Histol Histopathol (2009) 24: 1021-1027

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Tumor cell expression of podoplanin correlates with nodal metastasis in esophageal squamous cell carcinoma

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Summary. Podoplanin is a mucin-like glycoprotein expressed in the lymphatic endothelium. It has been suggested to play a role in lymphangiogenesis, since podoplanin deficient mice were found to have dilated malfunctioning lymphatic vessels and lymphedema. High podoplanin expression in tumor cells was found to correlate with lymph node metastasis and poor clinical outcome in patients with oral squamous cell carcinoma (SCC). However, the prognostic significance of podoplanin expression in esophageal SCC remains unexplored. Herein, we studied podoplanin expression in 59 patients who underwent surgical resection of esophageal SCC, with 43 of them preceded by preoperative concurrent chemoradiotherapy (CCRT). We found that high podoplanin expression strongly correlated with clinical nodal metastasis (cN1; p=0.0063), which was associated with short survival (p=0.012). However, there was no direct association between high podoplanin expression and short survival. We also found that lymphatic vessel invasion in the resected esophagus was strongly associated with pathological nodal metastasis (pN1; p=0.00092). Our results suggest that podoplanin could also play a role in tumor aggressiveness in esophageal SCC, as well as in oral SCC.

Key words: Podoplanin, Esophagus, Squamous cell carcinoma, Survival, Nodal metastasis

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Introduction

Podoplanin is a mucin-like transmembrane glycoprotein which is expressed in lymphatic endothelium but not in blood vessel endothelium (Breiteneder-Geleff et al., 1999; Kahn and Marks, 2002). Although the physiological function of podoplanin is still unknown, a possible role in lymphangiogenesis has been suggested, because podoplanin deficient mice were found to die of respiratory failure at birth, with a phenotype of dilated malfunctioning lymphatic vessels and lymphedema (Ramirez et al., 2003; Schacht et al., 2003). Recently, high level tumor cell expression of podoplanin was shown to correlate with lymph node metastasis and short survival in patients with oral squamous cell carcinoma (SCC) (Yuan et al., 2006). Although podoplanin expression has also been detected in tumor cells of esophageal SCC (Wicki et al., 2006), the prognostic significance of podoplanin expression level remains unexplored. To clarify the role of podoplanin in esophageal SCC, we investigated tumor cell expression of podoplanin in 59 patients of esophageal SCC who underwent surgical resection, with 43 of them preceded by preoperative concurrent chemoradiotherapy (CCRT).

Materials and methods

Patients

A total of 59 patients who underwent surgical resection of esophageal SCC within a period of 3 years were recruited for this study. Forty-three (73%) of them

received preoperative CCRT. The number of removed lymph nodes ranged from 1 to 26, with a median of 5. The number of positive lymph nodes ranged from 1 to 16, with a median of 1. The pathological stage was evaluated by pathological examination of resected esophagus and lymph nodes. The pre-treatment clinical stage was evaluated by imaging studies, including computed tomography scan and endoscopic ultrasonography. The clinicopathological characteristics of the patients are listed in Table 1.

Immunohistochemistry

Resected tumor tissue and adjacent normal esophageal tissue were fixed in 10% buffered formalin, dehydrated, and embedded in paraffin. Tissue sections were routinely stained with hematoxylin and eosin for pathological examination. Additional 4-µm tissue sections were taken, deparaffinized, and rehydrated through a series of graded ethanol for immunohistochemical study. The primary antibody used was mouse anti-human D2-40 monoclonal antibody (Dako, Glostrup, Denmark, 1:100). The immunohistochemical study was performed following a previously published protocol (Yuan et al., 2006). The lymphatic endothelial cells within the sections served as internal positive controls.

We also followed the previously published method to score the expression of podoplanin (Yuan et al., 2006). In brief, the slides were evaluated independently by two pathologists (C. H. and W.-Y. C.) without knowing the clinicopathological information. The staining intensity was graded on a scale of 0 to 3 (0=negative; 1=weak; 2=moderate; 3=strong), and the quantity of positive tumor cells was scored as 0 to 5 (0=0%; 1=1~10%; 2=11~30%; 3=31~50%; 4=51~80%; 5=81~100%). The data were then converted to an immunoreactive score (IRS) by multiplying the staining intensity and quantity

Table 1. Clinicopathological characteristics and podoplanin expression of cases included in the study.

Characteristic	Podoplanin expression				Total (n=59)	
	Low (n=34)		High (n=25)			
Age [a]						
Mean ± standard deviation	60 ± 14		51 ± 8		57 ± 13	
Median (minimum; maximum)	61 (38; 100)		51 (38; 63)		61 (38; 100)	
Gender (%)						
Female	0	(0)	1	(4)	1	(2)
Male	34	(100)	24	(96)	58	(98)
Tumor grade (%)						
Well differentiated	1	(3)	2	(8)	3	(5)
Moderately differentiated	28	(82)	16	(64)	44	(75)
Poorly differentiated	5	(15)	7	(28)	12	(20)
Lymphatic vessel invasion (%)						
Present	14	(41)	11	(44)	25	(42)
Absent	20	(59)	14	(56)	34	(58)
pT stage (%)						
pT1 + pT2	5	(15)	4	(16)	9	(15)
pT3 + pT4	29	(85)	21	(84)	50	(85)
pN stage (%)						
pN0	16	(47)	13	(52)	29	(49)
pN1	18	(53)	12	(48)	30	(51)
pM stage (%)						
pM0	33	(97)	21	(84)	54	(92)
pM1	1	(3)	4	(16)	5	(8)
Pathological (TNM) stage (%)						
I + II	14	(41)	13	(52)	27	(46)
III + IV	20	(59)	12	(48)	32	(54)
cT stage (%) [b]						
cT1 + cT2	9	(32)	7	(32)	16	(32)
cT3 + cT4	19	(68)	15	(68)	34	(68)
cN stage (%) [b]						
cN0	12	(46) [c]	2	(10) [c]	14	(30)
cN1	14	(54) [c]	19	(90) [c]	33	(70)
cM stage (%) [b]						
cM0	23	(92)	19	(95)	42	(93)
cM1	2	(8)	1	(5)	3	(7)
Clinical (TNM) stage (%) [b]						
I + II	15	(52) [d]	6	(27) [d]	21	(41)
III + IV	14	(48) [d]	16	(73) [d]	30	(59)

[a]: Age at diagnosis; [b]: Some cases excluded due to incomplete pre-treatment clinical staging; [c]: p=0.0063; [d]: p=0.079

scores. For cases with discrepant results, a consensus score was determined by simultaneous examination under a dual-head microscope.

Lymphatic vessel invasion (LVI) was also evaluated by microscopic examination of the slides. The presence of tumor cells in a podoplanin-positive vascular channel indicates LVI.

To assess the influence of preoperative CCRT on podoplanin expression, we also performed a podoplanin immunohistochemical study on 29 available pre-treatment tumor biopsy specimens in patients who received preoperative CCRT. The IRS scores were calculated in the same way as the resected specimens.

Statistical analysis

Difference in age between groups was assessed by the Mann-Whitney U-test. Differences in categorical data were assessed by a chi-square test or Fisher's exact test. Overall survival was analyzed by the Kaplan-Meier method and compared by log-rank tests. The influence of age on survival was analyzed using a Cox regression model. A p-value less than 0.05 was considered statistically significant. All statistical analyses were done using the WinSTAT® for Excel (R. Fitch Software, Bad Krozingen, Germany).

Results

Podoplanin expression in resected tumors

Podoplanin expression was observed in the endothelial cells of lymphatic channels and also commonly at the basal layer of the non-neoplastic squamous epithelium (Fig. 1). The IRS scores ranged from 0 to 12, with a median of 4. Therefore, an IRS score of 4 or larger was considered high podoplanin expression, whereas an IRS score of 3 or smaller was

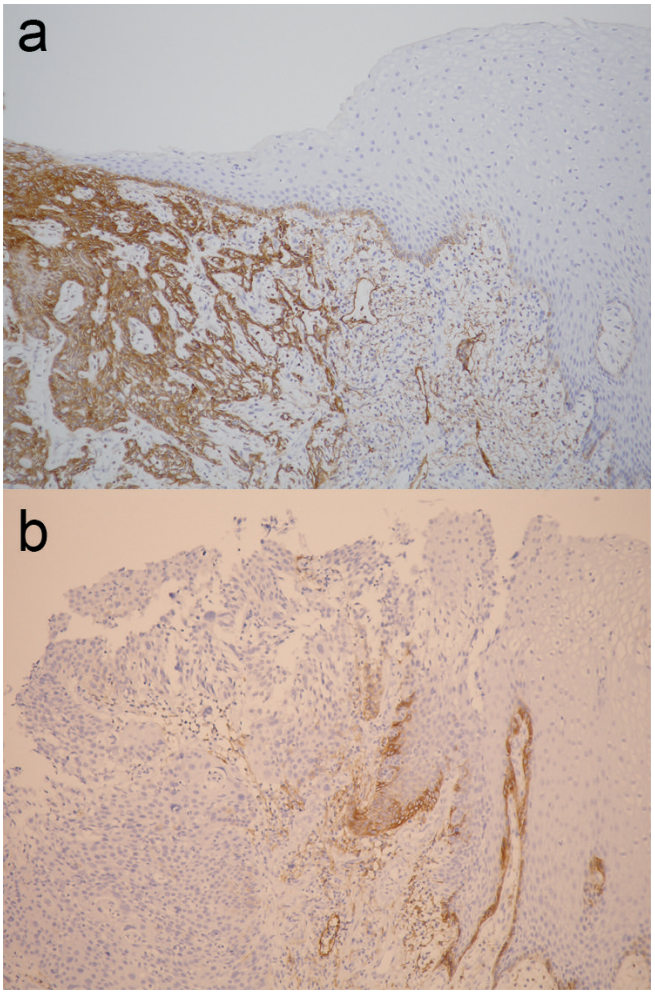

Fig. 1. Examples of high **(a)** and low **(b)** podoplanin expression in tumor cells of resected tumors (x100). Note the positive staining of lymphatic endothelial cells and the basal cells of adjacent non-neoplastic squamous epithelium. x 100

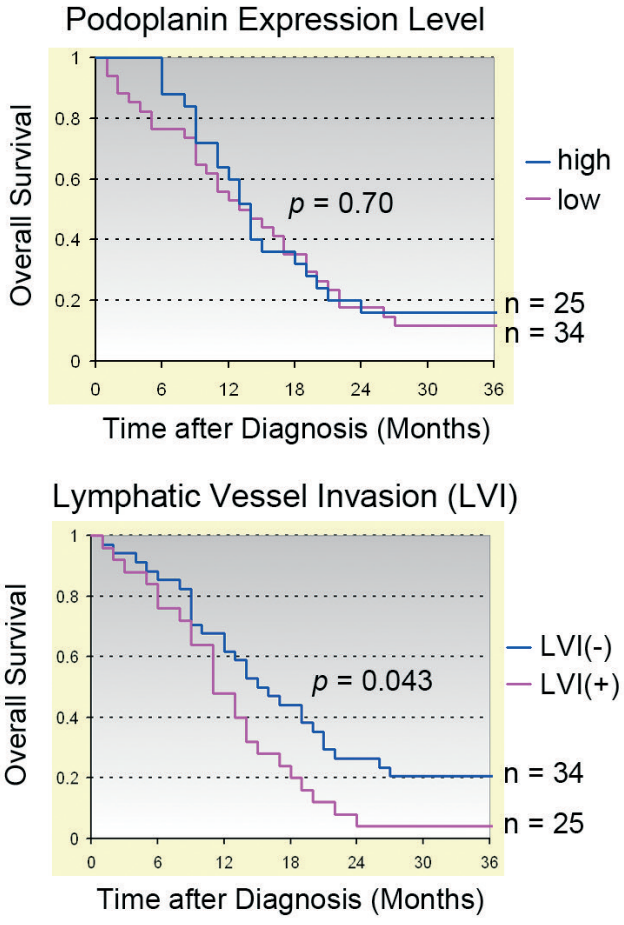

Fig. 2. Correlation between podoplanin expression level or lymphatic vessel invasion and survival.

considered low expression. Twenty-five (42%) of the 59 cases had high podoplanin expression in the tumor cells. The clinicopathological features of patients grouped by podoplanin expression level are listed in Table 1. We found that high podoplanin expression was strongly associated with clinical nodal metastasis (cN1; p=0.0063) but not with pathological nodal metastasis (pN1). A trend of more advanced clinical stage (clinical stage III and IV) was also found in patients with high podoplanin expressing tumors (p=0.079). None of the other clinicopathological characteristics showed a significant difference between patients with different podoplanin expression levels, except for a younger age in patients with high podoplanin expression (p=0.0069).

Survival

Fifty-two (88%) of the 59 patients died within 5 years after diagnosis of esophageal SCC, one died 71 months after diagnosis, and the other six were alive at the last follow up 80 to 105 months after diagnosis. Unlike the previous findings in oral SCC patients (Yuan et al., 2006), we found no direct correlation between podoplanin expression and survival of esophageal SCC patients (Fig. 2). Factors including pT stage, pTNM stage, cN stage and cTNM stage had significant influence on survival (Figs. 3, 4). All other factors evaluated, including age, gender, tumor grade, pN stage, pM stage, cT stage, cM stage and preoperative CCRT, had no significant influence on survival (data not shown).

Table 2. Correlation between lymphatic vessel invasion (LVI) and pathological or clinical nodal metastasis.

Nodal status	Lymphatic vessel invasion (LVI)			
	LVI (+) (n=25)		LVI (-) (n=34)	
Pathological N Stage (%)				
pN1	19	(76) [b]	11	(32) [b]
pN0	6	(24) [b]	23	(68) [b]
Clinical N Stage (%) [a]				
cN1	14	(78) [c]	19	(66) [c]
cN0	4	(22) [c]	10	(34) [c]

[a]: Some cases excluded due to incomplete pre-treatment clinical staging; [b]: p=0.00092; [c]: p=0.37

Table 3. Correlation of podoplanin expression between tumor biopsy samples and post-concurrent chemoradiotherapy (CCRT) resected tumors.

Podoplanin expression in biopsy samples	Podoplanin expression in post-CCRT resected tumors			
	High		Low	
High (%)	12	(80) [a]	3	(29) [a]
Low (%)	4	(20) [a]	10	(71) [a]

[a]: p=0.0054

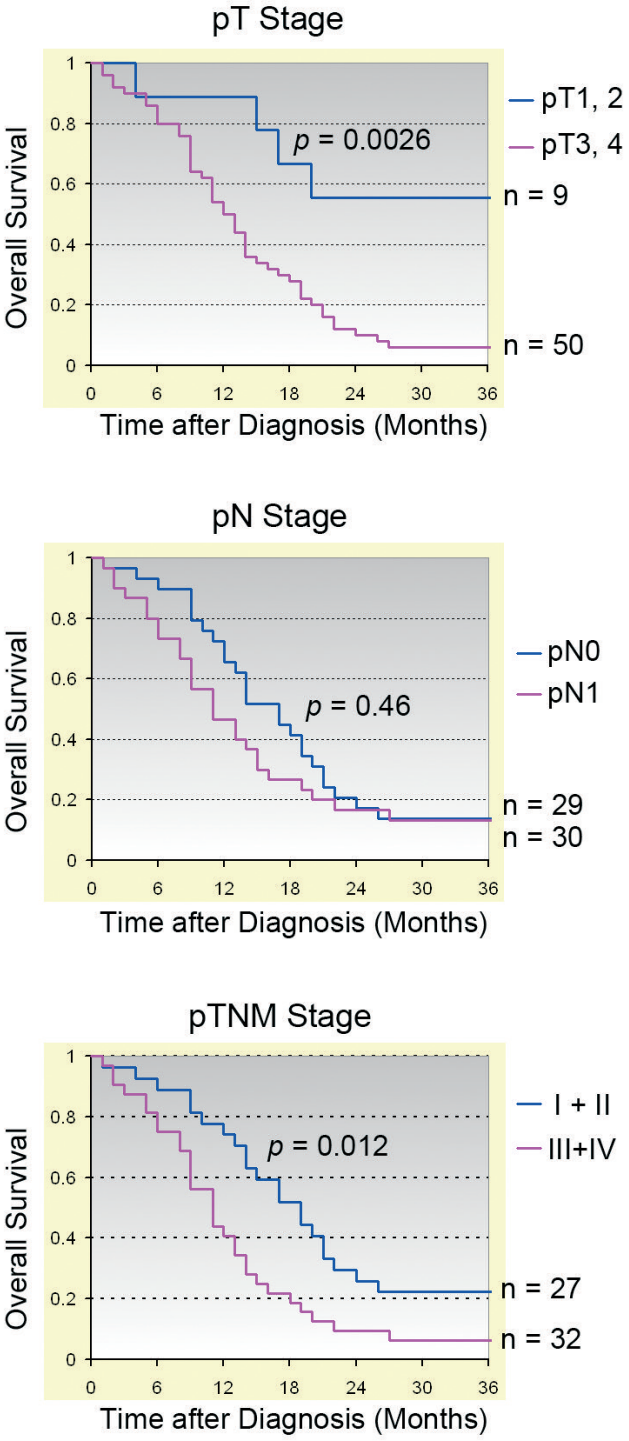

Fig. 3. Significant influence of pT stage and pTNM stage on survival.

Lymphatic vessel invasion (LVI) in resected specimens

Thirty (38%) of the 79 resected specimens had evidence of LVI (Fig. 5). We found a significant association between LVI and pathological nodal metastasis (pN1; p=0.00092). However, LVI did not correlate with clinical nodal metastasis (cN1, p=0.37) or high podoplanin expression (p=0.83). Of note, LVI was also associated with shorter survival (p=0.043, Fig. 2).

Podoplanin expression in tumor biopsy specimens

Fifteen (52%) of the 29 tumor biopsy specimens before CCRT showed high podoplanin expression (Fig. 6). There was a strong correlation of podoplanin expression levels between biopsy samples and post-CCRT resected tumors (Table 3; p=0.0054).

Discussion

Esophageal cancer is highly lethal, with an overall survival rate of 10-20%. Most patients who present with symptoms, such as dysphagia, have either locally advanced or metastatic disease (van Meerten and van der Gaast, 2005). At the time of diagnosis, two thirds of the patients had tumors considered inoperable due to patient comorbidities or tumor extension. Among therapeutic options, surgery is used most frequently to obtain locoregional control and long-term survival. Radiotherapy and chemotherapy could improve disease control by downstaging cancer and thereby increasing resectability (Mariette et al., 2007). As a result, preoperative CCRT is nowadays widely used in the treatment of patients with potentially resectable esophageal cancer (van Meerten and van der Gaast, 2005).

Podoplanin (T1α, aggrus and gp36) is a 38kDa type-1 transmembrane glycoprotein (Wicki and Christofori, 2007). In normal human tissue, it is expressed in lymphatic endothelial cells, renal podocytes (Breiteneder-Geleff et al., 1999), skeletal muscle, placenta, lung, heart (Martin-Villar et al., 2005), myofibroblasts of the breast and salivary glands,

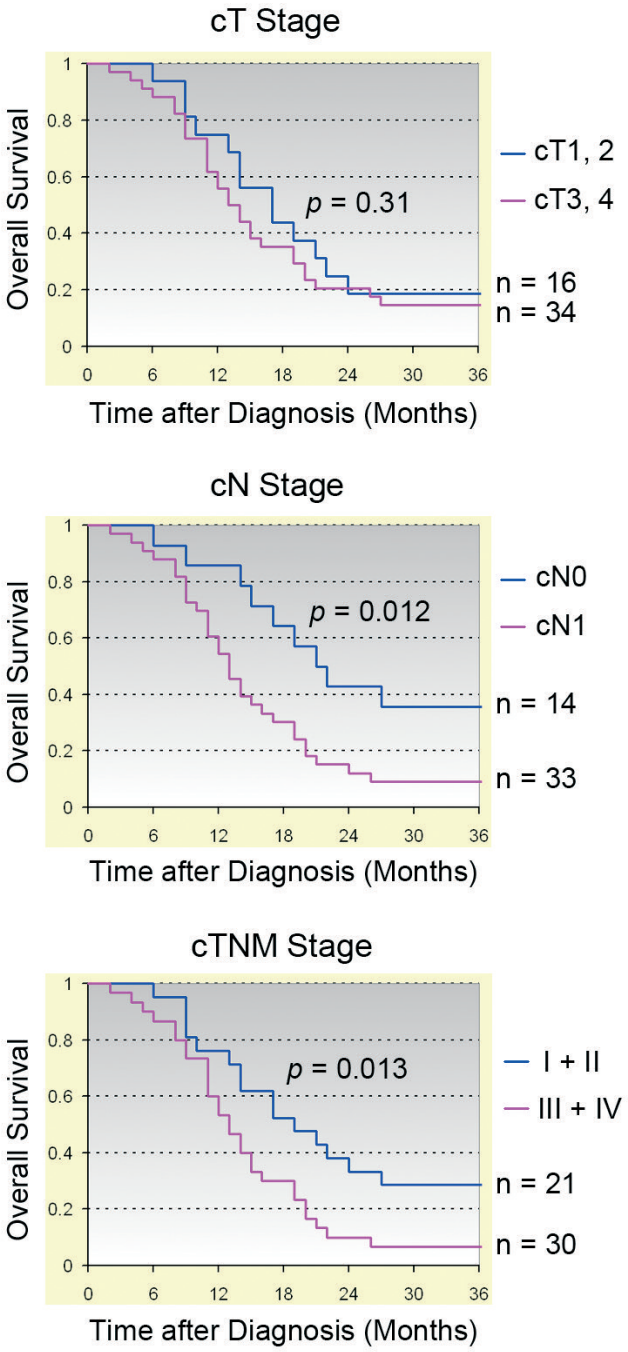

Fig. 4. Significant influence of cN stage and cTNM stage on survival.

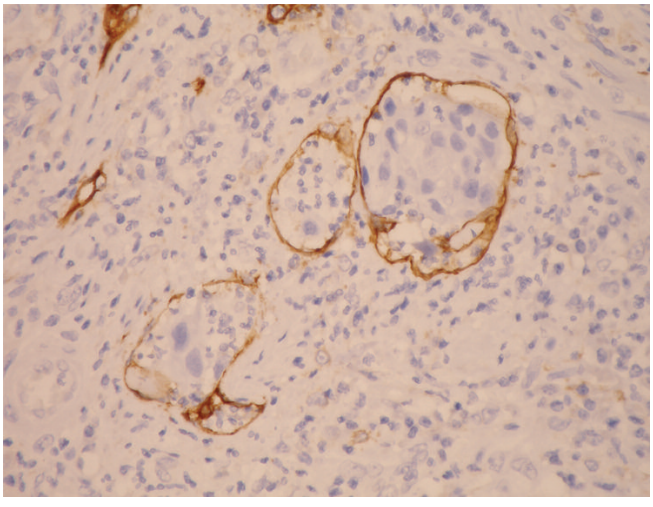

Fig. 5. An example of lymphatic vessel invasion showing tumor cells within podoplanin(+) lymphatic channels. x 400

osteoblasts, and mesothelial cells (Ordonez, 2006). Occasionally, focal expression of podoplanin can also be found in the basal layer of the human epidermis (Schacht et al., 2005). The physiological function of podoplanin remains unclear. A possible role in lymphangiogenesis has been suggested, since podoplanin-deficient mice were found to die at birth due to respiratory failure, with a phenotype of dilated, malfunctioning lymphatic vessels and lymphedema (Ramirez et al., 2003; Schacht et al., 2003). It is now widely used as a specific marker for lymphatic endothelial cells due to its expression by lymphatic endothelium but not by blood vessel endothelium (Breiteneder-Geleff et al., 1999; Kahn and Marks, 2002). Podoplanin has been utilized to assess lymphatic microvessel density (LMVD) and LVI in various cancers, such as non-small cell lung carcinoma (Kadota et al., 2008), invasive breast carcinoma (El-Gohary et al., 2008), colorectal adenocarcinoma (Matsumoto et al., 2007), and esophageal SCC (Mori et al., 2007; Nakayama et al., 2007). These studies, including the two on esophageal SCC, found a correlation of LMVD and LVI with lymph node metastasis, advanced stage or survival.

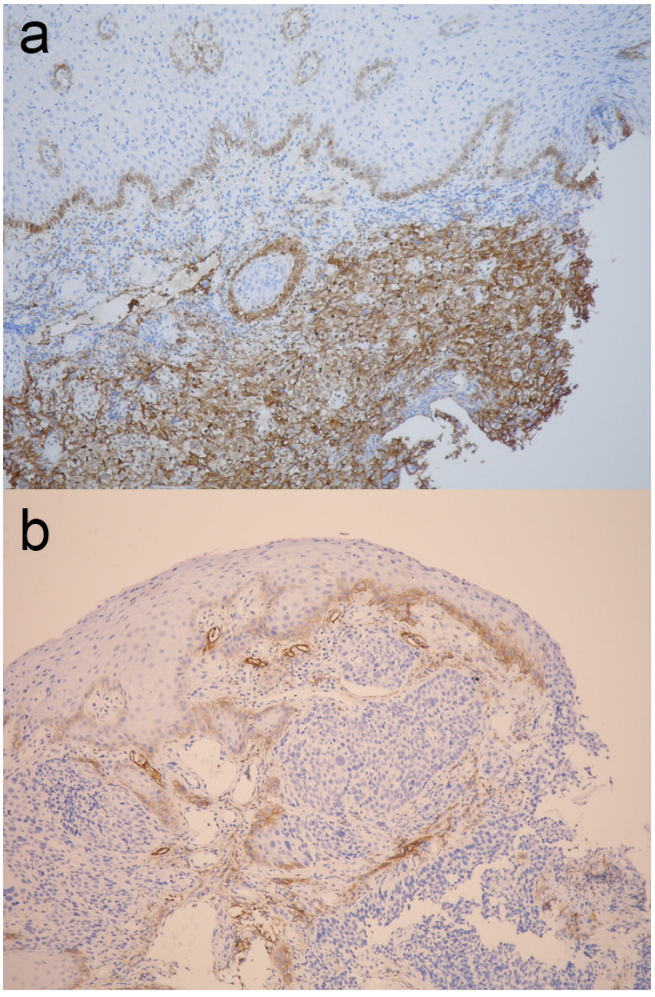

Fig 6. Examples of high **(a)** and low **(b)** podoplanin expression in tumor cells of biopsy specimens. x 100

It is of interest that podoplanin expression has also been shown in certain tumor cells, including SCC of the uterine cervix, skin, oral cavity and esophagus and germ cell tumors (Dumoff et al., 2005; Schacht et al., 2005; Yuan et al., 2006; Wicki et al., 2006). Recently, podoplanin expression in oral leukoplakia was found to be a novel marker for higher incidence of developing SCC, implying a role of podoplanin in tumorigenesis (Kawaguchi et al., 2008). In addition, podoplanin was found to be involved in a pathway of tumor cell migration and invasion in human SCC (Wicki et al., 2006; Wicki and Christofori, 2007). Podoplanin was capable of promoting invasion of tumor cell sheets into neighboring tissue, a process called collective cell migration, during which the pathway of epithelial-mesenchymal trasition was not initiated. This finding supports a role of podoplanin in tumor invasiveness. Another study showed an association of high tumor podoplanin expression with lymph node metastasis and short survival in oral SCC patients (Yuan et al., 2006), suggesting a possible influence of podoplanin on nodal metastasis. All these encouraged us to investigate the prognostic influence of podoplanin on esophageal SCC, since it has not been explored previously.

In our study, we found that high podoplanin expression strongly correlates with clinical lymph node metastasis (cN1; p=0.0063), which was associated with shorter survival (p=0.012). However, there was no correlation between podoplanin expression level and pathological nodal metastasis (pN stage). Since a large proportion (73%) of our patients underwent CCRT before surgery, the pN stage might have been underestimated due to elimination of metastatic tumor cells in lymph nodes. Therefore, the cN stage could be a better indicator of the pre-treatment nodal status of the disease.

It is of interest to see whether preoperative CCRT affects the tumor cell expression of podoplanin. We studied 29 pre-treatment tumor biopsy specimens and found that the podoplanin levels in biopsy samples correlated strongly with the post-CCRT resected tumors (p=0.0054; Table 3). The result suggested that no prominent change in podoplanin expression was induced by preoperative CCRT.

Similar to a previous study (Mori et al., 2007), we also found that the presence of LVI was strongly associated with pathological nodal metastasis (pN1; p=0.00092). However, no correlation between LVI and cN1 was found. Since tumor cells in lymphatic vessels and lymph nodes could have been equally affected by preoperative CCRT (i.e., both LVI and the pN stage represent a post-CCRT status), it is not surprising to see an assocation between LVI and the pN stage. This could also explain why LVI does not correlate with cN stage or podoplanin expression, since cN stage is assessed before

CCRT and podoplanin expression is not affected by CCRT.

We also found that the patients with high podoplanin expressing tumors tend to be younger, a finding which was not observed in the previous study on oral SCC (Yuan et al., 2006). Interestingly, a recent study found that podoplanin expression was more common in older patients with oral leukoplakia (Kawaguchi et al., 2008). The exact role of podoplanin in tumorigenesis and its possible impact on different age groups warrant further investigations.

In conclusion, this is the first study on the prognostic significance of tumor cell expression of podoplanin in esophageal SCC. We found a strong association of high podoplanin expression level with clinical nodal metastasis, which correlated with short survival. Our result suggests that podoplanin could also play a role in tumor aggressiveness in esophageal SCC, as well as in oral SCC.

Acknowledgements. This study was supported by grants from the Ministry of Education, Taiwan, ROC (EMRPD160111, to C. H.) and the Chang Gung Medical Research Program (CMRPG370021, to W.-Y. C.).

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Accepted February 16, 2009