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Original Article

Expression of ALCAM, CEACAM-6 in non-small cell lung cancer and its relationship with prognosis

Pengyi Yu[1], Hui Wang[2], Xiaoying Zhang[1]

[1]*Department of Cardiothoracic Surgery, The Third Affiliated Hospital of Soochow University, Changzhou, Jiangsu Province, P. R. China;* [2]*Department of Pathology, The Third Affiliated Hospital of Soochow University, Changzhou, P. R. China*

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Abstract: To explore the expression of ALCAM, CEACAM-6 in NSCLC relationship with pathological features and prognosis. We used immuno-histochemistry and Real-time PCR to detect the expression of ALCAM and CEACAM-6 in 46 NSCLC cases, and statistic analysis the relationship between ALCAM, CEACAM-6 and clinical features with prognosis. Results showed ALCAM and CEACAM-6 were high expression in NSCLC tissues, (76.0 and 69.5%, respectively), especially the ALCAM in squamous cell carcinoma (100%). The expression of ALCAM and CEACAM-6 in tumor tissue at phase T1 is significantly higher than at phase T2 and T3. ALCAM and CEACAM-6 were positive correlation in NSCLC. The median overall survival rate of patients with high expression of ALCAM and CEACAM-6 were better than that of patients with low expression of these proteins (P<0.05).

Keywords: NSCLC, ALCAM, CEACAM-6, prognosis

Introduction

Lung cancer is one of the most common malignant tumors on human. A 5-year survival rate of non small cell lung cancer (NSCLC) is still less than 20%. Therefore, to search lung cancer invading, metastasis and relapse, as well as determining the tumor marker were important objectives. Activated leukocyte cell adhesion molecule (ALCAM) and carcinoembryonic antigen-related cell adhesion molecule (CEACAM) belong to Ig-superfamily [1, 2]. They extensively exist in glycoprotein and participate in variety of pathological processes and are associated with the tumor differentiation and invasive [3]. Here, we will research the expression of ALCAM and CEACAM-6 in NSCLC and to detect the relationship between ALCAM, CEACAM-6 and NSCLC patient prognosis.

Patients and method

Patients

The study included 46 NSCLC patients treated in the Third Affiliated Hospital of Soochow University from 2009 to 2010. NSCLC samples were harvested from surgically resected specimens, and the paracarcinoma NSCLC tissues of 10 patients served as the negative control. The 46 NSCLC patients included 31 cases of male patients and 15 cases of female patients. All specimens were examined histologically according to the Japanese Classification of NSCLC by two pathologists independently. Disagreements were resolved by discussion. All patients were followed up for 5 years. Overall survival (OS) was measured from the date of surgery to the date of death. The experimental protocols were approved by the appropriate institutional review committee and met the guidelines of the responsible governmental agency. All patients didn't accept chemoradiotherapy and hormonotherapy before the surgery.

Immunohistochemistry (IHC)

Immunohistochemical staining was performed using the Elivsion™ method. The following primary antibodies were used in this study for ALCAM (Novus, USA), CEACAM-6 (Sigma, USA). The second antibody and diaminobenzidine tetra-hydrochloride (DAB) solution were provided by Amersham (Amersham, USA). All samples

Table 1. Sequence of ALCAM, CEACAM-6 and GAPDH primer

Name	Sequence (5'-3')
GAPDH Upstream primer	GGAAGGTGAAGGTCGGAGTC
GAPDH Reverse primer	CGTTCTCAGCCTTGACGGT
GAPDH Probe	FAM-TTTGGTCGTATTGGGCGCCTG-TAMRA
ALCAM Upstream primer	CTCCGCCACCGTCTTCAGG
ALCAM Reverse primer	TTGCCAAACATGAGATTCTGAGGT
ALCAM Probe	FAM-CCAGGCCTTGGATGGTATACTGTAAATTCAG-TAMRA
CEACAM-6 Upstream primer	AGGTGGACAGAGAAGACAGCAGAG
CEACAM-6 Reverse primer	AGAAGGTTAGAAGTGAGGCTGTGAG
CEACAM-6 Probe	FAM-ACCATGGGACCCCCCTCAGCC-TAMRA

were fixed in formalin solution and embedded in paraffin. Sections (3-4 mm) were dewaxed in xylene, dehydrated in ethanol, and incubated in 3% H_2O_2 for 15 min to destroy the activity of endogenous peroxidase. After incubation in 10% normal bovine serum for 10 min, each slide was incubated with the primary anti-bodies at 4°C overnight. Biotin-labeled mouse-rabbit immunoglobulin was chosen as the second antibody. The positive and negative controls were provided by the manufacturer.

Evaluation of immunostaining

The intensity (I) of staining was graded on a scale of 0-3+, with 0 representing no detectable staining and 3+ representing the strongest staining. Four strongest staining regions were randomly selected under a 40× field. In each of the four regions, the rate of positive cell staining (R) under a 400× field was calculated. R was defined as: 0, no staining; 1≤10% tumor cells with staining; 2, 11-50% tumor cells with staining; 3, 51-75% tumor cells with staining; and 4, 75% tumor cells with staining. Samples with scores <3 were considered as the negative and with scores >3 were considered as the positive. Histochemistry score = I×R. The mean optical density of ALCAM and CEACAM-6 was analyzed by Image-Pro Plus 6.0 software.

Real-time PCR

According to Package Insert of Triblue Kit, extracted RNA, and reversed transcribe to cDNA. PCR amplification conditions: The reaction system of PCR was 10× buffer 2.5 μl, 25 mmol/L $MgCl_2$ 2.5 μl, 10 mmol/L 4× dNTPs 0.5 μl, 100 μmol/L, 0.1 μl of upstream primer, 100 μmol/L 0.1 μl of reverse primer, 100 μmol/L 0.1 μl of probe, 5 U/μl Taq 0.25 μl of enzyme, 2 μl of specimen, PCR complementation to 25 μl. Thermal cycling condition is 95°C, 10 min; 95°C, 15 s and 60°C, 1 min; expanded to 40 cycles. 103-07 copy/ml standard substance was adopted to establish a standard curve, and measured the specimen of unknown concentration. The sequence of ALCAM, CEACAM-6, GAPDH (a housekeeping gene, internal standard) primer and probe was below (**Table 1**).

Statistical analysis

The correlations between the expressions of ALCAM, CEACAM-6 and clinico-pathological ch aracteristics were analyzed by the x^2 test. The correlations of the expression levels of ALCAM and CEACAM-6 were analyzed by Spearman correlation coefficients. The influence of these proteins on survival was assessed by the Cox proportional hazards model using the backward-LR method. Survival curves were plotted using the Kaplan-Meier method and the statistical difference was analyzed using the log-rank test. For all statistical analyses, SPSS 17.0 software (SPSS, Chicago, IL, USA) was used, and a significant difference was considered at $P<0.05$.

Results

Patients' characteristics

There were 31 males and 15 females in the postoperative patients with an age range of 43-76 years (median 60.6 years), of which 21 patients were Squamous carcinoma, and 25 patients were Adenocarcinoma. In addition, 16 patients had lymph node metastasis. According to the American Joint Committee on Cancer (AJCC) standard, all patients were staged from I to III in this study: there were 25 patients in stages I, 14 patients in stages II and 7 patients in stages III. Other clinicopathological features are shown in **Table 2**.

Table 2. ALCAM and CEACAM-6 expression and clinicopathological features

Clinicopathological features	ALCAM		x^2	*P* value	CEACAM-6		x^2	*P* value
	+	-			+	-		
Gender			3.65	0.06			3.08	0.08
Male	21	10			19	12		
Female	14	1			13	2		
Age			1.35	0.24			0.96	0.33
>60 years	22	9			23	8		
≤60 years	13	2			9	6		
Size			1.05	0.81			1.72	0.78
≤5 cm	15	3			11	7		
>5 cm	20	8			21	7		
Histological type			12.14	0.00*			1.07	0.30
Squamous carcinoma	14	0			13	8		
Adeno carcinoma	21	11			19	6		
T pathological staging			6.67	T1 vs T2+T3			4.49	T1 vs T2+T3
T1	22	2		0.01*	20	4		0.034*
T2	12	7			11	8		
T3	1	2			1	2		
Staging			1.89	I vs II+III			0.15	I vs II+III
I	21	4		0.17	18	7		0.63
II	8	6			8	6		
III	6	1			6	1		
Lymph node metastasis			0.73	0.39			0.34	0.56
Yes	11	5			12	4		
No	24	6			20	10		

*$P<0.05$.

Expression of ALCAM and CEACAM-6 protein in lung cancer tissue

ALCAM and CEACAM-6 positive staining was detected in 76% (35/46) and 69.6% (32/46), respectively. And the positive rates were significantly higher in lung cancer tissue than paracarcinoma normal tissues. No ALCAM and CEACAM-6 staining or only very weak staining was observed in the normal mucosa. Immunohistochemistry showed that the expression of ALCAM and CEACAM-6 were distributed mainly in the cytoplasm and membrane in the lung cancer cells (**Figure 1**).

Correlation between ALCAM and CEACAM-6 expression clinicopathological features

The positive rates of ALCAM and CEACAM-6 were significantly higher in tissue with T1 pathological staging than T2 and T3 pathological staging (ALCAM: T1 pathological staging, 47.8%; T2 and T3 pathological staging, 28.3%; $P = 0.01$. (CEACAM-6: T1 pathological staging, 43.5%; T2 and T3 pathological staging, 26.1%; $P = 0.034$). In addition, the expression of ALCAM was also positively associated with the histological type. The positive rate of ALCAM was higher in squamous carcinoma than in adenocarcinoma (45.6 vs. 30.4%, $P = 0.000$). However, no significant difference was observed in gender, age, tumor size, staging, lymph node metastasis ($P>0.05$), as shown in **Table 2**.

Correlation between ALCAM and CEACAM-6 in NSCLC

A positive correlation between ALCAM and CEACAM-6 expression (evaluated by mean optical density) was confirmed by Spearman correlation analysis. The correlation coefficients (r) were 0.645, (P = 0.000), as shown in **Table 3**.

Expression of ALCAM and CEACAM-6 mRNA in NSCLC

We also used Real-time PCR to analyses ALCAM and CEACAM-6 mRNA in NSCLC. The results

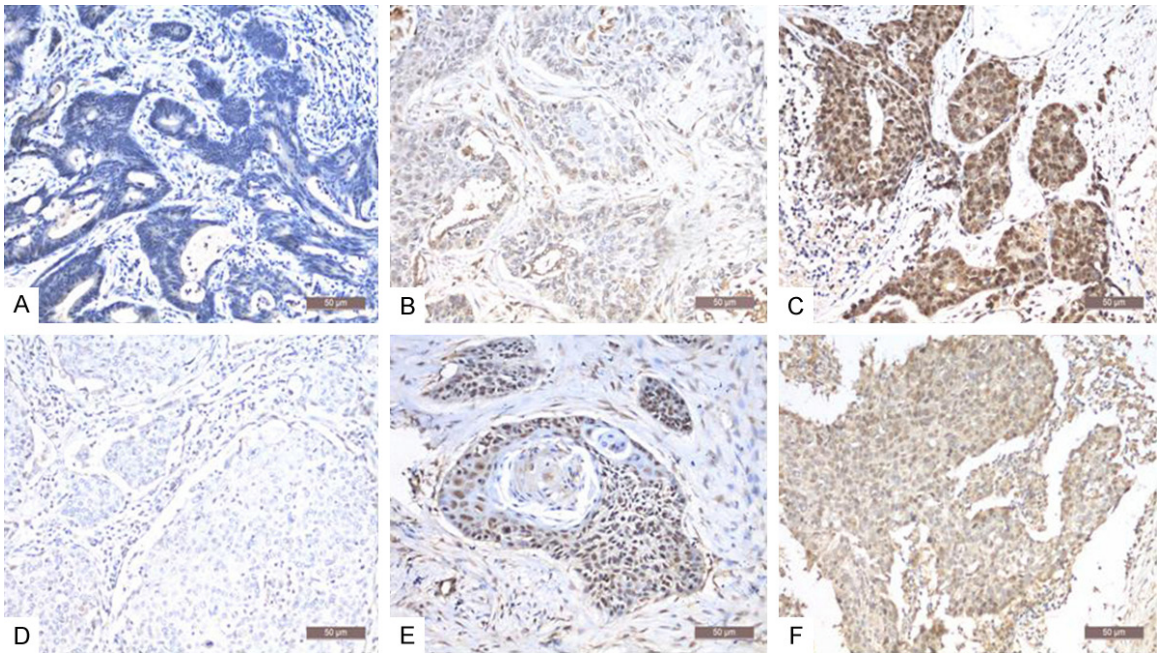

Figure 1. ALCAM and CEACAM-6 immunohistochemistry in lung cancer pericarcinomatous tissues; A. Negative expression of ALCAM; B. Weak positive expression of ALCAM; C: Strongly positive expression of ALCAM; D. Negative expression of CEACAM-6; E. Weak positive expression of CEACAM-6; F. Strongly positive expression of CEACAM-6 (×200).

Table 3. Correlation between ALCAM and CEACAM-6 expression in NSCLC

Spearman correlation analysis (n = 46)		CEACAM-6 expression		*r*	*P* value
		-	+		
ALCAM expression	-	9	2	0.665	0.000*
	+	5	30		

*$P<0.05$.

show that the expression of ALCAM and CEACAM-6 mRNA were significantly higher in cancerous tissue than pericarcinomatous tissue ($P<0.05$) (**Figure 2**). The expression of ALCAM mRNA was significantly higher in squamous cell carcinoma than in pericarcinomatous tissue ($P<0.05$) (**Figure 2A**). However, ALCAM and CEACAM-6 mRNA (in squamous carcinoma) was higher in adenocarcinoma tissue than in pericarcinomatous tissue, but was not statistically significant ($P>0.05$, **Figure 2**).

Survival analysis

All patients were followed up for more than 3 years. Cox regression univariate analysis showed that Histological type, T2 and T3 pathological staging, Lymph node metastasis, staging, ALCAM and CEACAM-6 low-expression were negative prognostic factors for OS ($P< 0.05$, **Table 4**). However, other factors such as age, gender, age and size had no effect on survival of patients ($P>0.05$). Moreover, Cox regression multivariate analysis confirmed that Histological type, T pathological staging, ALCAM and CEACAM-6 was independent prognostic factors hazard ratio (HR) = 1.55, 95% confidence interval (CI) = 10.48-1.89, $P = 0.042$; HR = 1.79, 95% CI = 21.33-2.65, $P = 0.015$; (HR) = 1.46, 95% confidence interval (CI) = 0.81-2.49, $P = 0.023$; (HR) = 1.75, 95% confidence interval (CI) = 1.22-3.69, $P = 0.026$, respectively, **Table 4**.

The Kaplan-Meier curves are shown in **Figure 3**. The median OS of patients with ALCAM-positive expression was significantly longer than that of ALCAM-negative cases (30.0 vs. 18.0 month, $P = 0.048$, **Figure 3A**). The median OS of patients with CEACAM-6-positive expression was also significantly longer than that of ALCAM-negative cases (31.0 vs. 19.0 month, $P = 0.035$, **Figure 3B**).

Discussion

Both of adhesion molecules ALCAM and CEACAM-6 belong to immunoglobulin gene

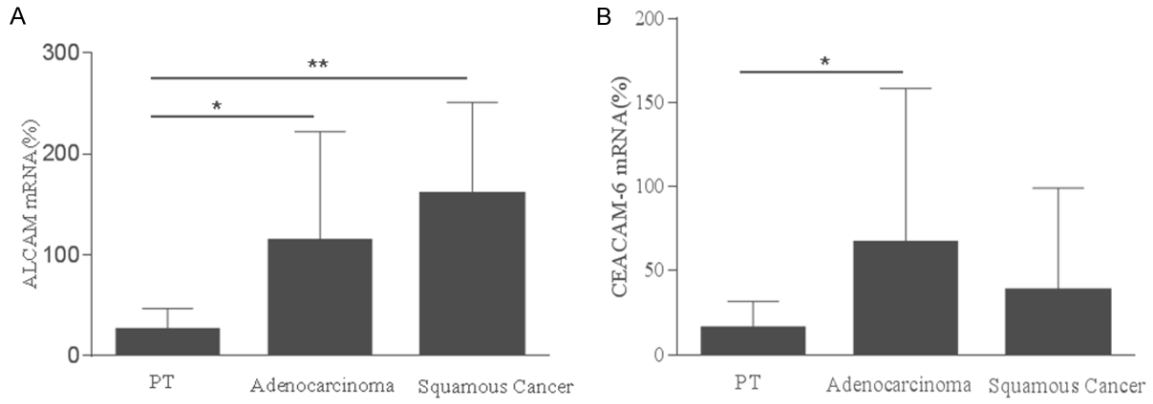

Figure 2. Expression of ALCAM and CEACAM-6 mRNA in NSCLC; A. ALCAM mRNA(both in squamous and adenocarcinoma carcinoma) was significantly higher than that in pericarcinomatous tissue in lung cancer ($P<0.05$), however, ALCAM mRNA in squamous carcinoma was higher than in adenocarcinoma carcinoma, but was not statistically significant ($P>0.05$); B. CEACAM-6 mRNA in adenocarcinoma carcinoma was significantly higher than in pericarcinomatous tissue ($P<0.05$), however, CEACAM-6 mRNA in squamous carcinoma was higher than that in pericarcinomatous tissue, but was not statistically significant ($P>0.05$); n = 46.

Table 4. Univariate and multivariate analysis of the clinicopathological and molecular features for overall survival

Factor	Univariate analysis		Multivaritae analysis	
	HR (95.0% CI)	*P* value	HR (95.0% CI)	*P* value
Gender				
Male vs. female	1.75 (0.68-1.99)	0.094	-	-
Age				
>60 years vs. ≤60 years	0.95 (0.81-1.26)	0.576	-	-
Size				
≤5 cm vs. >5 cm	1.08 (0.54-1.65)	0.315	1.42 (0.57-3.36)	0.22
Histological type				
Adeno vs. Squamous carcinoma	2.18 (0.94-4.89)	0.029*	1.55 (0.48-1.89)	0.042*
T pathological staging				
T1 vs. T2+T3	2.98 (2.13-4.08)	0.006*	1.79 (1.33–2.65)	0.015*
Staging				
I vs. II+III	2.33 (1.58-2.84)	0.005**	3.08 (2.68-3.59)	0.563
Lymph node metastasis				
Positive vs. negative	1.55 (1.06-2.63)	0.048*	2.02 (1.68-2.39)	0.256
ALCAM				
Positive vs. negative	1.51 (1.12-2.09)	0.039*	1.46 (0.81-2.49)	0.023*
CEACAM-6				
Positive vs. negative	1.84 (0.94-2.46)	0.024*	1.75 (1.22-3.69)	0.026*

superfamily, and can regulate the interaction between cell-cell and cell-matrix through regulation the adhesion of homophilic or cytophilic [4, 5]. Any change or deficiency of adhesion molecule may arouse changes on cell tight junction. All of this was closely associated with differentiation, invasive potential of malignant tumor. The purpose of the study was definite the expression of ALCAM and CAECAM-6 in NSCLC and the corresponding clinical significance and prognosis. ALCAM is a transmembrane glycoprotein with 583 amino acids, including signal peptidase with 27 amino acid, 500 extracellular domain, 24 transmembrane domain and 32 cytoplasm domain, extensively exists in human body tissues and organs [1].

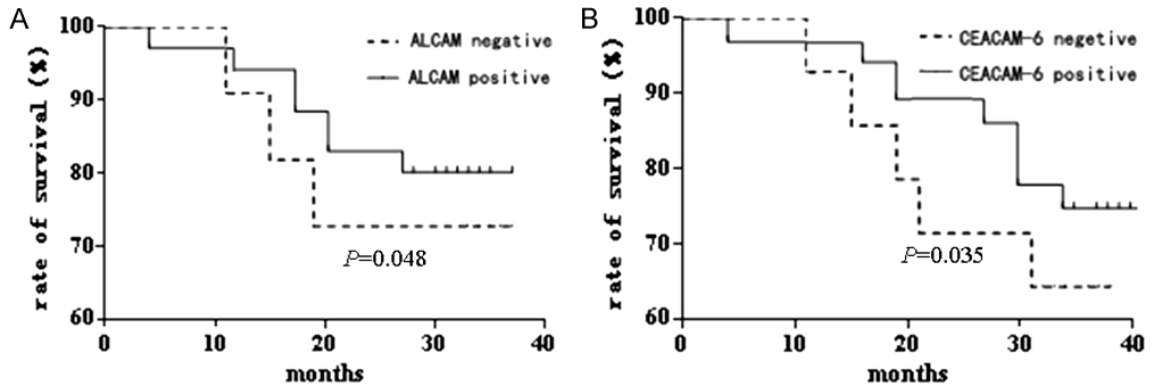

Figure 3. Survival curves for patients with different expression levels ALCAM, CEACAM-6; A. OS of ALCAM-positive patients was significantly longer than that of ALCAM-negative patients (30.0 vs. 18.0 month, $P = 0.048$); B. OS of CEACAM-6-positive patients was significantly longer than that of CEACAM-6-negative patients (31.0 vs. 19.0 month, $P = 0.035$); n = 46.

ALCAM participates in a variety of pathological processes through interaction between cells adhesion between homophilic or cytophilic [6]. It was reported that ALCAM was positive expression in much of malignant tumor including breast cancer, prostatic cancer, colon cancer, ovarian cancer, hepatocellular carcinoma, cutaneous malignant melanoma [4, 6, 7]. In addition, ALCAM was closely associated with tumor invasion and metastasis [8]. It was reported that the expression of ALCAM was negative correlation with extensive invasion of breast cancer, lymphatic metastasis and distant metastasis [6]. However, it was a controversy about the expression of ALCAM in lung cancer. It was reported that ALCAM may be a negative regulation factor of NSCLC and ALCAM can affect cell migration [9, 10]. The expression of ALCAM in cytomembrane can affect patients' prognosis. However, it was not clear about the impacts of ALCAM in cytoplasm on prognosis [11]. Our research showed that ALCAM was high expression in squamous cell carcinoma and was also associated with the Histological type and T pathological staging. Furthermore, the median OS of patients with ALCAM-negative expression was significantly poorer than ALCAM-positive cases, suggesting that in lung cancer, ALCAM can regulate tumor metastasis by regulating immune response and affect the tumor microenvironment. Perhaps the expression level of ALCAM has important diagnostic and prognostic value for lung cancer, which needs further investigation and verification.

CEACAM-6 is a cellular adhesion molecule. It achieves cell adhesion through dimerization of the N-terminal IgV domain. The crystal structure of the N-terminal dimerization domain of CEACAM-6 has been determined at 1.476103 resolutions. CEACAM-6 regulates cell proliferation, apoptosis, motility, morphogenesis, and microbial responses [5]. CEACAM-6 protein expression is down-regulated in several carcinomas and related to the progression-free five-year survival of lung adenocarcinoma patients [12-14]. Our research showed that CEACAM-6 presents high expression in lung cancer and was associated with T pathological staging. Moreover, our research also showed that the expression of ALCAM and CEACAM-6 were positive correlation.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Xiaoying Zhang, Department of Cardiothoracic Surgery, The Third Affiliated Hospital of Soochow University, 185 Juqian Street, Changzhou 213003, P. R. China. Tel: +86-519-68871272; Fax: +86-519-86621235; E-mail: zhangxy6689996@163.com

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