The EMT Status in the Primary Tumor Does Not Predict Postoperative Recurrence or Disease-free Survival in Lung Adenocarcinoma

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Abstract. *Background: The epithelial to mesenchymal transition (EMT) is an important contributor to the invasion and metastasis of epithelial cell-derived cancer. However, whether or not the expression of EMT-related molecules can be used as a biomarker for the prognosis of lung cancer has yet to be fully determined. Patients and Methods: Tumor specimens were collected from 183 consecutive patients who underwent a complete resection for lung adenocarcinoma. We analyzed the E-cadherin, gamma-catenin, vimentin, and fibronectin expression levels in the primary lung adenocarcinoma by immunohistochemical analysis. Results: A positive expression of E-cadherin, gamma-catenin, vimentin, and fibronectin was observed in 94 (51.4%), 82 (40.4%), 32 (17.5%) and 1 (0.5%) patient, respectively. A significant association between E-cadherin expression and the pathological stage, T status, N status, tumor grade, and carcinoembryonic antigen was identified. The rate of gamma-catenin positivity was higher in patients with a smoking history than in never smokers. Moreover, a significant correlation was observed between vimentin expression and the pathological stage, N status, and tumor grade. However, an association between EMT-related molecules and postoperative recurrence of lung adenocarcinoma is lacking. Based on a Kaplan-Meier analysis, the expression of EMT-related molecules is not associated with the survival of lung adenocarcinoma patients. Conclusion: The EMT status in the primary tumor does not predict postoperative recurrence or disease-free survival in lung cancer patients. Our findings indicate that immunocytochemical markers related to EMT do not provide relevant prognostic information about lung adenocarcinoma. Future research is therefore expected to clarify the clinical usefulness of EMT-related molecules.*

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Lung cancer is the leading cause of cancer-related death in the majority of countries worldwide (1). Non-small cell lung cancer (NSCLC) accounts for approximately 80% of all lung cancer cases, and the proportion of adenocarcinoma is notably increasing (2). Even if successful surgery is performed, many patients still develop tumor recurrence (3). As a result, it is important to evaluate the biological characteristics of NSCLC to identify the factors related to recurrence following surgery (4, 5).

The epithelial to mesenchymal transition (EMT) is characterized by the loss of epithelial cell junction proteins, such as E-cadherin and gamma-catenin, and the gain of mesenchymal markers, such as vimentin and fibronectin (6). The EMT is an important contributor to the invasion and metastasis of epithelial cell-derived cancer. Furthermore, the acquisition of EMT features has been associated with chemoresistance (7). Therefore, we hypothesized that a high level expression of EMT-related molecules may suggest strong malignant potential of the tumor. If this is the case, detection of the positive expression of these molecules might be useful to select the cancer patients who would most benefit from adjuvant chemotherapy after complete resection. However, whether or not the expression of EMT-related molecules can serve as a biomarker for the prognosis of lung cancer has yet to be determined.

This study aimed at evaluating the prevalence and prognostic implications of the immunoreactivity of representative EMT-related molecules in a retrospective series of 183 resected patients with adenocarcinoma of the lung.

Patients and Methods

Patients, clinical features, and follow-up. The Institutional Review Board of our university approved this study and informed consent for the use of the tumor specimens was obtained from all the patients or their legal guardians. Tumor samples were obtained from 296 patients with primary lung adenocarcinoma who had

undergone a surgical resection between 2003 and 2007 in our department. Nine of these patients had stage IV disease, and 25 underwent an incomplete resection. The tumor samples from 79 patients were too small to evaluate by immunohistochemical (IHC) staining for the E-cadherin, gamma-catenin, vimentin and fibronectin status. As a result, 113 patients were excluded from further analysis. Therefore, 183 tumor specimens were evaluated. All of the patients were Japanese, consisting of 102 males and 81 females in this series, with a median age of 70 years (range 23-88 years). The tumor stage was classified according to the TNM Classification for Lung Cancer (8). According to the pathological stage, 106 patients had tumors of stage IA, 39 of IB, 13 of IIA, 6 of IIB, 16 of IIIA and 3 of stage IIIB.

The patients were followed-up every month for the first postoperative year and at approximately 2- to 4-month intervals thereafter. The evaluations included a physical examination, chest roentgenography, a chemical blood analysis, and measurements of tumor markers. Chest and abdominal computed tomography, brain magnetic resonance imaging, and a bone scintiscan were performed every 6 months for 3 years after surgery. Additional examinations were performed if any symptoms or signs of recurrence were detected. Follow-up was conducted for all patients. The median follow-up period was 53.7 months. Twenty-seven (15.3%) patients had received adjuvant chemotherapy as follows: carboplatin plus paclitaxel (n=18), carboplatin plus gemcitabine (n=7), and tegafur–uracil (UFT) (n=2) (9, 10). At the last follow-up (in September 2011), 144 patients were alive and free of cancer, 11 patients had died of other causes without evidence of cancer, 8 patients were alive with recurrent cancer, and 20 patients had died of cancer. In total, 28 (15.3%) our of the 183 patients demonstrated disease recurrence after surgery. The majority of the sites of tumor recurrence were hematogenous metastases. Twenty-five cases had hematogenous (9 brain, 10 lung, 5 bone, and 1 adrenal metastasis) and six had locoregional (4 lymph node metastasis and 2 pleural dissemination) recurrences. Recurrent tumors in both the brain and bone in two cases, brain and adrenal gland in one, and bone and lymph nodes in one, respectively.

IHC staining of paraffin-embedded tumor samples. IHC staining was conducted using serial sections from the same paraffin-embedded blocks by previously described methods (11, 12). Briefly, all tissue specimens were formalin-fixed and processed similarly, according to the standard histology practices. A 3 μm thick formalin-fixed, paraffin-embedded tissue section was prepared from each specimen. All specimens were stained with hematoxylin–eosin for the histological diagnosis. The sections were briefly immersed in citrate buffer [0.01 mol/l citric acid (pH 6.0)] and then were incubated twice for 10 min at 121°C in a high-pressure sterilization oven for antigen retrieval. They were then incubated with antibody against E-cadherin (DAKO, Denmark) diluted at 1:2000; gamma-catenin (Dako) diluted at 1:200; vimentin (DAKO) diluted at 1:200; and fibronectin (DAKO) diluted at 1:100; in phosphate-buffered saline 60 min at room temperature (13). Thereafter, IHC staining was performed by the labeled polymer method (Histofine Simple Stain MAX-PO kit, Nichirei, Tokyo, Japan) according to the manufacturer's instructions (14). The positive controls for E-cadherin, and gamma-catenin were samples of the normal bronchial epithelium adjacent to the tumor, and the controls for vimentin and fibronectin were samples of malignant mesothelioma (7, 15). The negative controls used mouse IgG (Dako) instead of the primary antibody.

Evaluation of the stained specimens. Following the IHC detection of protein expression in each specimen, the percentage of immunoreactive tumor cells in five fields at ×400 selected randomly on one slide was recorded, and then the final proportion of positive tumor cells was determined as the average. To evaluate any correlations with the clinicopathological characteristics, these protein expression scores were divided into positive or negative groups by subdividing according to the percentage of nuclear staining using a cutoff of 50% (7). The slides were independently examined by two of the investigators (Y.C. and H.S.) who were blinded to the clinicopathological data. When a discrepancy was found between the two investigators, a consensus was reached via their simultaneous examination using a double-headed microscope.

Statistical analyses. Statistical significance was evaluated using the chi-square test or Fisher's exact test. The Kaplan-Meier method was used to estimate the probability of survival, and survival differences were analyzed by the log-rank test. A multivariate analysis was then performed according to Cox's proportional hazards model. The odds ratio (OR) and 95% confidence interval (95% CI) were calculated for each variable. Differences were considered to be statistically significant for p-values <0.05. The data were analyzed using the Stat View software package (Abacus Concepts, Inc., Berkeley, CA, USA).

Results

Detection of the EMT-related molecules and correlations among clinicopathological factors. E-Cadherin, gamma-catenin, vimentin, and fibronectin expression were observed in 94 patients (51.4%), 82 (40.4%), 32 (17.5%), and only 1 (0.5%) patient. A significant association between E-cadherin expression and the pathological stage, T status, N status, tumor grade, and tumor marker (carcinoembryonic antigen) was identified with the clinical factors. Positive expression of gamma-catenin was significantly more common in patients with a smoking history than in never smokers. Moreover, a significant correlation was observed between vimentin expression and the pathological stage, N status, and tumor grade. No significant association between fibronectin and clinicopathological characteristics was found (Table I).

Influence of the EMT-related molecules on postoperative recurrence. Positive E-cadherin expression was identified in 16 (64.0%) our of 25 patients and 78 (49.4%) our of 158 patients in patients with and without recurrence, respectively (p=0.174). Positive gamma-catenin and vimentin expression were also identified in 17 (68 %) and 7 (25%), and 65 (36.1 %) and 26 (16.1%) of the patients with and without recurrence, respectively (p=0.066 for gamma-catenin, p=0.255 for vimentin) (Table II). Thus, an association between EMT-related molecules and postoperative recurrence in lung adenocarcinoma is lacking.

The influence of EMT-related molecules on disease-free survival. The 5-year DFS rate in patients with negative and

Table I. *The relationship between E-cadherin/ gamma-catenin/ vimentin/ fibronectin expression and the clinicopathological characteristics.*

Variable	Category	No. of patients (n=183)	E-cadherin			Gamma-catenin			Vimentin			Fibronectin		
			+, 94 (51.4 %)	–, 89	*p*-Value	+, 82 (40.4 %)	–, 101	*p*-Value	+, 32 (17.5 %)	–, 101	*p*-Value	+, 1 (0.5 %)	–, 182	*p*-Value
Gender	Male	102	55 (53.9)	47	0.438	52 (51.0)	50	0.596	20 (19.6)	82	0.397	1	101	0.372
	Female	81	39 (48.1)	42		30 (37.0)	51		12 (14.8)	69		0	81	
Age (years)	<=70	90	49 (54.4)	41	0.729	38 (42.2)	52	0.489	18 (20.0)	72	0.379	1	89	0.308
	>70	93	53 (56.9)	40		44 (47.3)	49		14 (15.1)	79		0	93	
Pathology stage	IA	106	44 (41.5)	62	0.002	42 (39.6)	64	0.098	12 (11.3)	94	0.01	0	106	0.239
	IB-III	77	50 (71.4)	27		40 (51.3)	37		20 (26.0)	57		1	76	
T Status	T1a	76	30 (39.0)	47	0.006	29 (38.2)	47	0.127	10 (13.2)	66	0.194	0	76	0.398
	T1b-4	107	62 (60.4)	42		53 (49.5)	54		22 (20.6)	85		1	106	
N Status	Negative	152	75 (49.3)	77	0.011	66 (43.4)	86	0.084	22 (14.5)	130	0.018	1	151	0.651
	Positive	31	18 (58.1)	12		16 (51.6)	15		10(32.3)	21		0	31	
Smoking history	Never	74	35 (46.7)	40	0.289	26 (35.1)	48	0.03	11 (14.9)	63	0.442	0	74	0.409
	F/ C	109	59 (54.6)	49		56 (51.4)	53		21 (19.3)	88		1	108	
Tumor grade+	G1	91	40 (43.4)	52	0.02	37 (40.7)	54	0.393	10 (11.0)	81	0.029	0	91	0.272
	G2-G3	76	45 (61.6)	28		35 (46.1)	39		18 (23.7)	58		1	75	
CEA+	<2.5	126	58 (47.2)	65	0.016	52(41.3)	74	0.124	22 (17.5)	104	0.865	0	126	0.126
	≥2.5	54	33 (57.9)	24		29 (50.9)	25		10 (18.5)	44		1	53	

F/C: Former current, +: unclassified patients were excluded, CEA: carcinoembryonic antige.

Table II. *The relationship between E-cadherin, gamma-catenin, and vimentin expression and cancer recurrence.*

	E-Cadherin			Gamma-catenin			Vimentin		
Variable	+, n (%)	–, n	*p*-Value	+, n (%)	–, n	*p*-Value	+, n (%)	–, n	*p*-Value
With recurrence	16 (64)	9	0.174	17 (68)	11	0.066	7 (25.0)	21	0.255
Without recurrence	78 (49.4)	80		65 (36.1)	90		25 (16.1)	130	

positive E-cadherin expression was 89.1% and 82.0%, respectively (p=0.183). The 5-year DFS rate in those negative and positive for gamma-catenin expression was 80.7% and 68.1%, respectively (p=0.845). The 5-year DFS rate in patients who were negative and positive for vimentin expression was 76.7% and 67.9%, respectively (p=0.452) (Figure 1). Thus, the Kaplan-Meier survival curves demonstrated that the expression of EMT-related molecules was not associated with any statistically significant differences in survival for lung cancer patients. In addition, positive expression of EMT-related molecules was not associated with an increased risk of death based on the univariate analysis (Table III). A multivariate survival analysis also failed to demonstrate that E-cadherin, vimentin, and fibronectins expressions were independently associated with an increased risk for a poor DFS (Table IV).

Discussion

Our results are unique for several reasons: (i) we used a comparatively larger case series of 183 consecutive tumors; (ii) it was limited to the analysis of adenocarcinoma, which is relatively homogeneous; (iii) the method was based on simple IHC staining, which has the advantage of maintaining the morphology of the tissue, and minimizing sampling error. Our hypothesis was that deregulated EMT status can indicate aggressive behavior in lung adenocarcinoma. If the hypothesis had been proven correct, the detection of the EMT status might have allowed for the identification of the patients at a high risk for recurrence.

The present study demonstrated four major findings. Firstly, positive expression of E-cadherin was more common in patients with advanced-stage disease than these with early disease. However, a previous report showed that E-cadherin

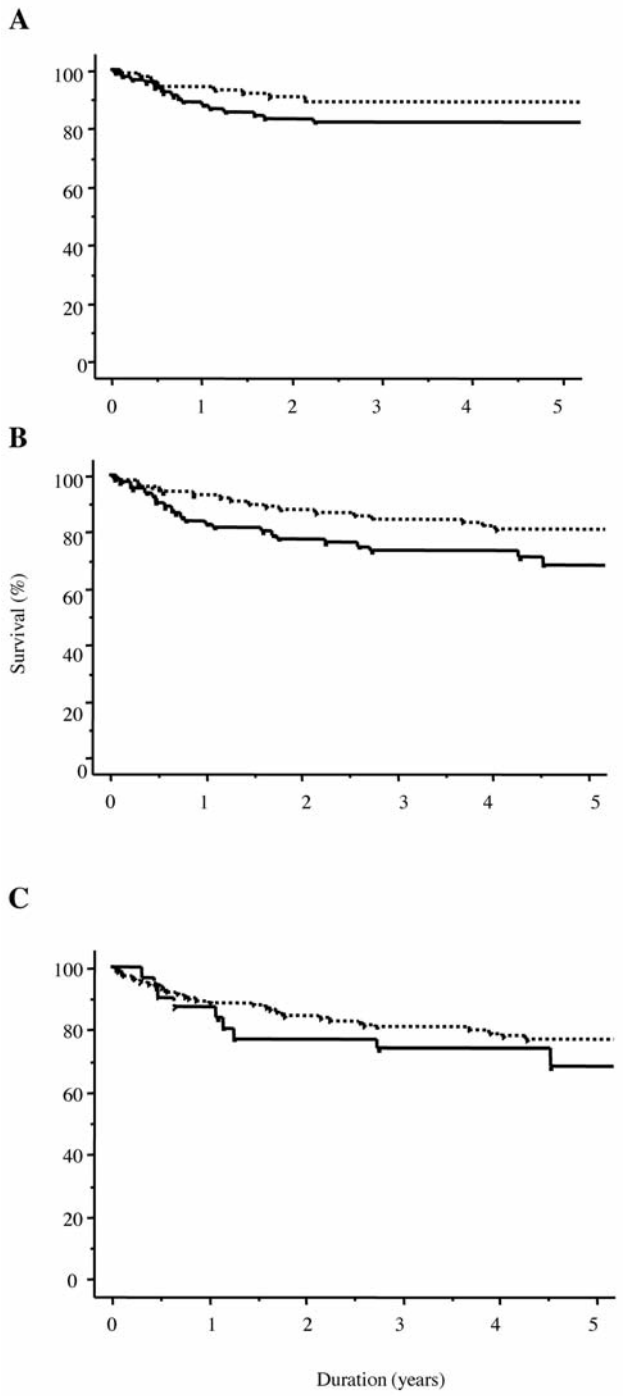

Figure 1. *Kaplan-Meier (disease-free survival) curves stratified by the expression of EMT-related molecules. The heavy and dotted lines indicate positive and negative expression of EMT-related molecules, respectively. A: The 5-year DFS rates in patients with negative and positive E-cadherin expression were 89.1% and 82.0% (p=0.183), respectively. B: The 5-year DFS rates in patients with negative and positive gamma catenin expression were 80.7% and 68.1% (p=0.845), respectively. C: The 5-year DFS rates in patients with negative and positive gamma-catenin expression were 76.7% and 67.9% (p=0.452), respectively.*

Table III. *The results of the univariate analysis using a proportional hazard model for the disease-free survival.*

Variable	Characteristic		95% CI	HR	*p*-Value
	Unfavorable	Favorable			
Gender	Male	Female	1.247-4.950	2.481	0.010
Age (years)	≤70	>70	0.907–3.155	1.692	0.098
T Status	1b-4	1a	1.144-4.525	2.278	0.019
N Status	Positive	Negative	2.481-8.621	4.608	<0.001
E-Cadherin	Positive	Negative	0.764-3.906	1.73	0.189
Gamma-catenin	Positive	Negative	0.924-3.125	1.701	0.088
Vimentin	Positive	Negative	0.634-2.770	1.326	0.454

95% CI: 95% Confidence interval, HR: hazard ratio.

Table IV. *The results of the multivariate analysis using a proportional hazard model for disease-free survival.*

Variable	Characteristics		95% CI	HR	*p*-Value
	Unfavorable	Favorable			
Gender	Male	Female	1.142-4.608	2.296	0.020
Age (years)	≤70	>70	1.042-6.280	1.739	0.089
T Status	1b - 4	1a	3.584-4.367	1.757	0.122
N Status	Positive	Negative	2.105-7.874	4.065	<0.001
E-Cadherin	Positive	Negative	0.519-2.841	1.215	0.654
Gamma-catenin	Positive	Negative	0.737-2.558	1.372	0.318
Vimentin	Negative	Positive	0.535-2.443	1.144	0.723

95% CI: 95% Confidence interval, HR: hazard ratio.

was a favorable prognostic factor in a univariate analysis by the log-rank test (16). Their data included 53 (29%) squamous cell carcinomas (SQ) out of 185 of NSCLC. There are significant differences in the incidence of E-cadherin expression between SQ and non-SQ NSCLC (16). Therefore, the discrepancy between this study and our present study may be attributable to differences in sampling.

Secondly, the frequency of positive expression of gamma-catenin was higher in smokers than in non-smokers. However, in vitro studies demonstrated that benzo(a)pyrene commonly found in cigarette smoke seems to induce EMT in lung cancer cells (17) and nicotine may also induce invasion, therefore, the EMT may contribute to the progression of lung cancer (18). The reason for the differences between our findings and those of in the vitro findings remains to be elucidated. Complicated molecular mechanism underlying the multiple and different stages of the transition might exist in human cancer *in vivo* (19).

Thirdly, positive expression of vimentin was more frequently recognized in advance stage cancer than early

cancer. These results seem to be reasonable because vimentin has been used as a representative mesenchymal marker. However, vimentin itself was not a predictive marker for survival in this study. It is likely that no single marker independently determines tumor prognosis, but rather, a combination of several markers may provide a more powerful method for predicting a patient's outcome.

Fourthly, we failed to discover significant correlations between the expression of EMT-related molecules and recurrence in lung adenocarcinoma. Furthermore, the expression of EMT-related molecules was also not associated with a poor DFS, which was consistent with another cohort study (11). On the other hand, Al-saad *et al*., showed that higher E-cadherin expression was a favorable prognostic indicator in NSCLC. However, that study included 57% SQ tumors (20). Lee *et al*., also showed that higher gamma-catenin, but not E-cadherin, expression was a favorable prognostic indicator in stage I NSCLC, including 20% SQ tumors (15). The discrepancies between these studies might be due to the population examined. In fact, we recently reported that gamma-catenin expression was found more frequently in SQ, and a multivariate survival analysis demonstrated that neither E-cadherin nor gamma-catenin expression were independently associated with an increased risk of poor survival in patients who underwent surgical resection of stage I NSCLC (11). High vimentin expression has been suggested to be an independent prognostic factor for poor survival in resected NSCLC (20). In contrast, another report of 113 resected NSCLC patients found no such correlation (21). Our results were consistent with the latter study. Ultimately, our findings suggest that the expression of EMT-related molecules is not likely to provide a suitable biomarker to identify candidate patients for adjuvant chemotherapy. Thus, EMT might result in metastasis, but the presence of EMT-related molecules in the primary tumor does not affect the course of the disease. In fact, acquired resistance of tumors to (EGFR-TKI) led to changes in the EMT status in a specific population (7). The present study has two limitations in its interpretation: its retrospective nature, and the fact that it was carried out at a single institution.

Conclusion

In summary, we have clearly demonstrated that the expression of EMT-related molecules does not have any impact on the prognosis of patients with lung adenocarcinoma. This evidence is not sufficient to alter any established clinical treatments or even to restrict the clinical trials of adjuvant treatments to predefined biological subsets of patients. Future research is necessary in order to clarify the biological role of EMT-related molecules to determine their full clinical usefulness. Thus, using a matched analysis between the primary tumor and metastatic lesions, such as lymph nodes might be necessary, because the information available from the primary lung tumor may not provide insight into the course of the disease.

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