Correlations between Carcinoembryonic Antigen, Epidermal Growth Factor and Leptin in Patients with Non-Small-Cell Lung Cancer

Cuihong Song[1,#], Jie Liao[2,#], Zihui Deng[1], Jinying Zhang[1], Hui Xue[1], Yongming Li[1], Chen Liang[1], Ming Han[1], Jianhua Li[1] and Guangtao Yan[1,*]

[1]*Research Laboratory of Biochemistry, Basic Medical Institute, General Hospital of PLA, 28 Fuxing Road, Beijing 100853, P.R. China*

[2]*Research Laboratory of Medical Experiment and Test Center, Basic Medical Institute, General Hospital of PLA, 28 Fuxing Road, Beijing 100853, P.R. China*

Abstract: *Objectives*: Carcinoembryonic antigen (CEA), epidermal growth factor (EGF) and leptin have been reported to be intimately intertwined in lung carcinogenesis. However, few studies have simultaneously examined these proteins in lung cancer and whether a correlation exist among them remains unclear. Here, we compared the levels of CEA, EGF and leptin in non-small-cell lung cancer (NSCLC) patients and controls and evaluated the possible associations among them.

Methods: 97 patients ranged from 30 to 83 years of age were studied. Serum CEA, EGF and leptin levels were determined following a standard protocol. The relationships between these proteins and clinicopathological factors were evaluated by Wilcoxon rank sum or Kruskal-Wallis H test. Spearman rank-correlation were used to determine the correlations among CEA, EGF and leptin. Co-expression of these proteins in NSCLC tissues was examined by immunofluorescence.

Results: Serum CEA and leptin levels in NSCLC patients were significantly higher compared to controls (both P = 0.000), but no statistically significant difference was found for EGF. CEA and EGF were not associated with the tumor-related factors, but leptin was strongly correlated with sex (P = 0.005). Significant correlations among these proteins were found when the patients were categorized into subgroups. Co-expresstion of these proteins was significantly enhanced with lung carcinogenesis.

Conclusions: CEA, EGF and leptin may interplay and play vital roles in the pathogenesis of NSCLC. Besides CEA**,** the leptin levels were also significantly higher in NSCLC patients than in controls. Determination of preoperative leptin levels may prove useful for screening and predicting NSCLC.

Keywords: Non-small-cell lung cancer, Carcinoembryonic antigen (CEA), Epidermal growth factor (EGF), Leptin, Immunofluorescence, Correlation.

1. INTRODUCTION

Lung cancer is the most malignant cancer today, accounting for more than 1.5 million new patients per year worldwide. Non-small-cell lung cancer (NSCLC) corresponds to 80-85 % of lung cancer cases with a 5-year survival rate of less than 15% [1].

Multifactorial in its nature, the etiology of lung cancer involves a variety of environmental factors and inherent parameters [2, 3]. Until date, several proteins or peptides have been reported to be intimately intertwined in the molecular pathophysiology of lung cancer. Among them, carcinoembryonic antigen (CEA), a glycosyl phosphatidyl inositol (GPI)-cell surface anchored glycoprotein, was originally developed as a prognostic or predictive marker in NSCLC by Ford *et al.* in 1981 [4]. Currently, many reports have described the significant correlation of preoperative CEA levels with prognosis in patients with early-stage lung cancer, especially NSCLC [5]. Another one, epidermal growth factor (EGF), first identified by Cohen in 1962 [6], was a potent mitogen peptide for a variety of cells, both from ectodermal and mesodermal origin [7]. The interaction of EGF with its receptor (EGFR) has been demonstrated to have strong stimulatory effect on cell migration, proliferation, differentiation, survival and angiogenesis. The EGFR is over-expressed in many kinds of human epithelial tumors including lung, head and neck, breast, and colorectal cancers. Till now, the EGF/EGFR system has been found to play critical roles in lung cancer carcinogenesis, mainly in NSCLC patients [8-10]. Besides, leptin, an ob gene-expression protein, secreted mainly by adipocytes, is currently considered to be involved in the development, progression and prognosis of several cancer types [11-13]. Nowadays, there are a few studies regarding the interplay between leptin/its receptor (ObR) and lung

*Address correspondence to this author at the Research Laboratory of Biochemistry, Basic Medical Institute, General Hospital of PLA, 28 Fuxing Road, Beijing 100853, P.R. China; Tel: +86-10-66937072; Fax: +86-10-68176512; E-mails: yan301@263.net, songch-82@163.com

[#]These authors contributed equally to this paper.

carcinogenesis, although with some ambiguous and even conflicting results [3, 14-16]. Among them, majority have found significant associations between reduced serum leptin levels and prognosis in lung cancer [15, 16]. On the contrary, there were quite few studies reported elevated serum leptin levels in NSCLC [3, 17].

As described above, CEA, EGF and leptin have all been reported to be intimately intertwined in lung cancer. However, to the best of our knowledge, no study untill now has simultaneously examined these three proteins in lung cancer and explored the possible associations among them. Accordingly, we presumed that study of the associations might contribute to fully elucidate the clinical significance and interplay of these proteins in NSCLC progression, and to clarify the role of the leptin/OB-R system in lung carcinogenesis.

Therefore, the aim of this study was to compare the preoperative levels of CEA, EGF and leptin in NSCLC patients with controls, to evaluate possible associations between their levels and clinicopathological variables, and to investigate the correlations among these proteins.

2. MATERIALS AND METHODS

2.1. Patients

This study was approved by the review board and ethics committee of General Hospital of the Chinese People's Liberation Army and was conducted in accordance with the Helsinki declaration. All patients provided written informed consent. Ninety-seven histologically confirmed NSCLC patients (median age 60, range 30–83 years, from October 31, 2007 to January 11, 2008) were enrolled for the present study. Histopathological diagnosis were made according to the World Health Organization Classification of Tumours (Pathology and Genetics of Tumours of the Lung, Pleura, Thymus and Heart, 2004). Sixty sex- and age-matched healthy individuals were recruited to take part in the study as a control group.

2.2. Blood Collection and Test

Blood samples from all individuals were collected *via* the cubital vein into tubes containing EDTA anticoagulant no later than 9 a.m. prior to receiving any treatment. After centrifugation at 3,000 rpm at a temperature below 4°C for 15 min, coded serum samples were stored at - 80°C until analysis.

Serum CEA levels were measured using electrochemiluminescence immunoassay (Elecsys CEA; Roche Diagnostics, Germany). Serum EGF levels were measured by an in-house radio-immunoassay. Serum leptin levels were measured using enzyme-linked immunosorbent assay (Murine Leptin ELISA Development Kit; PEPROTECH, Rocky Hill, NJ, USA). All the assays were performed according to the manufacturer's instructions.

2.3. Tissue Collection and Immunofluorescence

Histologically confirmed NSCLC tissues were obtained by biopsy with normal lung tissue excised at least 5 cm away from the tumor edge from the same individuals serving as control tissues. Immunofluore-scence was performed as described previously. In brief, after washed in PBS (3 times for 5 minutes each), the normal and lung cancer tissue sections were permeabilized with 0.1% Triton X-100 and blocked with 10% normal goat serum. For double-label immunofluo-rescence staining of leptin and CEA (or EGF), sections were first incubated with rabbit anti-leptin and mouse anti-CEA (or EGF) antibodies (Santa Cruz, 1:50 dilution in PBS) overnight at 4 °C, followed by incubation with Cy3-conjugated anti-rabbit and FITC conjugated anti-mouse secondary antibody (Sigma, 1:100 dilution in PBS) for 30 min at 37 °C, and nuclei were stained with DAPI (Vector Laboratories, Burlingame, CA, USA). The immunostained sections were mounted on cover glasses in fluorescent mounting medium and viewed using an immunofluorescence microscope [18].

2.4. Statistical Analysis

Statistical analysis was performed using Stata 7.0 software. After defining the contribution of our data, we identified that most variables did not accord with the normal distribution. Hence, all data are expressed as medians and interquartile ranges (IQR). Wilcoxon rank sum test and Kruskal-Wallis H test were used to compare the preoperative serum levels of CEA, EGF and leptin between NSCLC patients and controls, as well as the relationships between their levels and clinicopathological factors. Spearman rank-correlation coefficients were calculated to examine the correlations among serum levels of CEA, EGF and leptin. Two-tailed values of $P < 0.05$ were considered statistically significant.

3. RESULTS

3.1. Patients

As can be seen in Table **1**, median age of the NSCLC patients enrolled in the study was 60 years

(IQR range, 51-71 years). The majority of patients were > 40 years (93.8%) and male (75.3%). 17.5% of patients were squamous cell carcinoma (SQCC), 13.4% were adenocarcinoma (AdenoCa) and 69.1% had other types of lung cancer tumors. Surgical treatment was administered to 17.5% of the patients, and nodal metastasis occurred in 15.5%.

Table 1: Characteristics of NSCLC Patients Enrolled in this Study

Characteristic	No.	%
Age, years		
Median	60	
IQR	51-71	
≤ 40	6	6.2
41-60	46	47.4
> 60	45	46.4
Gender		
Male	73	75.3
Female	24	24.7
Histology		
SQCC	17	17.5
AdenoCa	13	13.4
Others	67	69.1
Surgical treatment		
Negative	80	82.5
Positive	17	17.5
Nodal stage		
N0	82	84.5
N1	15	15.5

Abbreviations: NSCLC, non-small-cell lung cancer; SQCC, squamous cell carcinoma; AdenoCa, adenocarcinoma; IQR, interquartile ranges.

3.2. Serum Levels of CEA, EGF and Leptin in Overall NSCLC Patients and Controls

As can be seen in Table **2**, compared with the control group, median serum levels of CEA, EGF and leptin were markedly increased in the NSCLC patient group. For the patient group, these data were 7.37, 1.67 and 10.06 ng/mL, respectively; For the control group, they were 2.08, 1.51 and 4.75 ng/mL, respectively. Data from this table also showed that serum CEA and leptin levels were significantly different between the patient and control group (both P = 0.000). However, no statistically significant difference was found for EGF.

3.3. Expression of CEA, EGF and Leptin in Normal and NSCLC Lung Tissues

To further investigate the clinical significance of CEA, EGF and leptin in lung cancer, we next detected their expressions in normal and NSCLC lung tissues using immunofluorescence assay. As shown in Figure **1**, compared with normal lung tissue, the expressions of CEA, EGF and leptin were markedly increased in NSCLC lung tissues. Moreover, the co-expressed leptin and CEA (or EGF) were significantly enhanced with the lung carcinogenesis. All these results indicated that CEA, EGF and leptin may play important roles in the development and progression of lung cancer.

3.4. Correlations between Serum CEA, EGF and Leptin Levels in NSCLC Patients and Clinicopathological Factors

Table **3** showed that serum CEA and EGF levels were not associated with the tumor-related factors, such as age, gender, histology and so on. (all P values>0.10). As expected, leptin was strongly correlated with gender (P = 0.005), but no correlations with other related factors.

3.5. Correlations between Serum CEA, EGF and Leptin Levels in NSCLC Patients

As can be seen in Table **4**, for overall NSCLC patients, leptin was strongly correlated with EGF (Spearman's r = 0.22, P = 0.028) although its correlation with CEA was weaker (Spearman's r = 0.17, P = 0.099). No correlation was found between CEA

Table 2: Serum CEA, EGF and Leptin Levels of Overall NSCLC Patients and Controls

Variables	No.	CEA (ng/mL)	P[a]	EGF (ng/mL)	P[a]	Leptin (ng/mL)	P[a]
		Median (IQR)		Median (IQR)		Median (IQR)	
Controls	60	2.08 (1.89-2.53)	**0.000**[b]	1.51 (0.90-2.04)	0.223[b]	4.75 (4.21-8.23)	**0.000**[b]
Patients	97	7.37 (6.62-9.59)		1.67 (1.26-2.08)		10.06 (6.66-14.31)	

Abbreviations: NSCLC, non-small-cell lung cancer; CEA, carcinoembryonic antigen; EGF, epidermal growth factor; IQR, interquartile ranges.
[a]Significant P values are indicated in boldface.
[b]Wilcoxon rank sum test.

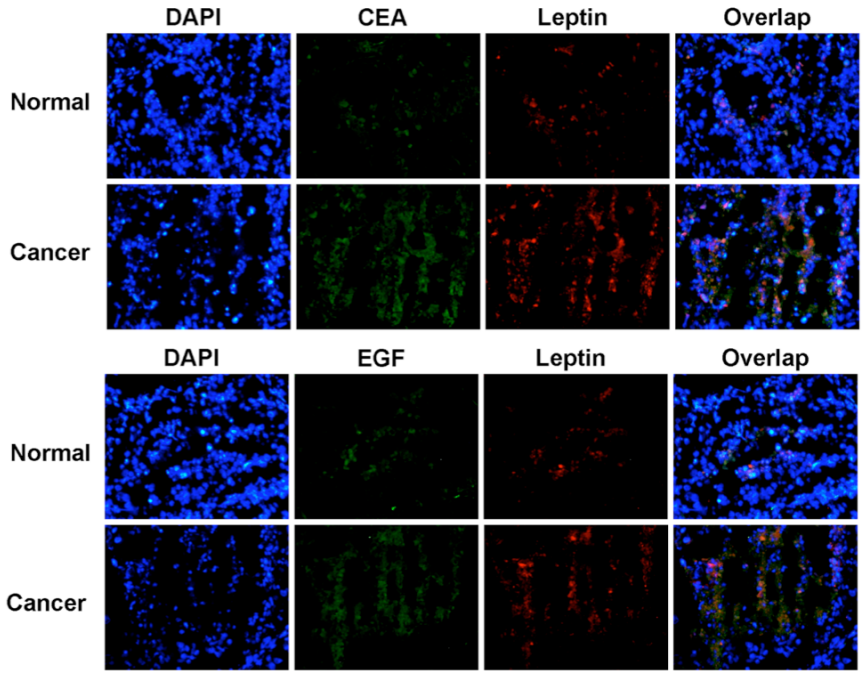

Figure 1: Immunofluorescent images of normal and NSCLC lung tissues stained for DAPI labeled nuclei (blue), CEA or EGF (green) and leptin (red). The images from each group are shown overlaid on the bottom row. Images were captured using an OLYMPUS IX71 fluorescence microscope and managed using Adobe Photoshop. Magnification ×200.

Table 3: Correlations between Serum CEA, EGF and Leptin Levels in NSCLC Patients with Clinicopathological Factors

Characteristic	CEA (ng/mL)	P[a]	EGF (ng/mL)	P[a]	Leptin (ng/mL)	P[a]
Variables	**Median (IQR)**		**Median (IQR)**		**Median (IQR)**	
Age, years						
≤ 40	8.78 (6.75-13.89)	0.636 [b]	1.53 (1.27-2.40)	0.874 [b]	11.64 (6.87-13.93)	0.897 [b]
41-60	7.74 (6.43-9.07)		1.62 (1.26-1.97)		10.03 (5.89-14.83)	
> 60	7.67 (6.70-9.59)		1.72 (1.30-2.13)		9.71 (6.79-13.86)	
Gender						
Male	7.42 (6.62-10.75)	0.464 [c]	1.70 (1.29-2.07)	0.957 [c]	8.64 (5.96-13.60)	**0.005** [c]
Female	7.26 (6.52-8.09)		1.63 (1.24-2.14)		13.02 (10.54-20.95)	
Histology						
SQCC	7.43 (6.08-11.35)	0.420 [b]	1.62 (1.32-1.99)	0.147 [b]	17.64 (7.99-23.29)	0.906 [b]
AdenoCa	7.12 (6.04-7.72)		1.67 (1.34-2.12)		10.94 (5.87-22.95)	
Others	7.44 (6.69-9.75)		1.63 (1.22-2.09)		9.61 (6.70-13.33)	
Surgical treatment						
Negative	7.39 (6.62-10.40)	0.547 [c]	1.62 (1.25-2.07)	0.585 [c]	10.43 (5.82-14.69)	0.360 [c]
Positive	7.26 (6.42-8.19)		1.70 (1.43-2.14)		9.80 (7.63-13.28)	
Nodal stage						
N0	7.42 (6.58-10.11)	0.996 [c]	1.61 (1.26-1.98)	0.428 [c]	10.24 (6.55-14.04)	0.121 [c]
N1	7.34 (6.83-8.14)		2.07 (1.54-2.25)		9.32 (7.17-23.08)	

Abbreviations: NSCLC, non-small-cell lung cancer; SQCC, squamous cell carcinoma; AdenoCa, adenocarcinoma; CEA, carcinoembryonic antigen; EGF, epidermal growth factor; IQR, interquartile ranges.
[a]Significant P values are indicated in boldface.
[b]Kruskal-Wallis H test.
[c]Wilcoxon rank sum test.

Table 4: Correlations between CEA, EGF and Leptin in Overall NSCLC Patients and Subgroups

Variables	Correlations between CEA and EGF		Correlations between CEA with Leptin		Correlations between EGF with Leptin	
	Spearman's r[b]	P[a]	Spearman's r[b]	P[a]	Spearman's r[b]	P[a]
Overall patients	0.02	0.873	0.17	0.099	0.22	**0.028**
Age, years						
≤ 40	0.43	0.419	0.43	0.419	-0.37	0.497
41-60	-0.21	0.161	0.07	0.659	0.38	**0.009**
> 60	0.19	0.217	0.28	0.058	0.17	0.275
Gender						
Male	0.14	0.231	0.26	**0.025**	0.22	0.067
Female	-0.47	**0.020**	-0.02	0.932	0.33	0.118
Histology						
SQCC	0.18	0.482	0.62	**0.008**	0.22	0.387
AdenoCa	-0.04	0.887	0.03	0.929	0.28	0.351
Others	0.00	0.995	0.06	0.649	0.18	0.139
Surgical treatment						
Negative	0.03	0.856	0.11	0.317	0.21	0.061
Positive	0.09	0.729	0.56	**0.019**	0.28	0.282
Nodal stage						
N0	0.04	0.715	0.18	0.115	0.25	**0.024**
N1	-0.06	0.820	0.01	0.975	0.06	0.833

Abbreviations: NSCLC, non-small-cell lung cancer; SQCC, squamous cell carcinoma; AdenoCa, adenocarcinoma; CEA, carcinoembryonic antigen; EGF, epidermal growth factor; IQR, interquartile ranges.
[a]Significant P values are indicated in boldface.
[b]Spearman rank-correlation.

and EGF (Spearman's r = 0.02, P = 0.873). For subgroups of the NSCLC patients, leptin was strongly correlated with EGF only in patients of 41-60 (Spearman's r = 0.38, P = 0.009). Leptin was strongly correlated with CEA in patients of male (Spearman's r = 0.26, P = 0.025), squamous cell lung cancer (Spearman's r = 0.62, P = 0.008) and postoperation (Spearman's r = 0.56, P = 0.019). About CEA and EGF, significant correlation was only found in patients of female (Spearman's r = -0.47, P = 0.020).

4. DISCUSSION

In the present study, we demonstrated that serum levels of CEA, EGF and leptin were higher in NSCLC patients compared to controls. Statistically significant differences were found between the two groups for CEA and leptin, but not for EGF. These findings indicated that besides CEA, leptin levels might also be used as predictive factors for lung cancer. This is the first study, to our knowledge, examining simultaneously serum levels of CEA, EGF and leptin in NSCLC patients.

Furthermore, we intuitively examined the expressions of CEA, EGF and leptin in normal and NSCLC lung tissues using immunofluorescence. We found that the expressions of CEA, EGF and leptin were significantly enhanced with the lung carcinogenesis, and more importantly, they are almost co-localized. These results indicated that CEA, EGF and leptin might be interrelated in lung cancer and that co-localization of these proteins might contribute to enhanced precision to identification and diagnosis in lung cancer. One previous study reported that leptin may promote the carcinogenesis and metastasis of breast cancer, possibly in an autocrine manner [19], which may also explain why the leptin expression was upregulated in lung cancer tissue (Figure **1**).

Regarding CEA and EGF, our finding of increased circulating levels in the patient group is in accordance with the majority of the related studies [4, 5, 8-10]. On the contrary, regarding leptin, our finding is in accordance with the minority of the related studies. Only few studies reported elevated levels of leptin.

Terzidis observed higher serum leptin levels in NSCLC cases and this results was attributed to direct or indirect effects mediated by cancer- or cachexia-related cytokines [3]. Another molecular study demonstrated that an overexpressing functional polymorphism in the leptin promoter gene was associated with an increased risk of lung cancer. Although no consecutive leptin determinations in the same patient were available in this study, there is an indication that elevated leptin levels were observed in stage I NSCLC patients [17].

In addition, we showed that serum CEA and EGF levels were not associated with the tumor-related factors, such as age, gender, histology and so on. Regarding leptin, as a cytokine mainly synthesized by adipocytes, we found it was strongly correlated with gender but no correlations with other related factors, which was consistent with the related researches everywhere [11-13].

According to the literature, CEA, EGF and leptin are all strongly intertwined in lung cancer. However, there is an obvious lack of literature reports whether a interplay exists among them in lung cancer. To the best of our knowledge, this is the first report demonstrating the correlations among these three proteins in NSCLC. Our results showed that for overall NSCLC patients, leptin was strongly correlated with EGF, which is in line with the results of previous studies [14, 20, 21], but its correlation with CEA was weaker. We have not found any statistically significant association between CEA and EGF. For the purposes of detailed presentation of the associations among CEA, EGF and leptin, consecutive analysis was performed in the subgroups of the NSCLC patients. We found that leptin was strongly correlated with EGF only in patients of 41-60, and leptin was strongly correlated with CEA in patients of male, squamous cell lung cancer and postoperation. About CEA and EGF, significant correlation was only found in patients of female. A possible explanation concerning these higher correlations above seem to be an elevated risk of lung cancer in these subgroups. These findings suggest that CEA, EGF and leptin levels are not irrelevant in NSCLC patients. Further studies of their correlations, not only in the circulating level but also in the lung tumour, would provide us with novel insight into their interplay during the development of lung cancer.

Several limitations should be addressed in this study. Firstly, our sample size (97 patients) was not large enough for comprehensive analysis. Secondly, although leptin is strongly associated with sex and BMI, we did not exclude the effects of them in each subgroup. Nevertheless, to the best of our knowledge, this is the first study to simultaneously examine CEA, EGF and leptin and evaluate the associations among them in NSCLC.

5. CONCLUSIONS

In this study, we for the first time simultaneously examined the preoperative serum levels of CEA, EGF and leptin and evaluated the correlations among them in NSCLC patients. Besides CEA, leptin levels were significantly higher in NSCLC patients compared with controls. Determination of preoperative leptin levels might provide useful predictive information for NSCLC. Moreover, leptin was strongly correlated with EGF but was weaker with CEA, no significant correlation was found between CEA and EGF. Future studies are required to thoroughly comprehend their possible interplay and significance in NSCLC progression.

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