Relation between recurrence and expression of proliferating cell nuclear antigen, sialyl Lewis^X, and sialyl Lewis^a in lung cancer

Blood vessel invasion, recurrence rate, and time to recurrence were examined in relation to the immunohistochemical expression of proliferating cell nuclear antigen, sialyl Lewis^X, and sialyl Lewis^a in 303 patients with lung cancer who had a curative resection from 1980 to 1991. Of these, 150 had adenocarcinoma, 117 had squamous, 21 had large-cell, and 15 had small-cell carcinoma. Significant differences were detected in the expression of proliferating cell nuclear antigen and sialyl Lewis^X between adenocarcinomas and the other histologic types; thus the subjects were divided into 150 with adenocarcinoma and 153 with non-adenocarcinoma. In those with adenocarcinoma, the frequency of blood vessel invasion was significantly higher in tumors with strong sialyl Lewis^X expression, and the disease-free survival of the patients with blood vessel invasion was significantly worse when their tumors strongly expressed both sialyl Lewis^X and proliferating cell nuclear antigen. In those with non-adenocarcinoma, on the other hand, tumors with strong expression of sialyl Lewis^a and proliferating cell nuclear antigen showed significantly higher frequencies of blood vessel invasion and worse disease-free survival. In patients with recurrent tumors, those with strong proliferating cell nuclear antigen expression showed a significantly shorter time to recurrence. We conclude that sialyl Lewis^X and proliferating cell nuclear antigen expression in adenocarcinoma and sialyl Lewis^a and proliferating cell nuclear antigen expression in non-adenocarcinoma may be an important determinant of recurrence. (J THORAC CARDIOVASC SURG 1994;108:329-36)

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Recurrence of lung cancer after radical resection is thought to result from minute metastatic foci that cannot be detected by current diagnostic techniques and are probably already present at the time of the operation. In addition to the recurrence itself, the time to recurrence is variable, reflecting the variable biologic behavior of the tumors. Distant metastasis is the major cause of death in patients with lung cancer. For a metastasis to form, invasion of blood vessels in the primary lesion, adhesion to

vascular endothelial cells in the target organ, and proliferation in the extracellular matrix after extravasation are considered to be essential. The ability to predict these risks from tumor tissues by means of molecular biologic techniques could offer an important contribution to treatment planning. Sialyl Lewis^X (SLX) and sialyl Lewis^a (CA19-9) are sialylated Lewis antigens, and tumor cells expressing these antigens have been reported to attach to the blood vessels by binding to E-selectin receptor expressed in activated blood vessels as its ligands.¹⁻⁶ Thus these carbohydrate antigens may have related to vascular invasion and adhesion to other organs at metastatic sites. Proliferating cell nuclear antigen (PCNA) is a nuclear protein that increases when deoxyribonucleic acid (DNA) is being synthesized.⁷ Thus PCNA may be an indicator of the time to recurrence after tumors extravasate from blood vessels. Because these antigens can be detected in formalin-fixed tissues by immunoperoxidase techniques, we have examined retrospectively the relation between

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Table I. Tumor characteristics in patients with lung cancer

	No.
Histologic type	
Adenocarcinoma	150
Squamous cell carcinoma	117
Large-cell carcinoma	21
Small-cell carcinoma	15
Stage	
I	140
II	36
III	90
IV	37
Recurrence	154
Blood-borne metastasis	111
Lung	36
Brain	19
Bone	19
Pleura	17
Liver	12
Skin	6
Adrenal gland	2

postoperative recurrence and the immunohistochemical expression of SLX, CA19-9, and PCNA.

Patients and methods

Three hundred three consecutive patients with lung cancer who underwent a curative tumor resection and lymph node dissection from 1980 to 1991 were included in this study. All tumors were classified according to the TNM staging system.⁸ Of the patients with non-small-cell carcinoma, none with stage I or II disease had received any adjuvant therapy. Patients with stage III or IV disease received postoperative chemotherapy or radiation therapy (5000 rad applied to the mediastinum). Those with small-cell carcinoma received preoperative and postoperative chemotherapy. Although the regimens of chemotherapy performed within the 11 years were not the same, they have not significantly affected survivals of the patients with either small-cell or non-small-cell carcinoma. All the patients were monitored bimonthly at the outpatient clinic, and the recurrences were confirmed by pathologic examination of specimens taken by bronchoscopic or biopsy procedures and by computed tomography or scintigraphy.

An indirect immunoperoxidase method that uses an antibody against PCNA (PC10; DAKO A/S, Grostrup, Denmark) and CA19-9 (Centcor, Malvern, Pa.) and the streptavidin-biotinyl peroxidase complex method that uses an antibody against SLX (FH6; provided by Otsuka Pharmaceutical, Tokyo) were performed on formalin-fixed tissue sections, as previously reported.⁹ In short, each diluted antibody solution (PCNA [1:10], CA19-9 [1:1], SLX [1:10]) was overlaid on the thin-sliced sections and incubated at room temperature overnight. Subsequently, the sections were incubated with a peroxidase-conjugated antihorse immunoglobulin G (Amersham, Buckinghamshire, United Kingdom) for PCNA and CA19-9. For SLX staining, biotinylated antimouse immunoglobulin solution

was overlaid and then covered with a streptavidin-biotinyl peroxidase complex (DAKO LSAB kit, Carpinteria, Calif.). Then the sections were stained with 0.02% 3,3'-diaminobenzidine and 0.06% sodium azide (Wako Pure Chemical, Osaka, Japan) and counterstained with 0.3% methyl green. Immunohistochemical staining was examined in at least 1000 cells over five high-power fields ($\times 1000$). The PCNA staining was graded as follows: (++) , 20% or more of the tumor cells had nuclear staining; (+) , less than 20% of the cells stained. Few tumors had no nuclear staining. The SLX and CA19-9 staining was graded as follows: (++) , 30% or more of the cells had membranous staining; (+) , 5% to 29% of cells had staining; (–) , less than 5% of cells stained. Negative controls were prepared by omitting the primary antibodies but using the same methods mentioned earlier. The histologic findings of blood vessel invasion (BVI) were examined by Victoria blue-hematoxylin and eosin staining.¹⁰ BVI was scored as positive by the identification of tumor cells in the lumen of at least one blood vessel surrounded by the elastic layer.

The pathologic findings were evaluated by two pathologists without knowledge of the clinical outcome. The frequency of BVI was evaluated statistically by the χ^2 test. Disease-free survival curves were calculated by the Kaplan-Meier method, and statistical evaluation was done by the log-rank test.

Results

Tumor characteristics in patients with lung cancer.

The histologic types, stages, recurrences, and sites of blood-borne metastases are summarized in Table I. Of the 154 patients with recurrent tumors, 111 (72%) had blood-borne metastases. The time to recurrence ranged from 1 to 87 months (median 12 months). The follow-up period for the 149 patients without recurrences ranged from 24 to 136 months (median 61 months).

Expression of PCNA, SLX, and CA19-9 according to histologic types (Table II). The frequency of PCNA (++) in patients with adenocarcinoma was significantly lower than in patients with squamous cell and small-cell carcinoma ($p < 0.01$, $p < 0.02$). Conversely, the frequency of SLX (++) in patients with adenocarcinoma was significantly higher than in patients with the other histologic types ($p < 0.01$). Because of the significant differences in PCNA and SLX staining, the patients were thereafter divided into two groups: 150 patients with adenocarcinoma and 153 with non-adenocarcinoma, which included squamous, large-cell and small-cell carcinoma.

Frequencies of blood vessel invasion according to expression of PCNA, SLX, and CA19-9 (Table III). In patients with adenocarcinoma, the frequency of BVI increased as the degree of SLX staining became stronger ($p < 0.01$). BVI was also significantly more prevalent in PCNA (++) tumors than in PCNA (+) tumors ($p < 0.05$). On the other hand, in patients with non-adenocarcinomas, the frequency of BVI increased as the degree of CA19-9 staining became stronger ($p < 0.01$). However,

Table II. Expression of PCNA, CA19-9, and SLX according to histologic types

Histology	No.	PCNA			SLX				CA19-9			
		(+)	(++)	%	(-)	(+)	(++)	%	(-)	(+)	(++)	%
Adenocarcinoma	150	91	59	39*†	22	41	87	58*	42	51	57	38
Squamous cell	117	36	81	69*	40	60	17	15*	33	41	43	37
Large cell	21	11	10	48	9	7	5	24*	12	5	4	19
Small cell	15	4	11	73†	15	0	0	0*	7	3	5	33

PCNA, Proliferating cell nuclear antigen; SLX, sialyl Lewis^x; CA19-9, sialyl Lewis^a. PCNA(+), less than 20% of the cells had nuclear staining; (++) , 20% or more were stained; SLX(-), CA19-9(-), less than 5% of the cells had membranous staining; (+), 5% to 29% were stained; (++) , 30% or more were stained.

**p* < 0.01.

†*p* < 0.02.

Table III. Frequencies of BVI according to expression of PCNA, SLX, and CA19-9

	PCNA		SLX			CA19-9		
	(+)	(++)	(-)	(+)	(++)	(-)	(+)	(++)
Adenocarcinoma (<i>n</i> = 150)								
BVI (-)	41	16	16	18	23	19	15	23
BVI (+)	50 (55%)†	43 (73%)†	6 (27%)	23 (56%)	64 (74%)*	23 (55%)	36 (71%)	34 (60%)
Non-adenocarcinoma (<i>n</i> = 153)								
BVI (-)	29	40	31	30	8	34	20	15
BVI (+)	22 (43%)	62 (59%)	34 (52%)	36 (55%)	14 (64%)	19 (36%)	28 (58%)	37 (71%)*

PCNA, Proliferating cell nuclear antigen; SLX, sialyl Lewis^x; CA19-9, sialyl Lewis^a; BVI, blood vessel invasion; non-adenocarcinoma, all squamous, large-cell, and small-cell carcinomas.

**p* < 0.01.

†*p* < 0.05.

no significant differences in the BVI frequencies were detected according to the PCNA and SLX staining.

Disease-free survival according to BVI, expression of SLX, CA19-9, and PCNA (Fig. 1). The 150 patients with adenocarcinoma were subdivided into those with BVI (-) tumors (*n* = 57); BVI (+) SLX (-, +) and PCNA (+) tumors (*n* = 21); BVI (+) SLX (++) or PCNA (++) tumors (*n* = 37); and BVI (+) SLX (++) and PCNA (++) tumors (*n* = 35). Similarly, the 153 patients with non-adenocarcinoma were subdivided into those with BVI (-) tumors (*n* = 69); BVI (+) CA19-9 (-, +), and PCNA (+) tumors (*n* = 12); BVI (+) CA19-9 (++) or PCNA (++) tumors (*n* = 45); and BVI (+) CA19-9 (++) and PCNA (++) tumors (*n* = 27). Disease-free survival was significantly better in patients with BVI (-) tumors than in those with BVI (+) tumors. Among adenocarcinomas with BVI (+) tumors, survival was significantly better in patients with SLX (-, +) and PCNA (+) tumors than in those with SLX (++) and PCNA (++) tumors (*p* < 0.03). Of non-adenocarcinomas with BVI (+) tumors, the disease-free survival of the patients with CA19-9 (-, +) and PCNA (+) tumors was superior to that of the patients with CA19-9 (++) and

Table IV. Chemical structure of sialyl Lewis^x and sialyl Lewis^a

Antigen	Structure
SLX	NeuAc α2→3Gal β1→4GlcNAcβ1→R 3 ↑ α1Fuc
CA19-9	NeuAc α2→3Gal β1→3GlcNAcβ1→R 4 ↑ α1Fuc

SLX, Sialyl Lewis^x; CA19-9, sialyl Lewis^a; NeuAc, acetyl neuraminic acid; Gal, galactose; GlcNAc, N-acetylglucosamine; Fuc, fucose.

PCNA (++) tumors (*p* < 0.05). The differences in survival were also the same when the patients with small-cell carcinoma were excluded.

Disease-free survival in the patients with recurrence according to PCNA expression (Fig. 2). In the 154 patients with recurrence, the disease-free survival curves were calculated according to PCNA expression. In both adenocarcinomas and non-adenocarcinomas, the disease-free survival of the patients with PCNA (+) tumors was

Fig. 1. Disease-free survival according to blood vessel invasion (*BVI*), expression of sialyl Lewis^x (*SLX*), and proliferating cell nuclear antigen (*PCNA*) in adenocarcinomas. ($p < 0.03$: *BVI* (+) *SLX* (-, +) *PCNA* (+) versus *BVI* (+) *SLX* (++) *PCNA* (++)).

significantly superior to that of the patients with *PCNA* (++) tumors ($p < 0.01$, $p < 0.01$). However, no differences in their survival were detected according to *SLX* or *CA19-9* expression.

Discussion

CA19-9 and *SLX* are sialylated Lewis antigens and often accumulate in the serum or tumor tissues of patients with carcinoma.^{11, 12} Chemical structures of *CA19-9* and *SLX* are shown in Table IV. The expression of these

antigens is thought to be due to the activation of abnormal fucosyl or sialyl transferases.

SLX staining in the adenocarcinomas was stronger than in the squamous, large-cell and small-cell carcinomas, as has been previously reported.¹³ However, *CA19-9* was stained almost equally in adenocarcinomas and non-adenocarcinomas, suggesting the difference in the pattern of expression of these two antigens between histologic types. As shown in Table IV, *CA19-9* and *SLX* are isomers in their structures and closely resemble each other.

Fig. 1. Cont'd. Disease-free survival according to blood vessel invasion (BVI), sialyl Lewis^a (CA19-9), and proliferating cell nuclear antigen (PCNA) in non-adenocarcinomas. $p < 0.05$: BVI(+) CA19-9(-,+) PCNA(+) versus BVI(+) CA19-9(++) PCNA(++).

Namely, CA19-9 is a sialyl-fucosyl type I chain carbohydrate, and SLX is a sialyl-fucosyl type II chain carbohydrate.^{11,12} The cause for the differences in CA19-9 and SLX expression in different histologic types remains unclear.

As to the BVI frequency and the disease-free survival, clear correlations with SLX and CA19-9 expression were observed in adenocarcinomas and non-adenocarcinomas. In the former, tumors with strong SLX expression had significantly higher frequencies of BVI, and patients with

BVI positive tumors with strong SLX and PCNA expression showed poorer disease-free survival. In non-adenocarcinomas, tumors with strong CA19-9 expression had higher frequencies of BVI, and patients with BVI positive tumors with strong CA19-9 and PCNA expression had poorer disease-free survival. Therefore, SLX in adenocarcinomas and CA19-9 in non-adenocarcinomas may be important prognostic factors for disease-free survival.

In the complex process of metastasis, the following steps are thought to be mandatory: Individual tumor cells

Fig. 2. Disease-free survival in patients with recurrent tumors according to expression of proliferating cell nuclear antigen (PCNA) in adenocarcinomas. $p < 0.01$: PCNA (+) versus PCNA (++).

first detach from the primary lesion and then invade the blood vessels. Next, they attach to the vascular beds in the target organs after migration in the blood. In both processes, adhesion to the blood vessels is necessary. Recently, it has been reported that E-selectin (ELAM-1, endothelial leukocyte adhesion molecule), which ordinarily participates in the recruitment of neutrophils during an inflammatory response, also mediates adhesion of tumor cells by recognition of SLX and CA19-9.¹⁻⁶ Our studies on the correlation of SLX and CA19-9 expression with BVI and recurrence could be explained from the adhesion of these antigens expressed on the tumor cells to E-selectin receptors expressed on the vascular endothelial cells. Others have reported that SLX expression is higher in metastatic tumors in the liver than in the primary tumors in human colon carcinoma¹⁴⁻¹⁶ and that SLX expression correlates with a higher potential of lung colonization in human lung adenocarcinoma cell lines.¹⁷

Although SLX and CA19-9 were both expressed in the adenocarcinomas, BVI frequency had no correlation with CA19-9 expression; thus, SLX and CA19-9 may compete with each other for the affinity to E-selectin. Others have reported that expression of H/Le^y/Le^b is inversely correlated with survival.¹⁸ H/Le^y/Le^b antigens bind to a receptor for cell motility and promote invasion and metastasis.¹⁹ SLX, CA19-9, Le^y, and Le^b are all tumor-associated carbohydrate antigens that accumulate in tumors. It may be considered that these have an important functional role in metastasis by aberrant fucosylation or sialylation in their structures.

In BVI positive tumors, PCNA expression was also related to the rate of recurrence, as was SLX and CA19-9. The role of PCNA in recurrence remains to be established. E-selectin may need to be expressed by vascular endothelial cells for tumor cells to adhere to them, and E-selectin is induced by interleukin-1 or tumor necrosis

Fig. 2. Cont'd. Disease-free survival in the patients with recurrent tumors according to expression of proliferating cell nuclear antigen (PCNA) in nonadenocarcinomas. $p < 0.01$: PCNA (+) versus PCNA (++) .

factor.¹ Some epithelial tumors such as breast cancer or leukemia are reported to secrete these cytokines.^{20, 21} To explain the role of PCNA, one can postulate that lung cancers that express PCNA also secrete some cytokines when trapped in microvessels and increase the likelihood of metastasis by inducing E-selectin expression.

The time to recurrence is thought to be related to the proliferative potential of tumors after they extravasate from the site of vascular arrest and lodge in the parenchyma. Our results showed that in the patients with recurrent tumors, those with tumors strongly positive for PCNA had a significantly shorter time to recurrence than those with tumors weakly positive for PCNA. Because PCNA is a nuclear protein that increases during DNA synthesis, PCNA may be a predictor of the time to recurrence.

In conclusion, SLX expression in adenocarcinomas and CA19-9 in non-adenocarcinomas may be useful for

predicting BVI and recurrence. PCNA expression may be useful for predicting recurrence and the time to recurrence in lung cancer.

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