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Clinical significance of epithelial–mesenchymal transition–related molecules in lung adenocarcinoma

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ABSTRACT

Background Epithelial–mesenchymal transition (EMT) refers to the biologic process in which epithelial cells are transformed into interstitial phenotypes by specific pathways. This transition plays an important biologic role in the process by which epithelium-derived malignant tumour cells acquire the ability to migrate and invade. We explored the relationship between EMT-associated molecules and patient-related clinical factors to determine whether any clinical characteristics could be used as biomarkers for EMT-related protein alterations in lung cancer—especially lung adenocarcinoma.

Methods Tumour specimens were collected from 80 patients with lung adenocarcinoma who underwent surgery or lung biopsy, with 4 patients being evaluated a 2nd time after re-biopsy. Expression of EMT-related proteins, including E-cadherin and vimentin, was evaluated by immunohistochemistry. We analyzed the relationship between clinicopathologic characteristics and expression level of the EMT markers.

Results Positive expression of E-cadherin was observed in 63 patients (79%), and vimentin, in 46 patients (57.5%). No significant relationships between E-cadherin or vimentin expression and smoking history, sex, age, driving gene mutations, or cell differentiation were identified. A significant correlation was observed between vimentin expression and pathologic stage. Of the 4 patients who were evaluated a 2nd time after re-biopsy, 3 showed the same EMT-related protein expression status as in the first analysis. In the remaining patient, E-cadherin had changed completely.

Conclusions Clinicopathologic factors in cancer patients did not help to diagnose EMT status in lung adenocarcinoma; however, TNM stage might be associated with vimentin expression.

Key Words Epithelial–mesenchymal transition, lung adenocarcinoma, E-cadherin, vimentin, clinicopathologic factors

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INTRODUCTION

Lung cancer is still one of the most common causes of cancer death worldwide and in China, although many efforts have been made to prolong survival for patients[1,2]. In non-small-cell lung cancer (NSCLC), and especially adenocarcinoma, the major subtype of lung cancer[3], overall survival is not optimistic. Molecular profiling studies have revealed that lung cancer is a complex disease with variable molecular mechanisms[4,5]. In patients with lung adenocarcinoma, infiltration and metastasis are the leading cause of death[6].

E-Cadherin, a member of the cadherin superfamily, is classified as a calcium-dependent cell–cell adhesion molecule[7]. In addition, E-cadherin connects the extracellular environment with the cytoskeleton and interacts with catenins such as α-catenin and β-catenin through its conserved cytoplasmic domain. As a result, E-cadherin is crucial for the maintenance of structural and functional integrity[8]. Abnormal expression of E-cadherin has been

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proved to play an important role in invasion and metastasis in a variety of human malignancies[9–12].

Vimentin is an intermediate filament protein in mesenchymal cells[13]. Vimentin regulates the cytoskeleton and cell adhesion molecules, and participates in adhesion, migration, invasion, and signal transduction for tumour cells and tumour-associated endothelial cells and macrophages[14,15]. Recent studies have shown that overexpression of vimentin in cancer correlates well with accelerated tumour growth, invasion, and poor prognosis[16].

Epithelial–mesenchymal transition (EMT) is an important biologic process that results in the loss of epithelial cell junction proteins such as E-cadherin and the gain of mesenchymal markers such as vimentin[17]. Research into EMT has demonstrated that epithelium-derived malignant tumour cells use EMT to acquire the ability to migrate and invade[17,18].

Although the effect of the markers of EMT has been described in recent years, the relationships between those markers and clinical factors in patients are still poorly understood. The aim of the present study was to evaluate the relationship between EMT-related markers and clinical characteristics in patients with lung adenocarcinoma. We also determined consistency of the expression of EMT-related molecules in patients who were evaluated a 2nd time after re-biopsy.

METHODS

Patients

We collected data including clinical characteristics, tumour stage, and cell differentiation for 80 patients with lung adenocarcinoma who underwent biopsy or surgical resection from February 2013 to December 2017 at Drum Tower Hospital Medical School of Nanjing University. Tumour samples were also obtained for the 33 men (41%) and 47 women (59%) in that group, who had a mean age of 61 years. The group included 59 never-smokers and 21 past or current smokers. Tumour stage was determined using the TNM classification for lung cancer, version 8, and according to pathologic stage, 16 patients (20%) had stage I disease; 6 (7.5%), stage II; 28 (35%), stage III; and 30 (37.5%), stage IV. Tumours were poorly differentiated in 24 patients (30%) and well or moderately differentiated in 34 (42.5%), with no relevant information about cell differentiation being available for 22 patients (27.5%). Table I presents details.

TABLE I Characteristics of the 80 study patients

Characteristic	Value [*n* (%)]
Sex	
Men	33 (41)
Women	47 (59)
Age group	
>61 Years	36 (45)
≤61 Years	44 (55)
Smoking status	
Yes	21 (26)
No	59 (74)
TNM stage	
I	16 (20)
II	6 (7.5)
III	28 (35)
IV	30 (37.5)
Cell differentiation	
Poorly	24 (30)
Well or moderately	34 (42.5)
No information	22 (27.5)
Driver mutation	
EGFR	43 (54)
ALK	5 (6)
EGFR and *ALK*	2 (2.5)
Wild type	30 (37.5)

Detection of Driver Mutations

Mutations in *EGFR* were examined using the amplification-refractory mutation system or next-generation sequencing in specimens of NSCLC. Rearrangements in *ALK* were evaluated by immunohistochemistry or next-generation sequencing.

Staining of Tumour Specimens

Immunohistochemistry for E-cadherin and vimentin was performed. The pathology examination was carried out by the Department of Pathology, Drum Tower Hospital Medical School of Nanjing University. Tumour samples were formalin-fixed and, using standard histology practices, serially sectioned. Slides were placed in a 60°C oven for 30 minutes, deparaffinized, and rehydrated in xylenes and graded ethanol solutions to water. Antigen retrieval was performed by a steamer method in which the specimens were placed in a solution of 0.01 mol/L EDTA (pH 8) for 30 minutes at 94°C in a steamer. Primary antibodies were applied overnight at 4°C and then incubated at room temperature with horseradish peroxidase conjugated with anti-mouse secondary antibody for each primary antibody. Appropriate positive and negative controls were used during the immunohistochemical analysis. The slices stained for E-cadherin and vimentin then underwent optical microscopy. Positive expression of the proteins was determined in the cell membrane (E-cadherin) and cytoplasm (vimentin). Results are reported as an immunoreactivity score[19]:

$$\text{Immunoreactivity score} = \text{SI} \times \text{PP}.$$

where SI is the staining intensity (classified as 0, negative; 1, weak; 2, medium; or 3, strong), and PP is the percentage of positive cells (defined as 0, negative; 1, 1%–10% positive cells; 2, 11%–50% positive cells; 3, 51%–80% positive cells; or 4, >80% positive cells). A positive staining result was defined as score greater than or equal to 4. Table II shows the details of the immunohistochemistry analysis.

TABLE II Expression of epithelial–mesenchymal transition–related molecules in 80 patients

	E-Cadherin		Total
	Positive	Negative	
Vimentin			
Positive	44	2	46
Negative	19	15	34
Total	63	17	80

Statistical Analyses

Statistical significance was evaluated using the chi-square or Fisher exact test, as appropriate. Differences were considered to be statistically significant at a p value less than 0.05. The data were analyzed using the IBM SPSS Statistics software application (version 19.0: IBM, Armonk, NY, U.S.A.).

RESULTS

Detection of Driver Mutations and EMT-Related Molecules

All patients were evaluated for driver mutations. Mutations in *EGFR* were present in 54% of patients (43 of 80), and *ALK* rearrangements were present in 6% (5 of 80). In addition, *EGFR* mutation and *ALK* arrangement were both present in 2 patients (Table I).

Figure 1 shows positive immunohistochemical expression of EMT-related molecules (E-cadherin and vimentin) and negative expression in liver metastasis (E-cadherin) and in lung adenocarcinoma (vimentin). E-Cadherin positivity was observed in 63 patients (79%), and vimentin positivity, in 46 patients (57.5%). Positivity for both E-cadherin and vimentin was noted in 44 patients (55%, Table II).

Correlation of EMT-Related Molecules with Clinical Characteristics

In the present study, positive expression of vimentin was significantly correlated with tumour pathologic stage (p = 0.007, Table III). However, the clinical factors considered—such as sex, age, smoking history, cell differentiation, and driver mutations—showed no correlation with vimentin expression ($p > 0.05$, Table III). Similarly, positive expression of E-cadherin was not significantly associated with the clinicopathologic characteristics collected, nor with pathologic stage (all $p > 0.05$, Table III).

To determine whether the two EMT-related biomarkers, when expressed simultaneously, behaved differently, we classified the expression of E-cadherin and vimentin into various levels. Expression levels of the proteins were significantly correlated with tumour pathologic stage (p = 0.007, Table IV). However, such expression was never found to be associated with driver mutations ($p > 0.05$).

Multiple Detections of EMT-Related Molecules

In 4 patients, the EMT-related molecules were evaluated a 2nd time after re-biopsy, with 2 of the samples being obtained from metastases. In 3 patients, expression results for both E-cadherin and vimentin were similar in the first and second samples. However, in 1 patient, E-cadherin was negative in liver at re-biopsy, but still positive in lung. Table V shows details of the analyses.

DISCUSSION

In recent decades, substantial progress has been made in all aspects of lung cancer, including screening, diagnostic evaluation, surgery, radiation therapy, and chemotherapy, as well as in therapies targeting the epidermal growth factor receptor and anaplastic lymphoma kinase, among others. Tyrosine kinase inhibitors are used in patients with *EGFR* mutations and *ALK* rearrangements. In addition, immunotherapy has also recently become a major therapeutic modality in NSCLC[20–22]. Despite that progress, 5-year survival in lung adenocarcinoma is less than 12%–15%[23].

Epithelial–mesenchymal transition is described as downregulation of the expression of E-cadherin (the epithelial cell marker) and upregulation of the expression of vimentin (the mesenchymal marker)[24]. Some research has suggested that EMT could be an important mechanism of tumour invasion[25,26]. In the present study, we collected data for 80 patients and analyzed relationships between the clinical characteristics of those patients and the EMT-related molecules to identify whether the EMT molecules have any special diagnostic value in lung adenocarcinoma. Deletions or insertions in exon 19 and point mutations in exons 18 and 21 in *EGFR* are common in patients with advanced-stage NSCLC and might contribute to metastasis to specific organs[27–29]. We therefore classified the study patients into groups with and without driver mutations, hypothesizing that EMT status might be associated with the driver mutations in lung adenocarcinoma. In addition, we evaluated multiple detections of EMT status to assess consistency.

All samples from our patients were evaluated for driver mutation status, and 54% were positive for an *EGFR* mutation. About 20% of patients with lung adenocarcinoma are reported to have an *EGFR* mutation; however, a prospective molecular epidemiology study demonstrated that the rate of *EGFR* mutation rose to more than 60% in non-smoking and Asian populations[30], and thus the *EGFR* mutation results in our study appear reasonable. We can do more to investigate the reasons for those results in future.

The incidence of *ALK*-positive NSCLC has been reported to be approximately 3%–7%, with no significant difference between Eastern and Western populations[31,32]. In our study, 5 of 80 patients (6%) were positive for *ALK* rearrangement, which corresponds with those prior reports.

Given our results, we have three main findings. First, positive expression of E-cadherin, a biomarker of epithelial cells, was approximately 79% in our patients, which corresponds to rates published in other reports[16,33]; however, we observed no associations between E-cadherin and any of the disease characteristics that we collected in our research, including pathologic stage, cell differentiation, and driver gene mutations. A previous report showed that downregulation of E-cadherin was more common in patients with advanced-stage disease than in those with early-stage disease (III/IV vs. I/II; odds ratio: 1.87; 95%

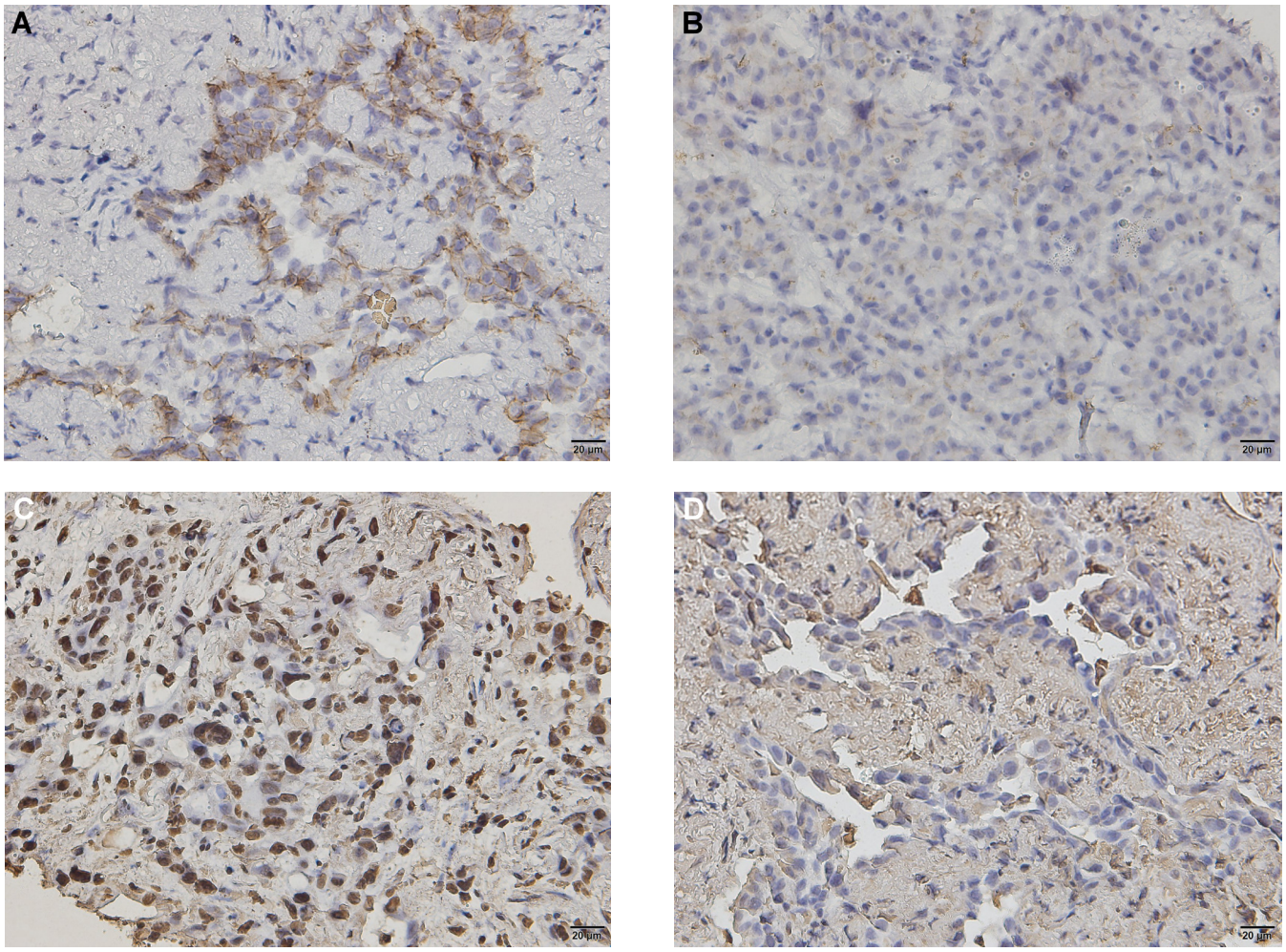

FIGURE 1 Positive immunohistochemical expression of epithelial–mesenchymal transition–related molecules in lung adenocarcinoma [(A) E-cadherin, (C) vimentin] and negative immunohistochemical expression of E-cadherin in liver metastasis and vimentin in lung adenocarcinoma [(B) E-cadherin, (D) vimentin].

confidence interval: 1.27 to 2.76; $p = 0.002$)[34], a result that seemed reasonable because E-cadherin has been used as a representative epithelial marker. Positive expression of E-cadherin was lower in tissue from poorly differentiated tumours than in tissue from well and moderately differentiated tumours ($p < 0.0001$)[35]. Our study might suggest that, although EMT is one of the most important causes of tumour metastasis, other factors could play crucial role in lung adenocarcinoma. Bulk tumour-cell migration has been reported to potentially be more common than EMT in lung carcinoma, and tumour cells were found to be able to migrate without changing to the mesenchymal phenotype called "epithelial migration type"[36].

Some studies have shown that reduction in E-cadherin expression is related to a decrease in sensitivity to epidermal growth factor receptor tyrosine kinase inhibitor and to *EGFR* status[37,38], results that are not consistent with the present study. No matter the kind of driver mutation, we found little association of such mutations with E-cadherin expression. We believe that it might be too simple to classify the biomarker as either positive or negative; further study into expression levels should be done.

Vimentin is reported to play an important role in EMT, being involved in the complex process of tumour metastasis and migration[39–41]. Although positive expression of vimentin was 57.5% in the present study, no significant relationship of vimentin with clinical factors such as sex, age, smoking history, driver gene mutations, or cell differentiation were observed in the study. But vimentin expression is supposed to be associated with tumour pathologic stage, a result supported by some studies[42,43]. We noted a higher trend of positive vimentin expression in early-stage disease ($p = 0.007$), which is contrary to the report by Chikaishi *et al.*[16]. But looking at specific tumour stages, we find that vimentin is downregulated and E-cadherin is upregulated in early-stage NSCLC. Our study results suggest that a mesenchymal–epithelial transition might take place in the early stages of tumour development. Results from the present study indicate that increased vimentin expression correlates positively with poorly differentiated

TABLE III Relationships of epithelial–mesenchymal transition–related molecules with clinical factors in lung adenocarcinoma

Characteristic	Pts (*n*)	E-Cadherin		*p* Value	Vimentin		*p* Value
		Positive	Negative		Positive	Negative	
Differentiation				1.000			0.263
Poorly	24	19	5		12	12	
Well or moderately	34	27	7		22	12	
No information	22	17	5		12	10	
TNM stage				0.052			0.007
I–II	22	21	1		18	4	
III–IV	58	42	16		28	30	
Sex				0.584			0.364
Men	33	25	8		17	16	
Women	47	38	9		29	18	
Age group				0.066			0.892
≤61 Years	44	38	6		25	19	
>61 Years	36	25	11		21	15	
Smoking status				0.981			0.286
Yes	21	16	5		10	11	
No	59	47	12		36	23	
Driving gene mutation				0.312			0.155
EGFR	43	32	11		25	18	
ALK	5	3	2		1	4	
EGFR and *ALK*	2	2	0		2	0	
Wild type	30	26	4		18	12	

Pts = patients.

TABLE IV Relationships of the expression levels of epithelial–mesenchymal transition–related molecules with clinical factors in lung adenocarcinoma

Characteristic	Expression type				*p* Value
	EC+, V+	EC+, V–	EC–, V+	EC–, V–	
TNM stage					0.007
I	14	1	0	1	
II	4	2	0	0	
III	16	7	2	3	
IV	10	9	0	11	
Driver mutation					0.47
EGFR	23	9	2	9	
ALK	1	2	0	2	
EGFR and *ALK*	2	0	0	0	
Wild type	18	8	0	4	

EC = E-cadherin (+, positive; –, negative); V = vimentin (+, positive; –, negative).

tumours (odds ratio: 2.133; 95% confidence interval: 1.664 to 2.735; $p < 0.001$)[44], although we observed no particular relevance of that finding. Studies have shown that vimentin is abnormally expressed in tumour cytoplasm[14,45], but in the present study, we found that nuclear vimentin was expressed in 27 patients [Figure 1(C)], and Luo *et al.*[46] reported positive expression of vimentin in cell nuclei in nasopharyngeal carcinoma. Thus, we suggest that abnormal localization of vimentin expression might be important in tumour metastasis. Still, it is hard to say that vimentin plays a key role in tumour migration and metastasis; further research is required to elucidate that hypothesis.

Second, to identify any interaction between the expression of E-cadherin and vimentin, we analyzed our results using various combinations of expression types. We observed a significant association between the various biomarker expression types and tumour stage ($p = 0.007$, Table IV). The various biomarker expression combinations were seen to be different in tumours of various stages, potentially suggesting that interactions between the EMT-related proteins might play a key role in tumour progression. Notably, we observed no significant difference in the combination of expression types for patients having driver mutations.

Third, 4 of our 80 patients were evaluated multiple times because of cancer progression. The first biopsy site in all 4 patients was the primary lung lesion. The rebiopsy sites depended on circumstances: in patients 1 and 3, the rebiopsy came from an intrapulmonary metastasis resulting from local progression; in patients 2 and 4, the rebiopsy came from a distant metastasis to the liver. Just 1 patient showed

TABLE V Multiple detections of epithelial–mesenchymal transition–related molecules in the study patients

Pt ID	Sample site[a]	Sex	Age (years)	Smoking status	*EGFR* mutation	Biopsy status		TNM stage	Cell differentiation
						E-Cadherin	Vimentin		
1	Lung primary Lung metastasis	Female	62	No	Exon 21	Positive Positive	Positive Positive	IB	Well or moderately
2	Lung primary Liver metastasis	Female	54	No	Exon 19	Positive Positive	Negative Negative	IV	Poorly
3	Lung primary Lung metastasis	Male	70	Yes	Exon 19	Positive Positive	Negative Negative	IV	Well or moderately
4	Lung primary Liver metastasis	Male	66	Yes	Exon 19	Positive Negative	Negative Negative	IV	Poorly

[a] For all 4 patients, the first biopsy site was the primary lung lesion. The re-biopsy site depended on circumstances. For patients 1 and 3, the sample came from an intrapulmonary metastasis resulting from local progression; for patients 2 and 4, the sample came from liver because of distant metastasis.

a change in E-cadherin expression—a result that seemed to be reasonable, because EMT occurs during tumour metastasis. That finding might suggest that tumour progression was related to EMT and that the downregulation of E-cadherin might lead to tumour metastasis. However, we could not draw a conclusion because the evidence was too weak. Although EMT happens during tumour progression and metastasis, research has not demonstrated a significant difference in EMT characteristics between primary cancers, lymphatic metastases, and cancer emboli[47]. Hence, results from the analysis of primary and metastatic lesions remain a matter of debate. Further studies of EMT-related proteins in primary and metastatic lesions are therefore required.

CONCLUSIONS

Our study showed that clinical characteristics do not have any significant relationship with the expression of E-cadherin in patients with lung adenocarcinoma. However, alteration in vimentin expression was associated with TNM stage in patients with lung adenocarcinoma. Some combination of expression types might be also associated with TNM stage. Although E-cadherin was downregulated in our study, the evidence was too weak to support any definitive conclusions. Further study about primary and metastatic lesions should be done to evaluate expression consistency.

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CONFLICT OF INTEREST DISCLOSURES

We have read and understood *Current Oncology*'s policy on disclosing conflicts of interest, and we declare that we have none.

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