Brief Report

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SCUBE3 overexpression predicts poor prognosis in non-small cell lung cancer

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Summary **Signal peptide-CUB-EGF-like domain-containing protein 3 (SCUBE3) is highly expressed in invasive lung cancers. In vitro investigation indicated that SCUBE3 may play a critical role in lung cancer invasion and metastasis. The current study immunohistochemically investigated the expression of SCUBE3 in 119 cases of non-small cell lung cancer (NSCLC) tumors and this study evaluated its clinical-pathological and prognostic significance. SCUBE3 was found to be up-regulated in NSCLC tissue samples compared to adjacent normal tissue. High SCUBE3 expression was noted in 84/119 (70.6%) of NSCLC tissue samples and was positively correlated with lymph node involvement (p = 0.001) and advanced stages of tumor/lymph node metastasis (TNM) (p = 0.014). Furthermore, high SCUBE3 expression was significantly associated with loss of the epithelial marker E-cadherin (p = 0.0015) and acquisition of expression of the mesenchymal marker vimentin (p = 0.005). Patients with high SCUBE3 expression had significantly a shorter survival time compared to patients with low SCUBE3 expression (p = 0.001), and SCUBE3 expression served as an independent prognostic factor for NSCLC patients. Results indicated that SCUBE3 might be involved in regulating the epithelial-mesenchymal transition (EMT) and malignant progression in NSCLC. Results also indicated that SCUBE3d may be a potential therapeutic target for lung cancers.**

Keywords: Non-small cell lung cancer, SCUBE3, epithelial-mesenchymal transition, prognosis, metastasis

1. Introduction

Lung cancer is the most commonly occurring type of cancer and the leading cause of cancer-related deaths worldwide. In China, the incidence of lung cancer is still rapidly increasing, and lung cancer mortality has increased by 465% over the past three decades (*1*). Non-small cell lung cancer (NSCLC) is the most frequent (approximately 85%) type of lung cancer. Due to the lack of effective biomarkers, at least 40% of patients with lung cancer are diagnosed in an advanced stage (*2*). Despite efforts at treatment, the prognosis for patients with NSCLC remains poor, with only a 5-year survival rate of 15% after patients have received the standard therapies (*3*). The pathological stage is currently the most commonly accepted prognostic factor for NSCLC but is not enough to significantly improve the management of patients. Therefore, reliable and independent prognostic or predictive markers are needed for further intervention.

Signal peptide-CUB-EGF-like domain-containing protein 3 (SCUBE3) is a secreted cell-surface glycoprotein that has been implicated in murine

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embryogenesis and development (*4*). Recently, Wu *et al*. (*5*) found that SCUBE3 was also highly expressed in extremely invasive lung cancers. Further investigation indicated that SCUBE3 may play a critical role in lung cancer invasion and metastasis, mainly *via* triggering the transforming growth factor-β (TGF-β) pathway and subsequently promoting tumor angiogenesis and the epithelial-mesenchymal transition (EMT) (*5,6*). These findings suggest that SCUBE3 might be a potential oncotarget for pharmacological intervention.

Recent studies have noted that methylation of SCUBE3 is significantly associated with an increased risk of cancer recurrence or death (*7*). Although *in vitro* studies have confirmed that SCUBE3 plays an important regulatory role in the development of lung cancer (*5,6*), its clinicopathological and prognostic significance and its association with EMT in NSCLC specimens have yet to be clarified. The current study immunohistochemically analyzed the expression of SCUBE3 in archived NSCLC tissue samples. Moreover, this study investigated the relationship between levels of SCUBE3 expression and expression of three EMT markers in NSCLC samples. Furthermore, this study assessed its potential clinicopathological and prognostic value in patients with NSCLC.

2. Materials and Methods

2.1. *Patients and samples*

Archived formalin-fixed paraffin-embedded (FFPE) NSCLC samples with corresponding adjacent non-tumor tissue were obtained from 119 patients undergoing surgery from January 2004 to December 2008. Clinical and pathological characteristics including gender, age, histological type, grade, stage, tumor size, differentiation, and status of lymph node metastasis are summarized in Table 1. None of the 119 patients received chemotherapy or radiation therapy before surgery. Survival was calculated from the date of surgery until the date of death or last follow-up appointment. The median follow-up was 39 months (range 4 to 89 months). This study was approved by local research ethics committees.

2.2. *Immunohistochemistry*

Immunostaining was performed as previously described. Briefly, 4 μm deparaffinized slices were incubated with 3% H_2O_2 in phosphate-buffered saline to block endogenous peroxidase. Antigen retrieval was performed by a combination of heat and pressure in sodium citrate buffer. Slides were incubated with rabbit polyclonal anti-SCUBE3 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA; at 1:100 dilution), mouse monoclonal anti-human E-cadherin antibody (Santa Cruz Biotechnology; at 1:100 dilution), and mouse monoclonal anti-human vimentin antibody (Santa Cruz Biotechnology; at 1:100 dilution). Detection was performed using the DAKO EnVision system (DAKO, Carpinteria, CA, USA) with diaminobenzidine (DAB) as the chromogen. Normal mouse IgG was used instead of the primary antibodies as a negative control.

2.3. *Evaluation of immunohistochemical results*

Immunostaining was evaluated by two independent pathologists who were blinded to clinical data. The percentage of SCUBE3-positive cells was determined and a score of 0 to 3 was assigned: 0 for 0%, 1 for 1-33%, 2 for 34-66%, and 3 for 67-100%. The intensity of SCUBE3 staining was scored from 0 to 3 for no staining, 1 for weak staining, 2 for moderate staining, and 3 for strong staining. SCUBE3 immunostaining results were scored by multiplying the percentage of positive cells by their intensity and expression was classified as low (0-3) or high (4-9). For selected EMT makers (E-cadherin and vimentin), immunohistochemistry staining was evaluated using the method described in a previous study: no staining or positive staining of < 10% of tumor cells was deemed to be negative, whereas positive staining of ≥ 10% of tumor cells was considered to be positive (*8*).

2.4. *Statistical analysis*

The association between SCUBE3 expression and clinicopathologic variables and between SCUBE3 expression and expression of EMT markers was examined using the chi-square test. Overall survival curves were calculated using the Kaplan-Meier method and were compared using the log-rank test. Factors with statistical prognostic significance in univariate models were included in multivariate analysis using a multivariate Cox regression model. Statistical analyses were completed using SPSS 16.0, and a p value of less than 0.05 was considered statistically significant.

3. Results and Discussion

3.1. *Immunohistochemical results for SCUBE3 and EMT markers in NSCLC tissue samples*

Representative immunohistochemical staining patterns observed for SCUBE3 and two EMT markers proteins are shown in Figure 1. Different levels of positive staining for SCUBE3 were noted mainly in the cytoplasm and membrane of cancer cells from NSCLC tissue samples. SCUBE3 immunostaining was not observed in the stroma of these tissue samples. High SCUBE3 expression was noted in 84/119 (70.6%) of NSCLC tissue samples and was noted in 18/119 (15.1%) of adjacent matched non-cancerous tissue samples

(Pearson's chi square test, $p < 0.001$).

The pattern of E-cadherin expression was predominantly cytoplasmic and membranous in the area of the tumor. Positive immunohistochemical staining for vimentin in cancer cells was observed in the cytoplasm of cancer cells or stromal fibroblasts in NSCLC tissue samples.

3.2. *Relationship between expression of SCUBE3 proteins and clinicopathological parameters in NSCLC*

The association between SCUBE3 expression and clinicopathological features of NSCLC is shown in Table 1. SCUBE3 expression was higher in patients with lymph node metastasis and subgroups with advanced stages of cancer than in patients with no lymph node metastasis or an early stage tumor; differences in the levels of SCUBE3 expression were statistically significant ($p = 0.001$ and $p = 0,014$, respectively). No significant associations with age, gender, tumor size, histological type, or grade were noted.

Table 1. Correlation of high SCUBE3 expression with clinicopathologic characteristics of NSCLC

Variable	No.	SCUBE3 expression		*p* value
		Low ($n = 35$)	High ($n = 84$)	
Age (years)				
<60	36	11	25	0.805
≥60	83	24	59	
Gender				
Male	93	28	65	0.753
Female	26	7	19	
Pathological type				
Squamous cell carcinoma	58	19	39	0.435
Adenocarcinoma	61	16	45	
Tumor size				
T1-T2	102	32	70	0.250
T3	17	3	14	
Lymph node status				
Negative	67	28	39	0.001
Positive	52	7	45	
Tumor grade				
G1-2	73	19	54	0.307
G3	46	16	30	
TNM stage				
1-2	83	30	53	0.014
3-4	36	5	31	
E-cadherin				
Negative	64	13	51	0.019
Positive	55	22	33	
Vimentin				
Negative	88	32	56	0.005
Positive	31	3	28	

3.3. *Correlation of SCUBE3 expression with expression of the EMT indicator proteins*

The relationship between SCUBE3 expression and expression of EMT indicator proteins was analyzed (Table 1). In the 119 NSCLC tissue samples, epithelial protein loss occurred at a rate of 52.9% for E-cadherin. In the same samples, expression of abnormal mesenchymal proteins occurred at a rate of 26.1% for vimentin. Results also indicated that high expression of SCUBE3 correlated with a loss of E-cadherin expression (Pearson's Chi-Square = 5.522, $p = 0.019$) and anomalous positivity of vimentin (Pearson Chi-Square = 7.864, $p = 0.005$) in clinical NSCLC samples.

3.4. *Survival analysis*

Survival was plotted using the Kaplan-Meier method. As seen in Table 2 and Figure 2, results revealed that patients with high SCUBE3 expression had a significantly shorter survival time than those with low SCUBE3 expression. Furthermore, loss of E-cadherin and acquired vimentin protein expression were also significantly associated with overall survival according to univariate analysis. SCUBE3 expression and these two genetic markers were included in a multivariate Cox regression model along with two other clinical prognostic factors (status of lymph node metastasis and stage). Results revealed that high SCUBE3 expression and a loss of E-cadherin expression were independent prognostic factors for a shorter survival time in patients with NSCLC (Table 2).

In this study, immunohistochemistry showed that SCUBE3 was up-regulated in NSCLC tissue samples compared to adjacent normal tissue, and

Table 2. Univariate and multivariate analysis of the prognosis for NSCLC

Variable	Univariate analyses HR for death (95% CI)	*p* value	Multivariate analysis HR for death (95% CI)	*p* value
Age	0.965 (0.515-1.809)	0.912		
Gender	0.951 (0.472-1.919)	0.889		
Pathological type	1.160 (0.650-2.069)	0.616		
Tumor size	1.669 (0.804-3.467)	0.169		
Lymph node status	3.546 (1.894-6.637)	< 0.001	2.426 (1.019-5.780)	0.045
Tumor grade	1.040 (0.578-1.872)	0.895		
TNM stage	2.611 (1.453-4.690)	0.001	1.054 (0.495-2.244)	0.891
E-cadherin	0.359 (0.188-0.687)	0.002	0.472 (0.244-0.914)	0.026
Vimentin	2.210 (1.210-4.039)	0.010	1.012 (0.520-1.968)	0.973
SCUBE3	4.520 (1.784-11.452)	0.001	2.962 (1.141-7.689)	0.026

HR: Hazard ratio; CI: Confidence intervals.

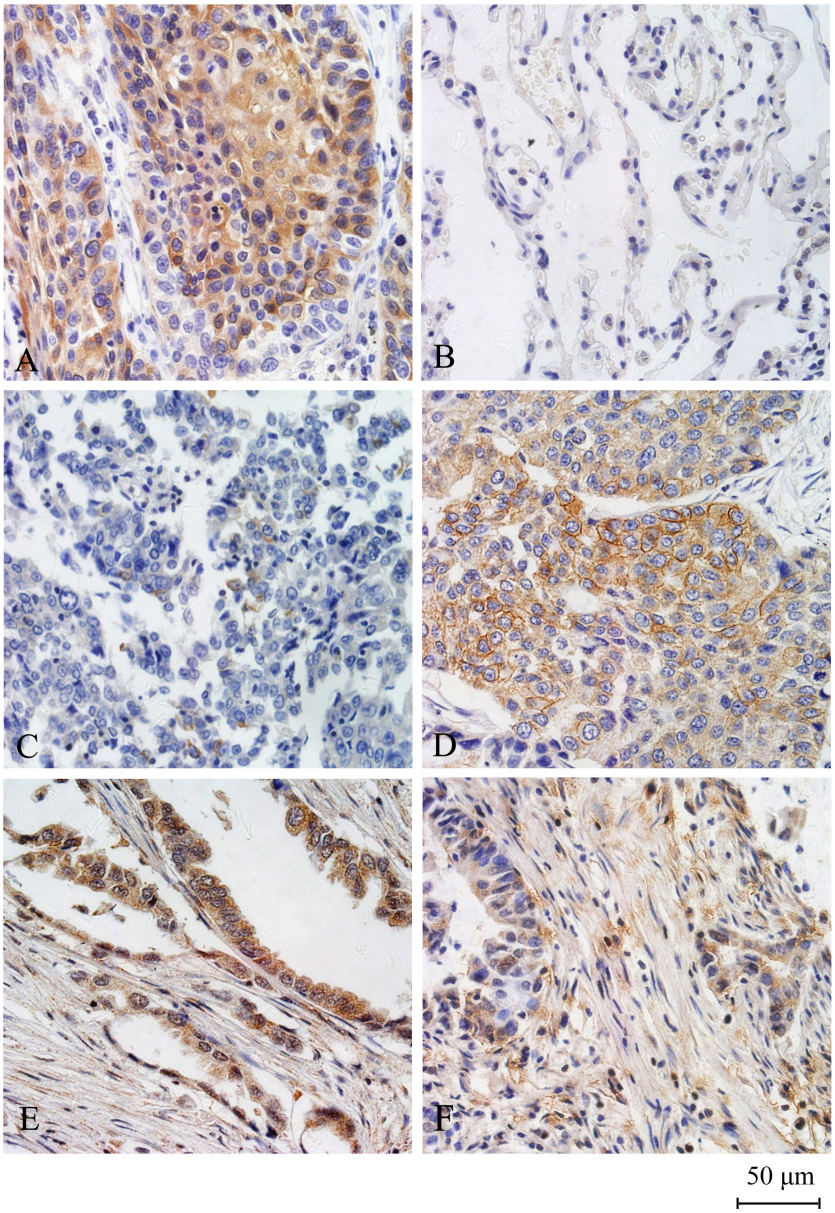

Figure 1. Representative immunohistochemical images of SCUBE3 and EMT markers. (A) High SCUBE3 expression in NSCLC tissue samples; **(B)** Negative SCUBE3 expression in adjacent lung tissue; **(C)** Loss of epithelial marker E-cadherin in NSCLC tissue samples; **(D)** Positive expression of E-cadherin in NSCLC tissue samples; **(E)** Acquisition of expression of the mesenchymal marker vimentin in NSCLC cells; **(F)** Vimentin expression was absent in cancer cells but present in stromal cells from NSCLC tissue samples.

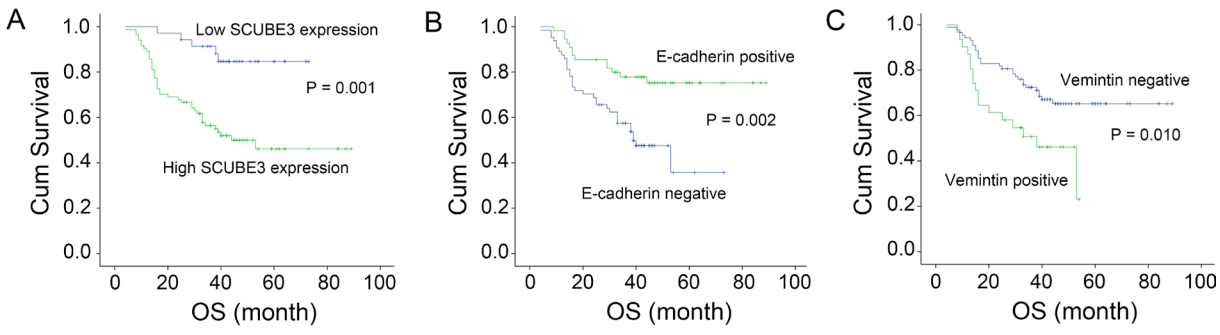

Figure 2. Kaplan-Meier survival curves in accordance with SCUBE3 expression and expression of EMT markers. (A) A subgroup with high SCUBE3 expression had a significantly shorter survival than a subgroup with low SCUBE3 expression; **(B)** Curves calculated for E-cad expression indicated that patients with decreased E-cadherin had a shorter survival time than patients who were positive for E-cadherin; **(C)** Curves calculated for vimentin expression indicated that a subgroup testing positive for vimentin had a shorter survival time than the negative control.

immunohistochemistry also indicated that high levels of SCUBE3 expression were associated with aggressive traits, shorter survival, and expression of EMT indicator proteins in NSCLC. Furthermore, high expression of SCUBE3 was shown to be an independent prognostic factor for patients with NSCLC. To the extent known, this is the first study that has focused on the clinicopathological and prognostic value of SCUBE3 expression and its association with EMT phenomena in clinical NSCLC tissue samples.

There is considerable proof that presence of the EMT phenomenon is related to more aggressive behaviors and shorter survival in lung cancer (*9-11*). The current results revealed that loss of E-cadherin and acquired vimentin expression were associated with outcomes in NSCLC, but E-cadherin was the only EMT marker that independently predicted prognosis. These findings are consistent with those of a previous study (*12*). Mounting *in vitro* evidence suggests that SCUBE3 plays an important role in regulating the EMT and progression in lung cancer. Wu *et al*. found that exogenous SCUBE3 treatment promoted lung cancer cell mobility and invasiveness. Through the C-terminal CUB domain, SCUBE3 binds to the TGF-β type II receptor and then induces Smad2/3 phosphorylation, increasing Smad2/3 transcriptional activity and up-regulating the expression of target genes involved in EMT and cancer progression. The current study expanded on those findings and this study indicated that high SCUBE3 was associated with lymph node metastasis and advanced stages of tumor/lymph node metastasis (TNM) in clinical NSCLC samples. In this study, the TNM stage failed to retain prognostic significance in multivariate analysis. This may be due to differences in the clinical status of patients and their tolerance of treatment. High levels of expression for SCUBE3 were found to be correlated with decreased E-cadherin protein expression and increases in vimentin protein expression in NSCLC. Therefore, the current results support the contention that the regulatory role of SCUBE3 in the EMT process accounts for its positive association with aggressive behaviors such as metastasis and poor prognosis.

Wu *et al*. and Chou *et al*. found that knockdown of SCUBE3 expression suppressed tumorigenesis and effectively inhibited the metastatic potential of NSCLC in an *in vivo* model. Microarray analysis revealed that SCUBE3-knockdown tumors had decreased expression of several genes involved in angiogenesis and EMT. Given the current findings with regard to the clinicopathological and prognostic significance of SCUBE3 in NSCLC, SCUBE3 might be a potential therapeutic target for lung cancer treatment, and particularly for the prevention of metastatic progression and invasion.

In conclusion, SCUBE3 was found to be closely associated with tumor progression and a poor prognosis in NSCLC. Highly expressed SCUBE3 could serve as an independent prognostic factor for NSCLC. Furthermore, results also indicated that SCUBE3 was correlated with expression of EMT-related genes in clinical NSCLC samples. SCUBE3 could serve as a potential therapeutic target in patients with lung cancers.

Acknowledgements

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