RESEARCH ARTICLE

Aberrant Expression of E-cadherin in Lung Tissues of Patients with Probable Lung Cancer

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Abstract

Introduction: This study assessed the relationship of E-cadherin mRNA and protein expression with the diagnosis of lung cancer with the aim of providing an auxiliary diagnostic method. Methods: Semi-quantitative nested RT-PCR and western blotting were applied to detect E-cadherin mRNA transcripts and protein, respectively, in 30 cases of diagnostic lung cancer, 30 cases of clinically suspected patients with lung cancer and 30 cases of other disease. Immunohistochemical staining was also used to detect E-cadherin. Results: Remarkably decreased levels of relative E-cadherin mRNA value and increased E-cadherin protein negativity were observed in probable lung cancer, when compared with possible lung cancer and others. With a threshold of 1.45, relative E-cadherin mRNA value showed a sensitivity of 90% and a specifity of 83% for the diagnosis of lung cancer. The combination of decreased relative E-cadherin mRNA value and negative E-cadherin protein increased the specificity and sensitivity. Conclusion: These data suggest that Chinese patients with diagnostic lung cancer have similar decreased levels of relative E-cadherin mRNA and E-cadherin protein value in the lung cancer tissues as in lung cancer patients in other countries. Measurement of relative E-cadherin mRNA and protein values in lung cancer tissues has potential for lung cancer diagnosis.

Keywords: Aberrant expression - E-cadherin - lung cancer diagnosis - lung tissue

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Introduction

Lung cancer was one of the most commonly diagnosed cancers, as well as the leading cause of cancer death in both males and females globally, it accounts for 13% (1.6million) of the total cancer cases and 18% (1.4million) of the cancer deaths in 2008 (Ahmedin Jemal et al., 2011; Kimman et al., 2012). Although numerous advances toward the diagnosis and treatment was discovered, the 5-year survival rate of patients with lung cancer remains lower. In order to improve the prognosis, it is necessary to better understand the underlying mechanism of this disease.

The cadherin superfamily of Ca^{2+}-dependent hemophiliac adhesion molecules play a critical role in intracellular adhesiveness and maintenance of normal and malignant tissue architecture via homotypic calcium-dependent interactions (Oda and Takeichi, 2011). The E-cadherin is the major component of this superfamily. E-cadherin-mediated cell adhesion requires intracellular attachment of this glycoprotein to the actin cytoskeleton (Mitselou et al., 2010; Yu et al., 2012). Cadherin associate with the cytoskeleton through cytoplasmic interactions with catenins.

Previous studies showed that the E-cadherin/catenin complex down-regulation in malignant disease is associated with tumor invasion, metastasis, and unfavorable prognosis (van Roy and Berx, 2008; Guo et al., 2011). Previous immunohistochemical studies have revealed the loss or aberrant expression of E-cadherin at the cell membrane is associated histopathological tumor characteristics (differentiation, aggressiveness, metastasis and poor prognosis) in different cancer types (Mitselou et al., 2010; Sun et al., 2011). In malignant tumors in general, E-cadherin and catenins are strongly expressed in well differentiated cancers that maintain their cell adhesiveness and are less invasive, whereas their expressions are reduced in poorly differentiated tumors which have lost their intercellular adhesion and show strong invasive behavior (Mir et al., 2012). Previously indicated that aggressive and infiltrating carcinoma cells are devoid of or show impaired expression of the E-cadherin-catenin complex, support the hypothesis that in a tumor mass, the cells with low or no E-cadherin will detach from the tumor and adopt an invasive phenotype (Umbas et al., 1992). In addition, highly metastasizing non-pulmonary carcinomas

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have been associated with low E-cadherin expression, loss of cadherin adhesion may promote cancer cell migration and subsequent dissemination of malignant cells also in lung cancer (Bukholm et al., 1998; Demirci et al., 2012). Reduced expression of E-cadherin and catenin appears as predictors of occult lymph node metastasis (Liu et al., 2001). E-cadherin has been found to have prognostic significance in lung cancer patients (Herbst et al., 2000). Although much information has been gathered about E-cadherin protein expression during development, organogenesis and tumor formation, little is known about the association between reduced expression of E-cadherin mRNA and protein with lung cancer patients at the molecular level. Particularly the relationship between changed E-cadherin expression and the clinical diagnosis, and how expression is activated and maintained in a developmentally and cell-type-specific manner. In this study, 30 cases of diagnostic lung cancer patients, 30 cases of clinically suspected patients with lung caner and 30 cases of other diseases were selected and analyzed in parallel for E-cadherin mRNA and protein expression. This study aimed to assess the association of E-cad mRNA levels and protein expression using nested RT-PCR and western blot analysis. According to the obtained data, we attempted to utilize E-cadherin mRNA and protein expression as an independent prognostic factor for the diagnosis of lung cancer.

Materials and Methods

Patients and samples

The study group was retrospectively selected from the patients in the First Affiliated Hospital of Kunming Medical College from December 2009 to July 2011. The selection criteria included i) complete surgical resection of the tumor (macroscopically and microscopically) as the initial treatment modality, ii) availability of adequate archival tissue for evaluation, iii) complete clinicopathologic data. None of the subjects received preoperative anticancer therapy. Samples were taken from lung cancer patients (30 cases) and the possible lung caner patients (30 cases) (the center of cancer tissues or pathogenic tissues within the diameter). The samples were flash frozen in liquid nitrogen and stored at -80°C. All cases were classified according to the histological classification of lung tumors specified by the World Health Organization (Brambilla et al., 2001).

Nested reverse transcription PCR (RT-PCR)

Total RNA from the tissue specimen were isolated using Trizol-GenClean (TianGene, China) according to the manufacturer's protocol. RNA quality and concentration were estimated spectrophotometrically at 260 nm. RT-PCR was carried out using RevertAidTM First Strand cDNA Synthesis Kit (Fermentas). The cDNA was synthesized from total RNA coupled with oligo(dT)18 primer and RevertAidTM M-MuLV Reverse Transcriptase. The PCR reaction was carried out in a 25 μl volume comprising 1×PCR buffer, 50 ng cDNA, 25 pmol of forward and reverse primers, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, and 3.0units of Taq DNA polymerase. Amplification conditions were: denaturation at 95°C for 5min, 25 cycles of 95°C for 60s, 66°C for 60 s, and 72°C for 60 s, followed by a final 10min extension at 72°C. The amplification reaction to the second round PCR and β-actin were performed in the same tube. The RT-PCR products were determined by electrophoresis on 2.0% agarose gel stained with ethidium bromide. The semi-quantitative of E-cadherin mRNA was evaluated according to the scan area ratio of E-cadherin/β-actin (Pharmacia, USA). Primer sets for E-cadherin and β-actin were designed according to the GenBank sequences. The RT-PCR react use the following primers: E-cad first round forward primer, 5'- GGCACAGATGGTGTGATTAC-3', E-cad first round reverse primer, 5'-GAGCACC TTCCATGACAGA-3', total 595bp; E-cad second round forward primer, 5'-AGTCA CGCTGAATACAGTGG-3', E-cad second round reverse primer, 5'-GGCAATGCGTT CTCTATCC -3', total 340bp; β-actin forward primer, 5'-CTTCCTGGGCATGGAGTC-3', β-actin reverse primer, 5'-GCCGATCCACACGGAGTA-3', total 232bp.

Western blots

Protein concentration was determined by the Bio-Rad Protein Assay (Bio-Rad, USA). Total proteins were separated on 15% SDS-PAGE gel for E-cad and GADPH detection. Separated proteins were transferred to the immun-blot PVDF membrane for protein blot (Bio-Rad, USA). The membranes were then blocked with 5% (w/v) deffated milk in PBST (phosphate buffered saline, pH 7.6, containing 0.05% Tween-20) before exposing to mouse monoclonal antibody against human E-cadherin (1:2500, R&D) or GADPH (1:1000, R&D). The blots were then incubated with a horseradish peroxidase-conjugated goat anti-mouse antibody (1:1000; Dako, Glostrup, Denmark). The signals were determined with ECL western blot substrate (Pierce).

Immunohistochemistry

The immunohistochemical method was according to the avidin-biotin-peroxidase complex and the staining evaluation method were described previously (Shibanuma et al., 1998). The reaction products were visualized with diaminobenzidine and the sections were counterstained with hematoxylin. Negative control staining was performed by the same class of immunoglobulin instead of the first antibody, showed negative results in all cases. The intensities and patterns of immunohistochemical staining with E-cadherin and GADPH in lung lesions were compared with those of normal lung tissues. Expression of E-cadherin and GADPH in each specimen were defined to be normal if more than 90% of the lung lesion cells were positively stained with the appropriate antibodies. If staining was distinctly weaker than that of normal epithelium, or if less than 90% of the intrabronchial lesion cells were positively stained, the expression was considered as down-regulation. Immunohistochemical staining was scored independently by two observers.

Statistical analysis

Because the Komlogorov-Smirnov test showed that the obtained results did not have a normal distribution

Table 1. Summary of the Main Clinical Features of Patients with Probable and Possible Lung Cancer

Clinical feature	Probable lung cancer (30)	Possible lung cancer (30)
Male(n)	19	17
Female(n)	11	13
Median age at onset (range,year)	59.5(20-74)	53.7(21-69)
Smoking (n,%)	22(73%)	19(63%)
Lung cancerous tissues type	30	
SCC	12	
Adc	15	
AdCa	3	

Table 2. Comparison of the Numbers of Relative E-cadherin mRNA Value and Negative E-cadherin Protein in Tissues Between Different Groups

Group	Negative E-cadherin	E-cadherin relative value
Probable lung cancer	19 (30)	1.03 ± 0.123[a,b]
Possible lung cancer	10 (60)	2.09 ± 0.519
Others	0(30)	2.27 ± 0.473

The values are expressed in mean±SD; a, significantly lower/higher than possible lung cancer (P<0.01); b, significantly lower/higher than others (P<0.01)

(P<0.05), analyses of the E-cadherin mRNA relative value among the different groups were performed using the rank sum test. The relationship between relative mRNA E-cadherin value and main clinical data on a population basis was calculated with the Spearman test. Optimal cutoff values were calculated using receiver operating characteristic analysis. All statistical analyses were performed using SPSS11.5 for windows.

Results

From December 2009 to July 2011, 90 suspected lung cancer patients were received in our hospital. Among them 40 were classified as clinical diagnostic lung cancer, 27 as possible lung cancer and the rest as others. The median ages at discovery of the diagnostic and possible patients with lung cancer were 59.5 years (range: 20-74 yeras) and 53.7 years (range: 21-69 years), respectively. The tumor histological subtypes of diagnostic lung cancer included 15 adenocarcinomas (Adc), 12 squamous (SCC) cell carcinomas, and 3 adenosquamous carcinomas (AdCa). No age and gender differences were observed between diagnostic and possible patients with lung cancer. 22 cases (73%) with the history of smoking in diagnostic lung cancer group, and 19 cases (63) in possible lung cancer group. There were no significant difference was found between diagnostic and possible lung cancer patients. The main clinical features of diagnostic and possible lung cancer cases are summarized in Table 1.

Analyses of the E-cadherin mRNA relative revealed widely distributed value ranges among the tested patients (Figure 1). In the group of diagnostic lung cancer, the E-cadherin mRNA relative levels ranged between 0.27 and 1.74 (median: 0.59), whereas in the groups of possible lung cancer and others, it ranged between 1.34 and 2.16 (median: 1.64) and between 1.51 to 3.42 (median: 1.83),

Table 3. Sensitivity and Specificity for Relative E-cadherin mRNA Value and E-cadherin Protein in the Diagnosis of Probable Lung Cancer

	Negative E-cadherin	E-cadherin <1.45	Negative E-cadherin and E-cadherin <1.45	Negative E-cadherin or E-cadherin <1.45
Probable lung cancer	28 (30)	27(30)	27 (30)	28 (30)
Others	5 (30)	5(30)	2 (30)	7(30)
Sensitivity (%)	93	90	90	93
Specificity (%)	83	83	93	77

Results are expressed as number of patients with raised specific protein/patients investigated

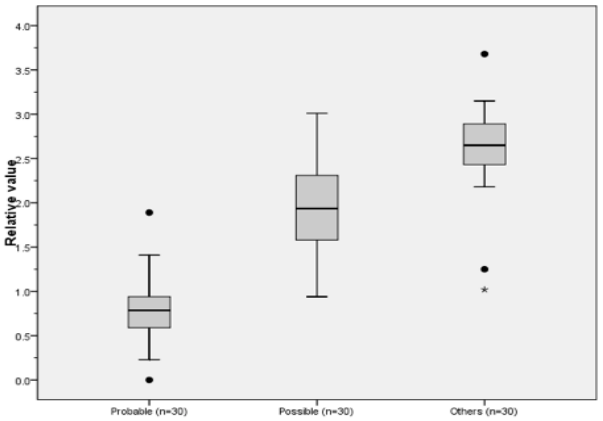

Figure 1. Scattergraphs of Relative E-cadherin mRNA Value of the Patients with Probable Lung Cancer Tissues, Possible Lung Cancer or Controls. The relative E-cadherin mRNA value were shown in Y axis. The case numbers were shown under each group. Boxes represent 25th, 50th and75th percentiles of the data. The length of the box is the interquartile range. The lower and whiskers represent minimum and maximum

respectively. The relative value of E-cadherin in diagnostic lung cancer cases (mean: 1.03±0.123) was lower than those of possible lung cancer cases (mean: 2.09±0.519, P<0.05) and others (mean: 2.27±0.473, P<0.01, Table 2). With the receiver-operating characteristic analysis, an optimal cutoff relative value of E-cadherin was suggested as 1.45 based on the comparison of the data from diagnostic lung cancer with that from others. With this cutoff value, more patients (90%, 27/30) in the group of diagnostic lung cancer showed significantly positive E-cadherin mRNA relative value, whereas only small portions of the patients in the gourps of possible lung cancer (37%, 11/30) and others (17%, 5/30) showed positive E-cadherin mRNA relative value. Statistical assays confirmed that the sensitivity, specificity o E-cadherin mRNA relative value in diagnosis of diagnostic lung cancer were 90% and 83%, respectively, which were equal to those of E-cadherin protein (Table 3). After combinating the E-cadherin mRNA relative value and E-cadherin protein together, both of the specificity and sensitivity for diagnosis of probable lung cancer were higher than taking the relative value of E-cadherin mRNA or E-cadherin protein alone (Table 3).

We also performed immunohistochemistry staining to profile the E-cadherin expression patterns in lung cells. The findings demonstrated typical membranous expression of E-cadherin in the others gourp, which

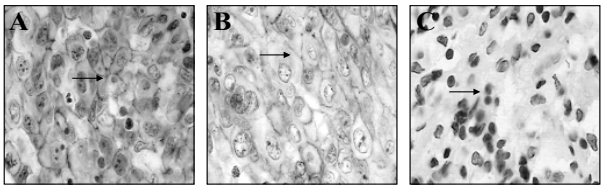

Figure 2. Immunohistochemical Staining of E-cadherin. A. A representative non-cancerous lung tissue sample staining showed strong staining of E-cadherin on basilar epithelial cells; B. A representative staining of suspected lung cancer tissue showed the weak E-cadherin expression. C. A representative staining showed the absent membranous E-cadherin. Original magnification, ×200

suggested that there were normal gap junction and cell adhesion among cells. However, almost all of the 30 cases of the probable lung cancer showed negative expression in cell membranes (Figure 2). The immunohistochemistry results are consistent with the mRNA and protein detection results.

Discussion

The homotypic cellular adhesion molecule E-cadherin is an important transmembrane glycoprotein for the organization of epithelial structure. E-cadherin can form homophilic interactions with E-cadherin molecules on neighboring cells in a Ca^{2+}-dependent way and is the main component of adherent junctions. By recruitment of α- and β-actin, the E-cadherin anchored to the actin cytoskeleton. Loss of E-cadherin expression occurs in a variety of human tumors and is hypothesized to be an important step in the progression from tumor formation to invasion and metastasis (Paras et al., 2008). Immunohistochemical studies have demonstrated that loss of E-cadherin expression is a frequent event in many types of carcinomas, however, CDH1 mutations are rare or absent (Hirohashi, 1998). Proposed epigenetic mechanisms for E-cadherin loss include alterations in the expression and/or function of the trans-acting factors that regulate CDH1 gene transcription, hypermethylation of the CDH1 promoter, and chromatin-mediated effects. A number of transcription factors may bind to CDH1 E-box elements to repress transcription, including the zinc-finger transcription factors Snail (Li et al., 2011), SLUG (Schmidt et al., 2005), δEF1/ZEB-1 (Shuang et al., 2007), SIP1/ZEB-2 (Comijn et al., 2001), and the basic helix–loop– helix factor E12/E47(Perez-Moreno et al., 2001). Hypermethylation of the CDH1 promoter has been postulated to play a critical role in the loss of E-cadherin expression observed in some primary tumors and cell lines without identifying CDH1 mutations. CDH1 promoter hypermethylation has been reported in Non-small cell lung cancer, prostate, thyroid, gastric, and other cancers (Tamura et al., 2000; Feng et al., 2008; Ramezani et al., 2012). The finding that treatment of cell lines with demethylating agents can, in some cases, restore E-cadherin expression suggests that in some tumors, promoter hypermethylation plays an important role in silencing of CDH1 expression. Mechanisms other than repression by certain transcription factors and promoter hypermethylation have been suggested to inactivate CDH1 expression in cancer. In particular, chromatin condensation has been proposed to play a role in the silencing of CDH1 expression in carcinomas (Hennig et al., 1995). The various proposed mechanisms of CDH1 silencing in cancer may not be mutually exclusive.

In this study, we performed semi-quantitative RT-PCR and western blot to examine the mRNA and protein expression of E-cadherin in 30 cases of probable (equal to 'diagnostic' in this study), possible and others patients. We observed decreased relative value of E-cadherin mRNA in Chinese patients with diagnostic lung cancer. Our results obtained in the present study showed reduction in the mRNA expression of E-cadherin in parts of the lung patients specimen (Table 2). The evidence for E-cadherin mRNA expression patterns reported in this study is consistent with those of others (Hirata et al., 2001; Stefanou et al., 2003).

In this study, 2 diagnostic lung cancer cases showed extremely low relative E-cadherin mRNA values (0.08 and 0.11). One patients was aged 65 years at discovery of the lung cancer and had the history of smoking for 50 years. The other patient aged 72 years, having the history of pneumoconiosis, and smoking for about 20 years. All of the tissues were taken approximately 4 months after the lung cancer was diagnosed. Based on the relative E-cadherin mRNA values of diagnostic patients with lung cancer and no-lung cancer, we proposed a cutoff value of 1.45, which shows high specificity and sensitivity for probable lung cancer diagnosis. Combinating the results of relative E-cadherin value with E-cadherin protein in lung cancer tissues will further increase the diagnostic specificity and sensitivity for probable lung cancer in China, even in the other countries.

Our findings of E-cadherin protein down-regulation in the vast majority of lung cancer are consistent with the hypothesis that E-cadherin plays an important role in the malignant phenotypes. We demonstrated that E-cadherin protein were abnormally expressed in the majority of lung cancer. Dysfunction of the cell-cell adhesion function caused by reduction of E-cadherin levels implies elevation of the potential ability of cancer cells to disperse, which is the probable early step of local invasion and distant metastasis.

We also examined the expression of E-cadherin using immunohistochemistry staining. We have found that even part of the detected lung cancer tissues showed complete negative immunohistochemistry staining of E-cadherin, which were consistent with our western blot results. At the same time, some results of western blot were consistent with nested RT-PCR indicating that the reduced E-cadherin protein levels may due to the lacking of mRNA transcription.

According to our clinical experience for many years, we found that the E-cadherin expression is closely related to the definite lung cancer cases. Based on the E-cadherin mRNA and protein expression level of probable patients with lung cancer and non-cancerous, we propose a combining results of E-cadherin mRNA with E-cadherin protein in lung tissues which will further increase the diagnostic specificity and sensitivity for probable lung cancer. We believe that this makes great sense for lung

cancer diagnosis, especially for the aspiration biopsy.

In conclusion, our investigation supports the hypothesis that the reduced expression, and particularly the loss of expression, of E-cadherin mRNA and protein may play an important role in the development and diagnosis of the malignant phenotypes in lung cancer. The combining abnormal expression of E-cadherin mRNA and protein might be common affairs in tumorgenesis and the progression of lung cancer. So the measurement of patient lung cancer tissues will supply additional reliable information for the diagnosis of lung cancer.

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The author(s) declare that they have no competing interests.

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