ANTICANCER RESEARCH *41*: 3401-3407 (2021)
doi:10.21873/anticanres.15127

The Prognostic Significance of Plakophilin-1 Expression in Esophageal Cancer

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Abstract. *Background/Aim: Plakophilin 1 (PKP1) expression is inversely related to cancer grade. This study aimed to evaluate whether PKP1 is a prognostic marker for esophageal cancer (EC). Materials and Methods: We tested immunohistochemically for PKP1 in squamous cell carcinoma EC specimens from 99 patients, including cytoplasmic (C), membrane (M), and nuclear (N) cellular areas, and analyzed their relationships with clinicopathological factors. Results: PKP1stains were stratified into strong and weak for all three cellular areas. Staining was inversely related to tumor depth (C: p=0.002, M: p=0.00007, N: p=0.02), lymph node metastasis (C: p=0.003, M: p=0.001, N: p=0.004) and pathological stage (C: p=0.0004, M: p=0.0001, N: p=0.006). Cytoplasmic and membrane staining were inversely related to vessel invasion. Patients with strong C stain had a better overall survival than those with weak C stains (p=0.01). Disease-free survival of patients with strong M stains was better than that of those with weak staining (p=0.01). Conclusion: Cytoplasmic and membrane PKP1 expression is a possible prognostic marker for EC.*

Esophageal cancer (EC) cases have been gradually increasing as the population's median age increases. EC is one of the 10 most common cancers and is responsible for more than half a million deaths worldwide (1).

In Japan, approximately 14,000 treated cases of EC are reported annually by the Japanese Society of Thoracic Surgery (2). Although the percentage of adenocarcinomas among lower ECs has been increasing, more than 90% of ECs in Japan are still squamous cell carcinoma. These cancers generally occur in elderly individuals who both smoke and drink alcohol and who have a high risk of developing other cancers, such as in the aerodigestive tract (3). The esophagus also has abundant lymphatic tissue in the lamina propria and the submucosa, so that lymph nodes can easily metastasize. Therefore, although advances in diagnosis and treatment have improved EC outcomes, EC is still considered to have a poor prognosis (4). Elucidation of EC carcinogenesis and identification of new biomarkers and therapeutic targets could improve this dismal pattern (5).

Changes in cell–cell adhesion are associated with tumor dedifferentiation and invasion. Plakophilins (PKP1, PKP2, and PKP3) are in the armadillo-like protein subfamily and are related to p120 (6). PKP1 is an important plaque component of major intercellular adhesion junctions, which act as fixation points for desmosomes, that is, intermediate filaments. Strong expression of PKP1, inhibits cell proliferation, colony formation, migration, and invasion, and enhances apoptosis. Its expression is inversely related to cancer grade (4). Although strong PKP expression has been observed in several cancers (*e.g.*, pharyngeal, colorectal, lung, and ovarian), no studies of its role in esophageal SCC have been reported so far. This study therefore investigated PKP1's expression and clinical significance in EC.

Materials and Methods

Study population. The subjects for this study were 99 patients who underwent radical resection for EC at Dokkyo Medical University Hospital from May 1, 2009 to December 31, 2018. We excluded patients who received preoperative chemotherapy. This study was approved by the Ethics Committee of Dokkyo Medical University Hospital (No: R-24-11J). Methods and experimental protocols were carried out in accordance with the approved guidelines of the Dokkyo Medical University Hospital. All patients gave their written informed consent preoperatively, for use of their specimens.

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Key Words: Esophageal cancer, squamous cell carcinoma, plakophilin-1, prognostic marker.

Table I. *Clinical characteristics of patients with esophageal cancer.*

Characteristics	All (n=99)
Age (mean), range	68.1±9.7
Gender	
Male	86
Female	13
Localization	
Upper	14
Middle	52
Lower	33
Depth of invasion	
T1a	37
T1b	26
T2	10
T3	24
T4a	1
T4b	1
Lymph node metastatis	
N0	75
N1	14
N2	5
N3	5
Stage	
0	16
IA	21
IB	24
IIA	5
IIB	11
IIIA	3
IIIB	14
IVA	5
Lymphatic invasion	32
Venous invasion	45
Status	
Survival	69
Death	30

Table II. *Relationship between cytoplasmic expression of PKP1 and clinicopathological parameters.*

	PKP1		*p*-Value
	Strong	Weak	
Age (mean), range	68.1±9.7	68.6±9.3	
Gender			0.509
Male	63	23	
Female	8	5	
Localization			0.459
Upper	12	2	
Middle	37	15	
Lower	22	11	
Depth of invasion			
T1a	33	4	0.002
T1b	20	6	
T2	4	6	
T3	12	12	
T4a	1	0	
T4b	1	0	
Lymph node metastatis			
N+	11	13	
N–	60	15	0.003
Stage			
0-1	52	9	0.0004
2-3B	17	16	
3C-4	2	3	
Lymphatic invasion			
+	16	16	
–	55	12	0.001
Venous invasion			
+	16	29	
–	42	12	0.18

Immunohistochemistry. Samples were fixed in buffered formalin and embedded in paraffin blocks from which 3-4-μm sections were cut for conventional hematoxylin and eosin staining (7). These sections were harvested from paraffin blocks, mounted on pretreated slices and stained, using the VECTASTAIN Elite ABC Rabbit IgG Kit (PK-6101) (Burlingame, CA, USA). Normal human esophageal tissue was used as a positive control. Several dilutions were tested; finally, we used a dilution of 1:200 (7). Sections were deparaffinized; for antigen retrieval the sections were heated in sodium citrate buffer (pH 6.0; LSI Medience RM102-C) (Chiyooda Ward, Tokyo, Japan) at 95˚C for 20 min in a microwave. They were cooled for 60-70 min until they returned to room temperature and were washed with water for 5 min. Next, they were treated with 0.3% hydrogen peroxide methanol for 30 min to elicit endogenous peroxidase activity and again washed with water for 5 min, then blocked with goat normal serum (Vectastain Elite ABC Rabbit IgG Kit) for 30 min at room temperature. Sections were incubated with primary antibodies for 60 min at room temperature and washed three times with phosphate-buffered saline (PBS) for 5 min, then incubated with secondary antibodies at room temperature for 30 min and washed three times with PBS for 5 min. Finally, they were nucleate-stained with hematoxylin for 1 min and rinsed in running water for 10 min.

Evaluation. We reported PKP+ cells as percentages of the total number of carcinoma cells. Immunostaining reactions were classified as homogeneous (50%-100%), focal (10%-50%), or negative (0%-10%); and further scored as 6 (strong brown), 5 (moderate yellow), or 4 (weak light yellow) among homogeneous samples, and 3 (strong brown), 2 (moderate yellow), or 1 (weak light yellow) among focal samples. A score of 0 indicated no staining; however, no samples in this study scored 0 (6). A score of 0-4 was considered weak and 5-6 was considered strong.

Statistical analysis. Statistical differences between categoric variables were determined using Fisher's exact test. Survival analysis was performed using the Kaplan–Meier method to evaluate survival time distribution.

Results

Patient characteristics. For the 99 patients, mean age was 68.1±9.7 years. The study cohort included 86 men and 13 women. Their tumor locations in the esophagus were upper:

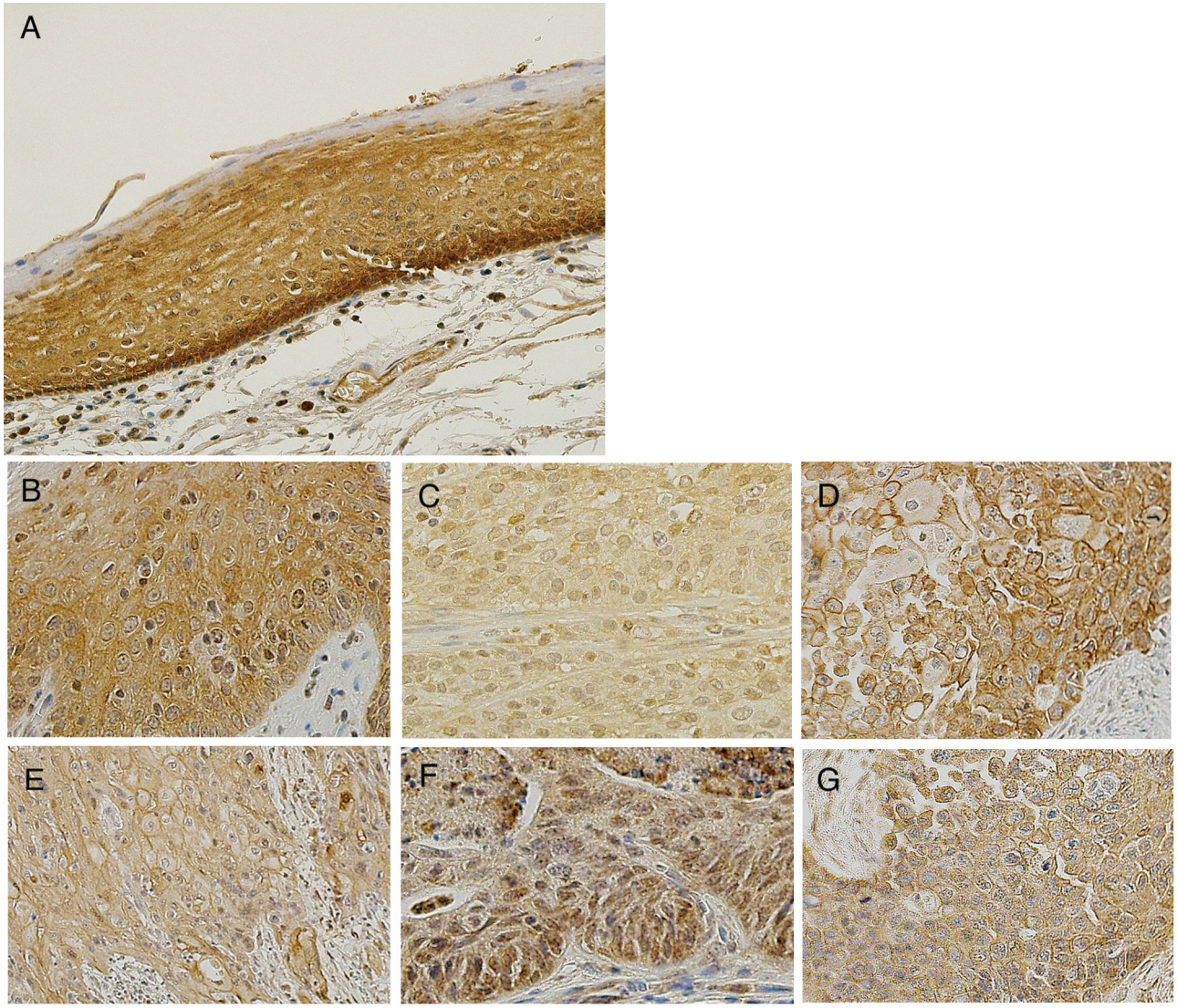

Figure 1. *Plakophilin-1(PKP-1) immunostaining in normal and cancerous esophageal tissues. (A) Strong cytoplasmic staining in normal epithelial cells (positive control). (B) Strong positive cytoplasmic staining in tumor tissue. (C) Weak positive cytoplasmic staining in tumor tissue. (D) Strong positive membrane staining in tumor tissue. (E) Weak positive membrane staining in tumor tissue. (F) Strong positive nuclear staining in tumor tissue. (G) Weak positive nuclear staining in tumor tissue.*

n=14, middle: n=52, and lower: n=33 cases. Clinicopathological factors are shown in Table I.

Immunostaining patterns. The PKP1 immunohistological results, along with positive controls, are shown in Figure 1. Although PKP1 expression is generally more confined to the nucleus than in the cytoplasm in adenocarcinoma, this study showed PKP1 expressed more frequently in cytoplasm.

Cytoplasmic PKP1 expression and clinicopathological factors. Among our samples, 71.7% (71/99) showed strong cytoplasmic PKP1 staining. Cytoplasmic PKP1 expression was inversely related to tumor invasion depth (p=0.002), lymph node metastasis (p=0.003), disease stage (p=0.0004) and lymphatic invasion (p=0.001; Table II).

Membrane PKP1 expression and clinicopathological factors. Of our samples, 45.5% (45/99) showed strong membrane staining. As with cytoplasmic expression, membrane PKP1 expression was inversely related to tumor invasion depth (p=0.00007), lymph node metastasis (p=0.001), disease stage (p=0.0001) and lymphatic invasion (p=0.001); and additionally,

Table III. *Relationship between PKP1 membrane expression and clinicopathological parameters.*

	PKP1		*p*-Value
	Strong	Weak	
Age (mean), range	68.6±9.5	68.2±9.7	
Gender			
Male	42	44	
Female	3	10	
Localization			
Upper	6	8	0.644
Middle	26	26	
Lower	13	20	
Depth of invasion			
T1a	28	9	0.00007
T1b	9	17	
T2	4	6	
T3	4	20	
T4a	0	1	
T4b	0	1	
Lymph node metastatis			
N+	4	34	0.001
N–	41	20	
Stage			
0-1	37	24	0.0001
2-3B	8	25	
3C-4	0	5	
Lymphatic invasion			
+	7	25	0.001
–	38	29	
Venous invasion			
+	12	33	0.001
–	33	21	

Table IV. *Relationship between nuclear expression and clinicopathological parameters of PKP1.*

	PKP1		*p*-Value
	Strong	Weak	
Age (mean), range	67.6±9.4	68.3±9.3	
Gender			
Male	43	43	
female	7	6	
Localization			
Upper	10	4	0.19
Middle	26	26	
Lower	14	19	
Depth of invasion			
T1a	23	14	0.02
T1b	15	11	
T2	6	4	
T3	6	18	
T4a	0	1	
T4b	0	1	
Lymph node metastatis			
N+	6	18	0.004
N–	44	31	
Stage			
0-1	38	23	0.006
2-3B	10	23	
3C-4	2	3	
Lymphatic invasion			
+	12	20	0.08
–	38	29	
Venous invasion			
+	20	25	0.31
–	30	24	

Table V. *Recurrent cases with distant metastases.*

Recurrent (n=20)	Cytoplasm PKP1		*p*-Value	Membrane PKP1		*p*-Value	Nucleus PKP1		*p*-Value
Distant metastases	Strong	Weak		Strong	Weak		Strong	Weak	
+	9	3	0.16	1	11	0.25	6	6	0.67
–	3	5		3	5		3	5	

to venous invasion (p=0.001). These relationships tended to be stronger than those for cytoplasmic expression (Table III).

Nuclear PKP1 expression and clinicopathological factors. Among our samples, 50.5% (50/99) showed strong nuclear PKP1 staining, which was inversely related to invasion depth (p=0.02), lymph node metastasis (p=0.004) and disease stage (p=0.006); but not to other tested factors (Table IV).

PKP1 expression and tumor recurrence. Among the 99 patients, recurrence was confirmed in 20 (20.2%). As PKP1 affects cell adhesion and migration, we investigated its effects on distant metastasis. Twelve of the 20 patients with recurrent disease had distant metastasis. Although patients with weak cytoplasmic expression tended to get metastasis, the other PKP1 staining patterns were not significantly related to distant metastasis (Table V).

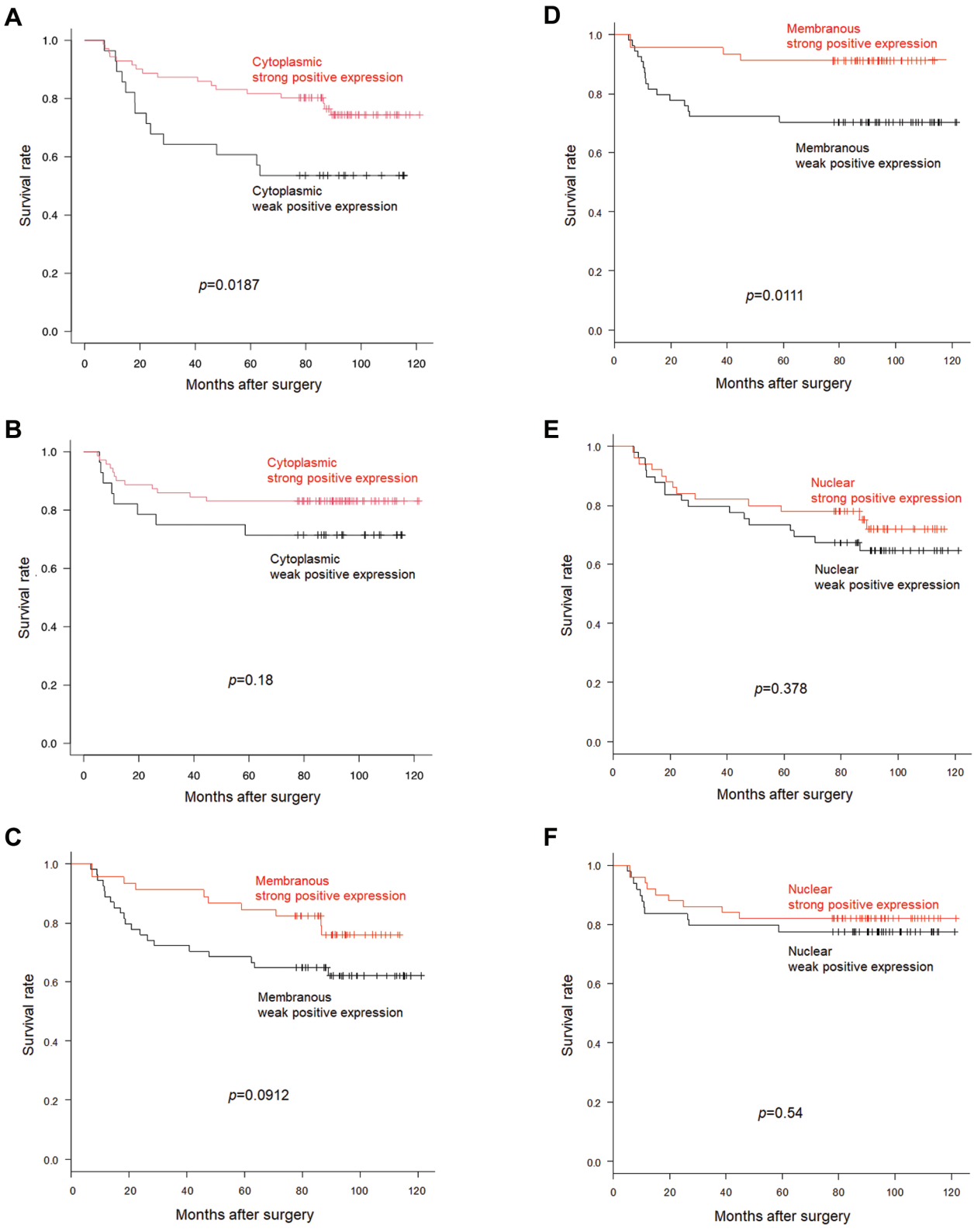

Figure 2. *Kaplan–Meier survival curves for each PKP1 staining status. (A) Overall survival (OS) by cytoplasmic staining status. (B) Disease-free survival (DFS) by cytoplasmic staining status. (C) OS by plasma membrane staining status. (D) DFS by plasma membrane staining status. (E) OS by nuclear staining status. (F) DFS by nuclear staining status.*

PKP1 staining patterns and patient survival. Overall survival (OS) and disease-free survival (DFS) were analyzed with respect to staining in all three cellular areas. With regard to cytoplasmic staining, 5-year OS of the strongly-staining group was significantly better (81.7%) than with the weakly-staining group (60.7%; p=0.0187; Figure 2A). However, 5-year DFS did not significantly differ between the strongly- and weakly- staining groups (p=0.18; Figure 2B).

As for membrane staining, 5-year OS did not significantly differ between the strongly- and weakly-staining groups (84.4%*vs*. 68.5%; p=0.0912; Figure 2C). However, DFS significantly differed between the groups (p=0.0111) (Figure 2D). Nuclear staining showed no significant differences between the two groups for both OS and DFS (Figure 2E and F).

Discussion

Several junctional proteins reportedly have important functions in carcinogenesis, tumor invasion and metastasis. Contact between epithelial cells is mediated by several types of cell–cell junctions, which comprise a complex array of transmembrane proteins and plaque proteins (8-12). Structurally, PKPs have a central core of 10 armadillo repeats flanked by N- and C-terminal domains. The PKP domains function as binding sites for various cellular proteins, including members of the cadherin family, desmoplakin, and actin and keratin filaments (6). They help link other desmosomal proteins and recruit intermediate desmosomal proteins, and thus confer stability and adhesion to cells and tissues during normal development (13-15). Previous studies have reported stronger PKP1 staining intensity in well-differentiated areas than in less differentiated areas (16, 17). Compared to normal epithelium, tumor desmosomes reportedly show reduced or absent PKP expression, especially of PKP1 and PKP3 (6).
In this study, cytoplasmic, membrane and nuclear staining for PKP1 indicated that its expression generally attenuated as the tumor progressed; that is, as staining intensity decreased, the tumor invaded deeper, lymph node metastasis spread more, and tumor stage worsened; venous infiltration also seemed to worsen. Distant metastasis tended to be more common in patients with weak PKP1 staining than in those with strong staining. As PKP1 is involved in cell adhesion, these results seem to be reasonable. In some disorders, PKP1 suppression leads to uncontrollable cell adhesion, and carcinogenesis may be provoked. If PKP1 is modified during carcinogenesis, the tumor may progress because of uncontrolled adhesion and migration. A proteomic analysis of oral cavity squamous cell carcinoma identified that the reduction of PKP1 was highly associated with poor disease-specific survival rate and a shorter time in developing distant metastasis (18). However, there are no reports that mention whether PKP1 decreases with tumor progression. As PKP1expression reflects tumor activity, it may plausibly be a predictor of EC progression and prognosis.

Of the other PKPs, elevated PKP2 has been associated with several tumor types (19, 20), mainly in adenocarcinomas, but has been reported in colorectal, prostate, oropharyngeal and bladder cancers and in gliomas. PKP3 is ubiquitously expressed in all layers of the epithelium except hepatocytes, and helps regulate protein synthesis, proliferation regulation, and transcription. It is observed in various types of cancers, including colon, lung, and bladder cancers. Neither PKP3 nor PKP2 were examined in this study. We intend to investigate these two PKPs in the future.

In conclusion, PKP1 inhibits cell proliferation, colony formation, migration, and invasion, and enhances apoptosis; its expression is inversely related to malignancy. The present study showed consistent results in terms of depth, lymph node metastasis, stage, and OS, and indicated that PKP1 may be a tumor suppressor and potential prognostic marker for EC.

Conflicts of Interest

The Authors declare no conflicts of interest with regard to this study.

Authors' Contributions

Study conception and design: Junki Fujita, Masanobu Nakajima. Acquisition of data: Hiroto Muroi, Maiko Kikuchi, Keisuke Ihara. Analysis and interpretation of data: Junki Fujita, Takeshi Yamaguchi, Masatoshi Nakagawa. Drafting of manuscript: Junki Fujita. Critical revision: Masanobu Nakajima, Shinji Morita, Takatoshi Nakamura, Takashi Tsuchioka, Kazuyuki Kojima.

Acknowledgements

The Authors thank the Edanz Group (https://en-author-services.edanz.com/ac) for editing a draft of this manuscript.

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Received March 31, 2021
Revised May 12, 2021
Accepted May 26, 2021