5 Hurwitz H, Fehrenbacher L, Novotny W, Cartwright T, Hainsworth J, Heim W, Berlin J, Baron A, Griffing S, Holmgren E, Ferrara N, Fyfe G, Rogers B, Ross R, Kabbinavar F: Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. N Engl J Med 2004;350:2335–2342.
6 Grothey A, Sargent D, Goldberg RM, Schmoll HJ: Survival of patients with advanced colorectal cancer improves with the availability of fluorouracil-leucovorin, irinotecan, and oxaliplatin in the course of treatment. J Clin Oncol 2004;22:1209–1214.
7 Grothey A, Sargent D: Overall survival of patients with advanced colorectal cancer correlates with availability of fluorouracil, irinotecan, and oxaliplatin regardless of whether doublet or single-agent therapy is used first line. J Clin Oncol 2005;23:9441–9442.
8 Falcone A, Ricci S, Brunetti I, Pfanner E, Allegrini G, Barbara C, Crino L, Benedetti G, Evangelista W, Fanchini L, Cortesi E, Picone V, Vitello S, Chiara S, Granetto C, Porcile G, Fioretto L, Orlandini C, Andreuccetti M, Masi G: Phase III trial of infusional fluorouracil, leucovorin, oxaliplatin, and irinotecan (FOLFOXIRI) compared with infusional fluorouracil, leucovorin, and irinotecan (FOLFIRI) as first-line treatment for metastatic colorectal cancer: the Gruppo Oncologico Nord Ovest. J Clin Oncol 2007;25:1670–1676.
9 Souglakos J, Androulakis N, Syrigos K, Polyzos A, Ziras N, Athanasiadis A, Kakolyris S, Tsousis S, Kouroussis C, Vamvakas L, Kalykaki A, Samonis G, Mavroudis D, Georgoulias V: FOLFOXIRI (folinic acid, 5-fluorouracil, oxaliplatin and irinotecan) vs FOLFIRI (folinic acid, 5-fluorouracil and irinotecan) as first-line treatment in metastatic colorectal cancer (MCC): a multicentre randomised phase III trial from the Hellenic Oncology Research Group (HORG). Br J Cancer 2006;94:798–805.
10 Masi G, Loupakis F, Salvatore L, Fornaro L, Cremolini C, Cupini S, Ciarlo A, Del Monte F, Cortesi E, Amoroso D, Granetto C, Fontanini G, Sensi E, Lupi C, Andreuccetti M, Falcone A: Bevacizumab with FOLFOXIRI (irinotecan, oxaliplatin, fluorouracil, and folinate) as first-line treatment for metastatic colorectal cancer: a phase 2 trial. Lancet Oncol 2010;11:845–852.
11 Montagnani F, Chiriatti A, Turrisi G, Francini G, Fiorentini G: A systematic review of FOLFOXIRI chemotherapy for the first-line treatment of metastatic colorectal cancer: improved efficacy at the cost of increased toxicity. Colorectal Dis 2011;13:846–852.
12 Masi G, Allegrini G, Cupini S, Marcucci L, Cerri E, Brunetti I, Fontana E, Ricci S, Andreuccetti M, Falcone A: First-line treatment of metastatic colorectal cancer with irinotecan, oxaliplatin and 5-fluorouracil/leucovorin (FOLFOXIRI): results of a phase II study with a simplified biweekly schedule. Ann Oncol 2004;15:1766–1772.
13 Trotti A, Colevas AD, Setser A, Rusch V, Jaques D, Budach V, Langer C, Murphy B, Cumberlin R, Coleman CN, Rubin P: CTCAE v3.0: development of a comprehensive grading system for the adverse effects of cancer treatment. Semin Radiat Oncol 2003;13:176–181.
14 Therasse P, Arbuck SG, Eisenhauer EA, Wanders J, Kaplan RS, Rubinstein L, Verweij J, Van Glabbeke M, van Oosterom AT, Christian MC, Gwyther SG: New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. J Natl Cancer Inst 2000;92:205–216.
15 Vaidyanathan G, Groman A, Wilding G, Fakih MG: Stop and go FOLFOX plus bevacizumab chemotherapy in the first-line treatment of metastatic colorectal cancer. Oncology 2010;79:67–71.
16 Tournigand C, Cervantes A, Figer A, Lledo G, Flesch M, Buyse M, Mineur L, Carola E, Etienne PL, Rivera F, Chirivella I, Perez-Staub N, Louvet C, Andre T, Tabah-Fisch I, de Gramont A: OPTIMOX1: a randomized study of FOLFOX4 or FOLFOX7 with oxaliplatin in a stop-and-go fashion in advanced colorectal cancer – a GERCOR study. J Clin Oncol 2006;24:394–400.
17 Aparicio J, Fernandez-Martos C, Vincent JM, Maestu I, Llorca C, Busquier I, Campos JM, Perez-Enguix D, Balcells M: FOLFOX alternated with FOLFIRI as first-line chemotherapy for metastatic colorectal cancer. Clin Colorectal Cancer 2005;5:263–267.
18 Hebbar M, Tournigand C, Lledo G, Mabro M, Andre T, Louvet C, Aparicio T, Flesch M, Varette C, de Gramont A, Oncology Multidisciplinary Research G: Phase II trial alternating FOLFOX-6 and FOLFIRI regimens in second-line therapy of patients with metastatic colorectal cancer (FIREFOX study). Cancer Invest 2006;24:154–159.
19 Recchia F, Candeloro G, Necozione S, Bratta M, Bisegna R, Rea S: Alternating XELFOX and XELFIRI in patients with metastatic colorectal cancer. Am J Clin Oncol 2008;31:323–328.

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

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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

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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,

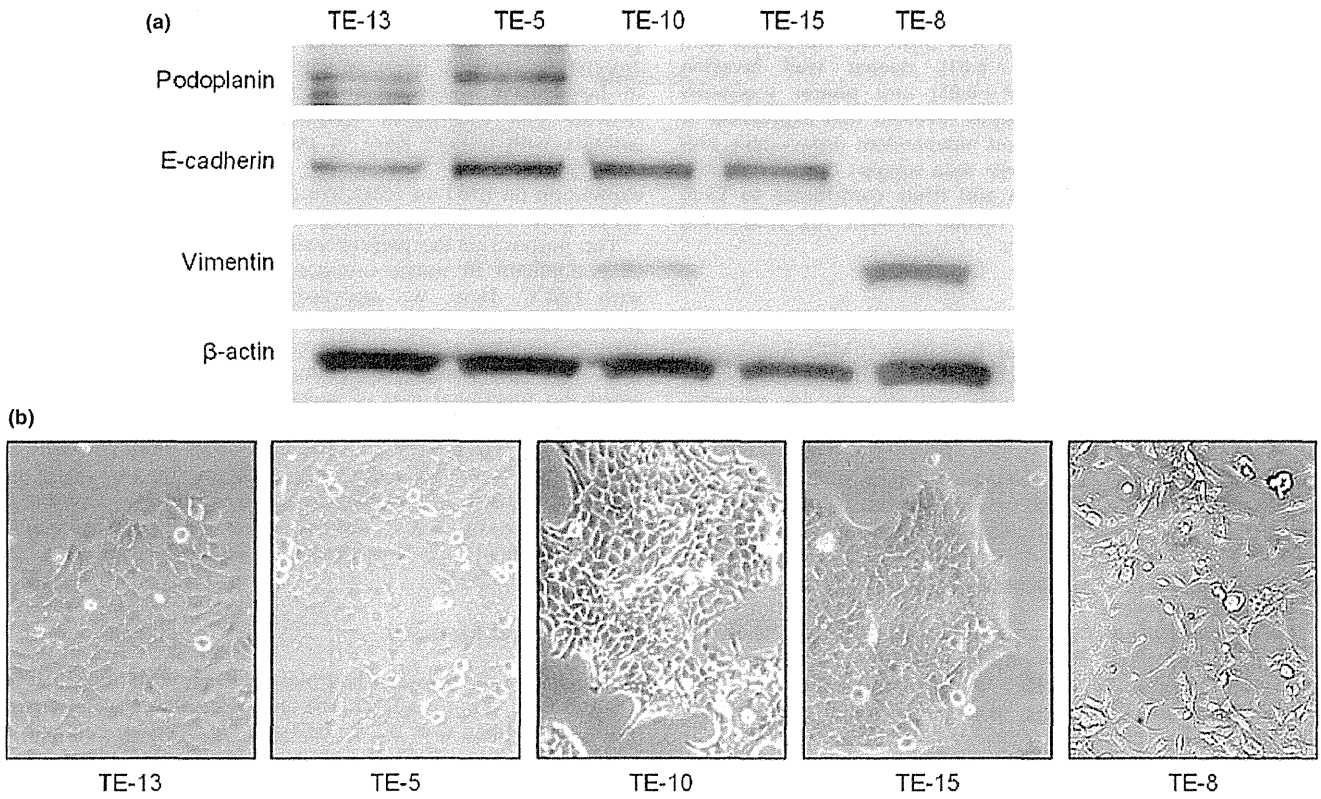

Fig. 1. Endogenous expression of podoplanin, E-cadherin and vimentin in ESCC cells. (a) Western blot analysis of podoplanin, E-cadherin and vimentin in ESCC cells (TE-13, TE-5, TE-10, TE-15 and TE-8). (b) Morphological observations of TE-13, TE-5, TE-10, TE-15 and TE-8 cells. Only TE-8 cells showed fibroblast-like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

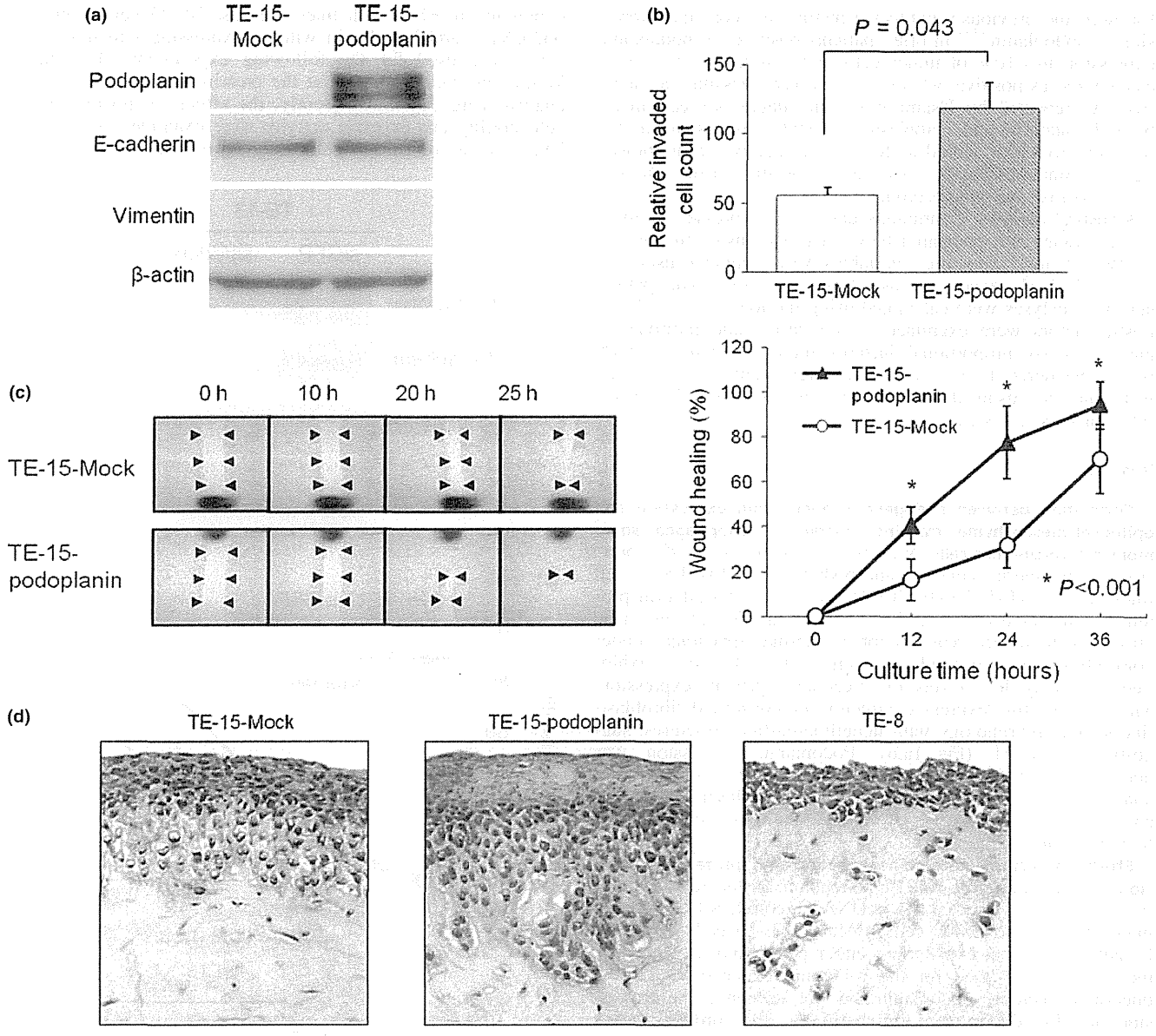

Fig. 2. Transduction of ectopic podoplanin promoted cell motility and invasion/migration activities. (a) Western blot analysis of podoplanin, E-cadherin and vimentin in podoplanin-transfected TE-15 cells (TE-15-podoplanin) and vector-transfected TE-15 cells (TE-15-Mock). (b) Matrigel invasion assay revealed 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

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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

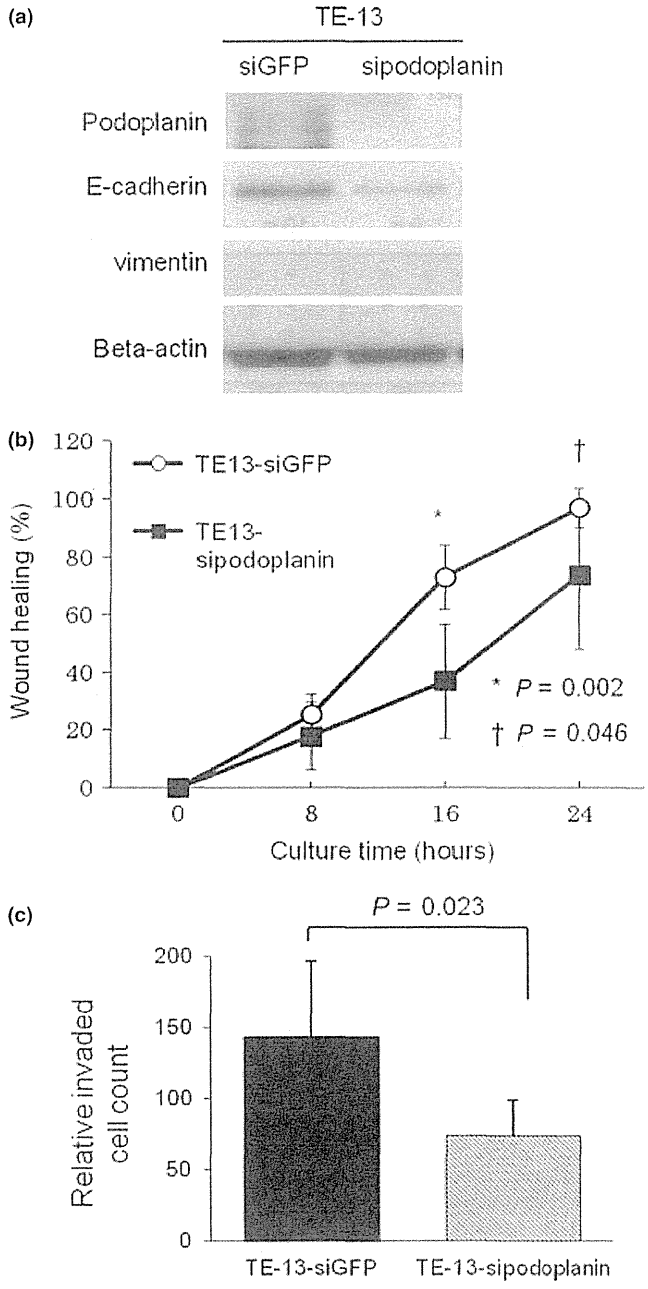

Fig. 3. Transfection with siRNA oligonucleotides targeting podoplanin mRNA or control in TE-13 cell line. (a) Western blot analysis of podoplanin, E-cadherin and vimentin expression after transfection of podoplanin knockdown TE-13 cells (TE-13-sipodoplanin) and GFP knockdown TE-13 cells (TE-13-siGFP). (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.

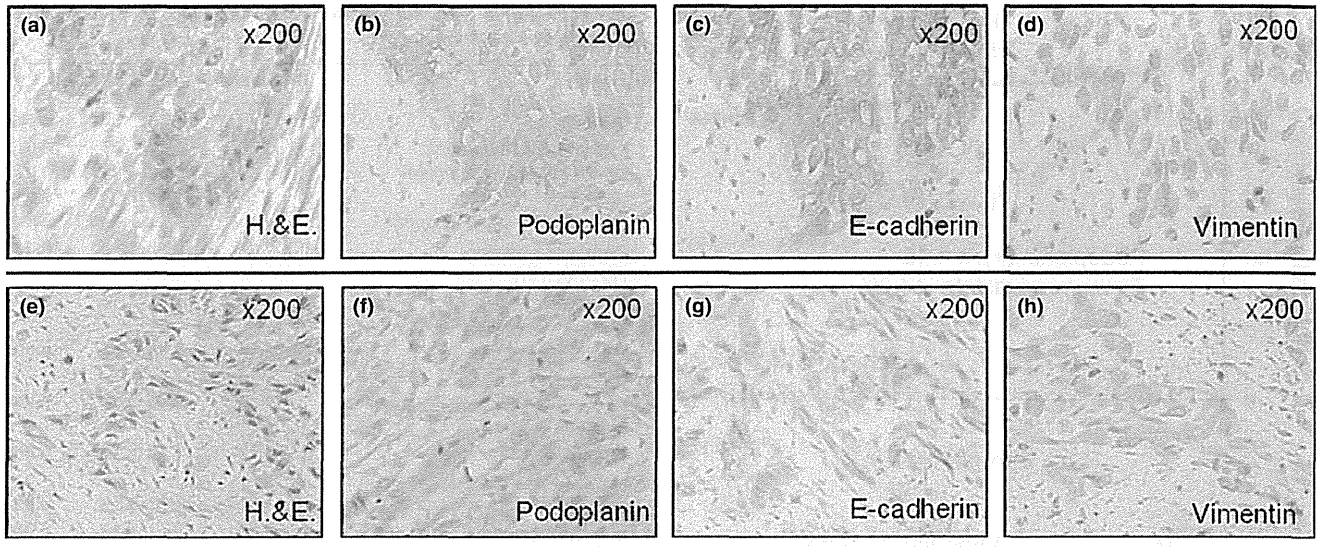

Fig. 4. Immunohistochemical expression of podoplanin, E-cadherin, and vimentin in ESCC samples. Invasive front of two representative cases are shown: (a–d) collective cell invasion sample; (e–h) single cell invasion sample. In the collective invasion sample (a–d), expression of podoplanin (b) and E-cadherin (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.

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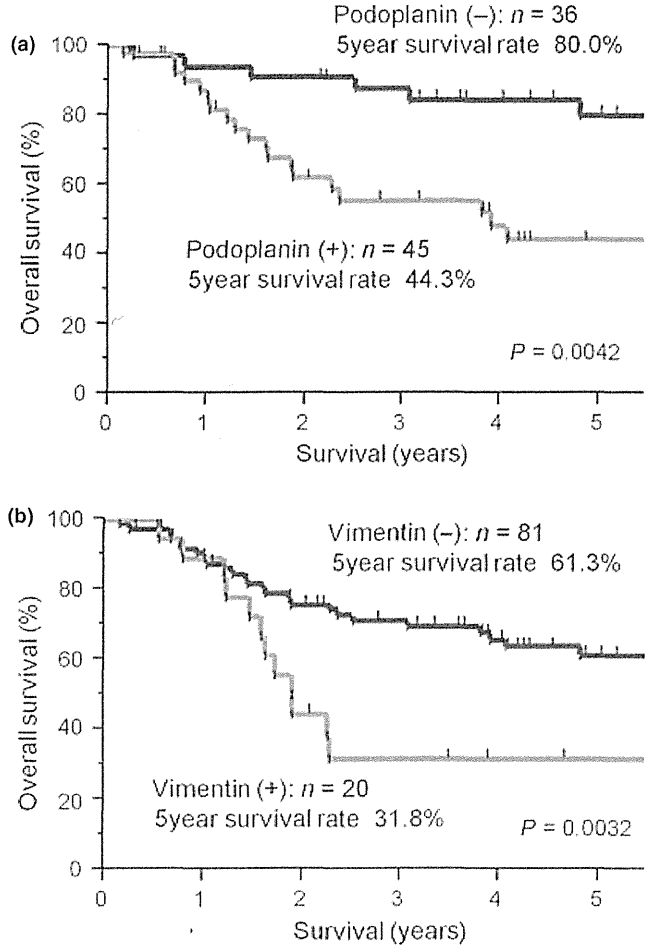

Fig. 5. The overall survival curve for ESCC patients. (a) The overall survival curve of vimentin-negative ESCC patients for positive versus negative expression of podoplanin. (b) The overall survival curve of all ESCC patients for positive versus negative expression of vimentin.

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.

References

1 Thiery JP, Sleeman JP. Complex networks orchestrate epithelial–mesenchymal transitions. *Nat Rev Mol Cell Biol* 2006; **7**: 131–42.
2 Davidson LA, Keller RE. Neural tube closure in Xenopus laevis involves medial migration, directed protrusive activity, cell intercalation and convergent extension. *Development* 1999; **126**: 4547–56.
3 Simian M, Hirai Y, Navre M, Werb Z, Lochter A, Bissell MJ. The interplay of matrix metalloproteinases, morphogens and growth factors is necessary for branching of mammary epithelial cells. *Development* 2001; **128**: 3117–31.
4 Friedl P, Gilmour D. Collective cell migration in morphogenesis, regeneration and cancer. *Nat Rev Mol Cell Biol* 2009; **10**: 445–57.
5 Christiansen JJ, Rajasekaran AK. Reassessing epithelial to mesenchymal transition as a prerequisite for carcinoma invasion and metastasis. *Cancer Res* 2006; **66**: 8319–26.
6 Friedl P, Hegerfeldt Y, Tusch M. Collective cell migration in morphogenesis and cancer. *Int J Dev Biol* 2004; **48**: 441–9.
7 Sahai E. Mechanisms of cancer cell invasion. *Curr Opin Genet Dev* 2005; **15**: 87–96.
8 Rorth P. Collective cell migration. *Annu Rev Cell Dev Biol* 2009; **25**: 407–29.
9 Schacht V, Dadras SS, Johnson LA, Jackson DG, Hong YK, Detmar M. Up-regulation of the lymphatic marker podoplanin, a mucin-type transmembrane glycoprotein, in human squamous cell carcinomas and germ cell tumors. *Am J Pathol* 2005; **166**: 913–21.
10 Wicki A, Christofori G. The potential role of podoplanin in tumour invasion. *Br J Cancer* 2007; **96**: 1–5.
11 Wicki A, Lehembre F, Wick N, Hantusch B, Kerjaschki D, Christofori G. Tumor invasion in the absence of epithelial–mesenchymal transition: podoplanin-mediated remodeling of the actin cytoskeleton. *Cancer Cell* 2006; **9**: 261–72.
12 Morita M, Yoshida R, Ikeda K *et al.* Advances in esophageal cancer surgery in Japan: an analysis of 1000 consecutive patients treated at a single institute. *Surgery* 2008; **143**: 499–508.
13 Rahadiani N, Ikeda J, Makino T *et al.* Tumorigenic role of podoplanin in esophageal squamous-cell carcinoma. *Ann Surg Oncol* 2010; **17**: 1311–23.
14 Tong L, Yuan S, Feng F, Zhang H. Role of podoplanin expression in esophageal squamous cell carcinoma: a retrospective study. *Dis Esophagus* 2012; **25**: 72–80.
15 Chuang WY, Yeh CJ, Wu YC *et al.* Tumor cell expression of podoplanin correlates with nodal metastasis in esophageal squamous cell carcinoma. *Histol Histopathol* 2009; **24**: 1021–7.
16 Kitao H, Kimura M, Yamamoto K *et al.* Regulation of histone H4 acetylation by transcription factor E2A in Ig gene conversion. *Int Immunol* 2008; **20**: 277–84.
17 Ikebe D, Wang B, Suzuki H, Kato M. Suppression of keratinocyte stratification by a dominant negative JunB mutant without blocking cell proliferation. *Genes Cells* 2007; **12**: 197–207.
18 Usami Y, Satake S, Nakayama F *et al.* Snail-associated epithelial-mesenchymal transition promotes oesophageal squamous cell carcinoma motility and progression. *J Pathol* 2008; **215**: 330–9.
19 Tarin D, Thompson EW, Newgreen DF. The fallacy of epithelial mesenchymal transition in neoplasia. *Cancer Res* 2005; **65**: 5996–6000.
20 Friedl P, Wolf K. Tumour-cell invasion and migration: diversity and escape mechanisms. *Nat Rev Cancer* 2003; **3**: 362–74.
21 Friedl P, Noble PB, Walton PA *et al.* Migration of coordinated cell clusters in mesenchymal and epithelial cancer explants *in vitro*. *Cancer Res* 1995; **55**: 4557–60.
22 Soulie P, Carrozzino F, Pepper MS, Strongin AY, Poupon MF, Montesano R. Membrane-type-1 matrix metalloproteinase confers tumorigenicity on nonmalignant epithelial cells.. *Oncogene* 2005; **24**: 1689–97.
23 Christiansen J, Rajasekaran AK. Biological impediments to monoclonal antibody-based cancer immunotherapy. *Mol Cancer Ther* 2004; **3**: 1493–501.
24 Green SK, Karlsson MC, Ravetch JV, Kerbel RS. Disruption of cell–cell adhesion enhances antibody-dependent cellular cytotoxicity: implications for antibody-based therapeutics of cancer. *Cancer Res* 2002; **62**: 6891–900.
25 Yamamoto M, Doki Y, Shiozaki H *et al.* Evaluation of the histologic effect of chemoradiation therapy for squamous cell carcinomas of the esophagus

doi: 10.1111/cas.12286

by assessing morphologic features of surgical specimens. *Dis Esophagus* 2000; **13**: 293–300.
26 Myashita M, Tajiri T, Sasajima K *et al.* Response to preoperative chemotherapy affects prognosis in esophageal cancer. *Dis Esophagus* 2003; **16**: 99–101.
27 Lowe KL, Navarro-Nunez L, Watson SP. Platelet CLEC-2 and podoplanin in cancer metastasis. *Thromb Res* 2012; **129**(Suppl. 1): S30–7.
28 Martin-Villar E, Megias D, Castel S, Yurrita MM, Vilaro S, Quintanilla M. Podoplanin binds ERM proteins to activate RhoA and promote epithelial-mesenchymal transition. *J Cell Sci* 2006; **119**: 4541–53.
29 Locascio A, Nieto MA. Cell movements during vertebrate development integrated tissue behaviour versus individual cell migration. *Curr Opin Genet Dev* 2001; **11**: 464–9.
30 Kaneko MK, Kunita A, Abe S *et al.* Chimeric anti-podoplanin antibody suppresses tumor metastasis through neutralization and antibody-dependent cellular cytotoxicity. *Cancer Sci* 2012; **103**: 1913–9.
31 Japanese Society for Esophageal Diseases. *Guidelines for the Clinical and Pathologic Studies on Carcinoma of the Esophagus*, 9th edn. Tokyo: Kanehara, 2007. [In Japanese.]

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).

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AKT Activation and Telomerase Reverse Transcriptase Expression are Concurrently Associated with Prognosis of Gastric Cancer

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Key Words
AKT · TERT · Telomerase · Prognosis

Abstract
AKT is a protein in the phosphatidylinositol-3 kinase (PI3K) pathway and associated with diverse pro-tumoral responses. Activation of the human telomere reverse transcriptase (hTERT) is one of AKT's tumorigenic effects. In this study, the significance of AKT phosphorylation and hTERT on prognosis of gastric cancer were examined. AKT activation by epidermal growth factor increased hTERT expression and telomerase activity. In contrast, AKT inactivation by inhibitors and knockdown decreased hTERT expression and telomerase activity in MKN28 gastric cancer cells. In 40 gastric cancer tissues, significant correlations were found among the levels of phosphorylated AKT (pAKT), hTERT expression, and telomer length. The pAKT levels or the levels of pAKT/hTERT were not associated with clinicopathological parameters, including stage and nodal metastasis. However, survival rates of the pAKT-high patients or the pAKT-high and hTERT-high patients were significantly poorer than those in other patients. These findings suggest that AKT and hTERT are good molecular targets for the treatment of gastric cancer.

Introduction

The telomere is a repetitive 'TTAGGG' sequence present at the ends of eukaryotic chromosomes to maintain and protect their integrity [1]. As cells divide, the telomere is shortened in length; thus, the length of the telomere behaves like a marker of the division limit for cells and/or for cell death [2]. In stem cells and cancer cells, the telomere is elongated by telomerase activity, which enables them to divide endlessly [3].

The catalytic subunit of human telomeras reverse transcriptase (hTERT) is responsible for telomerase activity

Abbreviations used in this article

PI3K	Phosphatidylinositol-3 kinase
hTERT	human telomerase reverse transcriptase
pAKT	phosphorylated AKT
EGF	epidermal growth factor
PTEN	phosphatase and tensin homolog deleted on chromosome 10
mTOR	mammalian target of rapamysin
NF	nuclear factor
EMT	epithelial-mesenchymal transition
VEGF	vascular endothelial growth factor

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and telomere elongation and is suppressed in differentiated cells [4]. In our previous study, we reported that telomere shortening is a significant factor for the induction of TERT expression in gastric mucosa [5].

AKT is a protein in the phosphatidylinositol-3 kinase (PI3K) pathway. Stimulation of receptor tyrosine kinases or G-proteins activates PI3K, which in turn activates AKT. AKT phosphorylation is maintained by heat shock protein 90, and AKT is dephosphorylated by protein phosphatase 2A. Thus, AKT is involved in signaling mediated by various growth factors and cytokines. In particular, insulin-like growth factor-1, epidermal growth factor receptor, and human epidermal growth factor receptor 2, which are important in cancer-progression, activate AKT [6, 7]. Hence, AKT is a one of biomarkers for predicting metastasis of human gastrointestinal cancer [8].

The phosphorylation of AKT modulates signals from phosphatase, tensin homolog deleted on chromosome 10 (PTEN), and the mammalian target of rapamycin to provide diverse effects on cells [9]. In this regard, AKT1 is recognized as an apoptotic inhibitor, which enhances cancer promotion. Phosphorylation via AKT inactivates Bcl-2 antagonist of cell death resulting in its dissociation from Bcl-2. Nuclear factor κB is also activated by AKT, which in turn up-regulates transcription of many survival genes [10]. AKT also induces angiogenesis through the up-regulation of vascular endothelial growth factor (VEGF) [11]. The AKT-microRNA regulatory network suggests that microRNA-mediated gene regulation interacts with the AKT signal pathway [12]. Hence, the expression of AKT is a pivotal tumorigenic factor and AKT is recognized as a relevant molecular target of cancer treatment [7].

The activity of hTERT is regulated by hTERT expression and phosphorylation. Protein kinase C and AKT can phosphorylate hTERT [13, 14]. AKT phosphorylation of hTERT induces intranuclear translocation of hTERT and, subsequently, activates hTERT. In contrast, ring finger protein 1, an E3 ubiquitin ligase, decreases the activity of hTERT by ubiquitylation [15].

In the present study, AKT phosphorylation is correlated with clinicopathological parameters such as TERT expression and telomerase activity in gastric cancer.

Materials and Methods

Cell Culture and Reagents

The human gastric cancer cell line MKN28 (kindly gifted from Professor Wataru Yasui, Hiroshima University, Japan) was maintained in Dulbecco's modified essential medium (Sigma Chemical Co., St. Louis, Mo., USA) containing 10% fetal bovine serum (Sigma Chemical Co.) at 5% CO_2 in air and 37°C. Wortmannin and triciribine were from Biovision LTD. (Milpitas, Calif., USA), and human epidermal growth factor was from Peprotec EC LTD. (Rocky Hill, N.J., USA).

Clinical Materials

Forty gastric tissues (approximately 30 mm^3) were randomly selected from cases diagnosed at the Department of Molecular Pathology, Nara Medical University, between 2001 and 2010. The tissues had been frozen quickly in liquid nitrogen and stored at −80°C. The tissue contents were confirmed by microscopic observation of the adjacent tissues, which were prepared for histopathological examination.

Short Interference RNA

FlexiTube short interference RNAs (siRNAs) for AKT was purchased from Qiagen Genomics, Inc. (Bothell, Wash., USA). All Stars Negative Control siRNA was used as control (Qiagen Genomics, Inc.). Cells were transfected with 50 nM siRNA for each gene using Lipofectamine 2000 (Invitrogen Corp., Carlsbad, Calif., USA) according to the manufacturer's instructions.

Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

Total RNA (1 μg) was used for cDNA synthesis with the ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan). qRT-PCR were performed on the StepOne Real-Time PCR System (Applied Biosystems, Foster City, Calif., USA) using the Fast SYBR Green Master Mix (Applied Biosystems) and analyzed by employing the relative standard curve quantification method. The PCR parameters were set according to the manufacturer's instructions and the beta-actin mRNA level was used as internal control. All amplifications were evaluated by melting curve analysis and PCR products were electrophoresed on 2% agarose gels. All PCRs were performed at least in triplicate. Primer sets were purchased from Santa-Cruz.

Immunoblot Analysis

Cell lysates were prepared as described previously [16]. Next, 25 μg (total protein) from lysates were electrophoresed on a 12.5% sodium dodecyl sulfate-polyacrylamide gel followed by electrotransfer to nitrocellulose membranes, which were subjected to immunoblot analysis. The membranes were incubated with primary antibodies and then probed with the appropriate peroxidase-conjugated secondary IgG antibodies (Medical and Biological Laboratories, Nagoya, Japan). Anti-tubulin antibody (LifeSpan Biosciences, Inc., Seattle, Wash., USA), anti-AKT antibody (Rockland Immunochemicals Inc., Gilbertsville, Pa., USA) and anti-phosphorylated AKT antibody (pSer463, Biorbyt, Cambridge, UK) were used as primary antibodies. The immune complex was visualized with the Enhanced Chemiluminescence Western-blot detection system (Amersham, Aylesbury, UK).

Enzyme-Linked Immunosorbent Assay (ELISA)

The lysates prepared as described above were also used for ELISA. Concentrations of pAKT and TERT were evaluated using specific ELISA kits, i.e., Akt (pS473) ELISA kit (Abcam, Cambridge,

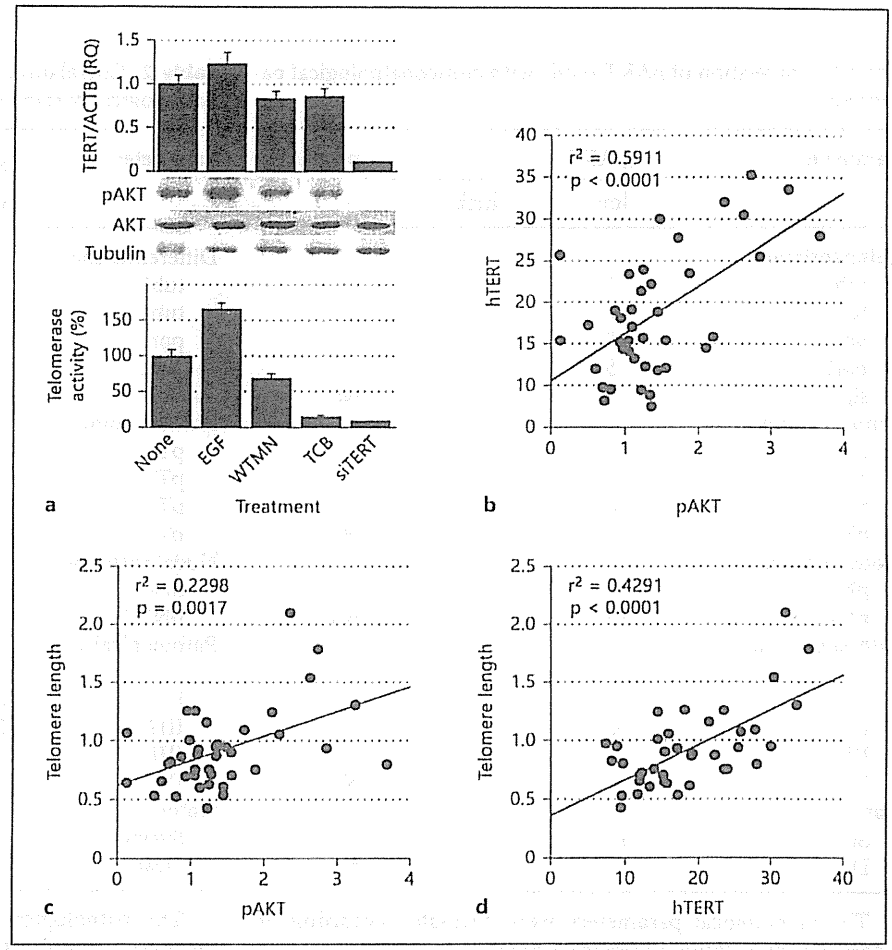

Fig. 1. Relationship between pAKT, hTERT, and telomerase activity. (**a**) Levels of pAKT protein and hTERT mRNA and telomerase activity were compared in MKN28 cells treated with EGF (10 ng/ml), wortmannin (10 nM), triciribine (10 μM), and siRNA against hTERT. Tubulin was used as loading controls. (**b–d**) Levels of pAKT protein and TERT mRNA and telomerase activity were compared in 40 gastric cancer cases. Levels of telomere length, pAKT and hTERT were represented as a relative value to that in peripheral blood lymphocytes, which was set to 1.0.

UK) and human telomerase reverse transcriptase (HTERT) ELISA kit (Oxford Expression Technology, Oxford, UK), according to the manufacturers' instructions.

Telomerase Activity and Telomere Length

Telomerase activity was examined by using the Quantitative Telomerase Detection Kit (US Biomax Inc., Rockville, Md., USA). The telomere length was examined by employing the TeloTAGGG Telomere Length Assay (Roche Applied Science, Indianapolis, Ind., USA). These kits were used according to the manufacturers' instructions.

Statistical Analysis

Statistical analyses of experimental data were carried out using the Spearman r test and ANOVA. The positivities of pAKT and TERT were compared using the two-tailed chi-squared test (InStat, Graphpad Software Inc., La Jolla, Calif., USA). Survival analysis was performed by using the Kaplan-Meier method along with the Logrank test. Univariate and multivariate analyses were calculated by using Cox's hazard model (SPSS Statistics, IBM Japan, Tokyo, Japan). Statistical significance was defined as a two-sided p value of less than 0.05.

Results

To examine the effect of AKT phosphorylation on hTERT expression and telomerase activity, MKN28 gastric cancer cells were analyzed after treatment under different conditions (fig. 1a). Epidermal growth factor stimulated AKT phosphorylation and increased hTERT expression and telomerase activity. In contrast, wortmannin, a PI3K inhibitor, triciribine, an AKT inhibitor, and siRNA-induced AKT knockdown inhibited AKT phosphorylation and decreased hTERT expression and telomerase activity. Total AKT protein levels were not affected by any treatment. These results suggest that AKT phosphorylation is associated closely with hTERT expression and telomerase activity.

Phospho-AKT (pAKT) levels, hTERT protein levels, and telomerase activity were examined in 40 cases of gastric cancer (fig. 1b–d). The pAKT level correlated with the hTERT level and telomerase activity, and the hTERT level correlated with telomerase activity ($p < 0.0001$, $p =$

Table 1. Correlation of pAKT levels with clinicopathological parameters

Parameter	pAKT		p value
	low	high	
Differentiation			
tub1	5	6	
tub2	8	5	
por1	0	2	
por2	5	5	
sig	2	2	NS
Primary tumor			
pT1	4	1	
pT2	3	3	
pT3	6	7	
pT4	7	9	NS
Nodal metastasis			
pN0	7	6	
pN1-2	13	14	NS
Pathological stage			
I	5	3	
II	4	4	
IIIA	6	5	
IIIB	5	3	
IV	0	5	NS
Status			
Survive	13	9	
Dead	7	11	NS

The pathological parameters were evaluated according to Japanese Classification of Gastric Cancer.

Table 2. Correlation of pAKT and hTERT levels with clinicopathological parameters

Parameter	pAKT and hTERT			p value
	both high	intermed	both low	
Differentiation				
tub1	2	8	1	
tub2	2	7	4	
por1	0	2	0	
por2	4	4	2	
sig	1	3	0	NS
Primary tumor				
pT1	1	1	3	
pT2	1	5	0	
pT3	3	8	2	
pT4	4	10	2	NS
Nodal metastasis				
pN0	2	6	5	
pN1-2	7	18	2	NS
Pathological stage				
I	1	4	3	
II	2	5	1	
IIIA	2	7	2	
IIIB	1	6	1	
IV	3	2	0	NS
Status				
Survive	3	14	5	
Dead	6	10	2	NS

The pathological parameters were evaluated according to Japanese Classification of Gastric Cancer.

0.0017, and $p < 0.0001$, respectively). These correlations were compatible to those found in figure 1a.

Next, pAKT levels were compared with clinicopathological parameters (table 1). Parameters in the pAKT-High cases showed no significant difference when compared with those in the pAKT-Low cases. However, survival analysis showed that the pAKT-High cases had a significantly poorer prognosis than pAKT-Low cases (fig. 2a, $p = 0.0498$).

Next, the cases were divided into 3 categories, i.e., pAKT high and TERT high (Both High), pAKT low and TERT low (Both Low), and other cases (Intermediate), to examine the concurrent effect of pAKT and hTERT on disease progression (table 2). As shown in figure 2c, the both high cases were the 9 highest cases of the product of pAKT by hTERT. In contrast, the both low cases were the 7 lowest cases of the product. These cases were distinguishable by distribution of the products. Parameters of the Both-High cases showed no significant differences compared to those of the Both-Low cases. However, the survival analysis showed that the Both-High cases had a significantly poorer prognosis than the Both-Low and Intermediate cases (fig. 2b, $p = 0.0339$), and the Both-Low cases had a significantly better prognosis than the Intermediate cases. Thus, the levels of pAKT and TERT could be useful prognostic markers.

Finally, the significance of pAKT levels or pAKT/hTERT levels was examined by univariate and multivariate analyses to compare stage and nodal metastasis (Table 3). Nodal metastasis emerged as an independent factor for the prognosis, whereas pAKT levels, pAKT/hTERT levels, and stage were dependent factors.

Discussion

In our study, the pAKT level or pAKT/hTERT levels showed no association with any clinicopathological parameters; however, the levels correlated well with disease prognosis.

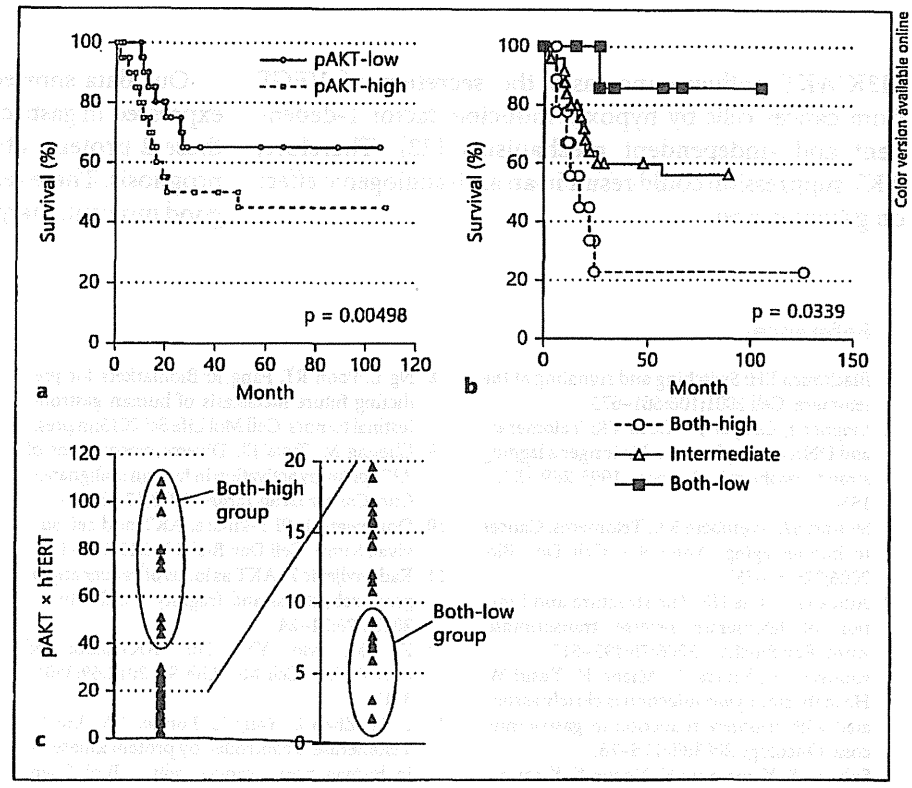

Fig. 2. Survival analyses of 40 gastric cancer cases. (**a**) Overall survival of in 20 cases with higher pAKT levels (pAKT High) was compared with that of 20 cases with lower pAKT levels (pAKT Low) by using the Kaplan-Meier method. (**b**) Overall survivals were compared between 9 cases with high AKT and high TERT (Both High), 7 cases with low pAKT and low TERT (Both High), and 24 cases with intermediate expressions (Intermed) by using the Kaplan-Meier method. (**c**) Distribution of the products of pAKT value by hTERT value in both high group (left panel) and both low group (right panel).

Table 3. Univariate and multivariate analyses of pAKT and/or hTERT levels

	Coefficient	95% CI	p value
Univariate analysis			
Stage	0.0252	0.005–0.524	0.0296
Nodal metastasis	7.613	1.727–33.562	0.0008
pAKT (High, Low)	2.453	0.945–6.368	0.0498
pAKT/hTERT	0.159	0.020–1.243	0.0198
Multivariate analysis			
Stage	1.077	0.236–4.916	0.9351
Nodal metastasis	0.132	0.028–0.627	0.0108
pAKT (high, low)	0.572	0.236–1.389	0.2172
pAKT/hTERT	0.793	0.267–2.353	0.7995

CI = Confidence interval. Both high, both low, intermed.

AKT is associated with cancer cell survival through altering Bcl-2 antagonist of cell death, p53, forkhead, nuclear factor κB, mammalian target of rapamycin, and PTEN [10] [17]. Moreover, dysregulated PTEN/PI3K/AKT signaling interacts with the Wingless-INT pathway to induce epithelial-mesenchymal transition (EMT), which is usually associated with cancer stem cell-phenotype and poor prognosis [18]. It has been recently reported that hTERT promotes transforming growth factor-β and β-catenin-induced EMT by inducing β-catenin nuclear translocation and its transcriptional activity for vimentin expression [19]. Therefore, PTEN/PI3K/AKT signaling enhances EMT and stem cell phenotypes. In the present study, the association of AKT phosphorylation, TERT expression, and telomerase activity was confirmed in MKN28 gastric cancer cells and tissues of 40 gastric cancer patients. These associations could result in poor prognoses in cases with high pAKT levels or high pAKT/hTERT levels. Multivariate analysis revealed that pAKT levels or pAKT/hTERT levels were dependent prognostic factors. The examination of more gastric cancer cases is required to confirm the hypothesis that the EMT/stem cell phenotype affects disease progression.

Angiogenesis is an essential phenotype for cancer progression [20]. VEGF expression is associated closely with neovascularization and cancer progression in many malignancies. The PI3K/AKT pathway is one of the inducers of a VEGF response, which includes other inducers such as mitogen-activated protein kinase (extracellular signal-regulated kinases or p38), Src, focal adhesion kinase, Rho family GTPases, and endothelial nitric oxide [21]. The

PI3K/AKT pathway increases the secretion of VEGF from cancer cells by hypoxia-inducible factor 1-dependent and -independent mechanisms [22]. Therefore, AKT suppression could result in an anti-angiogenic effect on gastric cancer.

Our data showed that AKT and hTERT were widely expressed in gastric cancer. The concurrent expression of these 2 proteins at high levels is associated with a poor prognosis. These results suggest that AKT and hTERT are good molecular targets for the treatment of gastric cancer.

References

1 Blackburn EH: Switching and signaling at the telomere. Cell 2001;106:661–673.
2 Lingner J, Cooper JP, Cech TR: Telomerase and DNA end replication: No longer a lagging strand problem? Science 1995;269:1533–1534.
3 Stewart SA, Weinberg RA: Telomeres: Cancer to human aging. Annu Rev Cell Dev Biol 2006;22:531–557.
4 Autexier C, Lue NF: The structure and function of telomerase reverse transcriptase. Annu Rev Biochem 2006;75:493–517.
5 Kuniyasu H, Kitadai Y, Mieno H, Yasui W: Helicobactor pylori infection is closely associated with telomere reduction in gastric mucosa. Oncology 2003;65:275–282.
6 Sukawa Y, Yamamoto H, Nosho K, Kunimoto H, Suzuki H, Adachi Y, Nakazawa M, Nobuoka T, Kawayama M, Mikami M, Matsuno T, Hasegawa T, Hirata K, Imai K, Shinomura Y: Alterations in the human epidermal growth factor receptor 2-phosphatidylinositol 3-kinase-v-AKT pathway in gastric cancer. World J Gastroenterol 2012;18:6577–6586.
7 Berg M, Soreide K: EGFR and downstream genetic alterations in KRAS/BRAF and PI3K/AKT pathways in colorectal cancer: Implications for targeted therapy. Discov Med 2012;14:207–214.
8 Ng L, Poon RT, Pang R: Biomarkers for predicting future metastasis of human gastrointestinal tumors. Cell Mol Life Sci 2013;in press.
9 Cheung M, Testa JR: Diverse mechanisms of AKT pathway activation in human malignancy. Curr Cancer Drug Targets 2013;13:234–244.
10 Downward J: PI 3-kinase, AKT and cell survival. Semin Cell Dev Biol 2004;15:177–182.
11 Radisavljevic Z: AKT as locus of cancer angiogenic robustness and fragility. J Cell Physiol 2013;228:21–24.
12 Xu M, Mo YY: The AKT-associated microRNAs. Cell Mol Life Sci 2012;69:3601–3612.
13 Li H, Zhao L, Yang Z, Funder JW, Liu JP: Telomerase is controlled by protein kinase Cα in human breast cancer cells. J Biol Chem 1998;273:33436–33442.
14 Kang SS, Kwon T, Kwon DY, Do SI: Akt protein kinase enhances human telomerase activity through phosphorylation of telomerase reverse transcriptase subunit. J Biol Chem 1999;274:13085–13090.
15 Kim JH, Park SM, Kang MR, Oh SY, Lee TH, Muller MT, Chung IK: Ubiquitin ligase MKRN1 modulates telomere length homeostasis through a proteolysis of hTERT. Genes Dev 2005;19:776–781.
16 Kuniyasu H, Oue N, Wakikawa A, Shigeishi H, Matsutani N, Kuraoka K, Ito R, Yokozaki H, Yasui W: Expression of receptors for advanced glycation end-products (RAGE) is closely associated with the invasive and metastatic activity of gastric cancer. J Pathol 2002;196:163–170.
17 Populo H, Lopes JM, Soares P: The mTOR signalling pathway in human cancer. Int J Mol Sci 2012;13:1886–1918.
18 Karamitopoulou E: Tumor budding cells, cancer stem cells and epithelial-mesenchymal transition-type cells in pancreatic cancer. Front Oncol 2012;2:209.
19 Liu Z, Li Q, Li K, Chen L, Li W, Hou M, Liu T, Yang J, Lindvall C, Bjorkholm M, Jia J, Xu D: Telomerase reverse transcriptase promotes epithelial-mesenchymal transition and stem cell-like traits in cancer cells. Oncogene 2012;advance online publication.
20 Fidler IJ: The pathogenesis of cancer metastasis: The 'seed and soil' hypothesis revisited. 2003;3:453–458.
21 Claesson-Welsh L, Welsh M: VEGFA and tumour angiogenesis. J Intern Med 2013;273:114–127.
22 Karar J, Maity A: PI3K/AKT/mTOR pathway in angiogenesis. Front Mol Neurosci 2011;4:51.
23 Japanese Gastric Cancer Association: Japanese classification of gastric carcinoma: 3rd English edition. Gastric Cancer 2011;14:101–112.

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MK-1 Expression in Gastric Carcinoma with Liver Metastasis

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Objective: The prognosis for gastric carcinoma patients with liver metastasis is very poor. This retrospective study investigated the prognostic significance of MK-1 expression in gastric carcinoma patients with liver metastasis.

Methods: Immunohistochemical staining using monoclonal antibody FU-MK-1 against MK-1 antigen was performed on paraffin-embedded tissues from 64 gastric carcinoma patients with liver metastasis. We attempted to determine the presence of any relationship between pathological prognostic factors and the expression of MK-1 in 64 gastric carcinoma patients with liver metastasis.

Results: MK-1 expression was found in 43 (67%) of 64 tumor samples. MK-1 expression was significantly higher in the intestinal type (73%) than in the diffuse type carcinoma (33%, $P = 0.049$). Multivariate analysis showed that MK-1 expression and lymph node metastasis were significant factors for overall survival. The difference between overall survival rates with positive or negative MK-1 expression was statistically significant as shown by Kaplan–Meier survival analysis ($P < 0.0001$; log-rank). In addition, the difference between cumulative disease-free survival rates with positive or negative MK-1 expression in gastric carcinoma patients with metachronous liver metastasis was statistically significant as well, as shown by Kaplan–Meier survival analysis ($P = 0.0006$; log-rank).

Conclusions: The prognostic significance of MK-1 expression as a biological tumor marker was demonstrated in a series of gastric carcinoma patients with liver metastasis. MK-1 positivity may be a reliable marker for predicting and taking measures to control liver metastasis after curative gastrectomy for gastric carcinoma.

Key words: FU-MK-1 – MK-1 – gastric carcinoma – liver metastasis

INTRODUCTION

Gastric carcinoma (GC) is one of the major causes of cancer-related death in the world, even though its incidence has decreased over the years (1). GC is the second most common cause of death due to cancer in Japan (2). Radical surgical treatment including lymph node dissection and adjuvant chemotherapy is used for patients with primary GC (3). But many patients suffer from a variety of metastatic patterns. Among these, liver metastasis (LM) of gastric cancer after curative gastrectomy is one of the most important factors for prognosis. This is found in 3–5% of patients with GC (4,5), predicting a 5-year survival of <10% (6), as current treatments are uncertain and ineffective. Considering the potential of adjuvant chemotherapy to improve the prognosis of GC with LM, it is imperative to identify any appropriate prognostic markers.

The monoclonal antibody (MAb) FU-MK-1, which recognizes the MK-1 antigen, was established by immunizing a mouse with cancerous ascites derived from a poorly differentiated adenocarcinoma of the stomach (7). The target

molecule of FU-MK-1 is encoded by the GA733-2 gene and the epitope for MAb FU-MK-1 is present on the distal half of the extracellular domain of the GA733-2 antigen. More than 20 antibodies directed against the GA733-2 antigen have been generated, including HEA125 for Ep-CAM, CO17-1A for 17-1A and FU-MK-1 for MK-1. However, because of the differences in their immunohistochemical reactivities, FU-MK-1 might recognize a different epitope group than that recognized by Ep-CAM and 17-1A (8). Some reports suggested that the epithelial cellular adhesion molecule is an attractive immunotherapeutic target to overcome the metastasis of a variety of epithelium-oriented cancers (9–11).

In this study, we attempted to determine the presence of any relationship between pathological prognostic factors and the expression of MK-1 in GC patients with LM.

PATIENTS AND METHODS

Patients

A total of 64 GC patients with LM after gastrectomy from 1994 to 2007 were included: 26 patients with synchronous LM and 38 patients with metachronous LM; whereas those with recurrent lesions in other locations as well were excluded. All patients underwent preoperative diagnosis, resection and postoperative follow-up in the Department of Surgery at Fukuoka University. Patients comprised 49 men and 15 women, ranging in age from 38 to 88 years (mean ± SD, 65.3 ± 9.9 years). Follow-up ranged from 1 to 147 months and the median was 27 months. CT scans were obtained with every visit.

Tumor Samples and Histologic Examination

Tissue samples were fixed in 10% formalin and cut into 3- to 4-mm slices. The tissue blocks were taken from the tumor and then routinely processed to paraffin sections (3 μm). All sections were stained with hematoxylin and eosin (H&E) and examined. The pathologic diagnosis and classification of the primary cancer were performed by a minimum of two pathologists according to the Japanese Classification of Gastric Carcinoma (12). Tumors were histologically classified into two types based on the predominant features according to the criteria of Lauren (13); well differentiated, moderately differentiated and papillary adenocarcinomas were categorized as intestinal type carcinomas; and poorly differentiated adenocarcinomas and signet ring cell carcinomas as diffuse type carcinomas. In all cases, additional staining using Victoria blue-H&E double stains (VB-HE) was performed for the evaluation of venous permeation by the carcinoma.

Immunohistochemical Analysis

Sixty-four tumor specimens were obtained at surgery. All specimens were formalin-fixed, paraffin-embedded and immunohistochemically stained with MAb FU-MK-1 as described previously (7). Stained slides were evaluated in all cases. An FU-MK-1 reaction was defined as the presence of specific staining on the surface membranes of tumor cells. The reaction was evaluated for membranous and occasional cytoplasmic staining. MK-1 expression was determined to be positive when more than 10% of tumor cells exhibited a positive reaction for the FU-MK-1 antibody. In all cases, additional immunostaining using MAb D2-40 was performed for the evaluation of lymphatic permeation by the carcinoma.

Statistical Analysis

The relationship between MK-1 expression and clinicopathologic factors was analyzed by using the χ^2 test, Fisher's exact probability test and Spearman's rank correlation test using StatView, version 5.0 (SAS Institute, Cary, NC). Survival curves were obtained by using the Kaplan–Meier method and compared by using the log-rank test. Multivariate analysis of prognostic factors was based on the Cox proportional hazards model. Results were considered significant if a two-sided P value of <0.05 was obtained.

RESULTS

The FU-MK-1 antibody did not react with the gastric foveolar epithelium, but it reacted strongly with the membranes of the gastric mucosa with intestinal epithelial metaplasia (Fig. 1A and B). MK-1 expression was found in 43 (67%) of 64 GC patients with LM samples. The expression was strongly and diffusely located in the cell membranes of carcinoma (Fig. 2A and B). No MK-1 expression was found in 21 (33%) of 64 GC with LM samples (Fig. 3A and B). These are the MK-1 expression of tumor cells with lymphatic or venous permeation by the carcinoma (Fig. 4A and B). D2-40 and VB-HE were performed for the evaluation of lymphatic or venous permeation by the carcinoma. The relationship between MK-1 expression and clinicopathologic variables is shown in Tables 1 and 2. In relation to tumor histologic type, MK-1 expression was significantly but marginally higher ($P = 0.049$) in the intestinal type (40 patients; 73%) than in diffuse type carcinomas (3 patients; 33%). The presence of lymph node metastasis or venous permeation was also correlated with MK-1 expression. However, MK-1 expression did not reach statistical significance for other clinicopathologic variables.

The mean survival time of the 64 patients was 26 ± 3 months (mean ± SEM), and no perioperative mortality occurred. The median survival time of the MK-1 positive group was 19 months, while that of the negative group was 38 months. The difference between the overall survival rates with positive or negative MK-1 expression was statistically significant as shown by the Kaplan–Meier survival analysis ($P < 0.0001$; log-rank) (Fig. 5).

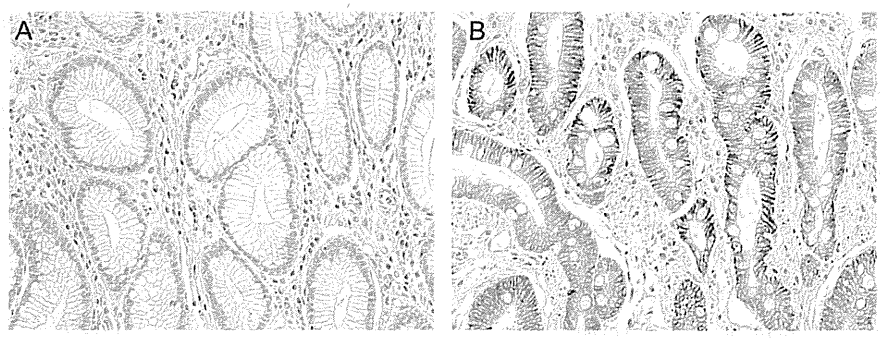

Figure 1. MK-1 expression in the gastric mucosa. (**A**) No MK-1 expression was shown in the normal gastric foveolar epithelium (×200). (**B**) MK-1 expression was shown in the membranes of the gastric mucosa with intestinal epithelial metaplasia (×200).

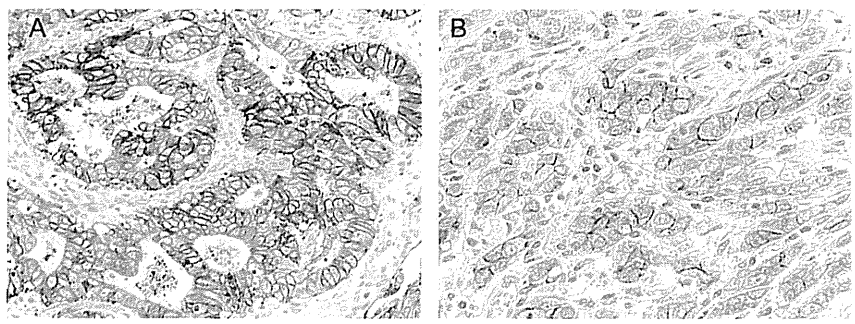

Figure 2. MK-1 expression was strongly and diffusely positive in membranes of tumor cells. (**A**) Intestinal-type carcinoma (×200) and (**B**) diffuse-type carcinoma (×200).

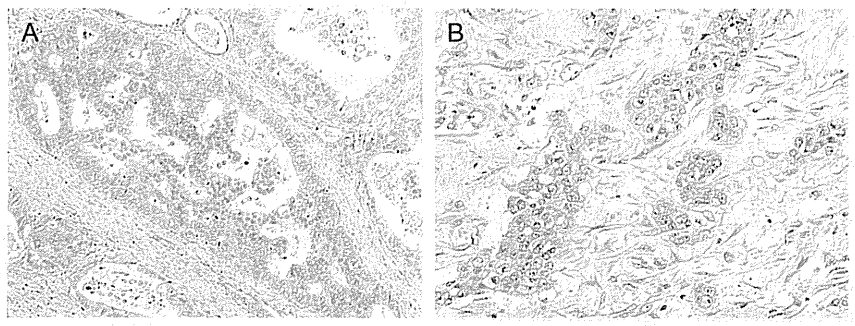

Figure 3. No MK-1 expression in gastric carcinoma. Most of the tumor cells lacked immunoreactivity. (**A**) Intestinal-type carcinoma (×200) and (**B**) diffuse-type carcinoma (×200).

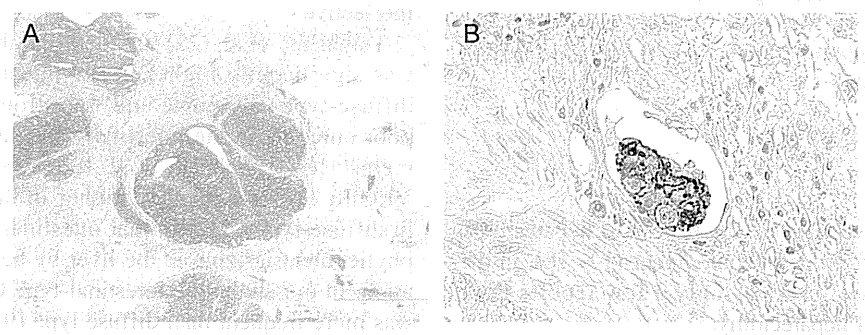

Figure 4. MK-1 expression with lymphatic and venous permeation by the carcinoma. (**A**) Venous invasion was present in the venous vessel (×40) and (**B**) lymphatic invasion in the lymphatic vessel (×200).

Table 1. Relationship of MK-1 expression with clinicopathologic variables in GC patients with LM

	Total	MK-1 expression		P
		Positive ($n = 43$)	Negative ($n = 21$)	
Sex[a]				
Male	49	30 (61%)	19 (39%)	0.11
Female	15	13 (87%)	2 (13%)	
Age[b] (years; mean, 65.5)				
≤65	29	17 (59%)	12 (41%)	0.18
65<	35	26 (74%)	9 (26%)	
Tumor size[b] (mean, 5.28cm)				
≤5.0 cm	27	20 (74%)	7 (26%)	0.99
5.0 cm<	37	23 (62%)	14 (38%)	
Macroscopic type[c]				
Early type	3	1 (33%)	2 (67%)	0.61
Borrmann type 1	4	4 (100%)	0	
Borrmann type 2	32	22 (69%)	10 (31%)	
Borrmann type 3	24	16 (67%)	8 (33%)	
Borrmann type 4	1	0	1 (100%)	
Histologic type[a] (Lauren classification)				
Intestinal type	55	40 (73%)	15 (27%)	0.049
Diffuse type	9	3 (33%)	6 (67%)	

[a]Fisher's exact probability test.
[b]χ^2 test.
[c]Spearman's rank correlation.

Table 2. Relationship of MK-1 expression with clinicopathologic variables in GC patients with LM

	Total	MK-1 expression		P
		Positive ($n = 43$)	Negative ($n = 21$)	
Depth of tumor invasion[a]				
T1a	0	0	0	0.85
T1b	3	1 (33%)	2 (67%)	
T2	29	21 (72%)	8 (28%)	
T3	30	19 (63%)	11 (37%)	
T4a	2	2 (100%)	0	
T4b	0	0	0	
Lymph node metastasis[b]				
Positive	50	35 (70%)	15 (30%)	0.37
Negative	14	8 (57%)	6 (43%)	
Lymph node metastasis stage[a]				
N0	14	8 (57%)	6 (43%)	0.57
N1	24	19 (79%)	5 (21%)	
N2	20	14 (70%)	6 (30%)	
N3	6	2 (33%)	4 (67%)	
Lymphatic permeation[b]				
Positive	30	20 (67%)	10 (33%)	0.93
Negative	34	23 (68%)	11 (32%)	
Venous permeation[b]				
Positive	49	32 (65%)	17 (35%)	0.76
Negative	15	11 (73%)	4 (27%)	

[a]Spearman's rank correlation.
[b]Fisher's exact probability test.

In addition, with respect to disease-free survival before metachronous LM, the median disease-free survival time of the MK-1 positive group was 9 months, while that of the negative group was 19 months. The difference between cumulative disease-free survival rates with positive or negative MK-1 expression was also statistically significant as shown by the Kaplan–Meier survival analysis ($P = 0.0006$; log-rank) (Fig. 6).

Univariate analysis showed that MK-1 expression and lymph node metastasis were significant factors for overall survival. Multivariate analysis also revealed that MK-1 expression and lymph node metastasis were prognostic factors for overall survival (Table 3).

DISCUSSION

While many studies have reported the benefit of hepatectomy for metastatic tumors from colorectal cancer (14–16), in the case of liver metastases from GC only a few reports have dealt with the results of hepatectomy.

Hepatectomy is a popularly curative therapy for patients with LM from colorectal carcinoma, presenting a 5-year survival rate of 30–50%. However, hepatectomy for GC metastasis has worse results with a 1-year survival rate of 15–50% and 5-year survival rate of 19% (17–21). Because GC metastases are often multiple, scattered and bilobular, recurring with a combination of various patterns, current treatments such as systemic or hepatic artery infusion chemotherapy and surgical therapy are uncertain and ineffective.

Yamashita et al. (22) reported that the microvessel density was significantly higher in the intestinal-type GC than in diffuse-type carcinoma and was strongly associated with a poor outcome in the intestinal-type carcinoma with hepatic metastases. Takahashi et al. (23) reported that VEGF and VEGFR expressions were higher in the intestinal-type than in diffuse-type GC, and that intestinal type tends to be exophytic, metastasizing to the liver by hematogenous dissemination. In our study, the intestinal-type GC (55 patients; 86%) was more frequent than diffuse type (9 patients; 14%).

Watanabe et al. (7) reported that MK-1 expression may be useful for classifying cancers of the stomach and for

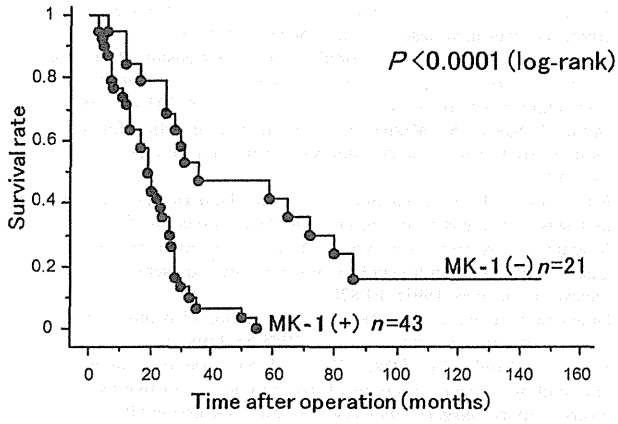

Figure 5. Correlation of MK-1 expression with prognosis. There is a significant correlation between MK-1 expression and prognosis.

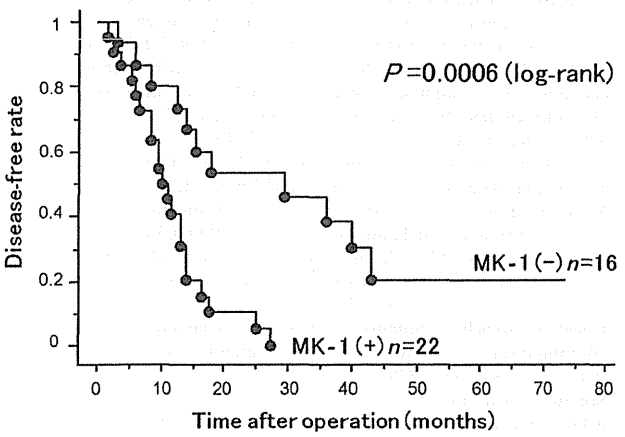

Figure 6. Correlation of MK-1 expression with disease-free rate. There is a significant correlation between MK-1 expression and disease-free rate.

Table 3. Multivariate prognostic parameters in GC patients with LM[a]

	Overall survival		
	P	Relative risk	95%CI
MK-1 expression	0.0023	0.340	0.170–0.680
Lymph node metastasis	0.0028	0.294	0.132–0.656

[a]Cox proportional hazards model.

detecting the metastatic foci of gastrointestinal adenocarcinoma in the lymph nodes and other organs. Hamada et al. (24) reported that MK-1 expression in carcinoma of the ampulla of Vater was significantly associated with histologic grades and was an independent prognostic factor of increased overall survival. Ikeda et al. (25) also reported similar results in gallbladder carcinoma. In our study, MK-1 expression was significantly higher in the intestinal-type than in diffuse-type GC (73 versus 33%). Multivariate analysis revealed that MK-1 expression and lymph node metastasis were prognostic factors for overall survival. We also show a significant relationship with poor overall survival and disease-free survival by Kaplan–Meier curves. Positive MK-1 expression was associated with a poor prognosis. We considered two reasons for this with biological background. First, Ep-CAM becomes part of a large nuclear complex containing transcriptional regulators β-catenin and Lef, which are both components of the wnt pathway (26). And it was associated with tumor cell proliferation, migration and invasion. Secondly, Ep-CAM was shown to interact directly with CD44v4-v7 and they supported tumor progression by promoting metastasis (27).

The relationship between lymph node metastasis and prognosis is well known (28). The high frequency of intestinal-type GC in our series of GC with LM may be consistent with the particular cell adhesion qualities of this histology, allowing easier penetration of the vascular wall at either the primary or metastatic tumor site and facilitating spread in the liver.

Ep-CAM is regarded as an antigen similar to MK-1 and there are some reports about Ep-CAM expression in GC. Songun et al. (29) reported that patients without the loss of Ep-CAM-expression by tumor cells had a significantly better 10-year survival rates compared with patients with any loss of expression. Went et al. (30) reported that on the GC microarray, 90.7% of the tumors expressed Ep-CAM on >70% of cells and 85.8% of the cases showed the highest level of staining intensity. No significant correlation of Ep-CAM expression between primary tumors, nodal spread or metastasis was found. Wengi et al. (31) reported that the average expression of Ep-CAM was higher in poorly differentiated tumor cells than in well and moderately differentiated tumor cells, and that Ep-CAM expression in patients with lymph node metastasis was significantly higher than that in patients without metastasis. In various reports about Ep-CAM expression by GC, results are inconsistent. In our study, the expression of Ep-CAM was positive in all patients with LM after curative gastrectomy. There is a discrepancy between previous studies using Ep-CAM and our results using MK-1. Because it is not clear why MK-1 expression differs in various organs or sites and different cancers, systematic analysis using a wide variety of normal human tissue and neoplasms is necessary.

In conclusion, the prognostic significance of MK-1 expression as a biological tumor marker was demonstrated in a series of GC patients with LM, predicting decreased median survival time and decreased median disease-free survival time. The prognostic significance of lymph node metastasis was similar. MK-1 positivity may be useful as a reliable marker for predicting and taking steps to control LM after curative gastrectomy for GC as an immunotherapeutic target.

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