Original Article
Transcription factors related to epithelial mesenchymal transition in tumor center and margin in invasive lung adenocarcinoma

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Abstract: The tumor microenvironment has many roles involving tumor progression, invasion and metastasis. The tumor cells at the tumor border loose epithelial properties and acquire mesenchymal features. This, epithelial-to-mesenchymal transition (EMT) has been suggested to be an important process for tissue and lymphovascular invasion. Pulmonary tissue samples from 15 patients with primary adenocarcinoma were evaluated with using immunofluorescence multi-staining the EMT-associated markers including E-cadherin and alpha-smooth muscle actin (α-SMA), and transcription factors including E-SNAIL and SLUG, and ZEB1. The data were analyzed in specific area, such as tumor center and tumor border. In this study we show that the invasive adenocarcinoma differentially expressed SNAIL and SLUG, and Zeb1 and it was associated with the loss of epithelial marker (E-cadherin) and gaining of mesenchymal marker (α-SMA) at the invasive border of lung carcinoma. The positive rates of SNAIL and ZEB1 were 26.7% and 0% in the tumor center and 40% and 20% in tumor margin, respectively. In addition, the expression of both SNAIL and ZEB1 at the border of tumor was observed in two cases (2/10). These two cases were associated with lymph node metastasis and advanced stage. The process of EMT has been suggested to be of prime importance for tissue and lymphovascular invasion. The process of EMT may be activated in the tumor border of lung adenocarcinoma. Related transcription factors, such as SNAIL and SLUG, and ZEB1, might be induced by paracrine effects of surrounded inflammatory cells and fibroblasts.

Keywords: Epithelial mesenchymal transition, lung adenocarcinoma, SNAIL, SLUG, ZEB1

Introduction

Primary adenocarcinoma in lung is leading cause of cancer death in worldwide and it accounts for the most common histological type among non-small cell lung cancer. The tumor recurrence and metastasis are the most common events encountered after resection of tumor that lead to mortality [1, 2]. The metastatic process starts with separation of cancer cells from the primary lesion, permeation of tumor cells into vessels, proliferation, and transmigration to the metastatic site [3]. Lymphovascular invasion represents the early phase of metastasis [4-6]. Therefore, the presence of lymphovascular invasion has been reported to be a predictor of recurrence and metastasis [7-9].

The epithelial-to-mesenchymal transition (EMT) is originally known as a process during embryonic development in which cells acquire mesenchymal phenotype and lose epithelial phenotype. However, EMT has emerged as a crucial phenomenon in carcinogenic process [10]. As EMT progresses, the tumor cells acquire motile and invasive phenotype [11-13].

The associations between EMT and local invasiveness as well as distant metastasis have been demonstrated in numerous studies [11-13]. Furthermore, a number of studies have reported that expression of molecules involved in EMT correlated with clinicopathological features in non-small cell lung cancer [14-22]. However, those studies cannot serve as a direct evidence of EMT because it is a transient pro-

Table 1. The clinicopathological features and differentially expressed of EMT-related markers in the tumor center and tumor margin of respective cases

No.	Sex	Age	Size (cm)	Stage	Differen-tiation	L. V. I.	Neural invasion	Node me-tastasis	Center				Margin			
									E-cad	SMA	SNAIL	ZEB1	Ecad	SMA	SNAIL	ZEB1
1	F	57	2.0 x 1.5	IA	Moderately	+	-	-	+	-	+	-	+	+	+	-
2	M	75	2.3 x 1.4	IA	Moderately	+	-	-	+	-	-	-	+	-	+	-
3	F	73	2.2 x 1.8	IA	Moderately	-	+	-	+	-	-	-	+	-	-	-
4	F	77	7.5 x 5.5	IIB	Moderately	-	-	-	+	-	-	-	+	-	-	-
5	M	82	2.8 x 2.2	IA	Moderately	-	-	-	+	-	-	-	+	-	-	-
6	M	71	1.7 x 1.5	IA	Poorly	-	-	-	+	-	+	-	+	-	+	-
7	F	78	2.3 x 1.9	IA	Moderately	-	-	-	+	-	-	-	+	-	-	-
8	F	45	1.5 x 1.3	IA	Moderately	-	-	-	+	-	-	-	+	-	-	-
9	F	51	2.7 x 2.2	IIIA	Moderately	+	-	+	+	-	-	-	+	+	+	+
10	M	76	2.8 x 2.5	IA	Moderately	-	-	-	+	-	-	-	+	-	-	-
11	F	46	1.8 x 1.4	IA	Well	-	-	-	+	-	-	-	+	-	-	-
12	F	69	1.9 x 1.5	IA	Well	-	-	-	+	-	-	-	+	-	-	-
13	F	51	1.7 x 1.5	IA	Well	-	-	-	+	-	-	-	+	-	-	-
14	F	56	4.8 x 2.1	IIB	Poorly	-	-	-	+	-	-	-	+	-	-	-
15	F	71	2.7 x 2.5	IIIA	Moderately	-	+	+	+	-	-	-	+	+	+	+

Abbreviation: L. V. I., Lymphovascular invasion.

cess. The loss of epithelial marker is a hallmark of EMT but it could be a simple result from anaplasia [10]. Therefore, this issue needs to be examined by another approach to demonstrate epithelial and mesenchymal features according to the region of tumor cells in associations with histology and immunohistochemistry.

In this study, we used immunohistochemical and double immunofluorescence staining method to examine the expression of EMT-related proteins including E-cadherin, α-SMA, SNAIL, and ZEB-1 at the invasive front of the tumor, compare with the center of the tumor. The objectives of the study are: (1) to demonstrate the existence of EMT in lung adenocarcinoma and (2) evaluate the frequencies and expressions of EMT-related proteins associated with lymphovascular invasion and metastasis.

Materials and methods

From January 2012 to June 2013, 15 patients underwent surgical resection of lung adenocarcinoma in our medical center were enrolled in this study. Clinical and pathological data were evaluated by reviewing the reviewing the pathological records. Patient consent was not required as this research was a retrospective chart review and personal identifiable information was not included. The average age of patients was 65.2 years. Preoperative chemotherapy or radiotherapy was not performed to any patients. The resected specimens are from 4 men (26.7%) and 11 women (63.6%), and there were 2 cases (13.3%) of early stage cancer (stage I and II) and 13 cases (86.7%) of advanced stage (stage III) adenocarcinoma. There was no stage IV lung adenocarcinoma case included in this study. The diagnosis of adenocarcinoma was based on histological assessment of resected specimen by microscopy with H&E stain. The histological classification and grade were assessed with using the WHO classification. One case (6.7%) was well differentiated adenocarcinoma, twelve cases (80%) were moderately differentiated adenocarcinomas, and two cases (13.3%) were poorly differentiated adenocarcinomas. Four patients (26.7%) had lymphovascular invasion, two patients had neural invasion, and two patients had metastatic lung adenocarcinoma in lymph node. The clinical parameters and histological assessments of the respective cases are summarized in **Table 1**.

Immunohistochemical staining of EMT markers

The formalin-fixed paraffin embedded tissues were cut into 4 μm sections and deparaffinized. The each section underwent immunohistochemical staining using rabbit polyclonal ZEB1 antibody (1:100, pH 6.0 Bethyl) and rabbit polyclonal anti-SNAIL + SLUG antibody (1:100, pH 6.0 Abcam). Only nuclear staining was assessed as positive or negative and the immunoreactivity in tumor center and tumor margin (invasion front) is analyzed separately.
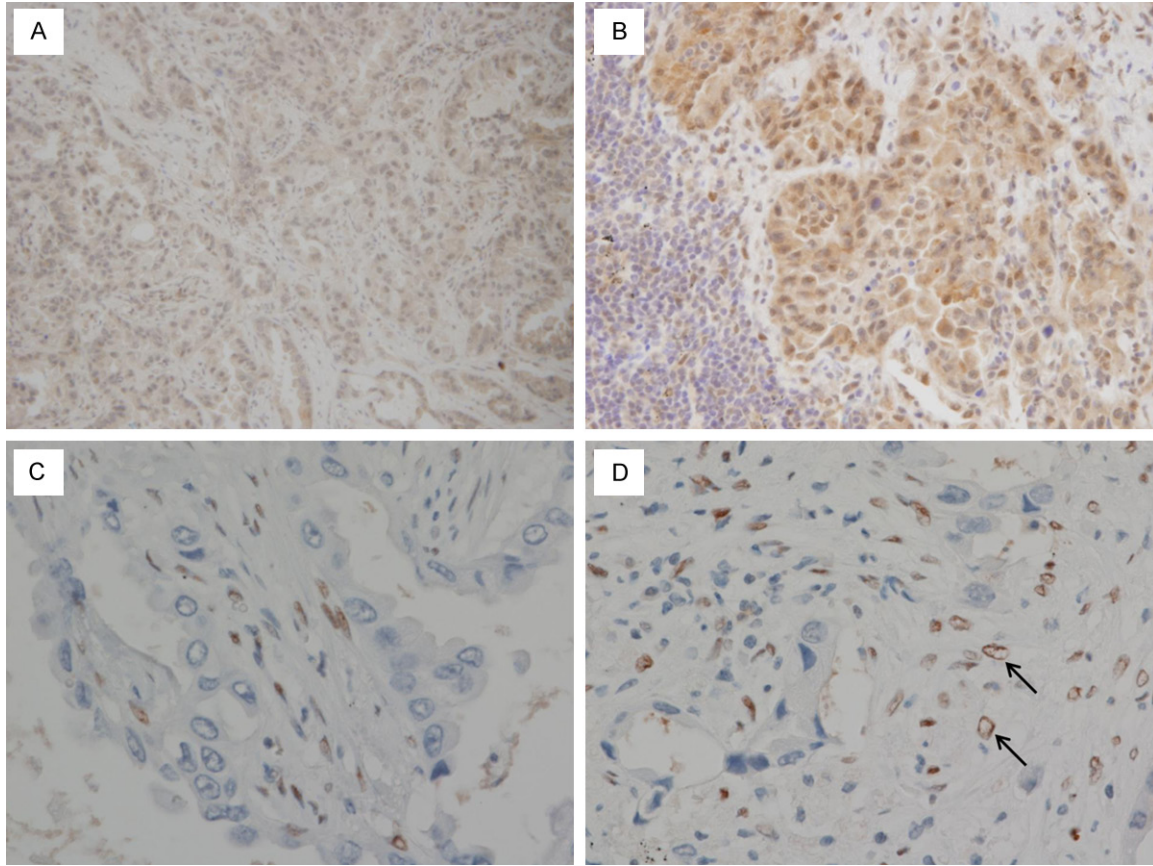
Figure 1. Immunohistochemical staining of SNAIL & SLUG (A, B) and ZEB1 (C, D). (A) In the center of tumor, SNAIL & SLUG is negative in both stromal cells and tumor. (B) The nuclear staining of SNAIL & SLUG is positive in tumor cells in tumor margin. (C) ZEB1 positive stromal cell are mostly observed in tumor center. (D) In tumor margin, ZEB1 is positive in tumor cells (marked with arrow), as well as in stromal cells.

Immunofluorescence staining of EMT markers

The formalin-fixed paraffin embedded tissues were incubated with anti-E-cadherin antibody (1:200, BD Biosciences, San Jose, CA, USA) and double staining one of the following antibodies: anti-Smooth muscle actin (1:400, Sigma-Aldrich, Saint Louis, MO, USA), anti-SNAIL + SLUG antibody (1:50, Abcam, Cambridge, MA, USA), and anti-ZEB1 antibody (1:50, Bethyl, Montgomery, TX, USA). To visualize the primary antibodies, slide were stained with secondary antibodies conjugated with FITC and TRITC. For each slide, counter stained with Hoechst to visualize the nuclei. All the stained slides were viewed and photographed under immunofluorescence microscope equipped with a digital camera.

Statistical analysis

All data collected from this study are analyzed with SPSS Amos 19.0 (IBM corp., Armonk, NY,

USA). The data were expressed as mean ± standard deviation for continuous variables and *P*-value < 0.05 was considered to be statistically significant. The Fisher's exact test or Pearson Chi-square test was used to compare differences in qualitative data.

Results

Fifteen patients who underwent surgical resection of adenocarcinoma of the lung are included in our study. The table shows the clinico-pathological features including age, sex, differentiation of tumor, tumor size, neural invasion, lymphovascular invasion, lymph node metastasis, and stage of tumor.

Immunohistochemical staining of ZEB1

The cells with any degree of nuclear staining were scored as positive. The nuclear staining of ZEB1 tumor center (**Figure 1C**) and tumor margin (**Figure 1D**) was observed in all (100%)
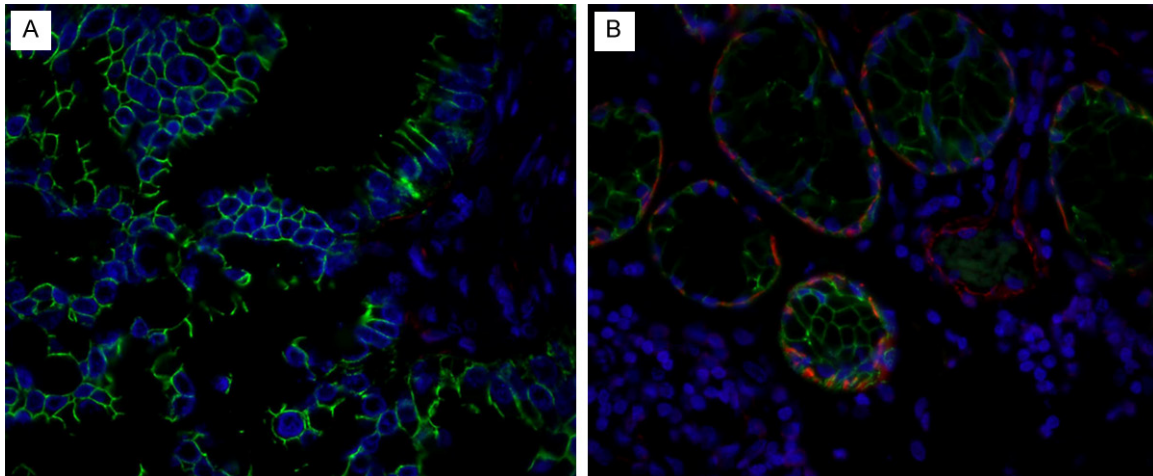
Figure 2. Double immunofluorescence staining of E-cadherin (detected in green) and α-SMA (detected in red) in the tumor tissue at the center (A) and the border (B) of the primary adenocarcinoma in lung. (A) E-cadherin positive cells are detected, but α-SMA is not detected in the center of the tumor; (B) Note that both E-cadherin positive and α-SMA positive cells are detected at infiltrating border of tumor. This is associated with the lymph node metastasis of the tumor cells. The blue staining represents the nuclei (original magnification X 400).

cases. However, the most positive cells with positive ZEB1 staining were spindle-shaped stromal element including endothelial, myofibroblast, fibroblast cells. Although some large cells with positive ZEB1 staining were observed, whether ZEB1 positive cells are from tumor cells or from stromal cells could not be differentiated only by immnuohistochemical stained slide.

Immunohistochemical staining of SNAIL and SLUG

Immunohistochemical staining of SNAIL & SLUG protein was detected at tumor center (**Figure 1A**) and tumor margin (**Figure 1B**) in all (100%) cases. However, some of the positive expression of SNAIL & SLUG was limited only in cytoplasm, not in nuclei. Cytoplasmic staining was disregarded. The nuclear expression of SNAIL & SLUG was observed in tumor center (n = 4, 26.7%) and tumor margin (n = 6, 40%). The staining patterns on nuclei were often focal and weakly stained. The nuclear staining was also identified in virtually all normal tissue of bronchial respiratory epithelium which was apart from the tumor.

Double immunofluorescence of E-cadherin and α-SMA in tumor center

In the center of the tumor, all the samples showed diffuse and strong expression of

E-caherin on the cell membrane (**Figure 2A**). However, α-SMA expression was not detected on tumor cells. The signal was only focally detected at the stromal vascular structures.

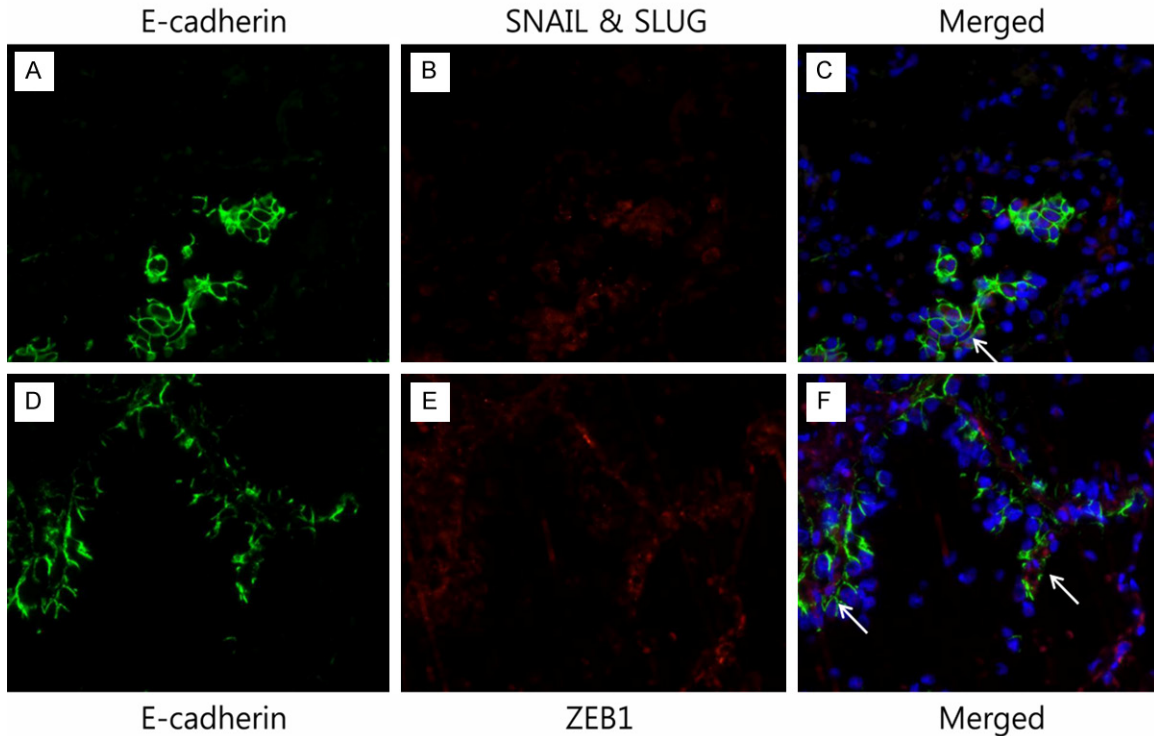*Double immunofluorescence of E-cadherin and α-SMA in tumor margin*

Although the expression was less strongly and focally on the cell membrane, a similar pattern of E-cadherin expression was observed at the margin of the tumor as in the tumor center. The focal intracytoplasmic α-SMA expression was observed in the three cases (20%) in tumor cells at invasion front (**Figure 2B**). All in these three cases, the coexpression of E-cadherin is observed where the cell expressed α-SMA in cytoplasm. As it was seen in tumor center, the signal of α-SMA was also detected at the stromal vascular structures.

The patients with E-cadherin expression and α-SMA expression at the tumor margin were correlated with lymph node metastasis (P = 0.029) and advanced stage (P = 0.029) but were unrelated with lymphovascular invasion (P = 0.081) and neural invasion (P = 0.371).

Double immunofluorescence of E-cadherin and SNAIL and SLUG in tumor center

In the center of the tumor, two samples (13.3%) showed focal but strong expression of SNAIL &

Figure 3. Merged images of double immunofluorescence multi-staining of the tumor tissue of lung adenocarcinoma. (A, D) anti-E-cadherin antibody, (B) anti-SNAIL antibody, and (C) merged image E-cadherin/SNAIL & SLUG. The co-expression of E-cadherin and SNAIL & SLUG is noted (marked with arrow). (E) Anti-ZEB1 antibody, and (F) merged image E-cadherin/ZEB1. The coexpression of E-cadherin and ZEB1 is noted (marked with arrow). The blue staining represents the nuclei (original magnification X 400).

SLUG in the nuclei. All in this two cases, the coexpression of E-cadherin is observed in the cell membrane where the SNAIL & SLUG are expressed in nuclei.

The patients with E-cadherin expression and SNAIL & SLUG expression at the tumor center were not correlated with neural invasion (P = 1.000), lymphovascular invasion (P = 0.371), lymph node metastasis (P = 1.000), or advanced stage (P = 1.000).

Double immunofluorescence of E-cadherin and SNAIL & SLUG in tumor margin

Five samples (30%) showed expression of SNAIL & SLUG in nuclei at the invasion front of tumor. All in these five cases, the expression of E-cadherin on cell membrane is detected where the cells expressed SNAIL and SLUG in nuclei (**Figure 3A-C**). The patients with E-cadherin expression and SNAIL and SLUG expression at the margin of tumor were correlated with lymphovascular invasion (P = 0.022), but were not

correlated with neural invasion (P = 1.000), lymph node metastasis (P = 0.095), and advanced stage (P = 0.095).

Double immunofluorescence of E-cadherin and ZEB1 in tumor center

In the center of the tumor, all the samples showed diffuse and strong expression of E-caherin on the cell membrane. However, ZEB1 expression was not detected on the nuclei of tumor cells.

Double immunofluorescence of E-cadherin and ZEB1 in tumor margin

Two samples (11.3%) expressed ZEB1 protein in nuclei at the margin of tumor. All in these two cases, the expression of E-cadherin on cell membrane is observed as well as ZEB1 expression in nuclei (**Figure 3D-F**).

The patients with E-cadherin expression and ZEB1 expression at the invasion front were correlated with lymph node metastasis (P = 0.010)

and advanced stage (P = 0.010), but not with neural invasion (P = 0.257) and lymphovascular invasion (P = 0.371).

Discussion

Primary lung adenocarcinoma is leading cause of cancer death in worldwide. The tumor invasion, recurrence, and metastasis are the most common events which lead to mortality of cancer patients. EMT has been recognized as crucial phenomenon during tumor cell invades the surrounding stroma. Recently, numerous studies reported that these complex processes are associated with EMT and it constitutes an important mechanism in the development of tumor invasiveness [23]. The clinical importance of SNAIL & SLUG expression is also well known for poor prognosis in various carcinomas, including ovarian carcinomas, urothelial carcinomas, hepatocellular carcinomas, breast cancer, and non-small cell lung carcinomas [22, 24-27].

The downregulation of E-cadherin expression is the most crucial characteristic of EMT development and ZEB1, SNAIL, and SLUG are the major transcription inhibitor of E-cadherin [28]. The loss of E-cadherin may cause loss of contact inhibition and detachment of intercellular connections. Many studies showed that the loss of E-cadherin expression can lead to loss of contact inhibition, infinite proliferation, de-differentiation, and loose intercellular connections which enhanced invasion and migration feature of cancer cells [29-31].

Assuming that EMT should preferably occur at the invasion front of tumor, and at the site of vessel invasion, we assessed the tumor center and tumor margin separately for EMT related proteins and transcription factors [32, 33].

On immunohistochemical study, our data showed that the nuclear expression of ZEB1 was observed in all cases both in tumor center and margin. The nuclear expression of SNAIL & SLUG was detected in tumor center (n = 4, 26.7%) and tumor margin (n = 6, 40%). Although the nuclear staining of SNAIL & SLUG was more frequently observed in the margin of tumor, the expression of transcription factors within the tumor was unexpectedly homogenous between both areas analyzed. This might be interpreted that distinguishing between stromal and tumor

cells is necessity. ZEB1 was also highly expressed in tumor-associated stromal cells [34]. It is unclear whether ZEB1-positive stromal fibroblasts may be derived from epithelial cancer cells through a bona fide ZEB1-dependent EMT [34]. An attractive hypothesis of candidates for ZEB1 inducer is that tumor infiltrating macrophage, which are abundantly detected at the tumor-host interface, express cytokines that may induce ZEB1 expression [35]. Consistent with our observations in our study, the nuclear ZEB1, SNAIL & SLUG protein expression in lung adenocarcinoma-associated stromal cells are observed in other studies. The expression of E-cadherin transcription repressors previously reported in tumor associated stromal cells [24, 34, 36-38]. This raise the possibility that expression of transcription repressor in stromal cell may influence lung adenocarcinoma and it need to be investigated in further study.

In present double immunofluorescence analysis of lung cancer patients, we investigated the expression of transcriptional repressor and EMT-related protein in primary lung adenocarcinoma. Our results show that all of tumor cells in patients with lung adenocarcinoma had E-cadherin expression in both tumor center and tumor margin, but had less E-cadherin expression in tumor margin.

In addition to the loss of E-cadherin expression in the invasion front of tumor, we detected α-SMA which is coexpressed with E-cadherin. In this study α-SMA is used as a marker of mesenchymal differention, which may indicate the occurrence of EMT. In the center of tumor, there was no expression of α-SMA in tumor cells. On the other hand, in the margin of tumor, 20% of the cases had intracytoplasmic α-SMA expression and this was correlated with lymph node metastasis (P = 0.029) and advanced stage (P = 0.029) but were unrelated with lymphovascular invasion (P = 0.081) and neural invasion (P = 0.371).

In our study, expression of SNAIL & SLUG was observed in 30% of the cases at tumor margin and this was reduced by 13.3% at the tumor center. The expression of SNAIL & SLUG in tumor margin was significantly associated with lymphovascular invasion. On the other hand, the expression of SNAIL & SLUG did not corre-

lated with any clinic parameter in tumor center.

We detected nuclear ZEB1 expression in 13.3% in tumor margin and no nuclear ZEB1 expression in tumor center. ZEB1 expression at the invasion front was correlated with lymph node metastasis ($P = 0.010$) and advanced stage ($P = 0.010$), but not with neural invasion ($P = 0.257$) and lymphovascular invasion ($P = 0.371$).

It has been demonstrated that increased α-SMA, ZEB1, and SNAIL & SLUG expression is observed in lung adenocarcinoma. The data suggest that expression of EMT related protein and transcription factor closely associated with enhanced invasion and lymph node metastasis of lung adenocarcinoma. However, it should be noted that this study examined in a relatively small sample of lung adenocarcinoma patients. Therefore, larger study is required to confirm the function of ZEB1, SNAIL & SLUG in lung adenocarcinoma.

Consistent with our observations, the data presented in this study suggest that EMT plays a role in lung adenocarcinoma and metastasis, Taken together, α-SMA, ZEB1, SNAIL and SLUG are overexpressed in the invasive front of tumors in some patients with lung adenocarcinoma suggesting that EMT occurs in the margin of tumor. Our data suggest that overexpression of α-SMA, ZEB1, and SNAIL & SLUG in tumor margin may play a critical role in EMT-induced invasion and metastasis in lung adenocarcinoma. Thus, ZEB1 and SNAIL & SLUG might be used as a predictive parameter for invasiveness and prognosis of lung adenocarcinoma. Furthermore, as our understanding of the underlying molecular processes in development of primary lung adenocarcinoma, elucidation of these interactions will be central to development of novel therapeutic strategies.

Acknowledgements

Disclosure of conflict of interest

None.

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