

# Clinicopathological Significance of E-cadherin and PCNA Expression in Human Non-small Cell Lung Cancer

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**OBJECTIVE** This study was designed to assess E-cadherin (E-cad) and proliferating cell nuclear antigen (PCNA) expression as well as their clinicopathological significance in human non-small cell lung cancers (NSCLCs). Possible molecular mechanisms of differentiation and metastasis of NSCLCs are discussed.

**METHODS** Immunohistochemical and immunofluorescence double staining were performed to examine the expression of E-cad and PCNA in 68 primary NSCLCs cases.

**RESULTS** The E-cad expression in squamous cell carcinomas and adenocarcinomas showed no significant difference. E-cad expression had a positive correlation with the histological-differentiated grade. A significant difference of E-cad expression was found between metastatic and non-metastatic groups. PCNA expression in squamous cell carcinomas and adenocarcinomas showed no significant difference. The PCNA expression had a reverse correlation with the histological-differentiated grade. A significant difference of PCNA expression was found between metastatic and non-metastatic groups. The E-cad and PCNA expression presented a reverse correlation.

**CONCLUSION** E-cad expression is not associated with the histological type of NSCLC, but is associated with differentiation and metastasis of the cancer. Down-regulation of E-cad expression affects the proliferation of cancer cells. Conjoint analysis of E-cad and PCNA expression is a good way to evaluate tumor biological behavior.

**KEY WORDS:** NSCLC, E-cadherin, PCNA, immunohistochemistry.

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## Introduction

Lung cancer is the leading cause of cancer deaths in both women and men, and its incidence has increased markedly in the past decade. Epithelial tumors, which present the majority of lung tumors, are classified primarily into two subgroups: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). NSCLC is composed of squamous cell, adeno-, large cell and adeno-squamous carcinoma. As the 5-year survival for lung cancer patients is currently 13%, mainly due to metastasis, the devastation caused by this single cancer type deserves special attention. Cell adhesion is a key process because it is directly related to the differentiation, architecture and normal tissue development. It has been postulated that changes in cell-cell and cell-matrix interactions are responsible for cancer cell transgression through normal tissue boundaries and their dispersion to distant sites<sup>[1]</sup>.

The interactions among cells or among cells and their substrate are mediated by adhesion molecules, a large number of which are involved in intercellular adhesion of epithelial and tumor cells. Among

cadherins, E-cadherin (E-cad) is the most important protein. E-cad, a 120-kDa transmembrane glycoprotein, is a calcium-dependent cell adhesion molecule that is expressed in epithelial tissues involved in formation and maintenance of the histoarchitecture. Reduced/absent expression of E-cad has been found in a variety of human carcinomas<sup>[2]</sup>. Even though recent immunohistochemical studies have shown that down-regulation of E-cad is associated with lung cancer, the relationship between the expression of E-cad and the histological type, differentiation, as well as proliferation degree of NSCLC is less understood.

This study was designed to determine the relationship between E-cad expression and the histological type, the differentiated level as well as metastasis of NSCLC. In addition, conjoint detection of the expression of proliferating cell nuclear antigen (PCNA) was performed to elucidate the possible molecular mechanism of how E-cad affects NSCLCs differentiation and metastasis.

## Patients and Methods

### Patients

Sixty-eight primary NSCLCs patients underwent surgical resection between 2004 and 2006 at the Hospital affiliated with Dalian University. The clinicopathological characteristics are shown in Table 1.

**Table 1. Summary of the clinicopathological characteristics of the lung cancer patients.**

Characteristic	Cases
Age (years $\pm$ SD)	59.6 $\pm$ 9.1 (range, 42~73)
Sex	
Male	42
Female	26
Type of carcinoma	
Squamous cell carcinomas	26
Adenocarcinomas	42
Histological differentiation	
Well	18
Moderately	34
Poorly	16
Lymph node metastasis	
Present	38
Absent	30

### Immunohistochemical analysis and image analysis

Paraffin sections, 4  $\mu$ m thick, were deparaffinized and rehydrated before being washed thoroughly in phosphate buffered saline (PBS, pH 7.2~7.6). Microwave heating in 10 mmol/L sodium citrate buffer (pH 6.0) for 6 min was used to retrieve the antigen followed by thorough PBS washing. Endogenous peroxidase was blocked by

0.3% hydrogen peroxidase in methanol for 30 min. The sections were then washed in PBS for 3~5 min. After blocking of nonspecific antibody-binding sites, the sections were incubated at 4°C overnight with mouse E-cad antibody (1:1000, BD Transduction Laboratories, US) as the primary antibody. After PBS washing, the sections were incubated with the secondary antibody, biotinylated anti-mouse immunoglobulin for 1 h, and then with avidin-biotin complex (ABC) for 1 h at room temperature<sup>[3]</sup>.

Antibody binding was identified with diaminobenzidine tetrahydrochloride (DAB) as the chromogenic substrate. Finally, the nuclei were counterstained with hematoxylin and dehydrated with an ascending series of alcohols, cleared through xylene and mounted. Immunostaining for the PCNA antigen was performed in a similar manner. Mouse PCNA antibody (1:1000; Vectorlaboratory, US) was used. For fluorescence immunohistochemistry double staining, rabbit E-cad antibody (1:200, Dako, US) and mouse PCNA antibody (1:1000, Vectorlaboratory, US) were mixed and used as the primary antibodies, then mixed secondary antibodies (anti-mouse and anti-rabbit) labeled with Alexa594 (red) and Alexa488 (green) (Invitrogen) were applied. Finally the sections were mounted with glycerin.

After the immunohistochemical reaction, images were captured using identical exposure times and camera settings. We measured the sum of staining intensity in 5 fields of view for each sample for each antibody using on Image J Plus system, and the values used for statistical analysis. The independent *t*-test, Spearman correlation and Person's correlation were determined by SPSS11.5 software package. Results were considered statistically significant at a *P* value of 0.05 or less.

## Results

### E-cad expression in NSCLC

The expression of E-cad was mainly located on the cellular membrane and partly in the cytoplasm near the membrane. The positive signal of E-cad expression in adenocarcinomas was stronger than that in squamous cell carcinomas. However, the difference between these two types was not significant (*P* = 0.34). On the other hand, it was observed that E-cad expression had a positive correlation with pathological differentiation of NSCLC. In other words, the expression of E-cad in well-differentiated tumors was significantly stronger compared to poorly differentiated (*P* = 0.003, Fig.1). The E-cad expression in the non-metastatic group was significantly stronger than that in the metastatic group (*P* = 0.002, Table 2).

### PCNA expression in NSCLC

The results showed no significant difference in PCNA expression between NSCLCS squamous cell carcino-

mas and adenocarcinomas. Furthermore, it was found that the expression of PCNA in well-differentiated tumors was weaker, but stronger in poorly differentiated tumors ( $P = 0.018$ , Fig.2). It also was found that the PCNA expression in the metastatic group was significantly stronger compared to the non-metastatic group ( $P = 0.001$ , Table 3). The E-cad and the PCNA expressions showed an inverse correlation ( $r = -0.51$ ,  $P = 0.002$ , Fig.3).

## Discussion

E-cad expression has been widely investigated in many

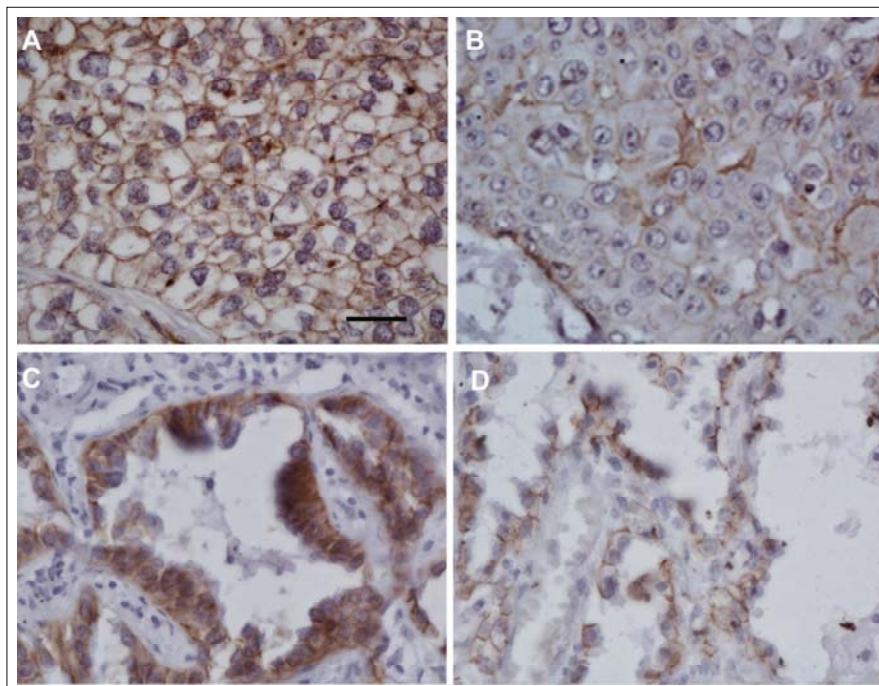
kinds of human cancers, such as those of the esophagus<sup>[4]</sup>, stomach<sup>[5]</sup>, colon<sup>[6]</sup>, liver<sup>[7]</sup>, and others<sup>[8,9]</sup>. In the past decade, several reports have dealt with the relationships between E-cad expression and proliferation, differentiation, metastasis, prognosis as well as other clinicopathological characteristics of lung cancers. However, correlations between E-cad expression and invasion/metastasis or survival are still controversial topics<sup>[10-12]</sup>. These inconsistent findings are thought to be attributable to the following reasons<sup>[13]</sup>: *i*) a majority of studies on lung cancer investigated NSCLC as a whole, but these included squamous cell carcinomas and adenocarcinomas in which average E-cad expression levels

**Table 2. The relationships between E-cad expression and clinicopathological factors.**

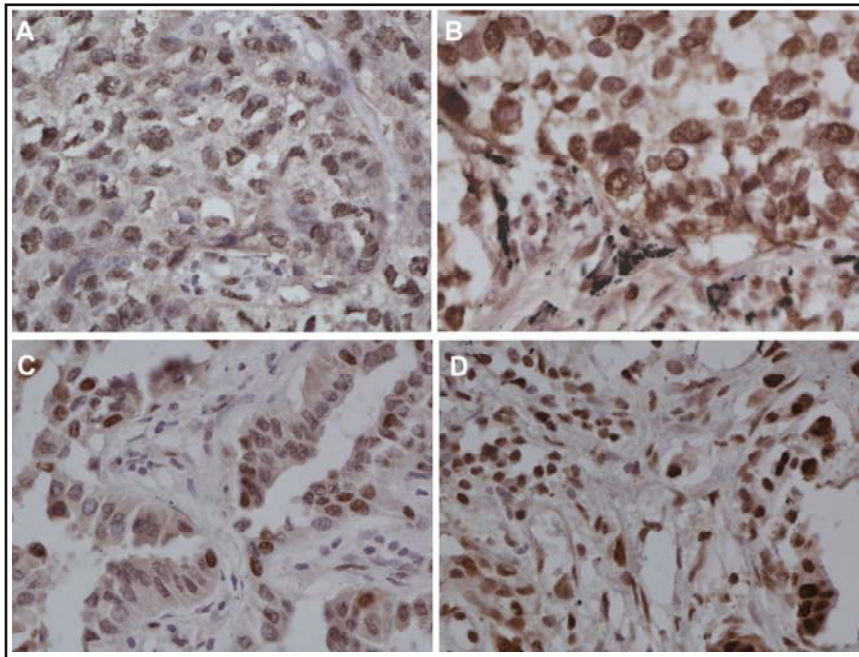
Clinicopathological factors	Cases	Mean $\pm$ SD	<i>P</i> value
Histological type			0.34
Squamous cell	26	138.4 $\pm$ 18.9	
Adenocarcinomas	42	179.0 $\pm$ 30.3	
Differentiation			0.003
Well	18	257.5 $\pm$ 65.4	
Moderately	34	138.3 $\pm$ 72.0	
Poorly	16	74.2 $\pm$ 38.6	
Lymph node metastasis			0.002
Present	38	97.5 $\pm$ 38.0	
Absent	30	227.3 $\pm$ 30.1	

**Table 3. The relationships between expression of PCNA and clinicopathological factors.**

Clinicopathological factors	Cases	Mean $\pm$ SD	<i>P</i> value
Histological type			0.49
Squamous cell	26	182.2 $\pm$ 67.8	
Adenocarcinomas	42	165.4 $\pm$ 65.8	
Differentiation			0.018
Well	18	147.3 $\pm$ 61.5	
Moderately	34	160.2 $\pm$ 55.5	
Poorly	16	230.6 $\pm$ 63.6	
Lymph node metastasis			0.001
Present	38	204.7 $\pm$ 58.3	
Absent	30	133.4 $\pm$ 52.6	



**Fig.1. Immunohistochemical distribution of E-cadherin in human lung cancers.** The A and B sections are from squamous cell carcinomas, C and D are from adenocarcinomas. The A and B sections are both from well-differentiated squamous cell carcinomas. The expression of E-cadherin was very weak in A section which was associated with lymph node metastasis, and very strong in B from a patient without any metastasis. The C section is from well-differentiated adenocarcinoma and D is from a poorly differentiated one. The expression intensity of E-cadherin in section C is stronger compared with poorly differentiated (D). Bar is 20  $\mu$ m.



**Fig.2. Immunohistochemical distribution of PCNA in various differentiated human lung cancers.** Section A is from a well-differentiated and B from poorly differentiated squamous cell carcinoma. In poorly differentiated squamous cell carcinomas (B), the expression of PCNA was more intense compared to well-differentiated tumors (A). Section C is from a well-differentiated and B from poorly differentiated adenocarcinoma. In poorly differentiated adenocarcinoma (D), the expression intensity of PCNA was more common and stronger compared to well-differentiated tumors (C). Bar is 20  $\mu$ m.

are different; *ii*) the expression of E-cad on cancer cell membranes has been evaluated using different criteria by different authors; *iii*) different antibodies against E-cad and different dilutions were used; *iv*) different ways of antigen retrieval or other staining procedures were employed, etc.

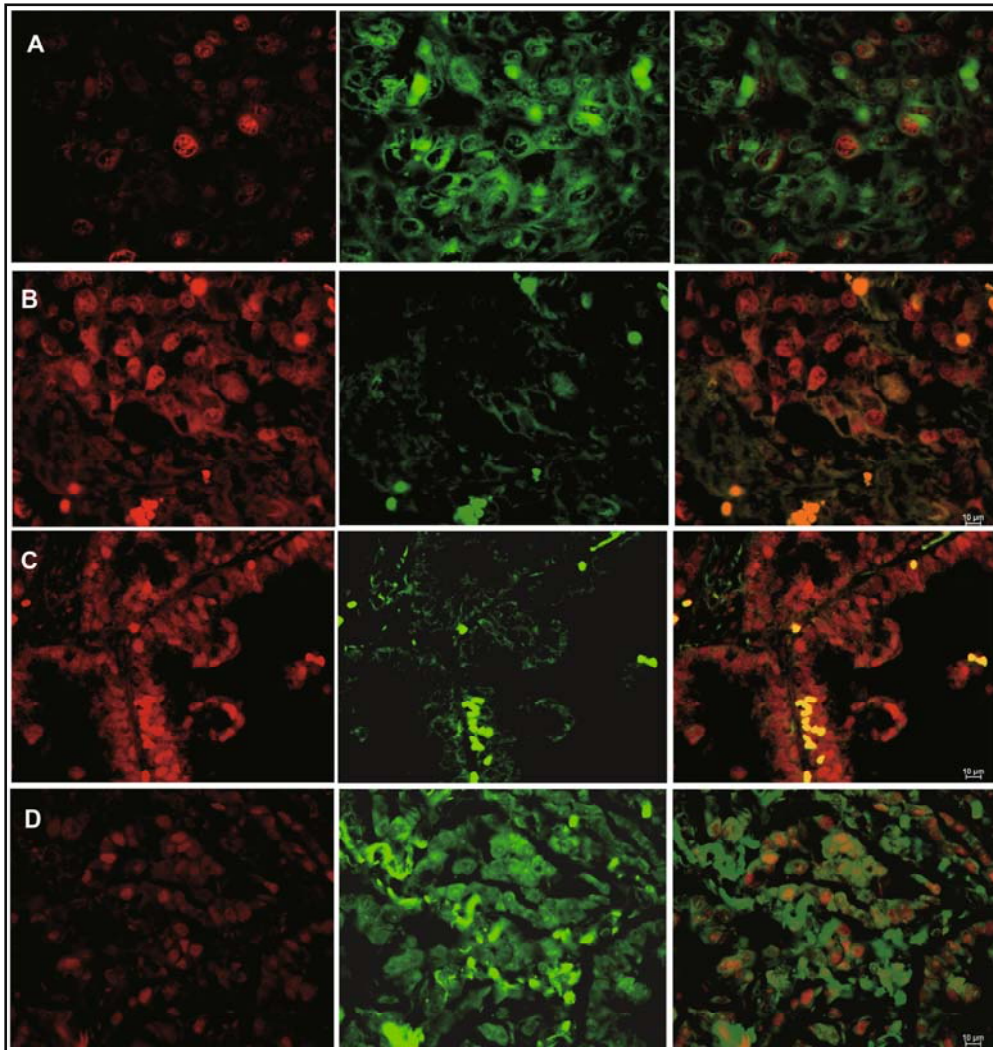
To circumvent the first factor, for example, we analyzed the relationship between the E-cad expression and histological type. After making sure no significant difference was found between these two types, we compared the difference in E-cad expression between metastatic and non-metastatic cancers. To circumvent the second factor and avoid subjectivity, we measured the intensity of each sample for each antibody by using an Image J Plus system based on impersonal criteria. Furthermore, we used two types of E-cad antibodies in order to get more exact results. We found that there was a significant correlation between E-cad expression and the degree of cancer differentiation as well as metastasis. E-cad expression was significantly lower in the poorly differentiated and metastatic group compared to the well- and moderately differentiated and non-metastatic groups. Therefore, E-cad expression is a reliable indicator that can reflect the level of differentiation and metastasis of lung cancers.

It is known that tumor growth and progression is closely related to activity of cellular proliferation. PCNA is a nuclear protein widely used for evaluation of tumor cell proliferation. The expression of PCNA has been

found significantly correlated with metastasis and relapse in many kinds of cancers, such as those of colon, thyroid gland and stomach<sup>[14]</sup>. As for lung cancer, it is widely thought that PCNA has high expression in poorly differentiated cancers and with those cancers associated with metastasis<sup>[15–18]</sup>. Our results supported these conclusions. However, up to now, it has been unclear whether the expression of PCNA correlates with the pathological lung cancer type. Some researchers have suggested that PCNA expression is different in various pathological types<sup>[19,20]</sup>, others proposed that PCNA had no relationship with the histological type of lung cancer, but only reflected the condition of cellular proliferation<sup>[21–23]</sup>. Our results were consistent with the latter concept.

In the present study, we also found that E-cad expression had an inverse correlation with the expression of PCNA. From Fig.3, we can understand that regardless of whether the tumor was a squamous cell carcinoma or adenocarcinoma, the samples with higher expression of E-cad had lower expression of PCNA and vice versa. These results indicated that low expression of E-cad is associated with tumor cell proliferation. St Croix et al.<sup>[24]</sup> considered that E-cad is not only a suppressor of metastasis, but also a kind of cell contact growth suppressor. Its ability to inhibit proliferation involves up-regulation of the cyclin-dependent kinase inhibitor p27. Therefore conjoint analysis of E-cad and PCNA expression is a reliable means to judge tumor biological behavior.

In summary, the results of our present study indi-



**Fig.3.** The expression of E-cad (green) and PCNA (red) in well- and poorly differentiated human squamous cell carcinoma and adenocarcinoma of the lung by immunofluorescent double staining. Sections A and B are from squamous cell carcinomas, C and D are from adenocarcinomas. A and D are from well-differentiated, B and C are from poorly differentiated tumors. Both squamous cell carcinoma and adenocarcinoma, in well-differentiated tumors (A and D) show a strong expression of E-cad, but less expression of PCNA. Conversely, poorly differentiated tumors (B and C) showed strong and more PCNA expression and poor E-cad expression. Bar is 10  $\mu$ m.

cate that the expression of E-cad has no significant correlation with the NSCLC histological type, but has a significant relationship with the differentiated grade and lymph node metastasis. Low expression of E-cad is associated with tumor cell proliferation. Conjoint analysis of E-cad and PCNA expression is a good way to evaluate tumor biological behavior. Further studies are necessary to elucidate the mechanism by which the down-regulation of E-cad expression is induced in lung cancer.

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