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# Downregulated vimentin and upregulated E-cadherin in T1 stage non-small-cell lung cancer: does it suggest a mesenchymal-epithelial transition?

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Lung cancer has been a major threat to human health worldwide. Vimentin (VIM) and E-cadherin (E-cad) are molecular markers that play important roles in epithelial-mesenchymal transition (EMT), which has been shown to be correlated with tumor progression. Herein, we investigated the associations between clinicopathological parameters and VIM/E-cad expression in primary T1 stage non-small cell lung cancer (NSCLC). Real-time PCR was performed on RNA extracted from tumor tissue isolated from 54 patients with T1 stage NSCLC. Immunohistochemistry was used to measure the protein levels of VIM and E-cad. The paired-samples t-test, independent-samples t- test, Kappa test, and nonparametric test were used to perform statistical analyses. The expression of VIM were lower ($P<0.001$) and that of E-cad was higher ($P<0.001$) in tumor tissue when compared with the normal tissue at the transcriptional level. The RNA of VIM in adenocarcinomas was significantly higher than that found in squamous cell carcinomas ($P<0.01$). Moreover, the level of metastasis observed in the lymph node was significantly higher than that observed in metastasis outside of the lymph node ($P<0.05$). Vimentin protein had a lower profile in the tumor tissue, and the protein level of E-cad was higher in the tumor tissue. The IHC score of VIM in tumor tissue was found associated with both the pathological type and smoke index, and the score of E-cad was found to be related to visceral pleura involvement. In conclusions, VIM is downregulated and E-cad is upregulated in T1 stage NSCLC. Our study results suggest that a mesenchymal-epithelial transition may take place in the early-stage of tumor development, and that EMT occurs when the tumor develops into a certain stage.

*Key words: non-small-cell lung cancer, vimentin, E-Cadherin, mesenchymal-epithelial transition, epithelial-mesenchymal transition*

Lung cancer is one of the most commonly diagnosed malignancies in the world. In 2012, there were 1.8 million people diagnosed with lung cancer, and 1.59 million patients died worldwide [1]. In addition, lung cancer ranks higher than other cancers in Chinese males, and is the third most common cancer in Chinese females [1]. For many years, lung cancer has been a major threat to global public health. To date, it is acknowledged that susceptibility genes combined with environmental factors are of importance in the development of lung cancer and that this combination contributes to lung cancer risk [2]. However, the exact mechanism of lung cancer has not yet been completely discovered. Lung cancer is divided into small cell lung cancer and non-small cell lung cancer (NSCLC) according to its pathological type. T1 stage NSCLC is considered as early lesions, and its prognosis is relatively good. Nevertheless, T1 stage NSCLC upholds the risk of metastasis, and its prognosis differs between patients. Therefore, elucidating the precise trait of T1 stage NSCLC is pivotal to improve clinical outcomes and warrants further investigation.

Intermediate filaments (IFs) are one of the three major fibrous polymers of the cell, and play a role as cytoskeletal scaffolds in the nucleus and cytoplasm [3]. These filaments are dynamically regulated, fully integrated within the cellular framework and interact with a range of cellular proteins

[4]. Furthermore, IFs have important functions in cellular migration [5], and are determining parameters in the cell invasiveness capacities, as their expression and composition could be used to evaluate the progression of cancers [6, 7]. Vimentin (VIM) is a type III of IF, and is a mesenchymal marker of epithelial-mesenchymal transition (EMT), which is related with the acquisition of invasive properties [8]. The expression of VIM correlates with the invasive ability of epithelial cancers [9]. It is found abnormally expressed in breast cancer [10], hepatocellular carcinoma [11], renal cell carcinoma [12], cervical cancer [13], gastric carcinoma [14], colorectal carcinoma [15], esophageal cancer [16], thyroid cancer [17], ovarian cancer [18] and prostate cancer [19]. However, the relationship of VIM to NSCLC, especially T1 stage NSCLC, remains unclear.

E-cadherin (E-cad) is a molecule that has been employed as a biological marker of EMT [20], in addition to VIM. However, as an epithelial marker, E-cad plays a different role in the EMT process. The functions of E-cad include maintaining intact cell-cell interactions and inhibiting cell mobility, invasion and metastasis in human cancer [21]. Downregulation of E-cad expression is regarded as an endpoint of EMT-inducing signaling pathways [22]. Although many studies have demonstrated its biological functions, the role of E-cad in early stage tumor is worth exploring.

Thus, we examined the expression and levels of E-cad in the tumor tissue and normal tissue of T1 stage NSCLC patients, and then analyzed the association of clinicopathological data and VIM expression to reveal the role of VIM, E-cad and EMT in lung carcinogenesis.

## Methods and patients

**Patients.** For this retrospective study, participant identification was initiated in February 2016. Medical records of patients with surgical treatment in the Zhejiang Cancer Hospital between August 2008 and October 2012 were collected. Cases for patients who were pathologically diagnosed as T1 stage NSCLC were selected, and those who met one of the following criterions were ruled out: multi-site tumor, had been pretreated with chemotherapy or radiotherapy, combined with other malignant diseases. Tumor grade and stage were defined according to the criteria of the World Health Organization (WHO) and the primary tumor, regional lymph nodes and distant metastasis (TNM) classification of the Union for International Cancer Control (UICC, eighth edition). This research was a retrospective study that used data solely from medical records and archived tissue samples, and was approved by the Medical Ethics Committee, Zhejiang Cancer Hospital (IRB-2015-176). Written informed consent was obtained from each patient before study enrollment.

**Tissue specimens.** Fifty-four T1 stage NSCLC patients were enrolled in the research. The specimens, including tumor and distal normal tissue, were collected under sterile conditions just after the lung tissues were removed, and were immediately snap frozen in liquid nitrogen. The specimens were stored at -80 °C for RNA extraction.

**RNA extraction and cDNA synthesis.** Total RNA was extracted from frozen sections of tumor and distal normal samples with a NucleoSpin® TriPrep kit (MACHEREY-NAGEL, Düren, Germany), according to the protocol provided by the manufacturer. RNA purity and concentration were detected by NanoDrop 1000 (Thermo Fisher Scientific, Waltham, MA, USA). Total RNA (1 μg) was used for cDNA synthesis by PrimeScript™ RT reagent Kit (TaKaRa, Dalian, Lianong, China), according to the instructions provided by the manufacturer.

**Quantitative real-time PCR analysis.** Amplification and melt curve analysis were performed using an ABI 7500 PCR system (Applied Biosystems). Reactions were carried out in a total volume of 20 μl, using a SYBR® Premix Ex Taq kit (TaKaRa). The primers for beta-actin (ACTB) were 5'-TGGCACCCAGCACAATGAA-3' (forward) and 5'-CTAAGTCATAGTCCGCCTAGAAGCA-3' (reverse). The primers for VIM were 5'-CAATGAGTCCCTGGAACGCC-3' (forward) and 5'-TCCAGATTAGTTTCCCTCAGGTTC-3' (reverse).

The program setting for the PCR cycles were as follows: denaturation step at 95 °C for 1 min, the samples ran 40 amplification cycles, each comprising denaturation (95 °C for 15 s), annealing and extension (60 °C for 1 min). All PCR reactions were run in triplicates and ACTB was used as a reference. Eight cases of cDNA were hybridized, packed and stored at 4 °C. Each PCR experiment used one pre-mixed template. For expression analysis, the experiment was designed to use the reference template as the control; thus, the relative quantification of gene expression in tested tissue was calculated using the equation: amount of target = $2^{-\Delta\Delta Ct}$, $\Delta\Delta Ct = (Ct^{gene} - Ct_{ACTB})$ test-$(Ct_{gene} - Ct_{ACTB})$ reference. To confirm the specificity and accuracy of the PCR experiments, the PCR products were electrophoresed on a 2% agarose gel and sequenced (Shanghai GeneCore BioTechnologies Company, Ltd., Shanghai, China).

**Immunohistochemistry.** Immunohistochemistry (IHC) staining was performed using standard IHC protocols. Tissue was cut to 5 μm thickness, deparafnized in xylene, and rehydrated through graded alcohol. Next, 0.01 M sodium citrate retrieval buffer (pH 6.0) at 125°C was used for antigen retrieval, and endogenous peroxidases were blocked using hydrogen peroxide. The sections were incubated with primary antibodies overnight at 4°C for VIM (rabbit monoclonal antibody, OriGene, Rockville, MD, USA) and E-cad (rabbit monoclonal antibody, Abcam, Cambridge, UK). Horseradish peroxidase detection was performed using SuperPicture™ detection system (OriGene), and PBS buffer solution was used to wash the sections. The sections were counterstained with hematoxylin, sealed with neutral gum, and analyzed by optical microscopy.

Immunohistochemistry was judged independently by two examiners. Distal normal tissue was used as a control. The immunoreactivity was assessed using the staining intensity score:

0 for negative staining, 1 for weak intensity, 2 for moderate intensity, and 3 for strong intensity of staining.

**Statistical analysis.** Paired-samples Student's t-test was used to compare the RNA level of VIM and E-cad between normal and tumor tissue. Independent student's t-test was employed to analyze the association between clinicopathological parameters and both VIM and E-cad expression. Kappa's test was used to analyze the correlation between the IHC results of VIM and E-cad. The criterion for statistical significance was set at P<0.05 and all analysis were performed with SPSS 19.0 software.

## Results

**Patient characteristics** were shown in Table 1. Patients with T1 stage NSCLC (40 males and 14 females) had a mean age of 61.2 years (range 38-79). Thirty-six of the patients were smokers, one-half of the subjects were classified as T1a NSCLC (tumor larger than or equal to 1 cm and smaller than 2 cm), and the remaining subjects were classified as T1b NSCLC. Twenty-four patients were diagnosed with adenocarcinoma and the remaining patients were diagnosed with squamous cell carcinomas. Lymph node metastasis was observed in 11 cases. The tumor cells in 20 patients were poorly differentiated compared to the remaining subjects, which had tumor cells that were moderately differentiated. In eight of the cases, there was evidence of visceral pleura involvement, while venous invasion was found in three cases.

**Table 1. Patient characteristics**

| Clinical and pathological parameters | Results |
|---|---|
| Sample size | 54 |
| Gender (Male) | 74.1% |
| Age(year) | 61.20±8.98 |
| Smoke index(≥20 pack-years) | 57.4% |
| Tumor diameter(cm) | 2.32±0.49 |
| Pathological type (adenocarcinoma) | 44.4% |
| Tumor differentiation (poor) | 37.0% |
| Visceral pleura involvement | 14.8% |
| Venous invasion | 5.6% |
| Lymph node metastasis | 20.4% |

Percentage was used for classified variables, and mean value ±standard deviation was used for continuous variables.

**Expression of VIM and E-cad.** In the tumor tissue, the mRNA level of VIM were significantly lower (0.52 vs. 1.46, 95%CI: -1.11~-0.77, P<0.001) (Figure 1A), and the mRNA

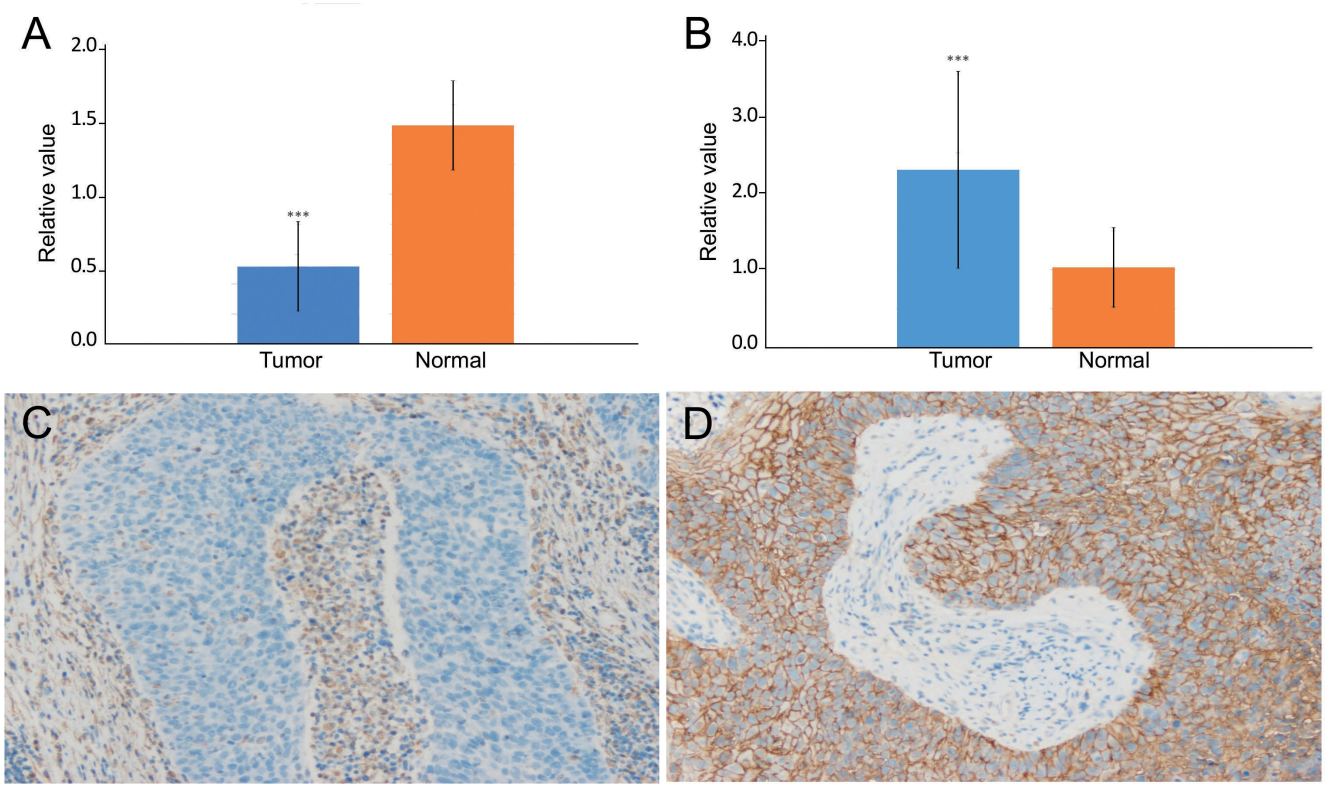


**Figure 1. The expression of VIM and E-cad. (A) RT-PCR showed VIM was downregulated in tumor tissue (p<0.001). (B) RT-PCR showed E-cad was upregulated in tumor tissue (p<0.001). (C) IHC showed a reduced expression of VIM was noted in the tumor tissue, and the expression was mainly detected in the cytoplasm. (D) IHC showed an increased expression of E-cad was noted in the tumor tissue, and the expression was mainly detected on the surface of cells. ***: p<0.001**

level of E-cad was significantly higher when compared with the normal tissue (2.26 vs. 1.01, 95%CI: 0.72~1.79, P<0.001) (Figure 1B). To further examine the expression of VIM and E-cad, IHC was performed in 46 of all 54 cases, and VIM protein was found mainly in the cytoplasm of the cells. Compared with paired normal tissue, the protein of VIM showed a lower profile in the tumor tissue (Figure 1C). As expected, E-cad was found mainly on the surface of cells (Figure 1D). Compared with paired normal tissue, the expression of E-cad was increased in the tumor tissue. Furthermore, the results of the Kappa test revealed that the protein levels of VIM and E-cad were correlated in the same tumor tissue (P=0.001, value of Kappa was 0.477).

**RT-PCR and clinicopathological parameters.** As shown in Figure 2A, VIM in adenocarcinomas was significantly higher than that in squamous cell carcinomas (0.64 vs. 0.42, 95%CI: 0.06~0.36, P=0.007). In addition, patients with lymph node metastasis had significantly higher VIM than patients without lymph node metastasis (0.69 vs. 0.48, 95%CI: 0.02~0.41, P=0.032). Other clinicopathological parameters, including age, smoke index, tumor diameter, differentiation and venous invasion, had no evident effect on VIM expression. Moreover, the RT-PCR results for E-cad revealed no correlation with the clinicopathological parameters.

**IHC scores and clinicopathological parameters.** The IHC score of VIM in tumor tissue was found associated with both the pathological type and smoke index (Figure 2B). The score in adenocarcinomas was relatively higher than that in squamous cell carcinomas (1.64 vs. 1.00, 95%CI: 0.05~1.23, P=0.035). In addition, patients with a smoke index of less than 20 pack-years had significantly higher IHC score of VIM than patients with a smoke index of greater than or equal to 20 pack-years (1.65 vs.1.04, 95%CI: 0.02~1.21, P=0.045). Other clinicopathological parameters had no evident effect on the IHC score of VIM.

The IHC score of E-cad in tumor tissue was found to be associated with visceral pleura involvement (Figure 2C). The score in patients with visceral pleura involvement was relatively lower than that in patients without visceral pleura involvement (4.50 vs.7.13, 95%CI: 0.36~4.90, P=0.030). Other clinicopathological parameters had no detectable effect on the IHC score of VIM.

## Discussion

The EMT process has been a central issue in oncological studies. This process is described as a loss of epithelial marker proteins and their functions such as junction and apical-basal polarity, and the reorganization of their cytoskeleton [23, 24]. Meanwhile, the upregulation of mesenchymal marker proteins enable the cells to gain the ability of migration and invasion during developmental morphogenesis [22, 23]. Recent studies have shown the EMT process results in endowing cancer cells with stem cell-like characteristics [25, 26], which enhances the ability of tumor cells to invade local tissues and resist immune-attacks [27]. Moreover, the EMT process is found to play an important role in tumor progression, including tumor dissemination, local invasion and metastasis [27-30].

The onset of EMT is associated with increased expression of VIM [31, 32], and it has been shown that the downregulation of E-cad is an essential EMT step [33]. However, in our study, we discovered VIM was downregulated in T1 stage NSCLC, both at the transcriptional and translational level. Furthermore, our studies found that protein levels of E-cad were increased in T1

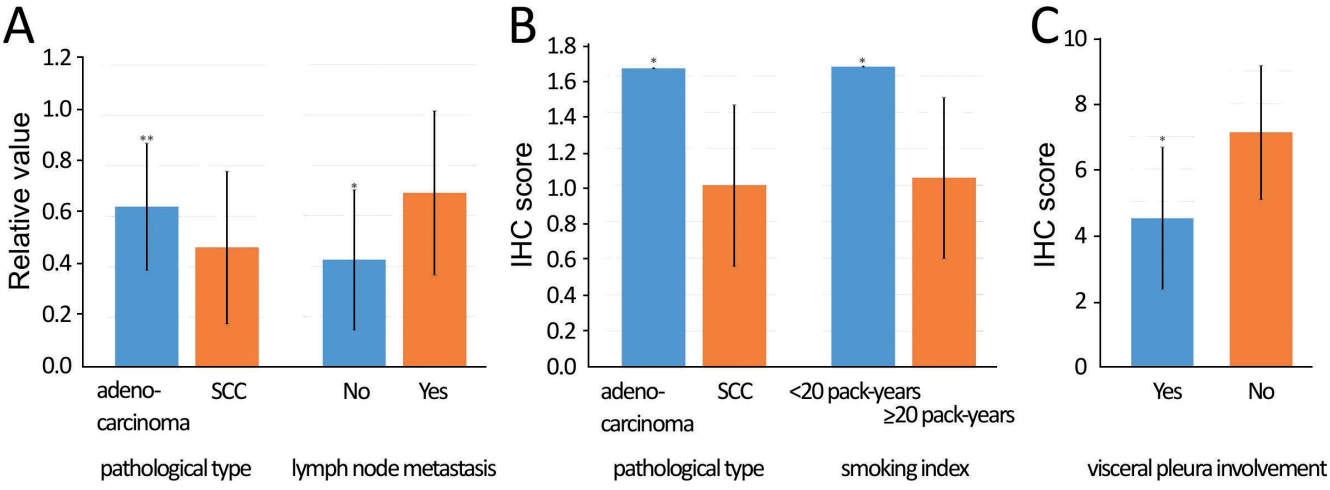


**Figure 2. The correlation of clinicopathological parameters and the expression of VIM and E-cad. (A) VIM in adenocarcinomas was relatively higher than in squamous cell carcinomas (p<0.01), and in lymph node metastasis was relatively higher than in no lymph node metastasis (p<0.05). (B) The IHC score of VIM in adenocarcinomas was relatively higher than in squamous cell carcinomas (p<0.05), and in smoke index <20 pack-years was relatively higher than in smoke index ≥20 pack-years (p<0.05). (C) The IHC score of E-cad in tumor with visceral pleura involved was relatively lower than in tumor without visceral pleura involved (p<0.01). *: p<0.05. **: p<0.01**

stage NSCLC. These results were in contrast with the previously reported profile of EMT. It is possible that the results of our study provide evidence that the EMT process does not occur in T1 stage NSCLC. Previous reports have also shown that the EMT process is the final step of tumor de-differentiation [34], and primary tumor may be one of the bases of the EMT process, as the tumor microenvironment has a significant effect on the EMT process [35].

It is well established that VIM is a mesenchymal marker [8], and E-cad is an epithelial marker [21]. The overexpressed epithelial marker and downregulated mesenchymal marker in T1 stage NSCLC may suggest a process of mesenchymal-epithelial transition (MET) in the tumorigenesis, as upregulation of E-cad is reported to be a mark for MET [36]. A previous study showed that the MET process is able to promote metastatic growth [37], and is essential for establishing macrometastases [38]. We infer that in the early stage or tumorigenesis of NSCLC, the MET process plays a major role. The downregulation of mesenchymal markers and upregulation of epithelial markers could promote the division of malignant cells and local invasion. Taken together, we believe that these regulatory products attribute to the vast number of patients diagnosed with early-stage NSCLC in the clinical setting.

To support our finding, we compared our results to other reports and showed that our inference is consistent with previous studies. Although Prudkin et al. [39] demonstrated both lung adenocarcinoma and squamous cell carcinoma had high levels of VIM expression, preneoplasia had significantly lower expression of VIM than normal tissue. An *in vitro* study revealed that mesenchymal markers were absent in precursor lesions, both hyperplastic and adenomatoid, and suggested that the EMT progress occurred at a later stage [40]. Liu et al. [41] reported VIM expression was found in later cancer stages as well.

The EMT process takes place only if the tumor has developed into a certain stage. Furthermore, the EMT process indicates reduced cell proliferation [37], and enhanced ability of tumor cells to metastasis [22]. The higher RNA level of VIM in tumors with lymph node metastasis in our study could be the symbol of EMT onset. We found that the RNA level of VIM in adenocarcinomas was relatively higher than that in squamous cell carcinomas, both at the transcriptional level and translational level, which may suggest that the EMT process is prone to occur in adenocarcinomas at an earlier stage than squamous cell carcinomas. In accordance with our clinical experience, the adenocarcinoma is more likely to have lymph node metastasis than squamous cell carcinoma during the early stage.

In addition, we found that the expression of E-cad was associated with visceral pleura involvement, as the level of E-cad in the tumor with visceral pleura involvement was relatively lower than in the tumor without visceral pleura involvement. It was reported that during lung carcinogenesis, the impaired expression of E-cad leads to strong invasive behavior [42]. Multiple studies suggested that in patients with NSCLC, reduced E-cad expression was significantly correlated with increased local invasion [43-45]. The lower profile of E-cad in visceral pleura involved NSCLC may suggest that the EMT process occurred in tumors with stronger invasive ability.

Reports show that VIM is associated with tumor differentiation. In prostate cancer, VIM is detected mainly in poorly differentiated cancers and is associated with bone metastases [27, 46]. Al-Saad et al. [47] revealed that the expression of VIM was significantly related with the tumor differentiation of NSCLC, and that high VIM levels were observed in 61% of poorly differentiated tumors, 39% of moderately differentiated tumors and no expression of VIM was observed in well-differentiated tumors. Similarly, we examined tumors at the early-stage as well as the late-stage. We found that VIM expression was not related to tumor differentiation in T1 NSCLC. This suggests that the differences of VIM in poorly, moderate and well-differentiated tumors may occur in a relatively late-stage. Furthermore, the role the EMT process plays in tumor differentiation is suggested as well, as VIM is a key marker of the EMT process.

VIM was reported as being overexpressed in various epithelial cancers [9], including NSCLC [27], and E-cad shows a low profile in most tumors, such as gastric cancer [48], breast cancer [49], and oral squamous cell carcinogenesis [50]. Thus, the object of these studies is not limited in cancer patients at the early-stage. As the EMT process takes place in tumor progression, the level of VIM in the tumor rises and the level of E-cad falls along with the tumor stage. Moreover, our findings are consistent with the results of previous reports.

In summary, this study found that in T1 stage NSCLC, VIM was downregulated and E-cad was upregulated. In addition, we found that the pathological type and lymph node metastasis were correlated with VIM at the transcriptional level, pathological type and smoke index were correlated with VIM at the translational level, and visceral pleura involvement was associated with E-cad at the translational level. Our results suggest that there may be a mesenchymal-epithelial transition that takes place in the early-stage of tumor development, and that EMT occurs when the tumor has developed into a certain stage. As the details surrounding the role of the MET process in tumorigenesis remain unclear, additional studies are warranted to elucidate the mechanism of action.

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