence status (**E** and **F**). Correlation between PDCD4/elf4A1 expression in tumor center and invasive front with metastasis status (**G** and **H**). * **, and *** represent that differences are considered statistically significant with $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

acid were demonstrated to inhibit the growth of head and neck cancers by inducing terminal differentiation,¹⁹ another study has shown that the expression of PDCD4

was induced in acute myelocytic leukemia (AML) cell lines while in the presence of all-trans retinoic acid.²⁰ Hence, PDCD4 restoration may be a novel approach for

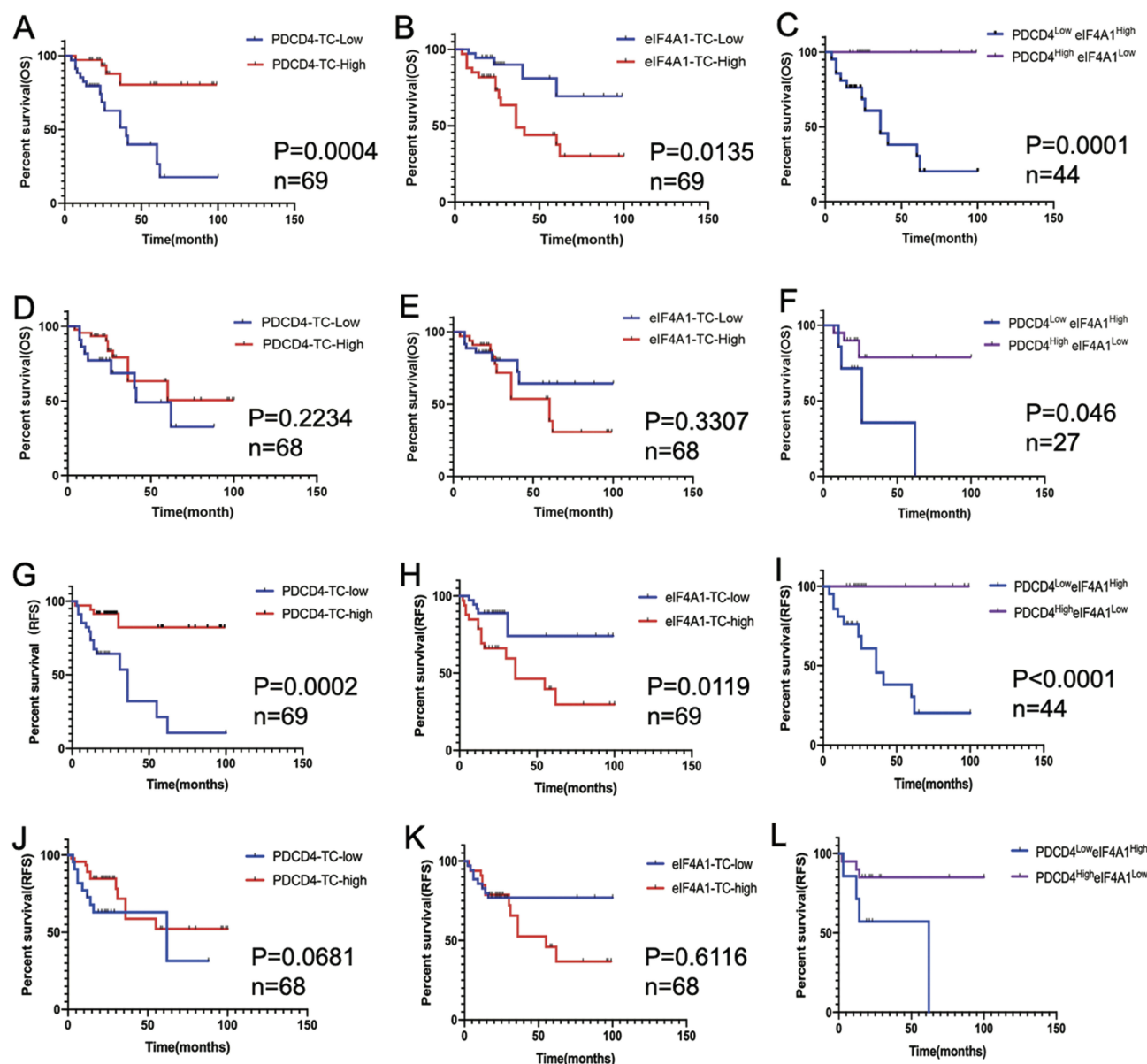


Figure 4 Kaplan-Meier survival curves for overall survival time of OSCC patients according to the expression of PDCD4 and eIF4A1 in TCs in tumor center (A–C) and invasive front (D–F). Relapse-free survival time of OSCC patients according to the expression of PDCD4 and eIF4A1 in TCs in tumor center (G–I) and invasive front (J–L).

differentiation cancer therapy in OSCC, leading to effective suppression of tumor development and improving the prognosis.

We observed an opposite effect on prognosis exerted by the expression of PDCD4 between TCs and TILs, it demonstrated the functional heterogeneity of PDCD4 in different cell types. Lingel et al found that deficiency of PDCD4 in CD8⁺T cell has been proved to attenuate effector functions, leading to abrogated anti-tumor responses.²¹ Shankaran et al also observed that increased IFN- γ levels in PDCD4-deficient CTLs could be responsible for an enhanced anti-tumor activity.²² Although we have not

classified lymphocyte subsets, the significance of PDCD4 expression in OSCC TILs is still consistent with anti-tumor role of low expressed PDCD4. So it is tempting to speculate that PDCD4 as a promising target in tumor immune microenvironment for therapeutic interventions.

In conclusion, the PDCD4/eIF4A1 signal expression level in early-stage OSCC was related to parameters reflecting primary tumor burden, including differentiation and WPOI at initial diagnosis. High expressed eIF4A1 or low expressed PDCD4 in TCs were correlated with a greater risk of recurrence, and also predict a poor outcome in early-stage OSCC. However, compared with TCs, the

Table 4 Prognostic Factors In The Cox Proportional Hazards Model For OS

Variables	OS	Univariate 95% CI	Sig.	HR	Multivariate 95% CI	Sig.
	HR					
Gender Male versus female	0.213	0.071–0.641	0.006*	1.033	0.295–3.618	0.959
Age <60 versus ≥60	0.307	0.251–1.565	0.317			
T-stage I versus 2	1.376	0.553–3.419	0.492			
Smoke No versus yes	0.577	0.28–1.396	0.222			
Differentiation Well versus moderate to poor	7.71	1.793–33.159	0.006*	1.316	0.217–7.986	0.765
WPOI I–III versus IV–V	4.271	1.416–12.882	0.010*	0.732	0.225–2.387	0.605
PDCD4 in TC in tumor center High versus low	0.178	0.060–0.531	0.002*	0.239	0.079–0.721	0.011*
PDCD4 in TIL in tumor center High versus low	6.594	1.935–22.469	0.003*	4.896	1.422–16.852	0.012*
PDCD4 in TC in invasive front High versus low	0.592	0.249–1.407	0.235			
PDCD4 in TIL in invasive front High versus low	3.14	1.039–9.499	0.043*	0.199	0.039–1.008	0.051
eIF4A1 in TC in tumor center High versus low	3.260	1.192–8.916	0.021*	1.735	0.593–5.076	0.314
eIF4A1 in TC in invasive front High versus low	1.535	0.635–3.711	0.341			

Note: *Represents that differences are considered statistically significant with $P < 0.05$.

Abbreviations: TC, tumour cells; TILs, tumor-infiltrating lymphocytes; PDCD4, programmed cell death 4; CI, confidence interval; OS, overall survival; HR, hazard ratio.

prognostic value of PDCD4 expression in TILs presents a converse phenomenon, suggesting that we should take the distinct expression pattern of PDCD4 into consideration when the PDCD4/eIF4A1 targeting therapeutics is administered to OSCC.

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Author Contributions

All authors contributed to data analysis, drafting or revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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