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Expressions of CD44s Is Associated with the Expression of Cyclooxygenase-2 in Non-Small Cell Lung Cancers

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Background : The overexpression of Cox-2 in tumors is important for tumor invasion, angiogenesis, resistance to apoptosis and the suppression of host immunity. Moreover, a tumor's CD44 expression plays an important role in tumor invasion and metastasis. We examined the expression of COX-2 and also CD44 and its variants as well as the biological implications and relationship between Cox-2 and the CD44 variants in non-small cell lung carcinoma. **Methods :** The expressions of Cox-2 and also CD44s and its variants (CD44v3 and CD44v6) were examined by performing immunohistochemistry on 98 surgical specimens. **Results :** The expressions of CD44s, CD44v3 and CD44v6 were significantly more frequent in squamous cell carcinoma specimens than in the adenocarcinoma (CD44s, p=0.033; CD44v3, p=0.007; CD44v6, p=0.022). The loss of CD44s and CD44v3 were significantly correlated with poor tumor differentiation (CD44s, p=0.03; CD44v3, p=0.011). Patients with Cox-2 positive-adenocarcinoma tumors had a significantly worse cumulative survival than did those adenocarcinoma patients without the Cox-2 (p=0.048). The expression of Cox-2 was significantly associated with the CD44s expression in non-small cell lung cancer, and especially in squamous cell carcinoma. **Conclusions :** These findings suggest that expression of CD44s is associated with the expression of Cox-2 in NSCLC, and especially squamous cell carcinoma.

Key Words : Carcinoma, Non-small-cell lung; Cyclooxygease-2; Antigens, CD44

Lung carcinoma is one of the leading causes of cancer-related death and this malady remains a major public health problem throughout the world. Although surgical resection can provide lung cancer patients with some hope of a cure, the long-term survival rate, even for the surgically treated cases, remains less than satisfactory. Tumor metastasis is a complex series of events in which cells migrate beyond the tissue compartments and spread to distant organ site. CD44 is a widely distributed transmembrane cell adhesion molecule that is the major cell surface receptor for hyaluronic acid.[1] It is associated with such diverse physiologic functions, as cell-to-cell and cell-to-matrix interactions as well as lymphocyte homing.[2] This variety of functions is attributed to the various isoforms that result from alternative splicing of at least ten variant exons.[3] Adhesion to the extracellular matrix, a critical initial step in the metastatic process, is CD44-dependent for several malignancies.[4,5]

The cyclooxygenase (Cox) enzyme has recently attracted to a great deal of attention because the prolonged use of aspirin has been associated with a 40-50% reduction for the risk of colon carcinoma.[6] Cox is the main target for many non-steroidal anti-inflammatory drugs (NSAIDs).

Two isoforms of the Cox enzyme share a more than 60% homology at the amino acid level, but the two isoforms have distinct physiologic functions. Cox-1 is constitutively expressed at various levels in the majority of tissues and it generally plays a role for tissue homeostasis. Cox-2 has a markedly different distribution pattern, and it usually remains undetectable in the majority of tissues, but it is readily inducible by an array of substances, including inflammatory mediators and mitogens.[7,8] Cox-2 is over-expressed in a variety of malignancies, including lung cancer.[9-12] The over-expression of Cox-2 in tumors is important for tumor invasion,[13] angiogenesis,[14] resistance to apoptosis[15] and suppression of the host's immunity.[16,17]

Since tumor CD44 expression plays an important role in tumor invasion and metastasis, it is hypothesized that CD44 expression may be important for the Cox-2 expression that is noted during human lung cancer invasion. Recently, Dohadwala *et al*. have indicated that increased invasiveness due to the Cox-2 expression was associated with an increased CD44 expression in several lung cancer cell lines.[18] We examined the expression of Cox-2

and CD44 and its variants, by performing immunohistochemistry, and we also examined the biological implications and relationship between Cox-2 and the CD44 variants in non-small cell lung carcinoma.

MATERIALS AND METHODS

Patient selection

We collected specimens from 98 lung cancer patients who were diagnosed as having squamous cell carcinoma or adenocarcinoma from January 1989 to January 2002. All the patients underwent surgical removal of the primary tumor by segmentectomy, lobectomy or pneumonectomy. Their clinical characteristics were reviewed by examining their hospital records. The resected tissue specimens were fixed in 10% buffered formalin and next embedded in paraffin. The histological sections cut from paraffin blocks were routinely stained with hematoxylin and eosin, and then histopathological examinations were performed. The clinical outcome of the patients was followed from the date of surgery to the date of death or to 1 December 2002. The mean follow-up time was 29 months (range: 1-120 months).

Tissue array slide

Tissue array slides were prepared for achieving effective immunological detection. We punched two representative foci of tissue cores (2.0 mm in diameter) from the original paraffin blocks and we inserted the cores into a new paraffin block that had 40 holes. Serial-sectioned slides were then prepared. Each tissue array slide allowed us to analyze 40 specimens at one time and it minimized variations during the staining process.

Immunohistochemistry for Cox-2 and CD44

Five-μm-thick sections from each tissue array block were subjected to immunohistochemical study with using the avidin-biotin peroxidase complex (ABC) technique. After the slides' antigen-retrieval in 10 mM citrate buffer was done with using a microwave oven for 10 min, the endogenous peroxidase activity was blocked with 5% hydrogen peroxide for 15 min. Incubation with the primary monoclonal antibodies that recognized either CD44s (A020; Chemicon International, CA, USA), CD44v3 (VFF-327v3; NeoMarkers, CA, USA), CD44v6 (2F10; Zymed, CA, USA) or Cox-2 (Cayman Chemicals Co., Ann Arbor, MI, USA) was performed overnight at 4℃. Tonsil tissue was used as a positive control for CD44s and its variants. As a positive control for Cox-2, we used a human colon carcinoma that is known to overexpress Cox-2. When more than 10% of the cancer cells showed strong membranous staining for the CD44 stains and cytoplasmic staining for Cox-2, the tumor was judged to exhibit the preserved expression of CD44 and it also was positive for Cox-2.

Statistical analysis

All statistical analyses were performed using a MedCalc 8.0.0.1 statistical software program. The relationships between the variables were examined using the χ^2 test. The cumulative survival rate was obtained using the Kaplan-Meier method. A probability value below 0.05 was considered statistically significant.

RESULTS

The median age of the 98 patients was 63.5 years (age range: 32 to 82 years). Seventy two patients were male and 26 were female (gender ratio 2.7:1). The histological diagnosis was as follows: 50 cases (51.0%) were squamous cell carcinoma and, 48 cases (49.0%) were adenocarcinoma. Table 1, 2 summarizes the clinicopathological features of the patients and the results of the CD44 and Cox-2 immunohistochemical stainings.

Of the 98 patients, 27 (27.5%), 12 (12.2%) and 41 (41.8%) patients showed preserved reaction for CD44s, CD44v3 and CD44v6, respectively. A positive reaction for CD44s, CD44v3 and CD44v6 was observed as membranous staining (Fig. 1, 2). The expression of CD44s, CD44v3 and CD44v6 was significantly more frequent in the squamous cell carcinoma specimens than in the adenocarcinoma specimens (CD44s, p=0.033; CD44v3, p=0.007; CD44v6, p=0.022). Poorly differentiated NSCLC showed a significant correlation with the loss of the CD44s and CD44v3 expression (CD44s, p=0.03; CD44v3, p=0.011) (Table 1). There were no significant correlations between the expression of CD44s and the two variants, and such clinicopathological parameters, as gender, stage, lymph node metastasis, pleural invasion and lymph/vascular invasion. For the squamous cell carcinoma, only the histologic differentiation was significantly correlated with the loss of CD44s and CD44v3 (CD44s, p=0.006; CD44v3, p=0.0003). No correlation was found between the expression of CD44s and its two variants, and the studied clinicopathological parameters for the adenocarcinoma. The cumu-

Table 1. Clinicopathological features and expression of CD44 in lung cancer

	No.	CD44s+ (%)	p-valvue	CD44v3+ (%)	p-valvue	CD44v6+ (%)	p-valvue
Sex							
Male	72	22 (30.5)		11 (15.3)		32 (44.4)	
Female	26	5 (19.2)	>0.05	1 (3.8)	>0.05	9 (34.6)	>0.05
Diagnosis							
SCC	50	19 (38.0)		11 (22)		27 (54)	
ADC	48	8 (16.7)	0.033	1 (2.1)	0.007	14 (29.2)	0.022
Differenitiation							
Well	29	13 (44.8)		8 (27.6)		13 (44.8)	
Moderate	52	12 (23.1)		3 (5.8)		24 (46.1)	
Poor	17	2 (11.8)	0.03	1 (5.8)	0.011	4 (23.5)	>0.05
Lymph node metastasis							
+	46	14 (30.4)		5 (10.9)		21 (45.7)	
–	52	13 (25.0)	>0.05	7 (13.5)	>0.05	20 (38.5)	>0.05
Stage							
I	14	2 (14.5)		2 (14.3)		4 (28.6)	
II	53	17 (32.1)		7 (13.2)		26 (49.1)	
III	31	8 (25.8)	>0.05	3 (9.8)	>0.05	11 (35.5)	>0.05
Pleural invasion							
+	21	6 (28.6)		2 (9.5)		10 (47.6)	
–	77	21 (27.3)	>0.05	10 (12.9)	>0.05	31 (40.3)	>0.05
Lymphovascular invasion							
+	33	7 (21.2)		3 (9.1)		10 (30.3)	
–	65	20 (30.8)	>0.05	9 (13.8)	>0.05	31 (47.7)	>0.05

SCC, Squamous cell carcinoma; ADC, Adenocarcinoma.

Table 2. Clinicopatholical features and expression of Cox-2 in lung cancer

	No. (%)	Cox-2		
		Positive	Negative	p value
Sex				
Male	72 (73.5%)	46	26	
Female	26 (26.5%)	20	6	>0.05
Diagnosis				
SCC	50 (51.0%)	30	20	
ADC	48 (49.0%)	36	12	>0.05
Differentiation				
Well	29 (29.6%)	22	7	
Moderately	52 (53.1%)	33	19	
Poorly	17 (17.3%)	11	6	>0.05
Lymph node metastasis				
Positive	46 (46.9%)	35	11	
Negative	52 (53.1%)	31	21	>0.05
Stage				
I	14 (14.3%)	6	8	
II	53 (54.1%)	37	16	
III	31 (31.6%)	23	8	>0.05
Pleural invasion				
Positive	21 (21.4%)	17	4	
Negative	77 (78.6%)	49	28	>0.05
Lymphovascular invasion				
Positive	33 (33.7%)	25	8	
Negative	65 (66.3%)	41	24	>0.05

SCC, Squamous cell carcinoma; ADC, Adenocarcinoma.

lative survival of the NSCLC patients was a slightly better survival for the CD44-positive group (including CD44s, CD44v3 and CD44v6), but the difference was not statistically significant (CD44s, p=0.913; CD44v3, p=0.146; CD44v6, p=0.0622). The cumulative survival in the adenocarcinoma and squamous cell carcinoma patients was not related to the expression of CD44s and its isoforms.

Sixty-six (67.3%) out of 98 patients were positive for Cox-2. The distribution of the Cox-2 positive cells was heterogeneous in a patchy or diffuse pattern. There were no significant correlations between the Cox-2 expression and such clinicopathological parameters, as gender, the subtype of lung carcinoma, differentiation, stage, lymph node metastasis, pleural invasion and lymph/vascular invasion (Table 2). For the squamous cell carcinoma, there were no significant differences in the frequency of Cox-2 expression with respect to the gender ratio, differentiation, lymph node metastasis, stage, pleural invasion and lymphatic/angio-invasion, respectively. For the adenocarcinoma, there were no significant relationships between the Cox-2 expression and clinicopathological parameters, except for the stage. A positive Cox-2 expression correlated with an advanced stage for adenocarcinoma (p=0.03). The cumulative survival for all the NSCLC and squamous cell carcinoma patients was slightly better for the Cox-2-negative group than for the Cox-2 positive group, but

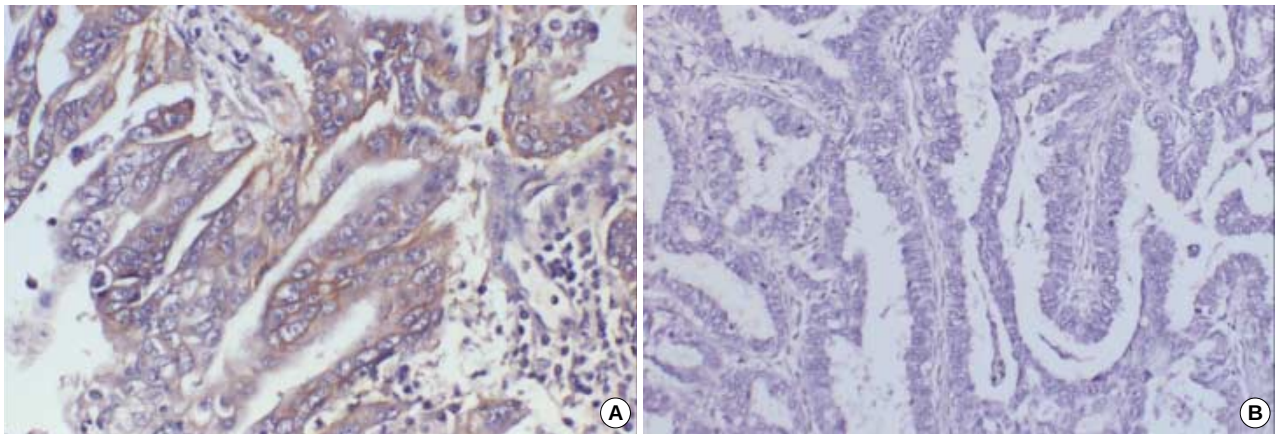

Fig. 1. Preserved membranous expression of CD44s (A) & loss of expression of CD44v3 (B) in adenocarcinoma are observed.

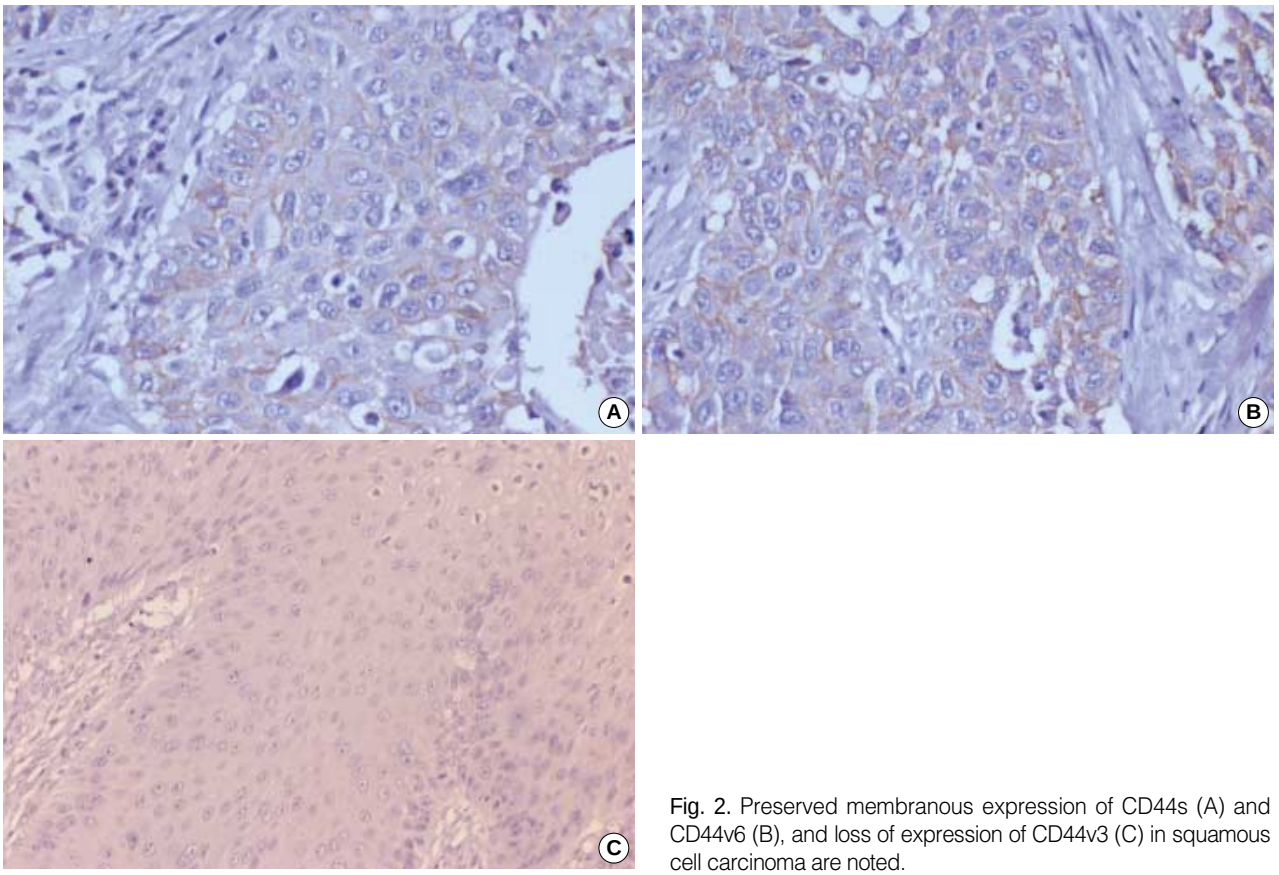

Fig. 2. Preserved membranous expression of CD44s (A) and CD44v6 (B), and loss of expression of CD44v3 (C) in squamous cell carcinoma are noted.

the difference was not statistically significant (NSCLC, p=0.08; squamous cell carcinoma, p=0.34). For adenocarcinoma, the patients with Cox-2 positive-tumors had a significantly worse cumulative survival than did those patients without a Cox-2 expression (Fig. 3, p=0.048). Patients showing a CD44s expression had a significantly higher expression of Cox-2 than did the CD44s negative patients with NSCLC (Table 3, p=0.005) and squamous cell carcinoma (p=0.03).

DISCUSSION

In this study, we observed a significant higher level of immunoreactivity for CD44s, CD44v3 and CD44v6 in the squamous cell carcinoma specimens than that in the adenocarcinoma specimens. These findings are in line with the previously published results,[19,20] and this appears to support the common view of the histogenesis of non-small cell carcinoma, *i.e.*, squamous cell car-

Table 3. Correlation between CD44 and Cox-2 expression in NSCLC

Cox-2	CD44s			CD44v3			CD44v6		
	+	−	p value	+	−	p value	+	−	p value
+	24	42		10	56		27	39	
−	3	29	0.005	2	30	0.207	14	18	0.78

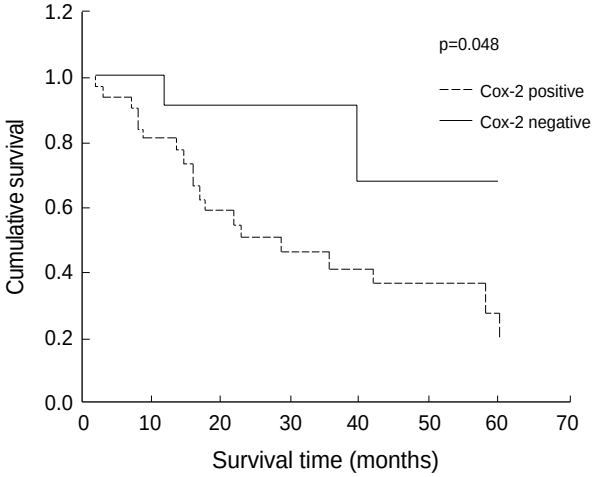

Fig. 3. Cumulative survival curve shows that adenocarcinoma reveals significantly decreased cumulative survival in Cox-2 positive patients (p=0.048).

cinomas arise from metaplastic squamous epithelium that progresses through dysplasia, carcinoma *in situ* and then invasive carcinoma, and the squamous metaplasia is thought to arise from the replacement of the ciliated epithelium by the bronchial basal cells that show a strong positive reaction for CD44. Adenocarcinomas are believed to arise from various epithelial cells, such as Clara cells, type II pneumocytes and the mucous goblet cells.

In our study, the reduced expression of CD44s and its variants tended to be higher in the patients having lymph node metastasis, but this was not significant (p=0.548). Miyoshi *et al*. have found that an increased CD44v6 expression was associated with lymph node metastasis,[21] while Pirinen *et al*. have shown that a reduced CD44v6 expression was associated with lymph node metastasis,[19] and Fasano *et al*. found no relationship between CD44v6 and lymph node metastasis.[22] The conflicting results concerning CD44v6 expression and lymph node metastasis may have resulted from the use of different primary antibodies and different study designs, or they were due to the different criteria that were used to evaluate the staining. Further extensive studies are needed to confirm the relation between the CD44 expression and lymph node metastasis.

In our study, histologic differentiation showed a significant correlation with the loss of the CD44s and CD44v3 expression. Resnick *et al*. have shown that the decreased expression of CD44s was associated with a lower grade, and this was supported by our study.[23]

Our study showed no association between the CD44 expression and the cumulative survival. Our results between the CD44 expression and patient survival were somewhat in conflict with the previously reported results. Pirinen *et al*. showed that the reduced expression of CD44s and CD44v3 was associated with a shortened survival in non-small cell lung carcinoma patients,[19] and Resnick *et al*. showed that the loss of CD44 variants' expression correlated with an increasing tumor stage and more aggressive tumor in lung cancer patients.[23] Tran *et al*. stated that the CD44s, v3 and v6 expressions did not correlate with tumor stage, recurrence and survival for non-small cell lung carcinomas patients.[20] Conflicting research results have also been seen for other cancers. The increased expression of CD44s and its splicing variants might be associated with an unfavorable clinical behavior in non-Hodgkin's lymphoma patients and gastric adenocarcinoma patients.[24,25] Conversely, the loss of CD44s expression is associated with the aggressive behavior of neuroblastoma, prostatic and laryngeal cancers.[26-28] Therefore, it is thought that tumor invasion and metastasis might be enhanced by both the increased cell-matrix interaction in tumors that show CD44 over-expression, and the decreased cell-cell interaction in tumors that show CD44 down-regulation.

Our study confirmed that an increased Cox-2 expression in adenocarcinoma could have prognostic significance, which is in keeping with previous report.[13]

A previous study showed the critical role of a tumor's Cox-2 expression for the regulation of CD44-dependent invasion in the human NSCLC cell line.[18] Sun *et al*. have shown that hyaluronate fragments and cross-linking of CD44 markedly enhanced the Cox-2 and thromboxane mRNA in macrophages.[29] Therefore, hyaluronate itself may serve to enhance Cox-2 in a tumor by a CD44-dependent pathway, and this maintains the Cox-2-dependent invasiveness. Ding *et al*. have recently reported that the expression of 15-hydroxyprostaglandin dehydrogenase (15-PGDH) was negatively related to the expression of CD44 and Cox-2.[30] We revealed that the over-expression of Cox-2 in NSCLC was related with the CD44 expression. Furthermore, we showed that CD44s was associated with Cox-2 over-expression among

the various CD44 isoforms.

Based on previous works and our studies, we suggest it is possible that the over-expression of Cox-2 in NSCLC may be through the up-regulation of CD44 expression, and a tumor's Cox-2 expression in NSCLC constitutes an important driving force for tumor invasion and the metastatic potential through CD44s induction in the lung cancer. However, as a tumor's Cox-2 activity has a multifaceted role in conferring the malignant and/or metastatic phenotypes, and as CD44-independent mechanisms may also be operative, further large scale investigations are needed to confirm the relationship between the Cox-2 expression and CD44s.

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