Cancer Science
The official journal of the Japanese Cancer Association

Podoplanin is expressed at the invasive front of esophageal squamous cell carcinomas and is involved in collective cell invasion

Yuichiro Nakashima,[1,5] Keiji Yoshinaga,[1] Hiroyuki Kitao,[2] Koji Ando,[1] Yasue Kimura,[1] Hiroshi Saeki,[1] Eiji Oki,[1] Masaru Morita,[1] Yoshihiro Kakeji,[3] Minako Hirahashi,[4] Yoshinao Oda[4] and Yoshihiko Maehara[1]

Departments of [1]Surgery and Science; [2]Molecular Oncology, Graduate School of Medical Sciences, Kyushu University, Fukuoka; [3]Division of Gastrointestinal Surgery, Department of Surgery, Graduate School of Medicine, Kobe University, Kobe; [4]Department of Anatomic Pathology, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan

(Received June 12, 2013/Revised September 3, 2013/Accepted September 9, 2013/Accepted manuscript online September 16, 2013/Article first published online October 22, 2013)

The expression of podoplanin is reportedly involved in collective cell invasion, which is independent from the epithelial–mesenchymal transition (EMT). We focused on the expression of podoplanin in esophageal squamous cell carcinomas (ESCC) and investigated the correlation of podoplanin and EMT-related markers, and evaluated its prognostic significance. Five ESCC cell lines were subjected to western blot analysis for podoplanin and EMT markers. The effects of podoplanin on EMT and carcinoma invasion were evaluated with wound healing assays, invasion assays and 3-D culture. Transfection of ectopic podoplanin into a podoplanin-negative ESCC cell line (TE-15) induced cell migration and invasive activity ($P < 0.001$ and $P < 0.05$, respectively) without downregulation of E-cadherin. In contrast, transfection of si-podoplanin RNA into a podoplanin-positive ESCC cell line (TE-13) reduced cell migration and invasive activity ($P < 0.05$). We reviewed 101 patients who had undergone esophagectomy for ESCC. Podoplanin expression was observed in 58 patients (57.4%), and positive expression was positively correlated with expression of E-cadherin ($P < 0.01$), deeper wall invasion ($P < 0.01$), venous invasion ($P < 0.05$) and poorer prognosis ($P < 0.01$). Multivariate Cox analysis revealed that expression of podoplanin was a significant and independent unfavorable predictor of survival ($P < 0.05$). These data suggest that podoplanin is significantly associated with and likely contributes to ESCC invasion in the absence of EMT. (*Cancer Sci* 2013; 104: 1718–1725)

The epithelial–mesenchymal transition (EMT) has important roles in the development of many tissues during embryogenesis. Moreover, similar cell changes are recapitulated during the development of carcinomas. Acquisition of the mesenchymal state, such as the fibroblastic phenotype, is accompanied by E-cadherin downregulation and upregulation of vimentin, enabling cells to dissociate from the epithelial tissue and migrate. These alterations result in changed adhesive properties, and the activation of proteolysis and motility that allow the tumor cells to metastasize and establish secondary tumors at distant sites.[1]

Collective cell migration is a second principal mode of cell movement. This mode differs from single cell migration in that cells remain connected as they move. Thus, cells migrate as cohorts, maintaining varying degrees of tissue organization. Collective cell migration of cohesive cell groups *in vivo* is particularly prevalent during embryogenesis and drives the formation of many complex tissues and organs.[2,3] A similar collective behavior, known as invasion, is displayed by many invasive cancer types.[4] It is becoming clear that collective cell invasion is involved in the dissemination of squamous cell carcinomas.[5–7] Imaging of the behavior of cancer cells placed in a 3-D culture and observations of clinical samples of advanced-stage carcinomas has revealed that epithelial-type cancer cells can spread as groups or sprouts.[8]

Podoplanin (PA2.26 antigen Aggrus, or T1α) is a type I transmembrane sialomucin upregulated in different types of cancer, such as squamous cell carcinomas and testicular germ cell tumors, suggesting a role for podoplanin in tumor progression.[9] In approximately 80% of human squamous cell carcinomas (lung, larynx, cervix, skin and esophagus), podoplanin is expressed at the invasive edge of the cancers.[10] Wicki *et al.* report that podoplanin promotes cancer cell invasion *in vitro* and *in vivo*. Podoplanin induces collective cell invasion in the absence of EMT.[11]

Esophageal carcinoma is the eighth most frequent cancer in the world. In Europe and the United States, most esophageal carcinoma patients have adenocarcinoma of the lower esophagus or gastroesophageal junction: so called Barrett's adenocarcinoma. In contrast, many patients with esophageal carcinoma in Japan have squamous cell carcinoma.[12] Several reports indicate that esophageal squamous cell carcinoma (ESSC) patients with a high level of podoplanin expression have a poor prognosis.[13–15] However, the effects of podoplanin expression on EMT or collective cell invasion in the setting of ESSC have not been considered.

The purpose of the present study was to determine the role of podoplanin in tumor invasion and prognosis for patients with ESCC. Here, we analyzed the expression and EMT-related functions of podoplanin in ESCC cell lines and in clinical samples of ESCC. We established TE-15 ESCC cell lines that stably expressed podoplanin and, thereby, investigated the functional role of podoplanin in cancer cell migration and invasion. We also established podoplanin knockdown TE-13 ESCC cell lines and investigated cell motility and invasive activity. In addition, we examined the relationship between podoplanin and EMT marker expression in 101 cases of ESCC and evaluated whether podoplanin expression could be considered a prognostic indicator for patients with ESCC.

Materials and Methods

Cell culture. Five established human ESCC cell lines (TE-5, TE-8, TE-10, TE-13 and TE-15) and MRI5 human embryonic fibroblasts (Riken Cell Bank, Tsukuba, Japan) were cultured in

[5]To whom correspondence should be addressed.
E-mail: naka0215@surg2.med.kyushu-u.ac.jp

doi: 10.1111/cas.12286

RPMI (Invitrogen, Carlsbad, CA, USA) with 10% FBS (Invitrogen). All cells were maintained at 37°C in 5% CO_2.

Western blot analysis. For total cell lysates, cells were lysed at 4°C in RIPA-plus buffer (50 mM Tris-HCl [pH8.0], 150 mM NaCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 0.5% sodium deoxycholate, 10% glycerol, 1% NP40, 0.1% SDS, 1 mM NaF, 2 mM $NaVO_4$, 0.1 mM PMSF, 1 mM DTT and protease inhibitor cocktail). The cleared protein lysates were separated by SDS-PAGE and electroblotted on polyvinylidene difluoride membranes (Invitrogen), and proteins were visualized with the appropriate primary and secondary antibodies and ECL Plus (GE Healthcare, Amersham, UK) on LAS-3000 (Fujifilm, Tokyo, Japan). Antibodies to podoplanin (mouse monoclonal, D2-40, 1:100; Nichirei Bioscience, Tokyo, Japan), E-cadherin (mouse monoclonal, 1:2500; BD Biosciences, Franklin Lakes, NJ, USA), vimentin (mouse monoclonal, 1:500; Dako Cytomation, Glostrup, Denmark) and β-actin (mouse monoclonal, 1:2500; Sigma-Aldrich, St. Louis, MO, USA) were used for the primary reaction.

cDNA cloning of podoplanin. The cDNA encoding the full-length open reading frame of human podoplanin was obtained by PCR using TE-13 cDNA as a template (GenBank accession No. NM_006474). The primer set for human podoplanin was as follows: 5′-ACCGAATTCACC-ATGCTGACTCCGCT CGGA-3′ and 3′-ACCGGATCCTTAGGGCGAGTACCTT CCCG-5′. The full length cDNA of human podoplanin was subcloned into the expression vector pcDNA3.1zeo-IRES-EGFP.[16]

Gene transfection. Vectors and empty vector controls were transfected into TE-15 cells using Lipofectamine LTX (Invitrogen) according to the manufacturer's instructions. A recombinant podoplanin-expressing TE-15 clone was established by selection in medium containing 200 μg/mL G418 (Nacalai Tesque, Kyoto, Japan).

siRNA transfection. A siRNA duplex (sense, 5′-GGAC CAUUGGAUCGAUAUUdTdT-3′) specific for human podoplanin (GenBank accession No. NM_006474) was designed at Takara Bio (Shiga, Japan). Takara Bio also provided the non-specific control siRNA duplex (sense, 5′-UCUUAAUCGC GUAUAAGGCTTdTdT-3′). TE-13 cells were transfected with siRNA oligonucleotides using Lipofectamine RNAiMAX (Invitrogen) following the manufacturer's protocol.

Wound healing assays. For the wound healing assay, cultured cells at almost 100% confluence were serum-starved for 12 h. After scratching the monolayer, cells were washed with PBS and then cultured in RPMI with 10% FBS. Each experiment was repeated five times.

Invasion assays. Cell invasion was determined with a Matrigel invasion assay using polycarbonate membranes (8.0 μm pore size) in the upper chamber of 24-well Transwell culture chambers coated with Matrigel (Falcon BD, Franklin Lakes, NJ, USA). Cells (1.0×10^4 per well) were placed in the upper chamber with 500 μL FBS-free medium, whereas the lower chamber was loaded with 750 μL medium containing 10% FBS. After 36 h of incubation, cells remaining inside the inserts were removed, and the cells that had traversed to the reverse side of the inserts were stained with hematoxylin and enumerated with light microscopy. Each experiment was repeated thrice.

3-D culture. Following previously described methods,[17] podoplanin-transfected cells (TE-15-podoplanin) and control cells (TE-15-mock) were used in 3-D cultures to examine the form of tumor cell invasion. MRC5 human embryonic fibroblasts (5×10^6/2 mL) were mixed into a neutralized type I collagen gel (10.5 mL; Cellmatrix type I-A, Nitta Gelatin,

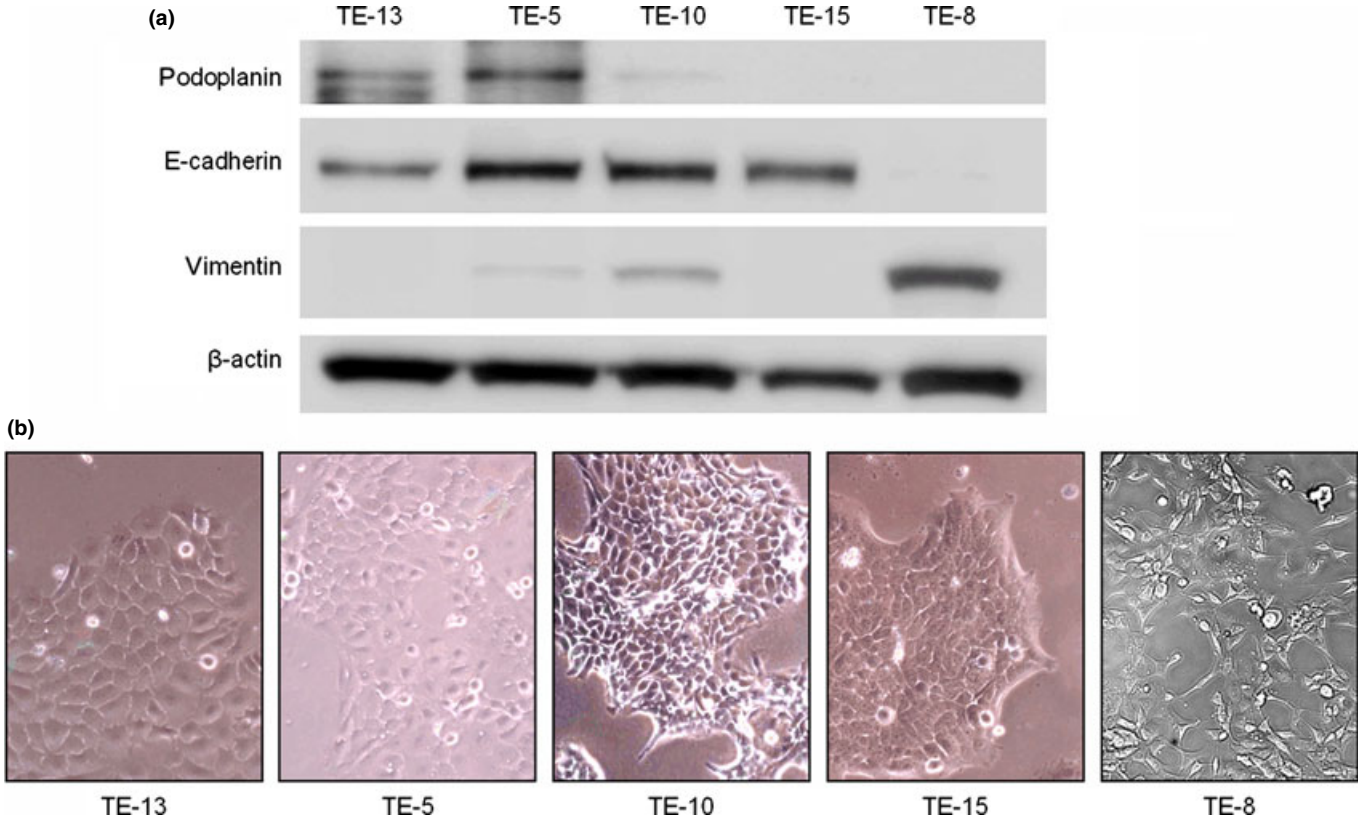

Fig. 1. Endogenous expression of podoplanin, E-cadherin and vimentin in ESCC cells. (a) Western blot analysis of podoplanin, E-cadherin and vimentin in ESCC cells (TE-13, TE-5, TE-10, TE-15 and TE-8). (b) Morphological observations of TE-13, TE-5, TE-10, TE-15 and TE-8 cells. Only TE-8 cells showed fibroblast-like morphology with lamellipodia-like structures.

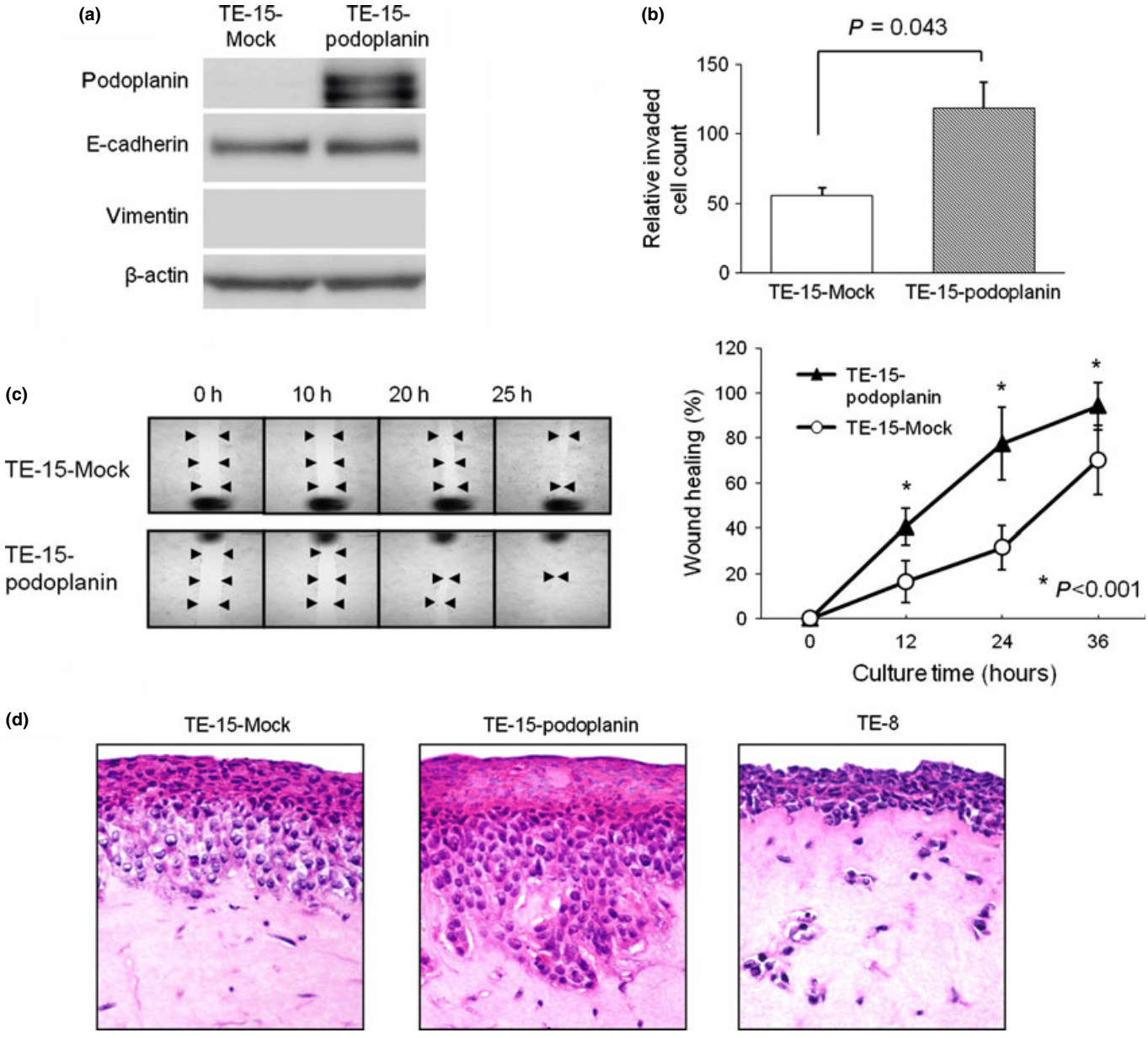

Fig. 2. Transduction of ectopic podoplanin promoted cell motility and invasion/migration activities. (a) Western blot analysis of podoplanin, E-cadherin and vimentin in podoplanin-transfected TE-15 cells (TE-15-podoplanin) and vector-transfected TE-15 cells (TE-15-Mock). (b) Matrigel invasion assay revealed significantly higher invasion/migration activities of podoplanin-transfected TE15 cells ($P = 0.043$). The experiment was done thrice. (c) Wound healing assay revealed significantly higher cell motility in podoplanin-transfected TE-15 cells ($P < 0.001$). The experiment was done five times. (d) 3-D culture showed that podoplanin-transfected TE-15 cells invaded deeper than did vector-transfected TE-15 cells. Moreover, carcinoma cells invaded the collagen matrix layer as multicellular clusters in a process termed collective cell migration and invasion. TE-8 cell showed single cell invasion in 3-D culture.

Osaka, Japan) following the manufacturer's recommendations. The cell suspension was aliquoted into six-well plates and allowed to harden for 30 min in a CO_2 incubator at 37°C. ESCC cells (2×10^6) were then dispensed onto each gel and allowed to incubate overnight. The following day, the hardened gels were detached from the plates and they were then placed on the mesh of cell strainers (BD Biosciences) so that the ESCC cells lay on the top of gel discs and the fluid level was adjusted to just below the upper edge of the gel. After 2 weeks of air–liquid interface culture, the gel discs were fixed in a phosphate-buffered formalin solution and embedded in paraffin, and vertical sections were stained with H&E.

Clinical samples. Surgically resected ESCC with no preoperative therapy were collected from 101 patients who underwent esophagectomy between 1994 and 2005 at the Department of Surgery and Science, Kyushu University Hospital, Japan. All of the tissue specimens were obtained after receiving the patients' written informed consent. All of the samples underwent H&E staining and were found to be squamous cell carcinomas upon histological examination.

Immunohistochemistry. Selected representative sections were immunostained with antibodies against podoplanin (mouse monoclonal, D2-40, 1:100 [Nichirei Bioscience]), E-cadherin (mouse monoclonal, 1:2500 [BD Biosciences]) and vimentin (mouse monoclonal, 1:500 [Dako Cytomation]).

To investigate the relationship between podoplanin expression and E-cadherin and vimentin expression, we observed the deepest invaded area, called the invasive front. We also

doi: 10.1111/cas.12286

followed the previously published method to score the expression of podoplanin.[13] In brief, patients positive for podoplanin expression in >10% of tumor cells at the invasion front were categorized as positive, whereas others were classified as negative. As reported by Usami *et al.*, the degree of cell membrane-E-cadherin and cytoplasmic-vimentin expression at the invasion front was scored as follows: a negative front showed <20% immunoreactivity, whereas a positive front showed ≥20% positive immunoreactivity.[18]

Statistical analysis. Continuous data were expressed as median and range and compared between groups using the Mann–Whitney *U*-test. Categorical variables were compared using the χ^2-test, Fisher's exact test and Student's *t*-test. The patient survival analyses were calculated using the log-rank test. Prognostic factors were examined by univariate and multivariate analyses (Cox proportional hazards model). *P*-values < 0.05 were considered to be statistically significant. These results were analyzed using the JMP 7 software program (SAS Institute, Cary, NC, USA).

Results

Correlation between endogenous podoplanin expression and epithelial–mesenchymal transition markers in esophageal squamous cell carcinomas cells. We investigated the level of expression of E-cadherin, vimentin and podoplanin in five ESCC cell lines (TE-13, TE-5, TE-10, TE-15 and TE-8). E-cadherin protein was detected in TE-13, TE-5, TE-10 and TE-15 cell lines, all of which showed typical cobblestone-like appearance under conventional culture conditions (Fig. 1a,b). TE-8 cells exhibited extremely low levels of E-cadherin protein expression. The cell line that expressed vimentin demonstrated fibroblast-like spindle morphology with lamellipodia-like structures, suggestive of EMT (Fig. 1a,b). Podoplanin expression was detected in TE-13 and TE-5 cell lines using western blot analysis. Those two cell lines showed E-cadherin expression and negative or weak expression of vimentin, and cobblestone-like appearance (Fig. 1a,b).

Effects of ectopic recombinant podoplanin protein on invasion. To determine the role of podoplanin in the regulation of the EMT, we transfected the pcDNA3.1zeo-IRES-EGFP-podoplanin vector into ESCC cells. Because TE-15 cells expressed E-cadherin without expraessing either podoplanin or vimentin, these cells were used for the following experiments. Transduction of ectopic podoplanin did not influence the expression of E-cadherin and vimentin at the protein levels (Fig. 2a).

We assessed the role of podoplanin on cell migration and invasive activity. In a Matrigel invasion assay, the number of exogenous podoplanin-transfected cells that invaded/migrated to the lower chamber was significantly increased compared to the control ($P = 0.043$; Fig. 2b). The wound healing assay revealed greater migration activity in podoplanin-transfected cells than in the control ($P < 0.001$; Fig. 2c). We next cultured podoplanin-transfected cells in 3-D cultures. After 16 days of an air–liquid interface culture, cells were fixed in formalin solution and embedded in paraffin, and vertical sections were stained with H&E (Fig. 2d). The 3-D culture method revealed greater invasion/migration activity in podoplanin-transfected cells than did the control, and both cell lines invaded the collagen matrix layer as multicellular clusters in a process termed collective cell migration and invasion. TE-8 cells showed single cell invasion in 3-D culture. TE-15-podoplanin showed significantly deeper depth of invasion (324.8 ± 69.1 μm) than TE-15-mock (228.0 ± 27.9 μm, $P = 0.0361$).

Suppression of podoplanin protein by RNAi technology. To address the role of podoplanin in the regulation of the EMT, we used siRNA oligonucleotides to suppress podoplanin expression in ESCC cell lines. Because TE-13 cells expressed podoplanin and E-cadherin without expressing vimentin, these cells were used for the following experiments. Podoplanin knockdown was confirmed at the protein level by western blot analysis (Fig. 3a). Subsequently, the effects of podoplanin on cell motility and invasive activity were examined. The wound healing assay revealed significantly lower migration activity in

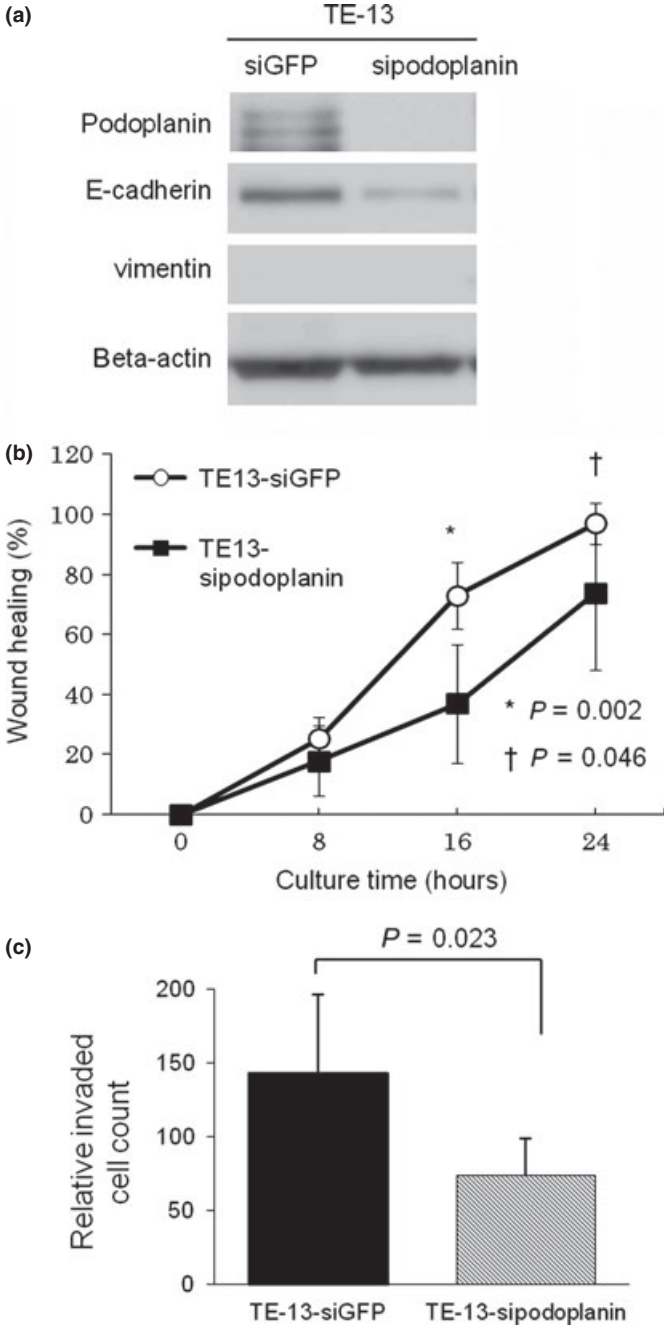

Fig. 3. Transfection with siRNA oligonucleotides targeting podoplanin mRNA or control in TE-13 cell line. (a) Western blot analysis of podoplanin, E-cadherin and vimentin expression after transfection of podoplanin knockdown TE-13 cells (TE-13-sipodoplanin) and GFP knockdown TE-13 cells (TE-13-siGFP). (b) Wound healing assay revealed significantly lower cell motility in podoplanin knockdown TE-13 cells ($P < 0.05$). The experiment was done five times. (c) Matrigel invasion assay revealed significantly lower invasion activities of podoplanin knockdown TE-13 cells ($P = 0.023$). The experiment was done thrice.

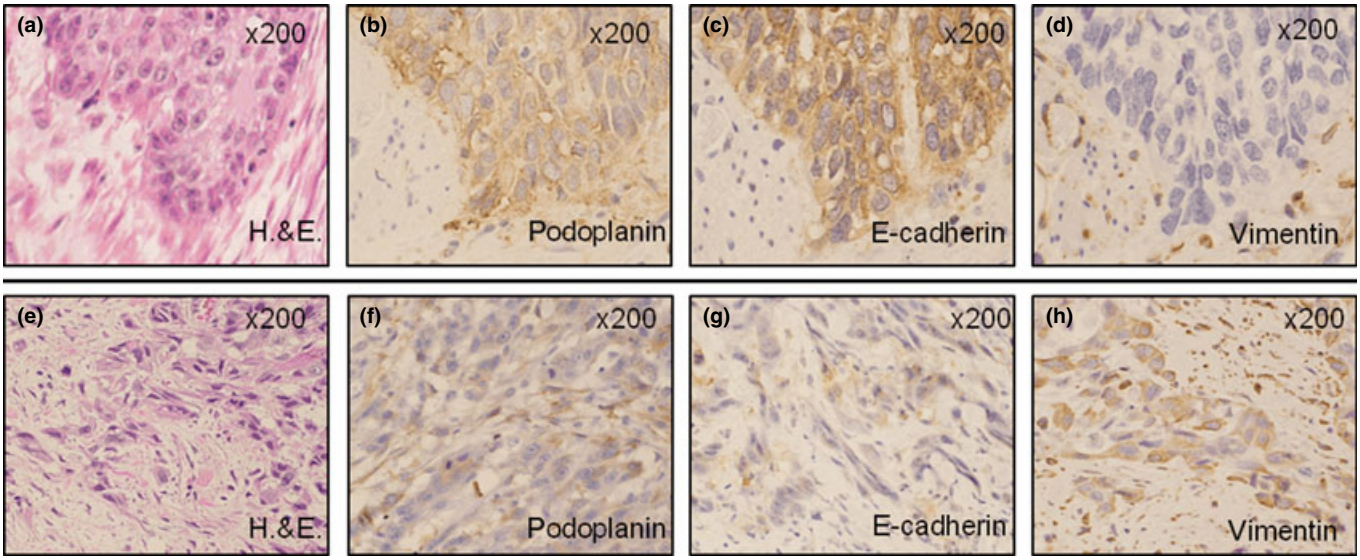

Fig. 4. Immunohistochemical expression of podoplanin, E-cadherin, and vimentin in ESCC samples. Invasive front of two representative cases are shown: (a–d) collective cell invasion sample; (e–h) single cell invasion sample. In the collective invasion sample (a–d), expression of podoplanin (b) and E-cadherin (c) was preserved, while vimentin expression was negative (d). In single cell invasion samples (e–h), immunoreactivities of podoplanin (f) and E-cadherin (g) were reduced, while vimentin expression was observed (h).

podoplanin-knockdown cells than in the control ($P < 0.05$; Fig. 3b). In the Matrigel invasion assay, the number of invading cells among knockdown cells was lower than that among the control cells ($P = 0.023$; Fig. 3c).

Expression of podoplanin, E-cadherin and vimentin in esophageal squamous cell carcinoma patients. We further investigated the expression of podoplanin, E-cadherin and vimentin at the invasive front of 101 ESCC samples. The invasive fronts of two representative cases are shown: Figure 4(a–d) is an example of collective invasion and Figure 4(e–h) is an example of single cell invasion sample. Of the 101 cases in the present study, 58 (57.4%) showed signals for podoplanin at the outer edge of the invading tumor cell nest (Fig. 4b). Positive staining for E-cadherin appeared in the cytoplasm of the cancer cells (Fig. 4c). Reduced expression of E-cadherin was observed in 19 of the 101 samples (18.8%; Fig. 4g). Positive staining of vimentin protein was observed in the cytoplasm of the carcinoma cells in 20 of the 101 samples (19.8%; Fig. 4h).

The correlations between podoplanin, E-cadherin and vimentin expression are shown in Table 1. In ESCC patients, podoplanin expression was positively correlated with preserved expression of E-cadherin ($P < 0.001$). There was no significant correlation between the expression of podoplanin and vimentin. A total of 13 cases showed double positive staining of podoplanin and vimentin in invasive fronts (Fig. S1).

Correlation among podoplanin expression, clinicopathological features and survival. The correlation between podoplanin expression and clinicopathological data is shown in Table 1. In 101 samples, podoplanin expression was correlated with aggressive wall invasion (deeper T2, $P = 0.002$) and positive venous invasion ($P = 0.015$).

Excluding 20 cases with positive expression of vimentin, the 5-year overall survival frequency for ESCC patients with positive and negative expression of podoplanin was 44.3% and 80.0%, respectively (Fig. 5a). Podoplanin expression in tumors was significantly associated with shorter postoperative overall survival ($P = 0.004$). The 5-year overall survival frequency for ESCC patients with positive and negative expression of vimentin was 31.8% and 61.3%, respectively (Fig. 5b). Vimentin expression in tumors was significantly associated with shorter postoperative survival ($P = 0.003$).

Table 1. Comparative analysis of the podoplanin expression, clinicopathological characteristics and the expression of E-cadherin and vimentin in esophageal squamous cell carcinoma

	n	Podoplanin expression		*P*
		Positive (%)	Negative (%)	
Total	101	58	43	
Age				
<64 years	43	24 (41.4)	19 (44.2)	0.778
≥64 years	58	34 (58.6)	24 (55.8)	
Gender				
Male	88	51 (87.9)	37 (86.1)	0.078
Female	13	7 (12.1)	6 (13.9)	
Histology†				
Well	22	17 (29.3)	5 (11.6)	0.074
Moderate	62	31 (53.5)	31 (72.1)	
Poor	17	10 (17.2)	7 (16.3)	
Depth of invasion†				
T1	50	21 (36.2)	29 (67.4)	0.002
T2–4	51	37 (63.8)	14 (32.6)	
Lymphatic invasion				
Absent	54	29 (50.0)	25 (58.1)	0.417
Present	47	29 (50.0)	18 (41.9)	
Venous invasion				
Absent	64	31 (53.5)	33 (76.7)	0.015
Present	37	27 (46.5)	10 (23.3)	
Lymph node metastasis				
Absent	50	27 (46.6)	23 (54.5)	0.490
Present	51	31 (53.5)	20 (46.5)	
E-cadherin				
Preserved	82	54 (93.1)	28 (65.1)	<0.001
Reduced	19	4 (6.9)	15 (34.9)	
Vimentin				
Positive	20	13 (22.4)	7 (16.3)	0.441
Negative	81	45 (77.6)	36 (83.7)	

†According to the *Guidelines for the Clinical and Pathological Studies on Carcinoma of the Esophageal Diseases*.[31] Moderate, moderately differentiated squamous cell carcinoma; poor, poorly differentiated squamous cell carcinoma; well, well-differentiated squamous cell carcinoma.

doi: 10.1111/cas.12286

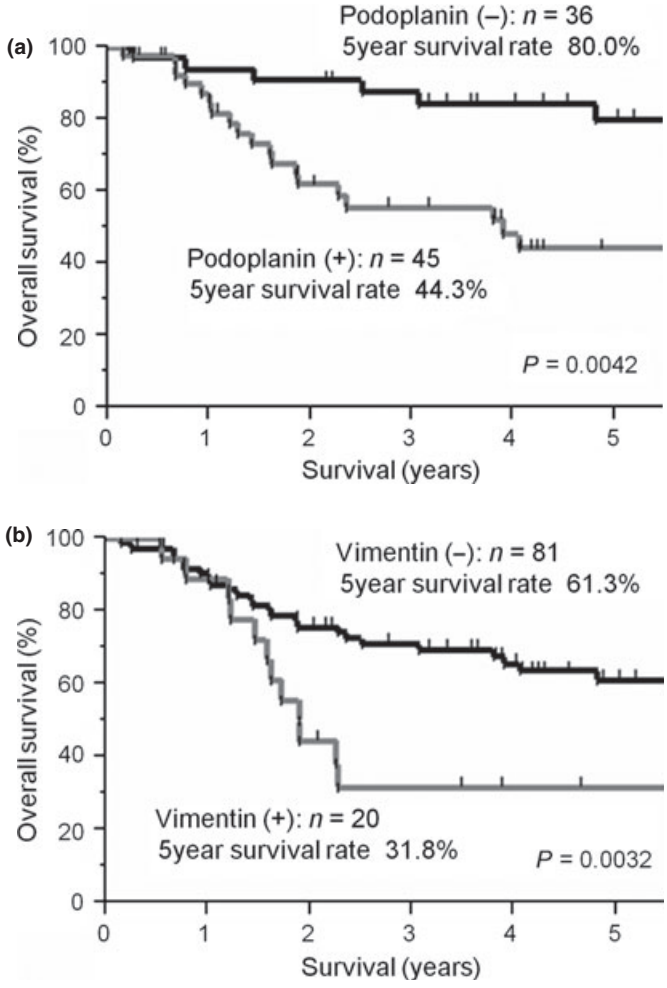

Fig. 5. The overall survival curve for ESCC patients. (a) The overall survival curve of vimentin-negative ESCC patients for positive versus negative expression of podoplanin. (b) The overall survival curve of all ESCC patients for positive versus negative expression of vimentin.

In univariate analysis, the parameters that significantly affected survival were depth of invasion, lymphatic invasion, venous invasion, lymph node metastasis, and expression of podoplanin and vimentin (Table 2). Multivariate analysis showed that the depth of invasion and podoplanin expression were independent prognostic factors for poor survival in ESCC (Table 2).

Table 2. Relative risks for death in 101 patients with esophageal squamous cell carcinoma

Variables	Risk ratio	95% CI	*P*
Univariate			
Age (<64 *vs* ≥64 years)	0.99	0.96–1.03	0.675
Gender (female *vs* male)	0.69	0.30–0.59	0.073
Histology (moderate + poor *vs* well)†	2.16	0.99–4.34	0.052
Depth of invasion (T2–4 *vs* T1)†	7.71	3.60–18.47	<0.001
Lymphatic invasion (present *vs* absent)	3.50	1.80–7.14	<0.001
Venous invasion (present *vs* absent)	4.16	2.15–8.28	<0.001
Lymph node metastasis (present *vs* absent)	3.76	1.94–7.66	<0.001
Podoplanin (positive *vs* negative)	2.52	1.30–5.19	0.006
E-cadherin (negative *vs* positive)	1.93	0.90–9.54	0.074
Vimentin (positive *vs* negative)	2.66	1.31–5.14	0.008
Multivariate			
Histology (Moderate + poor *vs* well)†	1.92	0.82–4.19	0.129
Depth of invasion (T2–4 *vs* T1)†	4.25	1.64–11.76	0.003
Lymphatic invasion (present *vs* absent)	1.54	0.64–3.77	0.337
Venous invasion (present *vs* absent)	1.32	0.60–2.97	0.175
Lymph node metastasis (present *vs* absent)	1.76	0.78–4.12	0.737
Podoplanin (positive *vs* negative)	2.16	1.05–4.65	0.036
Vimentin (positive *vs* negative)	1.38	0.60–3.07	0.447

†According to the *Guidelines for the Clinical and Pathological Studies on Carcinoma of the Esophageal Diseases*.[31] CI, confidence interval; moderate, moderately differentiated squamous cell carcinoma; poor, poorly differentiated squamous cell carcinoma; well, well-differentiated squamous cell carcinoma.

Discussion

In our histopathological analysis of the invasive front of ESCC, reduced expression of E-cadherin was observed in only 19 cases (18.8%), and positive expression of vimentin was observed in only 20 cases (19.9%) out of a total of 101 patients. This expression ratio of vimentin was comparable to a previous report.[18] It is particularly interesting that complete EMT phenotypes, which showed both reduced expression of E-cadherin and positive expression of vimentin, were observed in only three cases (3.0%). The low incidence of vimentin expression indicates that EMT is not necessarily required for the process of invasion and metastasis of all ESCC cases.

Many advanced cancers possess molecular and morphologic characteristics indicative of well-differentiated epithelia, including high levels of E-cadherin expression.[5] Moreover, some reviews question the role of EMT in cancer, citing a lack of evidence of this phenomenon *in vivo*.[5,19] Invasive carcinoma could invade surrounding tissues as multicellular aggregates or clusters in a process known as collective cell invasion.[20] Using a 3-D collagen matrix and time lapse video microscopy, Friedl *et al.*[21] show that clusters of squamous cancer cells could detach from the site of the primary cancer and invade as independent aggregates within the adjacent extracellular matrix. MDCK cells with ectopic expression of membrane-type-1 MMP aggregates were able to enter lymphatic and blood vessels, which is consistent with observations that clusters of metastatic cells from a variety of cancers can be detected in the circulation.[22]

In our histopathological analysis of the invasive front of ESCC, podoplanin expression was positively correlated with preserved expression of E-cadherin, deeper wall invasion and higher rates of venous invasion. In addition, podoplanin expression was an independent adverse prognostic factor. These clinical findings indicate that podoplanin is related to ESCC invasion and malignancy in the absence of EMT. In two of three reports that examined immunohistochemical expression of podoplanin in ESCC, podoplanin expression was significantly correlated with lymph node metastasis.[14,15] Another report showed no significant correlation with podoplanin expression and lymph node metastasis.[13] In the present study, no significant correlation was observed between podoplanin expression and lymph node metastasis. The conflicting results might be a result of the the range of dissected lymph nodes differing between facilities. We found that in ESCC cell lines, podoplanin expression accelerated cell motility and invasive activity, results similar to a previous report.[13]

Furthermore, we found that podoplanin promoted cell motility and invasive activity without changes in molecular phenotype (such as the expression levels of E-cadherin and vimentin), or alterations in epithelial morphology. These results are similar to those of a previous study on MCF7 breast cancer cell lines.[11] In fact, analysis of clinical samples demonstrates that podoplanin expression is associated with collective cell invasion.

Epithelial junctions in well-differentiated metastatic carcinomas can form physical barriers that restrict the access of drugs or antibodies to the sites of cancers.[23,24] Based on ESCC patients' responses to preoperative chemotherapy, some investigators have shown that responsive patients had poorly differentiated ESCC while non-responsive patients harbored greater numbers of well-differentiated ESCC.[25,26] Rahadiani *et al.*[13] report that ESCC cells were more vulnerable to topotecan, 5-FU and cisplatin after podoplanin knockdown than were control cells. Podoplanin elicits powerful platelet aggregation and is the endogenous ligand for the platelet C-type lectin receptor, CLEC-2, which itself regulates podoplanin signaling.[27] Aggregated platelets coat tumor cells during their transit through the bloodstream and mediate adherence to vascular endothelium, protection from shear stresses, evasion from immune molecules, and release of an array of bioactive molecules that facilitate cancer cell extravasation and growth at metastatic sites.[27]

There is evidence that podoplanin can also promote single cell invasion of MDCK cells, thus contributing to EMT-mediated cell motility.[28] The molecular dissection of collective and single cell invasion is a relatively new topic in cancer research, whereas numerous efforts in the past (as well as current work) have attempted to distinguish these two pathways during embryonic development. TGFβ family members (such as Nodal), FGF and Wnt signaling cadherin cell adhesion molecules contribute to the collective migration of vertebrate embryonic tissue.[29] Yet other TGFβ family members (such as BMP), Snail family members, FGF and Wnt play a role in embryonic single cell migration.[29] Thus, it seems that several factors capable of inducing cell migration and invasion can activate both collective and single cell migration and invasion. Details of the interaction between podoplanin and preserved E-cadherin expression are still unclear. In the present study, the expression level of E-cadherin protein was decreased after knockdown of podoplanin in TE-13 cells. Meanwhile, that of E-cadherin protein was not changed after transfection of podoplanin in TE-15 cells. Further research is required to unravel the molecular circumstances that modulate the effect of pro-migratory factors in target cells and determine the resulting invasion pattern.

Anti-human podoplanin antibodies (NZ-1 and NZ-8) possess high binding affinities and can neutralize platelet aggregation and antibody-dependent cellular cytotoxicity.[30] Targeted therapy against podoplanin-expressing cancers may be useful as a novel immunotherapy.

In conclusion, the present study showed that the forced expression of podoplanin was sufficient to promote collective cell invasion by engrafted ESCC cell lines. ESCC cells with podoplanin expression invaded without losing epithelial morphology or E-cadherin expression and without inducing expression of vimentin. Clinical specimens from ESCC patients demonstrated that podoplanin-expressing cells were capable of metastasizing despite the retention of E-cadherin expression. These observations belie the assumption that a complete transition to a mesenchymal phenotype is required for invasion and metastasis of carcinoma cells. Rather, collective cell invasion combined with podoplanin expression may represent an alternative mechanism that contributes to advancing malignancy in ESCC.

Disclosure Statement

The authors have no conflict of interest to declare.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. The case of double positive expression of podoplanin and vimentin. (a) H&E. The cancer cells invaded as cohorts are positive for podoplanin (b), while the cancer cells showed single cell invasion are positive for vimentin (d). Both carcinoma cells are negative for E-cadherin (c).