Original Article

Expression of the desmosome-related molecule periplakin is associated with advanced stage and poor prognosis of esophageal squamous cell carcinoma

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Background: Our previous report showed that periplakin (PPL), a member of the plakin family of proteins, was expressed in all the normal esophageal squamous cells, except the Ki67$^+$ basal cell layer; however, PPL-negative cells were observed in esophageal squamous cell carcinoma (ESCC) tissue samples and the proportion of PPL-positive areas in tumors showed considerable variation. In this study, we analyzed the relationships between PPL expression in tumors and the clinicopathological features of ESCC.

Methods: PPL expression was evaluated by immunostaining ESCC samples from 70 patients who underwent surgery and we classified the samples into PPL-negative and PPL-positive groups based on the proportion of PPL-positive areas in tumors, and the relationships among PPL expression, clinical features, and the ESCC prognosis were analyzed. ESCC cell line KYSE270 cells were stably transfected with the PPL expression vector and their growth was assessed by colony formation assay and subcutaneous xenografts in athymic mice.

Results: As found in our previous study, decreased PPL staining intensity was observed in all of the cancer tissues analyzed, compared with that observed in paired normal esophageal mucosae; however, the PPL-positive group (the percentage of PPL-positive tumor cells was >20% on immunostaining) exhibited positive associations with the pT classification (larger primary tumor) (P=0.029), pN classification (lymph node metastasis) (P=0.0462), and advanced stages of cancer (P=0.0253). High PPL expression was observed in all of 26 lymph node metastases analyzed. Furthermore, patients with PPL-positive tumors showed poor postoperative prognosis compared to those with PPL-negative ones. Forced expression of PPL in the KYSE270 ESCC cell line induced a stratified structure and colony formation. In addition, PPL-transfected cells formed larger tumors in nude mice than mock-transfected cells, suggesting that relatively high PPL expression in tumors may facilitate cell-cell adhesion by desmosome formation and eventual cell growth *in vivo*.

Conclusions: PPL expression was generally reduced in ESCC compared with paired non-cancer tissue; however, relatively high levels of PPL expression in tumors correlated with tumor progression, lymph node metastasis, advanced stage cancer, and a poor prognosis.

Keywords: Desmosome; esophageal squamous cell carcinoma (ESCC); periplakin (PPL); prognosis

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Introduction

Esophageal squamous cell carcinoma (ESCC) is an aggressive form of cancer and is one of the most frequent cancers worldwide, particularly in Asian countries (1). Environmental factors, such as smoking and alcohol consumption, are risk factors for ESCC in Western countries (2), whereas the consumption of hot beverages is a major risk factor in Eastern countries (3). Environmental risk factors affect the condition of the epithelial mucosa, inducing mutations and epigenetic changes, which accumulate and induce mucosal dysplasia, and eventually developing into invasive squamous cell carcinoma.

Stratified squamous epithelial cells have many spines that intermingle through dense desmosomes. Periplakin (PPL), a 195-kD membrane-associated protein, is a member of the plakin protein family, which comprises desmoplakin, bullous pemphigoid antigen, and envoplakin (EVPL) (4). Plakin family proteins connect cytoskeletal elements to form desmosomes, intercellular-junction complexes (4,5). PPL expression is observed in keratinized and non-keratinized epithelial cells of the epidermis, urinary bladder, and the oral, esophageal, and cervical mucosae (4). In epidermal epithelial cells, PPL forms stable heterodimers with EVPL. PPL-EVPL heterodimers are localized in desmosomes, the interdesmosomal plasma membrane, intermediate filaments, and the plasma membrane (6).

Although PPL is ubiquitously present in normal squamous cells, PPL-knockout mice do not exhibit obvious abnormalities (7). Furthermore, a past study revealed significant downregulation of PPL in human esophageal cancer tissue and scarce expression in advanced-stage cancers by proteome analyses of cancer tissues compared to adjacent non-cancer tissues (8,9). The decreased expression of PPL was also found in advanced-stage urinary bladder cancer (10). In addition, PPL knockdown is associated with reduced cellular movement and adhesion in pharyngeal cancer cells (11). In a previous study, we determined that PPL is essential for desmosome formation and for the stratified growth of esophageal squamous cells. Although PPL was expressed in all normal esophageal squamous cells, except the $Ki67^{+}$ basal cell layer, we noticed the presence of PPL-negative cells in ESCC tissues and the proportion of PPL-positive areas in tumors showed considerable variation. We also observed that ESCC cell lines with forced PPL-expression often accumulate into multiple layers, whereas the mock-transfected cells form a monolayer sheet with no stratification (12). In this study, we analyzed the relationships among PPL expression and the clinicopathological features of ESCC tumors. We collected detailed clinical data from 70 patients with ESCC and retrospectively analyzed PPL expression, related clinical features, and ESCC prognosis. Our present study demonstrated that high levels of PPL expression in ESCC are associated with tumor progression, lymph node metastasis, advanced stage cancer, and a poor prognosis. Furthermore, we also observed that increased PPL expression facilitated tumor cell growth *in vivo*, probably due to PPL-promoted cell-cell adhesion. This mechanism may explain why the PPL-positive group was associated with tumor progression, lymph node metastasis, advanced stage cancer, and a poor prognosis.

Methods

Patients

We enrolled 70 patients with histologically-confirmed ESCC diagnoses. Of these, 46 patients that underwent esophagectomy or endoscopic submucosal dissection from January 2013 to August 2015 at the National Center for Global Health and Medicine (NCGM) provided informed consent before sample collection. This study was approved by the NCGM research ethics committee [121 and 1484]. We also analyzed formalin-fixed, paraffin-embedded sections of surgical specimens, from the remaining 24 patients who were treated between April 2008 and December 2012, by immunohistochemical staining. From these patients, consent was obtained retrospectively in accordance with the dictates of the NCGM research ethics committee [1622].

We retrieved clinicopathological parameters including tumor stage (according to the *TNM Classification of Malignant Tumors*, *7th edition* published by the Union for International Cancer Control) from hospital records. Patients were followed for 1–5 years (until death or until September 7, 2016).

Immunohistochemical analysis

As previously described (12), formalin-fixed paraffin-embedded sections of surgical specimens from ESCC patients were stained with an anti-human PPL antibody (Sigma-Aldrich, Inc., St. Louis, MO, USA), and hematoxylin was used for counterstaining. Antigen retrieval was performed by autoclaving the sections in 10 mM

sodium citrate buffer. The ImmPACT™ DAB peroxidase substrate kit (Vector Laboratories, Burlingame, CA, USA) was used for diaminobenzidine staining. For double staining of PPL and Ki67, the MACH2 Double Stain 2 Kit (Biocare Medical, Concord, CA, USA) and the Vulcan Fast Red Chromogen Kit 2 (Biocare Medical) were used to detect signals of anti-Ki67 antibodies (MIB-1, Dako, Glostrup, Denmark). All slides were reviewed by two observers who were blind to clinical and pathological data. Based on the proportion of PPL-positive areas in the malignant tissue, we classified ESCC cases into PPL-negative (the percentage of PPL-positive tumor cells was ≤20% on immunostaining) and PPL-positive groups (the percentage of PPL-positive tumor cells was >20%). Intraobserver reliability of the PPL staining results showed no statistically significant differences. The two observers exhibited high interobserver reliability.

Cell lines and culture

Human ESCC cell line KYSE270 was purchased from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). For passage or to detach cells, we used TrypLE Express (Invitrogen). For the colony formation assays, stably transfected KYSE270/Vector cells and KYSE270/PPL cells (12) (200 cells) were seeded in each well in 24-well plates. After incubation for 3 or 8 days, the colonies were stained with crystal violet. At day 3, four images were captured from each assay and the number of colonies was counted. At day 8, we performed quantification using NIH ImageJ software measuring the area covered with cells as a percentage of the whole captured square area.

Cell growth as subcutaneous xenografts in athymic mice

All experiments were performed according to the Institutional Guidelines for the Care and Use of Laboratory Animals in Research with prior approval from the Animal Experimentation Committee of NCGM [16080]. The male BALB/c nude (nu/nu) mice were purchased from CLEA Japan Inc. (Tokyo, Japan) and maintained under pathogen-free conditions in the NCGM animal facility. Transfection of PPL was prepared as previously described (12). Briefly, human HaloTag® expression vector (pFN21A HaloTag® CMV Flexi® Vector, used for mock-transfection) and human PPL HaloTag® ORF clone in pFN21A (Promega, Madison, WI, USA) were transfected into KYSE270 cells using a lipofectamine LTX reagent (Life Technologies, Inc., Rockville, MD, USA). Stably transfected KYSE270/Vector cells and KYSE270/PPL cells (5.0×10^6 or 5.0×10^5) were subcutaneously inoculated in the right and left flanks of 6-week-old BALB/c nude mice, respectively. Tumor size was measured with calipers. Tumor volume was determined by long (a) and short (b) diameters with height (c), calculated as volume = a × b × c ×3.14/6.

Statistical analysis

Each tumor was classified based on its location, size, pathology, the condition of the lymph nodes, and the degree of metastasis (pTNM, 7th edition, 2009). Univariate analyses were performed using one-way ANOVA to compare the expression of PPL for age, the Fisher exact test for gender, tumor location, pT status, pN status, pM status, and disease stage. Survival curves were calculated by the Kaplan-Meier product-limit estimate method and examined using the Log-rank (Mantel-Cox) procedure. Data were expressed as mean ± SD, and results were compared by unpaired Student's *t*-tests. Statistical analyses were performed with the Prism 5 statistical program (GraphPad Software, Inc., La Jolla, CA, USA). All tests were two-tailed, and P values <0.05 were considered significant.

Results

Associations between PPL immunoreactivity and ESCC clinicopathological features

To analyze the relationships among PPL expression and the clinicopathological features of ESCC tumors, the PPL expression levels in tumors was evaluated by immunostaining using formalin-fixed, paraffin-embedded sections from 70 ESCC patients whose demographics are summarized in *Table 1*. Consistent with other studies (8) and our previous study (12), intense PPL staining was observed at the cell membranes of all of the normal squamous cell epithelium (100% positive) in all cases examined (*Figure 1A*). On the other hand, half of 70 ESCC tumors were almost totally PPL-negative (*Table 1*), while others contained more than 40% PPL-expressing cells (*Figure 1A*). There were clear differences between them. In cases with PPL-positive tumors, localization in tumor cells was not limited to the cell membrane, but was also found in the cytoplasm. Further, PPL-positive cancer cells often expressed Ki67, indicating cell proliferation (*Figure 1B*). This was in sharp contrast to normal mucosa, where

Table 1 Clinicopathological features of PPL-positive or PPL-negative ESCC

Characteristics	Number of patients (%)			P value
	Total	PPL-negative	PPL-positive	
Number of patients	70	35 (50.0)	35 (50.0)	–
Mean age ± SD (years)	–	68.83±8.81	69.49±9.71	0.7677
Sex				
Male	58 (82.9)	27 (77.1)	31 (88.6)	0.2046
Female	12 (17.1)	8 (22.9)	4 (11.4)	
pT classification				
T1	28 (40.0)	20 (57.2)	8 (22.8)	0.029*
T2	9 (12.9)	4 (11.4)	5 (14.3)	
T3	28 (40.0)	9 (25.7)	19 (54.3)	
T4	5 (7.1)	2 (5.7)	3 (8.6)	
pN classification				
N0	36 (51.4)	21 (60.0)	15 (42.8)	0.0462*
N1	18 (25.7)	8 (22.8)	10 (28.6)	
N2	13 (18.6)	3 (8.6)	10 (28.6)	
N3	3 (4.3)	3 (8.6)	0 (0.0)	
pM classification				
M0	65 (92.9)	33 (94.3)	32 (91.4)	0.6426
M1	5 (7.1)	2 (5.7)	3 (8.6)	
Cancer stage[§]				
I	28 (40.0)	17 (48.6)	11 (31.4)	0.0253*
II	14 (20.0)	10 (28.6)	4 (11.4)	
III	23 (32.9)	6 (17.1)	17 (48.6)	
IV	5 (7.1)	2 (5.7)	3 (8.6)	
Tumor location				
Upper thoracic	16 (22.9)	5 (14.3)	11 (31.4)	0.1707
Middle thoracic	28 (40.0)	17 (48.6)	11 (31.4)	
Lower thoracic	26 (37.1)	13 (37.1)	13 (37.1)	

*, P<0.05; [§], based on the Union for International Cancer Control, 7th edition.

proliferating cells labeled with Ki67 were limited to the basal layer and did not express PPL (*Figure 1B*). Based on the proportion of PPL-positive areas in the malignant tissue, we classified the ESCC cases as either PPL-negative (the percentage of PPL-positive tumor cells was ≤20% on immunostaining) or PPL-positive (the percentage of PPL-positive tumor cells was >20%). Univariate analysis revealed no differences between the PPL-negative and PPL-positive groups with respect to gender, age, tumor location or distant metastasis. However, we found that relatively high levels of PPL expression in tumors (PPL-positive group) were associated with pT classification (larger primary tumor), pN classification (lymph node metastasis) and advanced stages of cancer (*Table 1*). PPL was expressed in all of the 26 lymph node metastases analyzed (*Figure 1C*), indicating that high PPL expression was strongly associated with lymph node metastasis of ESCC. We combined members of the small groups *(Table 1)* as a large group for comparison to avoid false results. Once again, the PPL-positive group showed correlations with pT classification (T1–2 *vs.* T3–4) and advanced stages of cancers (I and II *vs.* III and IV). PPL-positive tumors tended to be associated with lymph node metastases (N0 *vs.* N+) but this association was not statistically significant (P=0.1513) (*Table 2*).

Association between PPL immunoreactivity and a poor prognosis

Patients with tumors that had higher PPL expression had significantly poorer postoperative prognoses than patients with tumors that had lower PPL expression evaluated by immunostaining (*Figure 2*).

Expression of PPL promoted tumor growth

To understand the significance of PPL expression in ESCC, we performed colony formation assay using ESCC cell line KYSE270 cells with induced expression of PPL used in our previous study (12). At day 3, there were no differences in the number of colonies between *PPL* transfectants and mock transfectants (*Figure 3A*). However, PPL transfectants formed larger colonies than mock transfectants at day 8 (*Figure 3B*). We confirmed that PPL transfectants often

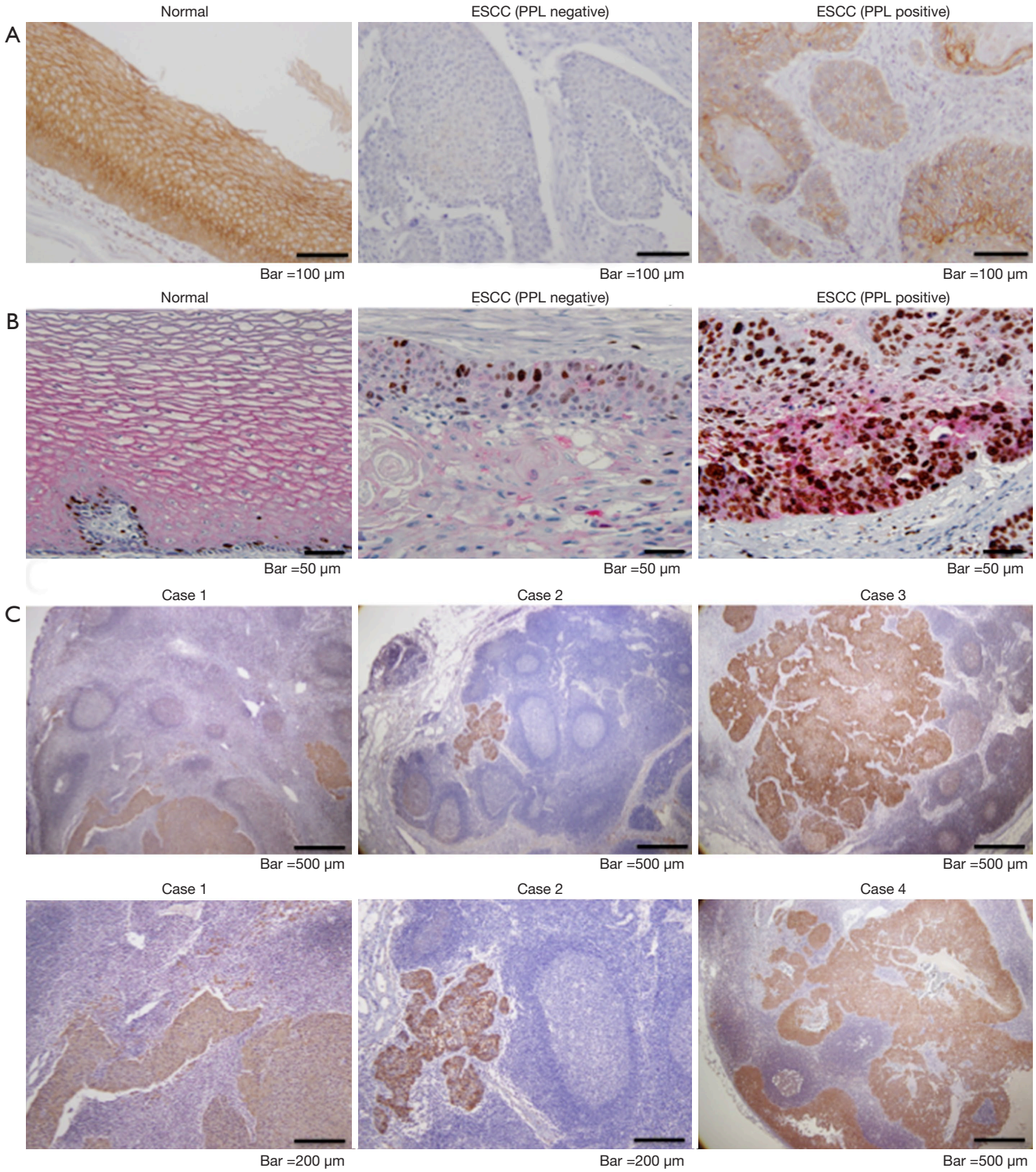

Figure 1 High PPL expression in proliferative ESCC and lymph node metastases. (A) Immunohistochemical analysis of PPL expression in noncancerous tissue and in ESCC specimens. Representative staining of PPL-negative (20% or less of the tumor cells are PPL-positive) or PPL-positive (more than 20% of the tumor cells are PPL-positive); (B) representative images produced from formalin-fixed, paraffin-embedded samples of ESCC and adjacent normal mucosa double stained with anti-PPL (pink) and anti-Ki67 (dark brown); (C) immunohistochemical analysis of PPL expression in lymph node metastases of ESCC. PPL, periplakin; ESCC, esophageal squamous cell carcinoma.

Table 2 Correlations between PPL immunoreactivity and pT, pN, and stage

Characteristics	Number of patients (%)			P value
	Total	PPL-negative	PPL-positive	
Number of patients	70	35 (50.0)	35 (50.0)	
pT classification				
T1, T2	37 (52.9)	24 (68.6)	13 (37.1)	0.0084*
T3, T4	33 (47.1)	11 (31.4)	22 (62.9)	
pN classification				
N0	36 (51.4)	21 (60.0)	15 (42.9)	0.1513
N1, N2, N3	34 (48.6)	14 (40.0)	20 (57.1)	
Cancer stage[§]				
I, II	42 (60.0)	27 (77.1)	15 (42.9)	0.0034*
III, IV	28 (40.0)	8 (22.9)	20 (57.1)	

*, P<0.05; [§], based on the Union for International Cancer Control, 7th edition.

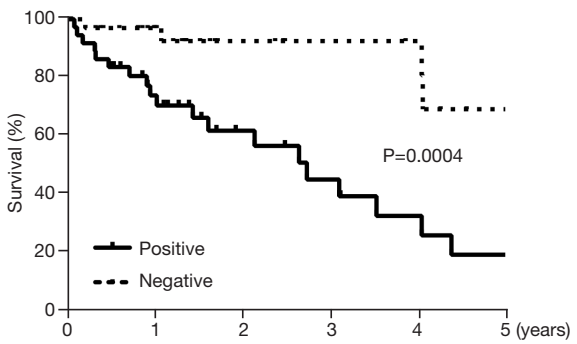

Figure 2 High PPL expression correlated with poor prognosis. Kaplan-Meier curves of overall ESCC patient survival. There was a significant difference between the survival of PPL-positive and PPL-negative patients. PPL, periplakin.

accumulated and formed stratified layers of ~2–3 cells in colonies, while mock transfectants formed a monolayer sheet with no stratification, consistent with our previous study (*Figure 3C*). We next tested whether PPL expression affected cell growth *in vivo* using a xenograft model of tumor formation in BALB/c nude mice. Because the results of colony formation assay seemed to suggest that PPL expression enhanced clonogenicity of ESCC, we inoculated BALB/c nude mice with different number of transfectants. When mice were injected with a relatively large number of cells (5×10^6 cells), the growth of the PPL transfectants was almost the same as that of the mock transfectants (*Figure 3D*). In contrast, PPL transfectants grew significantly larger than mock transfectants *in vivo* when a relatively small number of cells (5×10^5 cells) were inoculated, indicating that high PPL expression in tumors may promote clonogenicity *in vivo*, probably due to their capacity to survive and proliferate in an anchorage-independent way by facilitating cell-cell adhesion with desmosome formation. Thus, high PPL expression may eventually promote tumor growth *in vivo*. This feature may relate to observations of PPL expression and poor prognosis.

Discussion

We previously reported that PPL, a desmosome protein, was downregulated in ESCC and it was associated with relatively high DNA methylation, compared with paired background mucosa. These results suggested that *PPL* may be methylated and silenced during tumor progression. However, in this study, we analyzed more tumors and unexpectedly found that higher PPL expression in tumors was associated with advanced tumor size, lymph node metastasis, advanced tumor stage, and a poor prognosis. Consistent with our previous study, PPL was expressed in all the normal esophageal squamous cells, except the $Ki67^+$ basal cell layer; however, PPL-negative cells were observed in ESCC tissue samples and the frequency of PPL-positive cells in tumors were less than those in normal mucosa. Further, cytoplasmic localization of PPL was observed in tumors. It has been reported that PPL was cleaved by caspase 6 in keratinocytes after induction of apoptosis (13). Increased expression of caspase 6 in ESCC, compared to expression in normal esophageal mucosa, was also demonstrated (14), which may explain the unusual subcellular localization of PPL in tumors. *In vitro* expression of PPL has been shown to induce desmosome formation and to facilitate cell-cell adhesion and cell stratification in some ESCC cell lines (12). In this study, we found that ESCC cells with forced expression of PPL gained the ability to form larger colonies than those of mock-transfected cells *in vitro*. Further, PPL expression accelerated the tumor growth rate *in vivo* in a xenograft model of tumor formation using BALB/c nude mice. PPL induction promoted ESCC cell growth *in vivo*, indicating an oncogenic function of

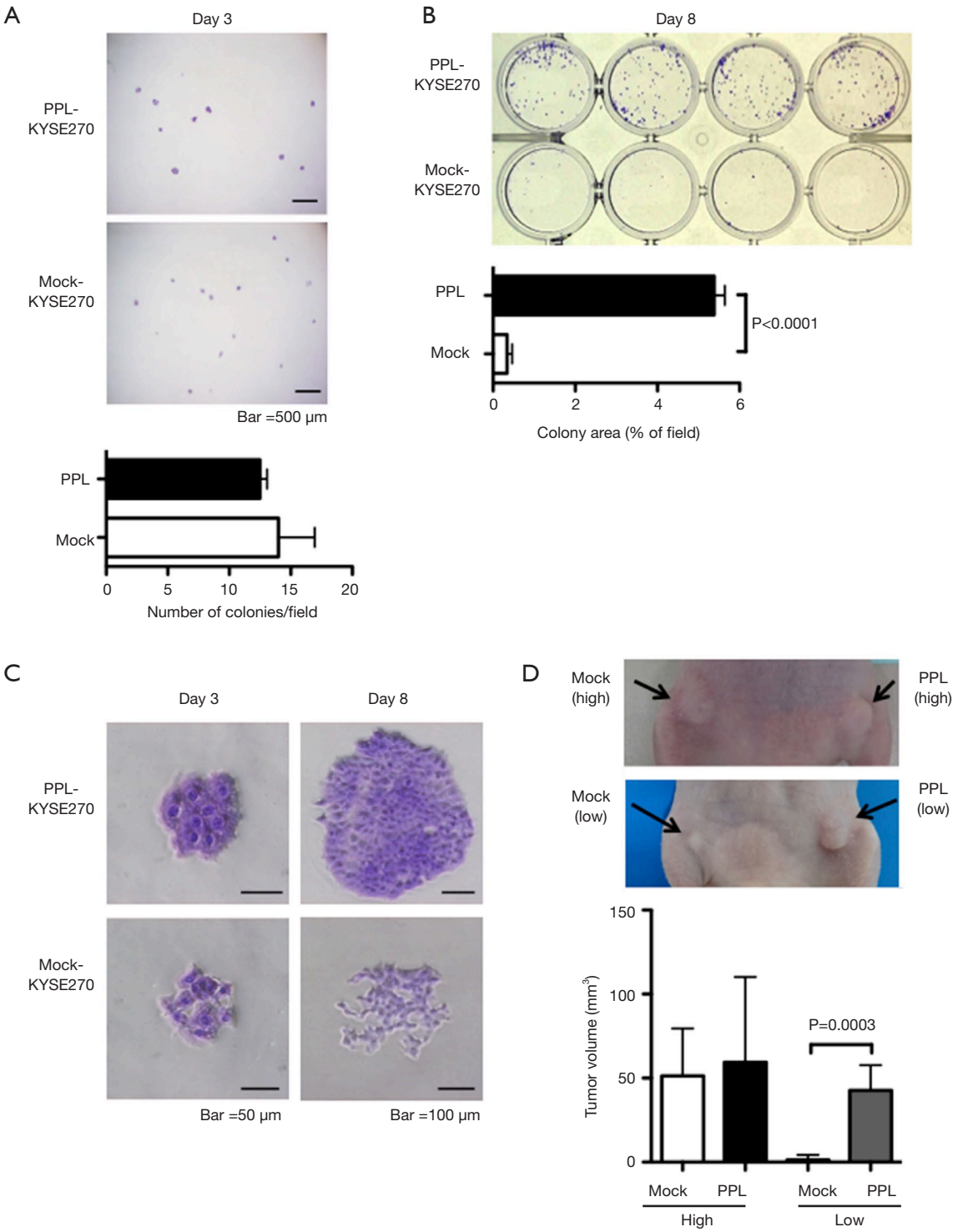

Figure 3 High PPL expression promoted tumor growth. Colony formation of *PPL*-transfected KYSE270 cells and mock-transfected KYSE270 cells. Cells were cultured for 3 (A) and 8 days (B), respectively. Colonies were visualized by crystal violet staining; (C) representative images of colonies; (D) representative images of subcutaneous xenografts (upper panels). Nude mice were subcutaneously inoculated with *PPL*-transfected KYSE270 in the left flank and mock-transfected KYSE270 in the right flank. Two weeks after inoculation, tumor volumes were measured. PPL, periplakin.

PPL. This may appear to conflict with the fact that PPL is generally less expressed in cancer tissues, compared to adjacent normal tissue. As shown in *Figure 1B*, the critical difference between PPL-expressing cells in normal and malignant tissues is that PPL-positive cells never express a proliferating marker Ki67 in normal mucosa, but often do express it in tumors. When only $Ki67^+$ cells are compared, PPL expression appears highly upregulated in tumors. This indicates that in tumors, the system controlling PPL expression is significantly disturbed. We also speculate that PPL-positive tumors and PPL-negative tumors have different carcinogenic histories. PPL-negative tumors may have developed by losing the differentiating features of squamous cells. Low PPL expression and the lack of a desmosome may limit tumor growth in the normal squamous cell mucosal layer, which has tight cell-cell adhesion, resulting in a propensity towards an intramucosal-type cancer. In contrast, PPL-positive cancer cells may gain the ability to self-proliferate without losing differentiating features, such as the desmosome or matrix adhesion (12). This could promote penetration between squamous cells, further into submucosal layers. This enhanced penetration may be the cause of metastasis and poor prognoses in PPL-positive patients. In this sense, we think it is necessary to separate gene expression changes associated with carcinogenesis (differences between cancerous and normal tissues) from gene expression changes associated with tumor progression (differences related to advanced tumor stages and prognoses).

ESCC is characterized by high malignancy and a poor prognosis. Our studies demonstrate that PPL tumor expression levels could be used as a clinical marker to predict prognoses. Currently, multimodal therapy is performed for ESCC, but, in this study, we have not analyzed the relationships between preoperative chemotherapy or irradiation efficacy and PPL expression. Further studies, including these analyses, as well clarification of the biological roles of PPL in established cancer cells, will refine the value of PPL detection as a clinical prognostic marker of ESCC.

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Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/tcr.2018.01.03). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). This study was approved by the NCGM research ethics committee [121 and 1484]. Informed consent was obtained retrospectively in accordance with the dictates of the NCGM research ethics committee [1622].

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