Prolyl isomerase Pin1 expression predicts prognosis in patients with esophageal squamous cell carcinoma and correlates with cyclinD1 expression

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Abstract. Esophageal carcinoma is one of the most lethal tumors, and identification of prognostic factors for patients with this disease is important. Propyl isomerase Pin1 is overexpressed in some human cancers and thought to be an important regulator of cyclinD1. However, the relationships between Pin1 expression and clinicopathologic features in patients with esophageal squamous cell carcinoma (SCC) have not been explored. Here, we investigated the role of Pin1 in association with cyclinD1 in esophageal SCC progression and its clinicopathological significance. The expressions of Pin1 and cyclinD1 were examined immunohistochemically in surgical specimens from 119 esophageal SCC patients. The expression levels of Pin1 and cyclinD1 in 6 esophageal SCC-derived cell lines were compared with those in an immortalized human esophageal cell line by Western blotting. Pin1 overexpression was correlated with lymph node metastasis (P=0.0384), and its expression was related to cyclinD1 expression. Pin1 expression was correlated with poor prognosis in esophageal SCC patients (P=0.0044), and found to be an independent prognostic factor (P=0.0277). Pin1 was overexpressed in 5 of 6 esophageal SCC-derived cell lines compared with immortalized esophageal keratinocytes. Moreover, the Pin1 level was correlated with the cyclinD1 level in 4 of the 6 cell lines. In conclusion, Pin1 expression is correlated with cyclinD1 expression and may be a useful prognostic factor for esophageal SCC.

Introduction

Despite recent progress in cancer diagnosis and treatment, esophageal cancers still have relatively high mortality rates (1). Moreover, lymph node metastasis occurs more frequently in esophageal cancers than in other gastrointestinal malignancies, thereby resulting in a poor outcome, even in patients detected at an early stage (2,3). However, recent advances in molecular biology have revealed that various oncogenes and tumor suppressor genes are related to the development and progression of esophageal cancer. Amplification of the *cyclinD1* gene is a well-known genetic change and its overexpression is closely related to the invasiveness of cancer cells as well as the patient outcome (4,5). However, the details of the influence of cyclinD1 on this disease remain unclear.

Phosphorylation of proteins on serine/threonine residues that precede proline (pSer/Thr-Pro) is a major intracellular signaling mechanism for regulating cell proliferation and transformation (6,7). The pSer/Thr-Pro motifs present in a certain subset of phosphoproteins are specifically isomerized by the peptidyl-prolyl cis-trans isomerase Pin1. This post-phosphorylation isomerization can lead to conformational changes in the substrate proteins and show profound effects on their catalytic activities, dephosphorylation, protein-protein interactions and subcellular localization (8-11). Pin1 is essential for mitotic progression and required for the DNA checkpoint (12-15). Therefore, Pin1 plays an important role in cell cycle regulation.

It has been demonstrated that Pin1 is overexpressed in some human cancers and that its expression is closely correlated with the level of cyclinD1 in human breast and oral cancers (14-17). Furthermore, Pin1 expression is correlated with tumor development and poor prognosis in patients with human prostate cancer (18). Up-regulation of Pin1 has been shown to potentiate the function of several oncogenic pathways. Pin1 elevates *cyclinD1* gene expression by activating the c-jun/AP-1 and ß-catenin/TCF transcription factors (14,15). Taken together, these results suggest that Pin1 in association with cyclinD1 plays important roles in oncogenesis. However, the effects of variations in Pin1 expression in esophageal squamous cell carcinoma (SCC) remain unknown. In the present study, we used immunohistochemistry to examine the relationships between Pin1 expression and clinicopathologic features in esophageal SCC patients who underwent potentially curative surgery. Moreover, we explored the details of the influence of cyclinD1 in association with Pin1 expression in esophageal SCC.

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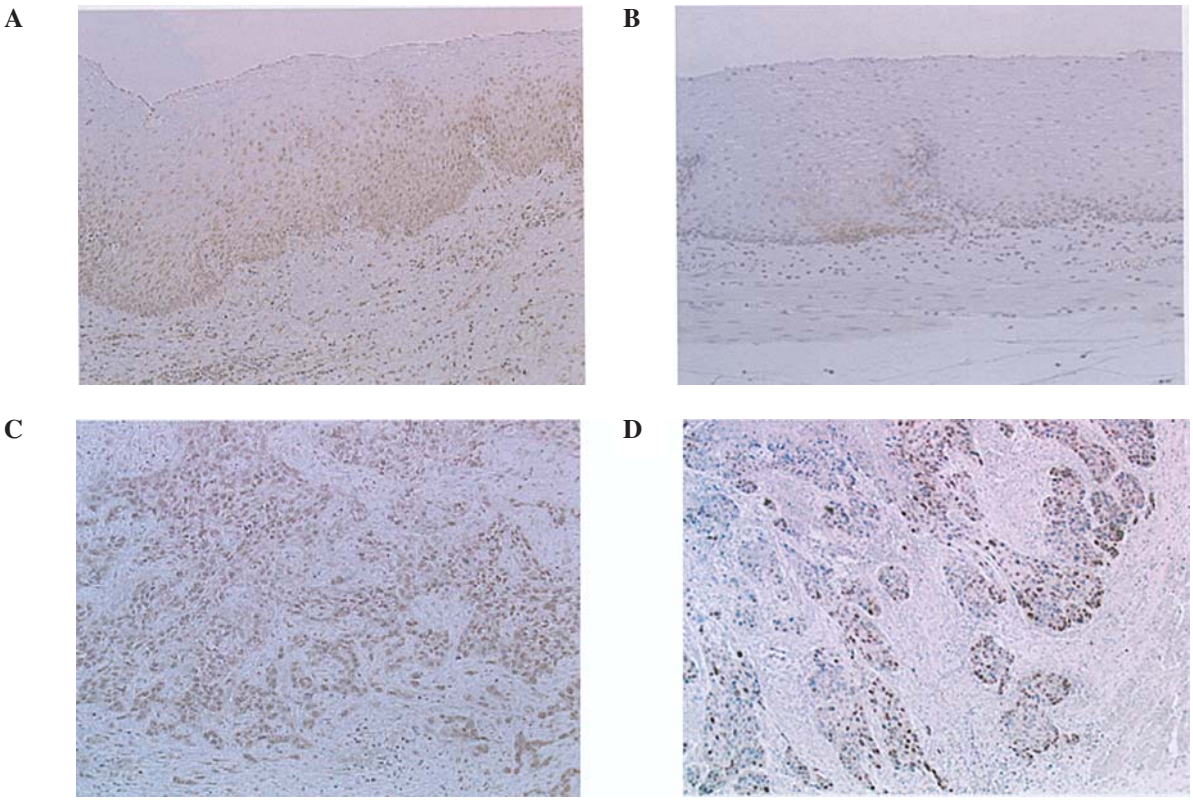

Figure 1. Photographs of tissue sections immunostained for Pin1 and cyclinD1 (x100). (A), Pin1 is predominantly detected in the nuclei of the terminally differentiated basal keratinocytes in normal esophageal epithelium. (B), CyclinD1 is partially detected in the nuclei of cells in the basal regions in normal esophageal epithelium. (C), Pin1 is predominantly present in the cell nuclei and also detected in the cytoplasm at the invasive front of the carcinoma. (D), CyclinD1 is detected in the nuclei of tumor cell nests in esophageal squamous carcinoma.

Materials and methods

Patients. Surgical specimens were obtained from 119 patients (103 males and 16 females) with esophageal SCC who underwent potentially curative surgery without preoperative therapy at the Department of General Surgical Science, Gunma University Graduate School of Medicine, between 1983 and 2002. The age of the patients ranged from 40 to 78 years with a mean age of 62.2 years. Tumor stage was classified according to the fifth edition of the TNM classification of the International Union Against Cancer (19). All of the distant metastastic lesions were lymph nodes.

Immunohistochemistry for Pin1 and cyclinD1. Resected specimens were fixed with 10% formaldehyde and embedded in paraffin blocks. Immunohistochemical staining of the sections was performed by the standard avidin-biotin peroxidase complex method described previously (20,21). Briefly, the sections were incubated with anti-Pin1 polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at a dilution of 1:200 and anti-cyclinD1 monoclonal antibody (clone P2D11F11, Novocastra Laboratories, Ltd., Newcastle, UK) at a dilution of 1:50 and counterstained lightly with hematoxylin. A negative control was prepared by substituting normal rabbit and mouse serum for each primary antibody. No staining was detected in any control section.

Evaluation of Pin1 and cyclinD1 expression. Pin1 immunostaining was evaluated visually and semi-quantified by two of the authors (M.F. and Y.F.) in a coded manner, and then scored for the degree of expression. Normal squamous mucosa was always used as a positive control to ensure the quality of the immunostaining (18). Pin1 was classified as high (staining in 67-100%) or low (0-67%) based the percentage of the tumor cells that were immunopositive and also the intensity of the staining.

The cyclinD1 staining was classified as high when >10% of the tumor cells were positive, and low when ≤10% of the tumor cells were positive, as described in previous studies (22,23).

Cell culture. Six established cell lines derived from esophageal SCC and one immortalized human esophageal cell line were used: TE-series 2, 8, 13, 14 and 15 (gift from Dr T. Nishihira, Tohoku University, Japan) (24), T.T (JCRB0262, gift from Dr K. Takahashi) and CHEK-1 (gift from Dr H. Matsubara). This latter cell line was established by transduction of human papillomavirus type 16 E6/E7 into primary cultures of human esophageal keratinocytes (25). The TE-series and CHEK-1 were cultured in RPMI-1640 medium (Sigma, St. Louis, MO) containing 10% fetal bovine serum and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin); T.T was cultured in a 1:1 Dulbecco's modified Eagle's medium and Ham's F-12 medium (Sigma) containing 10% fetal bovine serum and antibiotics as described above.

Cell extraction and Western blotting. Lysates from exponentially growing cell lines were prepared in a buffer comprising

20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 1% aprotinin and 1 mM phenylmethylsulfonyl fluoride and subjected to Western blotting, as described previously (20). The protein concentrations were determined with a BCA Protein Assay Kit (Pierce, Rockford, IL, USA). A 30-μg aliquot of protein from each cell line was subjected to electrophoresis on a 10% Ready-Gel (Bio-Rad, Tokyo, Japan) followed by electroblotting onto a Hybond enhanced chemiluminescence nitrocellulose membrane (Amersham Pharmacia Biotech, Bucks., UK). The proteins were immunoblotted using anti-Pin1 (Santa Cruz Biotechnology) and anti-cyclinD1 (Novocastra Laboratories) antibodies. An anti-ß-actin (Sigma) antibody served as the control.

Statistical analysis. Statistical analysis was performed using the χ^2 test, Fisher's exact test, and the Mann-Whitney. Survival curves of the patients were calculated using the Kaplan-Meier method, and analysis was performed using the log-rank test. The prognostic factors were examined by univariate and multivariate analysis (proportional hazard regression model). Statistical significance in this study was set as $P<0.05$.

Results

Immunohistochemistry for Pin1 and cyclinD1. In normal squamous epithelium of the esophagus, Pin1 immunostaining was predominantly detected in the nuclei of the terminally differentiated basal keratinocytes (Fig. 1A), and cyclinD1 immunostaining was partially detected in the nuclei of cells in the basal regions (Fig. 1B). In primary esophageal SCC, Pin1 staining was predominantly present in the nucleus but also detected in the cytoplasm (Fig. 1C). In the periphery of tumor cell nests, cyclinD1 staining was detected in the nuclei (Fig. 1D).

Correlations between the Pin1 and cyclinD1 expression and the clinicopathologic findings. The mean Pin1 expression in esophageal SCC was 60.2%, while that in the surrounding normal squamous epithelium was 51.2%. Pin1 expression was high in 38 of the 119 (31.9%) patients, and low in the remaining 81 (68.1%). The correlations between the clinicopathologic characteristics of the esophageal SCC patients and the Pin1 expression in their tumors are summarized in Table I. There was a significant correlation between Pin1 expression and regional lymph node metastasis (P=0.0384), but no significant correlations with patient age, gender, tumor location, differentiation, depth of invasion, pathologic stage or distant lymph node metastasis. CyclinD1 expression was high in 62 of the 119 (52.1%) patients and low in the remaining 57 (47.9%). There were no significant correlations between cyclinD1 expression and the clinicopathologic characteristics of the esophageal SCC patients (Table II). However, Pin1 expression was correlated with cyclinD1 expression (P=0.0146; Table III).

Prognostic significance of Pin1 and cyclinD1. The 5-year survival rate of patients with high Pin1 expression was significantly lower than that of patients with low Pin1 expression (35 vs. 61%, P=0.0044; Fig. 2A). The 5-year survival rate of patients with high cyclinD1 expression was significantly lower than that of patients with low cyclinD1 expression (41 vs. 65%, P=0.0318; Fig. 2B).

Moreover, according to a multivariate analysis using a Cox proportional hazards model, Pin1 and cyclinD1 were independent prognostic factors of overall survival (P=0.0277 and 0.0211, respectively; hazard ratios, 2.038 and 2.067, respectively, Table IV).

Pin1 and cyclinD1 protein levels in esophageal SCC-derived cell lines. The Pin1 and cyclinD1 protein levels in 6 esophageal

Table I. The correlation between clinicopathologic characteristics and Pin1 expression.

Parameters	Pin1 low (n=81)	Pin1 high (n=38)	Total	P-value
Age (mean ± SD; yrs)	62.6±1.0	61.3±1.4		0.4544
Sex				0.6077
Male	71 (68.9)	32 (31.1)	103	
Female	10 (62.5)	6 (37.5)	16	
Location				0.8676
Upper	10 (62.5)	6 (37.5)	16	
Midthoracic	52 (69.3)	23 (30.7)	75	
Lower	19 (67.9)	9 (32.1)	28	
Differentiation				0.3398
Well	19 (65.5)	10 (34.5)	29	
Moderate	32 (62.7)	19 (37.3)	51	
Poor	30 (76.9)	9 (23.1)	39	
TNM classification[a]				
T				0.6793
T1	37 (74.0)	13 (26.0)	50	
T2	10 (66.7)	5 (33.3)	15	
T3	30 (62.5)	18 (37.5)	48	
T4	4 (66.7)	2 (33.3)	6	
N				0.0384
N0	42 (77.8)	12 (22.2)	54	
N1	39 (60.0)	26 (40.0)	65	
M				0.8900
M0	69 (68.3)	32 (31.7)	101	
M1	12 (66.7)	6 (33.3)	18	
Stage				0.2693
I	27 (73.0)	10 (27.0)	37	
II	27 (75.0)	9 (25.0)	36	
III	15 (53.6)	13 (46.4)	28	
IV	12 (66.7)	6 (33.3)	18	

SD, standard deviation (%). [a]International Union Against Cancer TNM classification of malignant tumors.

Table II. The correlation between clinicopathologic characteristics and cyclinD1 expression.

Parameters	CyclinD1 low (n=57)	CyclinD1 high (n=62)	Total	P-value
Age (mean ± SD; yrs)	63.6±1.1	60.9±1.4		0.0966
Sex				0.2089
Male	47 (45.6)	56 (54.4)	103	
Female	10 (62.5)	6 (37.5)	16	
Location				0.1600
Upper	5 (31.3)	11 (68.7)	16	
Midthoracic	35 (46.7)	40 (53.3)	75	
Lower	17 (60.7)	11 (39.3)	28	
Differentiation				0.5802
Well	16 (55.2)	13 (44.8)	29	
Moderate	22 (43.1)	29 (56.9)	51	
Poor	19 (48.7)	20 (51.3)	39	
TNM classification[a]				
T				0.4727
T1	22 (44.0)	28 (56.0)	50	
T2	10 (66.7)	5 (33.3)	15	
T3	22 (45.8)	26 (54.2)	48	
T4	3 (50.0)	3 (50.0)	6	
N				0.2480
N0	29 (53.7)	25 (46.3)	54	
N1	28 (43.1)	37 (56.9)	65	
M				0.7501
M0	49 (48.5)	52 (51.5)	101	
M1	8 (44.4)	10 (55.6)	18	
Stage				0.7354
I	17 (45.9)	20 (54.1)	37	
II	20 (55.6)	16 (44.4)	36	
III	12 (42.9)	16 (57.1)	28	
IV	8 (44.4)	10 (55.6)	18	

SD, standard deviation (%). [a]International Union Against Cancer TNM classification of malignant tumors.

Table III. The correlation between Pin1 and cyclinD1 expression.

	Pin1		
	High	Low	P-value
CyclinD1			
High	26	36	0.0146
Low	12	45	

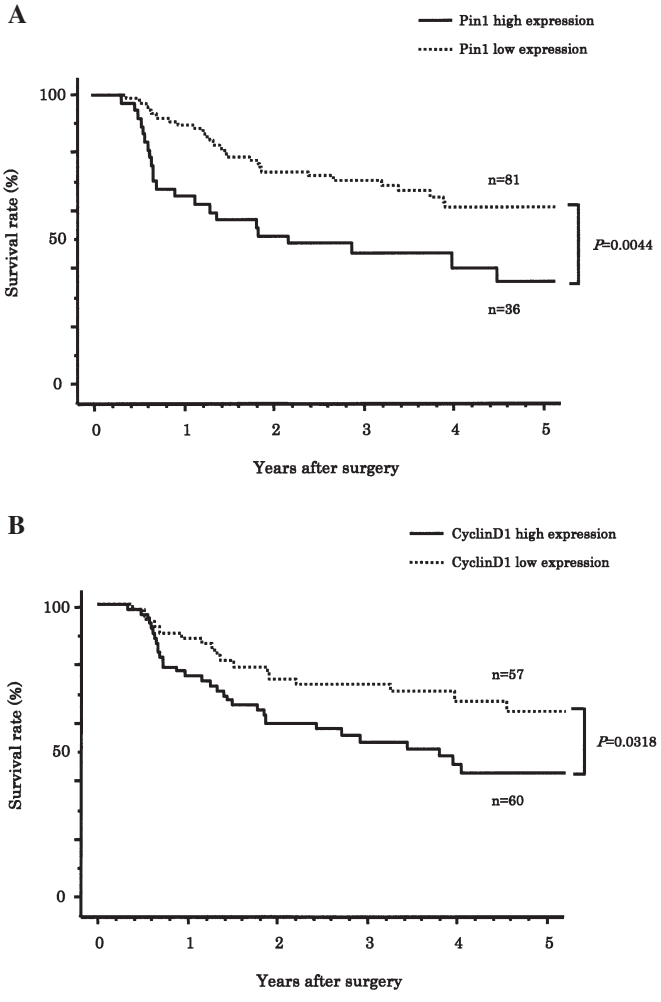

Figure 2. Overall postoperative survival rates according to the Pin1 and cyclinD1 expression. The P-value was determined using the log-rank test. (A), Patients with high Pin1 expression have a significantly more unfavorable prognosis than patients with low Pin1 expression (5-year survival rates: high expression, 35% vs. low expression, 61%, P=0.0044). (B), Patients with high cyclinD1 expression have a significantly more unfavorable prognosis than patients with low cyclinD1 expression (5-year survival rates: high expression, 41% vs. low expression, 65%, P=0.0318).

SCC-derived cell lines were compared with those in an immortalized esophageal keratinocyte cell line (CHEK-1) by Western blotting. Compared with CHEK-1, Pin1 was expressed at a high level in 5 of the 6 esophageal SCC cell lines, with TE-13 being the exception. Moreover, cyclinD1 was expressed at a high level in 2 cell lines (TE-2 and TE-13), and at a slightly higher level in 3 cell lines (TE-8, TE-15 and T.T). Four of the cell lines (TE-2, TE-8, TE-15 and T.T) showed a positive relationship between the Pin1 and cyclinD1 expression levels, while the other 2 cell lines (TE-13 and TE-14) did not (Fig. 3).

Discussion

Pin1 has been shown to play an important role in oncogenesis (14-18). It is overexpressed in human breast and oral cancers,

Table IV. Multivariate analysis of risk factors affecting survival rate.

Risk factor	Reference factor	Hazards ratio	P-value
Histological grading[a]	G1, G2 vs. G3[b]	1.920	0.0377
Primary tumor (T)[a]	T1, 2 vs. T3, 4	3.646	0.0006
Regional lymph nodes metastasis (N)[a]	Negative vs. Positive	5.719	0.0198
Distant lymph nodes metastasis (M)[a]	Negative vs. Positive	2.058	0.0477
Stage grouping[a]	I vs. II, III, IV	0.680	0.6801
CyclinD1	Negative vs. Positive	2.067	0.0211
Pin1	Negative vs. Positive	2.038	0.0277

[a]International Union Against Cancer TNM classification of malignant tumors. [b]G1, well differentiated; G2, moderately differentiated; G3, poorly differentiated.

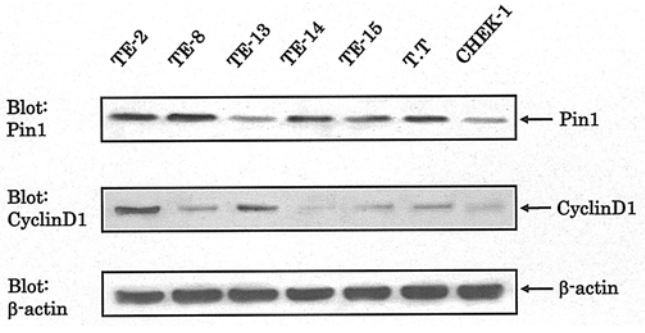

Figure 3. Western blotting of cell extracts from 6 esophageal squamous carcinoma-derived cell lines and an immortalized human esophageal cell line (CHEK-1). The expressions of Pin1 (18 kDa), cyclinD1 (36 kDa) and ß-actin (42 kDa; control) are shown.

and correlated with tumor development and poor prognosis in patients with human prostate cancer (16-18). However, the effects of variations in Pin1 expression in esophageal SCC remain unclear. Therefore, we used immunohistochemistry to investigate the correlations between Pin1 expression and pathologic tumor variables in patients with esophageal SCC. Pin1 was predominantly present in the nuclei of esophageal SCC cells, and higher at the invasive front of tumors where the proliferative activity is high (26). Comparisons of Pin1 expression and clinicopathologic features revealed that Pin1 overexpression was positively correlated with lymph node metastasis (P=0.0384), but not with any other commonly used clinicopathologic features. Moreover, patients with high Pin1 expression had a significantly more unfavorable prognosis (P=0.0044), and a multivariate analysis revealed that Pin1 expression was an independent prognostic factor (P=0.0277). Furthermore, Western blotting showed that Pin1 was over-expressed in 5 of 6 esophageal SCC-derived cell lines compared with the level in immortalized esophageal keratinocytes. These results suggest that high levels of Pin1 expression may influence tumor progression and lead to a poor prognosis in esophageal SCC.

The strong relationship between the level of Pin1 expression and the clinical outcome of esophageal SCC suggests that Pin1 is involved in the progression of this disease. Pin1 over-expression activates multiple steps in oncogenic signaling pathways. For example, Pin1 collaborates with Ras signaling to increase the transcriptional activity of c-Jun toward cyclinD1 (14). Pin1 also activates ß-catenin, which can induce the transcription of both cyclinD1 and c-Myc (15). Furthermore, Pin1 can directly bind and stabilize cyclinD1 (27). Pin1 is also involved in the DNA damage response, through modulation of p53 functions upon genotoxic stress (28,29). Although cyclinD1 and p53 are well-known to be associated in tumor progression and poor prognosis in patients with esophageal SCC, the details of their influences, as well as the effects of Pin1, remain unclear.

Initially, we hypothesized that cyclinD1 expression may serve as a prognostic factor, since amplification and over-expression of cyclinD1 can allow cancer cells to traverse the G0 to G1 and/or G1 to S transitions. Amplification of the *cyclinD1* gene has been reported in 22-58% of esophageal carcinomas, and this can provide useful prognostic information (30-34). Moreover, cyclinD1 has been reported to be involved in tumor progression of esophageal carcinoma, and its over-expression detected by immunohistochemistry is a useful prognostic factor (32). Similar to the findings of previous reports, our data demonstrate that cyclinD1 expression is correlated with poor prognosis in esophageal SCC patients (P=0.0318) and acts as an independent prognostic factor (P=0.0211), although no significant correlations were observed between cyclinD1 expression and the clinico-pathologic characteristics. However, the molecular mechanisms responsible for cyclinD1 overexpression remain unknown. In the present study, Pin1 expression showed a positive correlation with cyclinD1 expression in esophageal SCC by immunohistochemistry (P=0.0146) and in 4 of 6 cell lines by Western blotting. Moreover, considering previous findings that Pin1 induces *cyclinD1* gene expression (14-18), it is likely that Pin1 up-regulates the expression level of cyclinD1 and is involved in tumor progression of esophageal SCC. On the other hand, our data showed that Pin1 expression was not correlated with p53 expression by either immunohistochemistry or Western blotting, although it is known to modulate p53 activation during the DNA damage response (data not shown)

(28,29). However, since p53 plays an important role in oncogenesis, the role of Pin1-mediated p53 activation in esophageal carcinogenesis needs to be clarified in future studies.

In conclusion, Pin1 overexpression may affect the tumor development of esophageal SCC, especially in relation to cyclinD1 expression, and be associated with poor prognosis. Although Pin1 may be the most important regulator of cyclinD1 in esophageal SCC, it also regulates the activities of other proteins (35,36). Therefore, further studies on the effects of Pin1 are necessary to elucidate the participation of other Pin1-regulated factors in aggressive esophageal SCC.

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