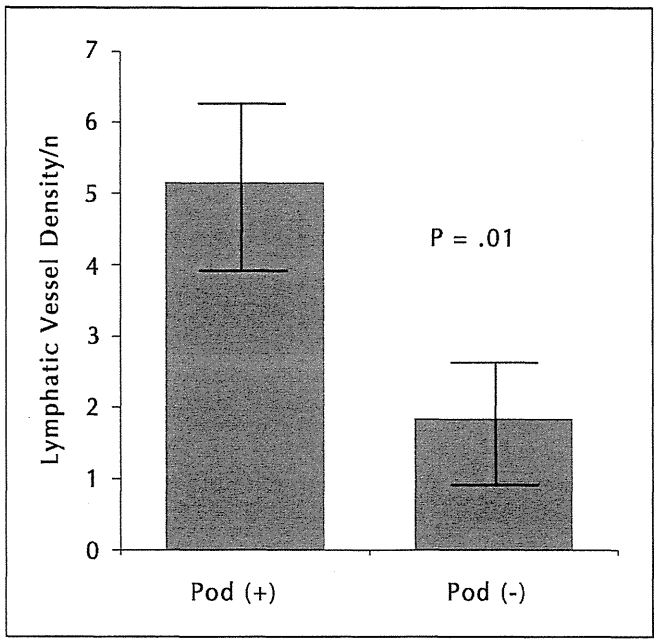

Figure 2. *The mean numbers of lymphatic vessels in the stroma with podoplanin (Pod) positive (+) and negative (−) expression. The numbers were counted in the area of a ×20 (1.3 mm²) objective. Lymphatic vessel densities in the podoplanin-positive stroma were higher than those in podoplanin-negative stroma (P = .01, t tests).*

(87 of 94 cases) lacked cancerous stromal cells, which is not an unanticipated result considering the histologic features of renal cell carcinoma. Therefore, all cases of renal cell carcinoma were excluded from further analysis. Ultimately, 784 cases were analyzed. The number of cases eventually extracted for final analysis is listed in Tables 1 and 2 along with their clinical data.

Upon examination of multiple cancer TMA, the distribution of immunohistochemical status in each cancer type was determined (Table 3). The most frequent expression was found in colon cancer (90%), followed by stomach cancer (82%), cancer of the biliary tract (73%), and pancreatic cancer (73%). Associations between podoplanin expression and T status ($P < .001$), lymph node metastases ($P < .001$), and stage ($P < .001$) were found. Podoplanin expression was observed in 49% of T2 to T4 cancers compared with 26% of T1, 52% of cancers with lymph node metastases compared with 39% of cancers without metastasis, and 50% stage II to IV cancers compared with 28% of cancers in stage I. Associations between podoplanin expression and lymphatic invasion ($P = .02$) and venous invasion ($P < .001$) were also observed (Table 4).

Upon examination of lung cancers in lung cancer TMA including all histologic types, there were no significant associations between podoplanin expression and T status ($P = .06$), lymph node metastases, or stage. However, when the analysis was limited to pulmonary adenocarcinomas (n = 157), podoplanin expression was associated with lymph node metastases ($P = .05$), lymphatic invasion ($P = .006$), venous invasion ($P = .008$), and trended with stage ($P = .06$) (Table 5). No association was observed in squamous cell carcinoma (n = 88). Case numbers in other histologic types were insufficient for statistical analysis.

Follow-up data were only available for non–small cell lung cancer. Survival analysis demonstrated podoplanin expression in stroma correlated with increased risk of death for non–small cell lung cancer ($P = .02$) (Figure 3, A). After adjustment for sex, age, and stage by the Cox proportional hazards regression model, the association

Table 2. Clinicopathologic Characteristics of the Lung Cancer Patients

	No. of Cases	No. Cases With Follow-up
Sex		
Male	182	122
Female	84	59
Age, y		
Mean ± SD	65.6 ± 9.3	64.9 ± 9.2
Stage		
I	146	97
II	54	44
III	62	37
IV	4	3
Tumor type		
Adenocarcinoma	157	107
Squamous cell carcinoma	88	70
Large cell carcinoma	12	0
Adenosquamous carcinoma	8	4
Small cell carcinoma	1	0

Table 1. Clinicopathologic Characteristics of the Patients in Multiple Cancer Tissue Microarray[a]

	Sex		Age	Tumor Stage, No.			
	Male, No.	Female, No.	(mean ± SD), y	I	II	III	IV
Prostate	31	. . .	68 ± 5.8	0	12	15	2
Thyroid	11	57	55 ± 16.3	15	11	39	0
Bladder	22	7	67 ± 9.3	11	4	9	4
Breast	1	75	57 ± 12.3	23	39	6	0
Lung (AD)	45	37	65 ± 9.7	49	16	14	3
Uterine body	. . .	29	59 ± 12.2	15	6	6	2
Stomach	42	13	67 ± 10.7	7	13	27	6
Ovary	. . .	37	54 ± 13.2	20	1	15	1
Liver	16	6	64 ± 10.0	5	6	2	5
Colon	46	32	69 ± 11.3	7	27	40	4
Lung (SCC)	60	2	66 ± 9.3	34	22	6	0
Biliary tract	38	34	68 ± 11.5	7	11	8	46
Pancreas	14	7	68 ± 10.1	0	0	14	5

Abbreviations: AD, adenocarcinoma; SCC, squamous cell carcinoma.

[a] Numbers of cases listed are after exclusion due to tissue loss, lack of cancerous stroma, or negative result of validation for antigen retardation.

Table 3. Podoplanin Expression in Stromal Spindle Cells Using Multiple Cancer Tissue Microarray						
		Total Score, No.				
	Total, No.	0	1	2	3	Positive Rate, %
Colon	77	1	7	14	55	90
Stomach	55	1	9	19	26	82
Biliary tract	66	1	17	16	32	73
Pancreas	22	1	5	11	5	73
Bladder	29	7	7	2	13	52
Lung (SCC)	68	13	29	15	11	38
Liver	22	5	9	6	2	36
Breast	76	18	36	18	4	29
Uterine body	29	1	20	7	1	28
Prostate	31	7	16	8	0	26
Ovary	37	13	16	5	3	22
Lung (AD)	82	36	32	9	5	17
Thyroid	68	54	14	0	0	0
Total	662	158	217	130	157	43

Abbreviations: AD, adenocarcinoma; SCC, squamous cell carcinoma.

Table 4. Association Between Podoplanin Expression and Clinicopathologic Factors in Multiple Cancer Tissue Microarray				
	No.	Positive, No. (%)	Negative, No. (%)	*P* Value
T1	150	39 (26)	111 (74)	<.001
T2–T4	486	239 (49)	247 (51)	
N0	382	149 (39)	233 (61)	<.001
N1–N3	240	124 (52)	116 (48)	
Stage I	193	54 (28)	139 (72)	<.001
Stage II–IV	447	225 (50)	222 (50)	
Ly (−)	128	39 (30)	89 (70)	.02
Ly (+)	164	72 (44)	92 (56)	
v (−)	166	44 (27)	122 (73)	<.001
v (+)	121	67 (55)	54 (45)	

Abbreviations: Ly, invasion to the lymphatic vessel; v, invasion to the blood vessel.

with survival was not significant. In patients with adenocarcinoma, expression was associated with increased risk of death ($P < .001$) (Figure 3, B), and Cox proportional hazards modeling showed persistent significance in lung adenocarcinoma ($P = .01$) (Table 6). In squamous cell carcinoma, however, the expression was not associated with prognosis (Figure 3, C).

COMMENT

In this study we found that podoplanin was expressed in myofibroblasts of desmoplastic stroma in a great variety of cancer cell types. Expression of podoplanin within desmoplastic stroma correlated with T and lymph node status. In examining lung cancer specifically, we demonstrated that podoplanin expression is not statistically correlated with stage and other factors when all histologies are considered. Subset analysis, by histology, demonstrated podoplanin expression in the stroma associated with adenocarcinoma is correlated with T status, lymph node status, stage, and patient survival and remained statistically significant with multivariate analysis. We examined podoplanin expression in tumor cells, but the associations between podoplanin expressions in cancer cells and clinicopathologic factors or patients' survival in lung cancer were not found.

In our cohort of non–small cell lung cancer, we only observed the survival significance of podoplanin expression in pulmonary adenocarcinoma but not in pulmonary squamous cell carcinoma. Biologically there appears to be a difference in the stromal cells between adenocarcinoma and squamous cell carcinoma. As for utility, podoplanin expression in adenocarcinomas may have some value in guiding therapy as well as in being a predictive marker of survival. For example, detection of stromal podoplanin expression in a preoperative small biopsy might function to guide the extent of lymph node dissection at the time of definitive resection.

Although expression of different molecules has been widely explored in tumor cells, expression within the stroma has received little attention.[7,26,27] Our results support a unique model in which podoplanin expression in the stromal myofibroblasts is associated with lymph node metastases and lymphatic invasion. They indicate that podoplanin expression in myofibroblasts may be

Table 5. Association Between Podoplanin Expression and Clinicopathologic Factors in Lung Cancer Tissue Microarray								
	All Cases				Adenocarcinoma			
	No.	Positive, No. (%)	Negative, No. (%)	*P* Value	No.	Positive, No. (%)	Negative, No. (%)	*P* Value
T1	113	32 (28)	81 (72)	.06	80	14 (18)	66 (83)	.09
T2–T4	153	60 (39)	93 (61)		77	22 (29)	55 (71)	
N0	161	51 (32)	110 (68)	.22	96	17 (18)	79 (82)	.05
N1–N3	105	41 (39)	64 (61)		61	19 (31)	42 (61)	
Stage I	146	44 (30)	102 (70)	.13	93	16 (17)	77 (83)	.06
Stage II–IV	120	47 (39)	73 (61)		64	20 (31)	44 (69)	
Ly (−)	111	36 (32)	75 (68)	.96	60	6 (10)	54 (90)	.006
Ly (+)	116	38 (33)	78 (67)		77	22 (29)	55 (71)	
v (−)	140	36 (26)	104 (74)	.008	91	12 (13)	79 (87)	.008
v (+)	82	36 (44)	46 (56)		42	14 (33)	28 (67)	

Abbreviations: Ly, invasion to the lymphatic vessel; v, invasion to the blood vessel.

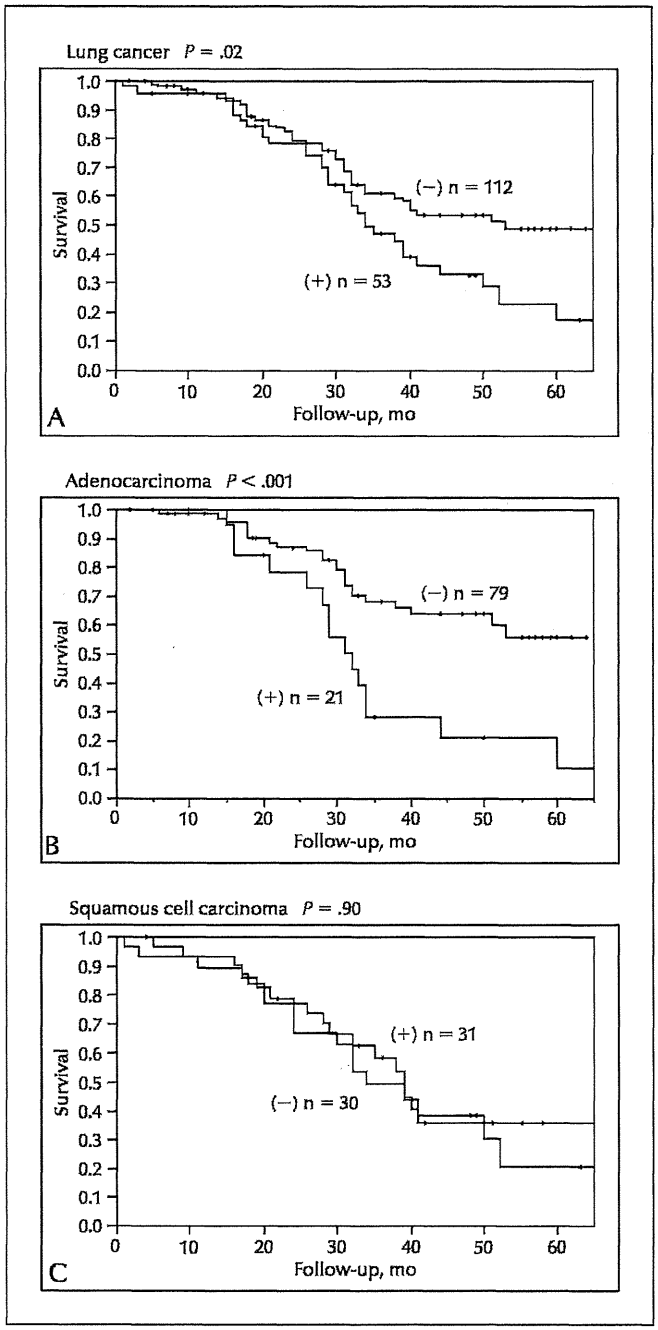

Figure 3. *Survival analysis of podoplanin expression in cancerous stromal cells in lung cancer patients. Kaplan-Meier (KM) curves comparing survival between cases with podoplanin negative (−) in cancerous stroma and those with podoplanin positive (+). A, KM curve in non–small cell lung cancer cases. B, KM curve in adenocarcinoma cases. C, KM curve in squamous cell carcinoma cases.*

associated with lymphangiogenesis and lymphatic spread of cancer cells. Tumor lymphangiogenesis has been reported as a prognostic indicator and a predictor of lymph node metastases in non–small cell lung cancer,[28] bladder cancer,[29] colorectal cancer, and head and neck cancer.[25,30] Aishima et al[18] reported the antibody D2-40, against human podoplanin, stained myofibroblasts and was associated with lymphatic metastasis in intrahepatic cholangiocarcinoma.

Table 6. Multivariate Analysis with Cox Proportional Hazards Model for Prediction in Lung Cancer Patient With Adenocarcinoma

	Hazard Ratio (95% Confidence Interval)	*P* Value
Podoplanin		
Negative	1	.01
Positive	1.72 (1.13–2.60)	
Age, y		
<60	1	.42
≥60	0.86 (0.60–1.69)	
Sex		
Female	1	.57
Male	1.12 (0.75–1.69)	
Stage		
I	1	<.001
II	1.46 (0.89–2.33)	
III	2.78 (1.80–4.35)	
IV	2.20 (0.86–4.31)	

Kawase et al[19] also reported the prognostic significance of stromal podoplanin expression in lung adenocarcinoma, consistent with our findings. However, they presented no data in squamous cell carcinoma or other common cancers. They additionally observed that expression of podoplanin in carcinoma-associated fibroblasts was not found in noninvasive adenocarcinoma.[19] We similarly observed that in 9 cases with predominant bronchioloalveolar features, all demonstrated podoplanin-negative stromal cells.

We showed that the mean numbers of lymphatic vessels in the cancerous stroma with positive podoplanin staining was higher. The results suggest the hypothesis that podoplanin expression in myofibroblasts predicts prognosis due to lymphangiogenesis associated with stromal podoplanin expression. The hypothesis is partially supported by the data in the study by Kawase et al[19] in which cases with high-grade podoplanin expression in myofibroblasts was associated with lymphatic invasion. In contrast, Aishima et al[18] reported similar data examining expression of vascular endothelial factor C in tumor cells and D2-40/podoplanin in myofibroblasts for intrahepatic cholangiocarcinoma. They showed that lymphatic vascular density is not associated with patients' survival or vascular endothelial factor C expression in intrahepatic cholangiocarcinoma; however, they did not examine stromal podoplanin expression in reference to lymphatic vascular density. They concluded that lymphangiogenesis does not play a direct role in lymph node metastasis. The clinical significance of lymphangiogenesis in relation to lymph node metastasis is controversial and may differ by cancer type.[31] According to the report by Renyi-Vamos et al[28] published recently, lymphangiogenesis in non–small cell lung cancer significantly correlated with lymph node metastasis, which supports our findings.

There are few reports describing molecular expression in stromal cells in relationship to patients' prognosis[19,30,32–44]; however, most of those reports use the term *activated fibrosis*[45] and do not define the phenotype of the stromal cells. Using SMA and desmin staining in conventional whole specimen analysis, we confirmed that most of the podoplanin-positive stromal cells are myofibroblasts. In contrast, myofibroblasts and/or fibroblasts in inflammatory lung diseases were negative for podoplanin expres-

sion (data not shown). Myofibroblasts within wound sites have been demonstrated to express podoplanin, similar to carcinoma-associated fibroblasts.[45] Orimo et al[45] described that carcinoma-associated fibroblasts enhanced tumor angiogenesis and exhibited increase of SMA expression as well as contractility, which indicates that the fibroblasts they described were myofibroblasts. Proliferation of myofibroblasts in peritumoral areas appears with invasion by the adenocarcinoma tumor cells and may play an important role in lymphangiogenesis.[19,46]

The origin of myofibroblasts in cancerous stroma remains unclear. One theory is that they are derived from bone marrow. Alternative models suggest that they may be tumor cells with epithelial mesenchymal transition or endothelial cells with endothelial mesenchymal transition.[47–49] Additional analysis to unveil the pathogenesis of myofibroblasts in cancerous stroma is needed.

The importance of tumor stroma to tumor behavior is well demonstrated. Tumor stroma consists of a complex admixture of cells and extracellular matrix. Approaches where tumor stroma is evaluated as a homogenous unit are inadequate. The different cell types that compose the tumor stroma have biologic significance and require interrogation at the cellular level and are not amenable to evaluation at the homogenous tissue level. Evidence of this is the contribution of bone marrow–derived stem cells contributing to tumor cells and tumor stroma, with cells that have phenotype characteristics of vascular endothelium, fibroblasts, and myofibroblasts.[48,49] We anticipate that cytomorphologic analysis is necessary to identify the exact cell types contributing to the tumor stroma biology. Biologic events in stroma are potential molecular targets for therapy. Modulation of stromal cells derived from the normal host has advantages over targeting multimutated genetically unstable tumor cells. Tumor cells routinely acquire multiple mutations. A tumor may contain different clonal populations with different phenotypes, different gene expression patterns, and differences in response to the environment.[50] Currently, most anticancer drugs target tumor cells. One exception is bevacizumab (Avastin), a drug that targets stromal vessels within cancerous stromal tissues. Additional studies on the role of podoplanin in lymphangiogensis may help the development of new drugs targeting stromal tumoral vessels.

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