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Original Article

The expression of ERβ2, Bcl-xl and Bax in non-small cell lung cancer and associated with prognosis

Junfeng Liu[1*], Zhaoming Nie[1*], Yiyan Lei[2], Shibin Yang[3], Zhaoguo Liu[2]

Departments of [1]Pathology, [2]General Thoracic Surgery, [3]Gastrointestinal Surgery, The First Affiliated Hospital of Sun Yat-sen University, Guangzhou, People's Republic of China. [*]Equal contributors.

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Abstract: Lung cancer is now the leading cause of related death in the world, non-small lung cancer (NSCLC) in predominant type of lung cancer. In this study, we mainly discuss expression, distribution, and prognostic significance of ERβ2, Bcl-xl and Bax in NSCLC. The expression of ERβ2, Bcl-xl and Bax were detected by immunohistochemistry (IHC), and then the staining was evaluated and correlated with clinic and pathologic characteristics, overall survival (OS). ERβ2, Bcl-xl and Bax were localized in NSCLC, and they were over-expressed all in NSCLC ($P<0.05$) compared with BPL tissues. IHC results showed that ERβ2, Bcl-xl and Bax were not correlated with gender, age, smoking index, histological type, regional lymph node metastasis, whereas it was correlated with TNM staging of patients. In a Kaplan Meier analysis, the higher expression of ERβ2, Bcl-xl and Bax was correlated with good OS. ERβ2, Bcl-xl and Bax may be prognostic factors in NSCLC and useful to clinic trials.

Keywords: Estrogen receptors beta, non-small cell lung cancer, Bcl-xl, Bax

Introduction

Lung cancer is now the leading cause of cancer-related mortality worldwide [1]. Non-small-cell lung cancer (NSCLC) is the most common type of the lung cancer. It accounts for about 85% of the lung cancer cases throughout the world and the five-year overall survival rate is almost 15% [2]. Through its therapy has seen much improvement, the prognosis of NSCLC patients remains poor. Even in early stages with no nodal or other metastatic involvement, there is little advancement in regards to distant recurrence and subsequent mortality [3]. In recent years, ERβ was found as an important maker in the disease progression of some cancers, including some estrogen targeted organ cancers, such as prostate cancer and breast cancer, even some no estrogen targeted organ cancers, such as colon cancer and lung cancer [4-9]. Some studies suggested that ERβ may be a good prognosis maker for NSCLC, which is related to inhabit its progression [10, 11]. ERβ is one class of estrogen receptors that has five protein isoforms, including ERβ1, ERβ2, ERβ3, ERβ4 and ERβ5. The expression level, distribution and function of them in organs are different [8, 9, 12-14]. In the breast cancers, ERβ2 could predict better prognosis [15]. In our previous studies, we found ERβ2 maybe had important function in NSCLC. But there are still some controversies on mechanisms of ERβ2 in tumors, which is necessary to be explored further.

The B-cell lymphoma (BCL) protein family is well known as an important role in the intrinsic apoptotic signaling pathway [16]. In normal conditions, pro-apoptotic members like Bax and Bak are sequestered and inhibited by anti-apoptotic factors like BCL-xl, Bax and BCL-2. But their fate may be changed when apoptotic stimuli like DNA damage or massive protein aggregation occur, then their expression level may be disorder and out of control [17]. The studies reported that high Bcl-2 levels seem to correlate with a good clinical outcome [18]. In contrast, anti-apoptotic proteins (bcl-2, bax and bcl-xl) are overexpressed in different tumor entities [19], high Bcl-xL expression has been shown to correlate with lower tumor differentiation and poorer overall patient survival [20]. And low expression of Bax may be associated with the poor outcome in patients with non-

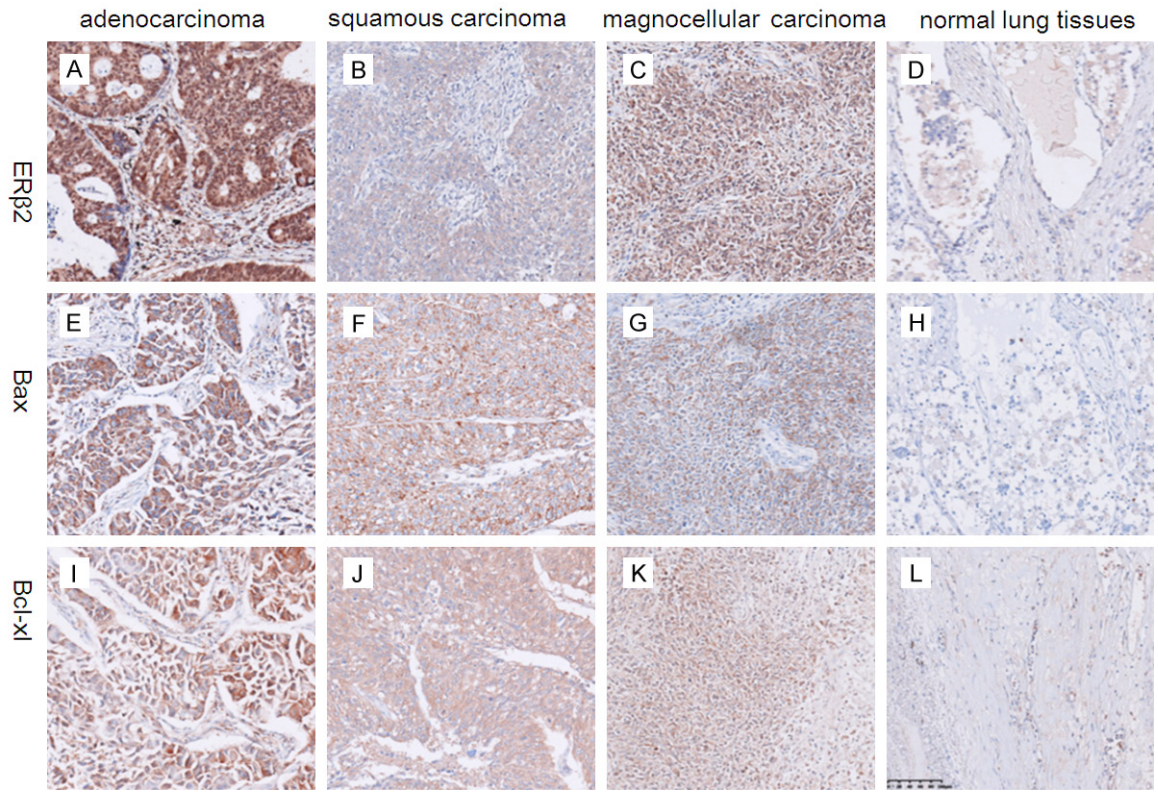

Figure 1. Immunohistochemical analyses of ERβ2, bcl-xl and Bax in NSCLC tissue and BPL tissue.

squamous NSCLC [21]. Since anti-apoptotic proteins have always been described as being redundant, we underline the necessity of a better understanding of their relevance and commitment in lung cancers.

In our study, our study is mainly to detect the expression of ERβ2 and Bcl-xL, Bax via immunohistochemical staining, and observe the distribution in NSCLC. We would analyze the relationship between ERβ2, Bcl-xL and the pathology would be analyzed, the survival. The results obtained from this study maybe provide useful clinical data into the treatment in NSCLC.

Materials and methods

Human tissues

148 samples including 108 NSCLC (adenocarcinoma (AC), squamous carcinoma (SC) and magnocellular carcinoma (MC)) and 40 benign pulmonary lung tissues (BPL) were taken upon surgical resection in the first affiliated hospital of Sun Yat-sen university from 2009 to 2011. All the samples were made into paraffin-embedded tissue specimens after normal dehydration and dehydration processing. All patients selected had no liver, kidney or endocrine diseases, and had not received radiotherapy, chemotherapy or estrogen therapy before surgical resection. The relevant clinic and pathological data included patients' age, gender; smoking index; tumor staging (TNM); histological type; regional lymph node metastasis; overall survival (OS).

Reagents and materials

Anti-ERβ2 antibody and anti-Bcl-xL antibody were purchased from Cell Signaling Company (Danvers, MA, USA), and then anti-Bax antibody was purchased from Santa Cruz Company (California, USA). NovoLink Polymer Detection System was obtained from Leica Microsystems Company (Wetzlar, Germany). EDTA buffer (pH 8.0), PBS, 3% H_2O_2 reagent and other reagents were prepared by ourselves.

Immunohistochemical analysis

Paraffin-embedded NSCLC and normal tissue sections were cut into 3 μm and adhered into slides. The slides were dewaxed by xylene and rehydrated with a series of graded alcohols and washed with water for three times, followed by

Table 1. The different analyses of the ERβ2, Bcl-xl and in Bax NSCLC and BPL expression

Allred score	ERβ2		Bcl-xl		Bax	
	NSCLC	BPL	NSCLC	BPL	NSCLC	BPL
-	47	31	52	27	59	34
+	24	5	23	7	18	4
++	21	3	18	4	15	1
+++	16	1	15	2	16	1
Positive Rate (%)	44.48	22.50	51.85	32.50	45.37	15.00
X^2 Value	13.522		4.393		11.529	
P Value	<0.001		0.027		0.001	

Table 2. Correlation of cytoplasm and nuclear ERβ2 with clinic pathologic parameters

Characteristics	ERβ2		*P* Value
	-	+	
GENDER			
Male	34	36	0.108
Female	13	25	
Age (years)			
<55	13	26	0.080
≥55	34	35	
Smoking index			
<400	34	47	0.367
≥400	13	14	
Histological type			
Squamous cell carcinoma	16	22	0.793
Adenocarcinoma	25	32	
Large cell carcinoma	6	7	
Regional lymph Node metastasis			
N0	29	34	0.411
N1-3	18	25	
TNM staging			
IA-IIB	32	22	0.001
IIIA-IV	15	39	

heat-induced antigen retrieval with EDTA buffer (pH 8.0). Subsequently, the slides were blocked by 3% H_2O_2 reagent for 5 minutes, and then were stained by the NovoLink Polymer Detection System, according to the manufacturer's protocol. The main primary antibodies have been used: anti-Bax antibody, anti-ERβ2 antibody and anti-Bcl-xL. Negative controls were generated by omitting the primary antibody.

Immunoreactive score

The immunoreactive score (IRS) was evaluated by three independent and experienced examiners. Expression status was dichotomized using Allred score >3 as cutoff. Cytoplasmic and nuclear staining was determined, where for negative -; + for weak; ++ for moderate; +++ for strong staining.

Statistical analysis

All statistical analyses were analyzed by statistical software SPSS 22.0 (SPSS Inc., Chicago, USA). The associations of expressions of ERβ2, Bcl-xl and Bax were conducted with Spearman correlation S(r). Differences between high and low expression were compared through X^2. Multiple linear regression analysis was used to analyze the relationship between ERβ2, Bcl-xl, Bax and the factors of clinical pathology.

OS were analyzed initially by Kaplan-Meier plots (Log-rank test). The *P* Value of <0.05 was considered statistically significant.

Results

Expression of ERβ2, Bcl-xl and Bax in NSCLC and benign pulmonary tissues

To assess the expression level of ERβ2, Bcl-xl and Bax, immunohistochemistry were used in 108 cases NSCLC and BPL paraffin-embedded tissues. Expression status was dichotomized as negative (Allred score <3) and positive (≥3) based on statistical assessment. Positive ERβ2 expression was observed in 61 out of 108 (44.48%) in NSCLC, whereas positive Bcl-xl and Bax expression was detected in 56 out of 108 (51.85%) and 49 out of 108 (45.37%) in NSCLC respectively. Compared with expression of ERβ2, Bcl-xl and Bax in BPL, it was significantly higher in NSCLC respectively (**Figure 1**).

Meanwhile, the correlations among ERβ2, Bcl-xl and Bax were analyzed. The analysis result showed that there was a significant positive correlation between ERβ2 and Bcl-xl in NSCLC, and the similar results were observed between ERβ2 and Bax, Bcl-xl and Bax (**Table 1**).

Expression of ERβ2, Bcl-xl and Bax in relation to clinic and pathologic characteristics

The correlations between the expression of ERβ2, Bcl-xl and Bax and clinic and pathologic

Table 3. Correlation of cytoplasm and nuclear ERβ2 with clinic pathologic parameters

Characteristics	Bax		*P* Value
	-	+	
GENDER			
Male	38	32	0.542
Female	21	17	
Age (years)			
<55	20	19	0.372
≥55	38	30	
Smoking index			
<400	43	38	0.370
≥400	16	11	
Histological type			
Squamous cell carcinoma	20	18	0.941
Adenocarcinoma	31	26	
Large cell carcinoma	7	5	
Regional lymph Node metastasis			
N0	35	28	0.569
N1-3	23	19	
TNM staging			
IA-IIB	35	19	0.026
IIIA-IV	24	30	

Table 4. Correlation of cytoplasm and nuclear ERβ2 with clinic pathologic parameters

Characteristics	Bcl-xl		*P* Value
	-	+	
GENDER			
Male	36	34	0.235
Female	16	22	
Age (years)			
<55	19	20	0.544
≥55	22	36	
Smoking index			
<400	36	35	0.133
≥400	16	21	
Histological type			
Squamous cell carcinoma	18	20	0.561
Adenocarcinoma	30	27	
Large cell carcinoma	4	9	
Regional lymph Node metastasis			
N0	28	35	0.171
N1-3	24	19	
TNM staging			
IA-IIB	31	23	0.041
IIIA-IV	21	33	

Table 5. Spearman correlation S(r) between expression of ERβ2, bcl-xl and Bax

Patients	ERβ2 vs. Bcl-xl	Bcl-xl vs. Bax	Bax vs. ERβ2
P Value	0.017	<0.001	0.001

characteristics were analyzed. The expression of ERβ2 was not correlated with gender, age, smoking index, histological type, regional lymph node metastasis, whereas it was correlated with TNM staging of patients (**Table 2**). The similar results of were Bcl-xl and Bax obtained simultaneously, the expression of Bcl-xl and Bax was not associated with any other known clinic pathologic indicators except TNM staging of patients (**Tables 3**, **4**). Spearman correlation analysis showed that the expression of ERβ2, Bcl-xl and Bax were correlative (**Table 5**).

Correlation of the expression of ERβ2, Bcl-xl and Bax with OS

Kaplan-Meier survival analysis was used to assess survival with respect to ERβ2, Bcl-xl and Bax expression. Kaplan-Meier survival analysis showed that ERβ2 higher expression was significantly associated with the better OS (P= 0.022), and the results of Bcl-xl and Bax expression were similar (P=0.032 and 0.016) (**Figure 2**).

Discussion

Lung cancer is the most malignancy in many countries and it has become one cause of cancer death in world, surpassing gastric cancer [22, 23]. Lung cancer has two types, one is small lung cancer, the other is non-small lung cancer (NSCLC). As we know, NSCLC is now the predominant type of lung cancer, which includes lung adenocarcinoma, lung squamous carcinoma and large cell carcinoma. In the previous articles, we have reported the role of estrogen receptor β2 (ERβ2) in the progress of non-small lung cancer, but we have not found the associations between ERβ2 and other proteins [24].

In this study, we mainly discuss the associations ERβ2 and the other two proteins, and this is firstly aimed at elucidating the expression and prognostic of ERβ2, bcl-xl and bax in NSCLC. Our results suggest the importance of evaluating ERβ2, bcl-xl and bax immunoreac-

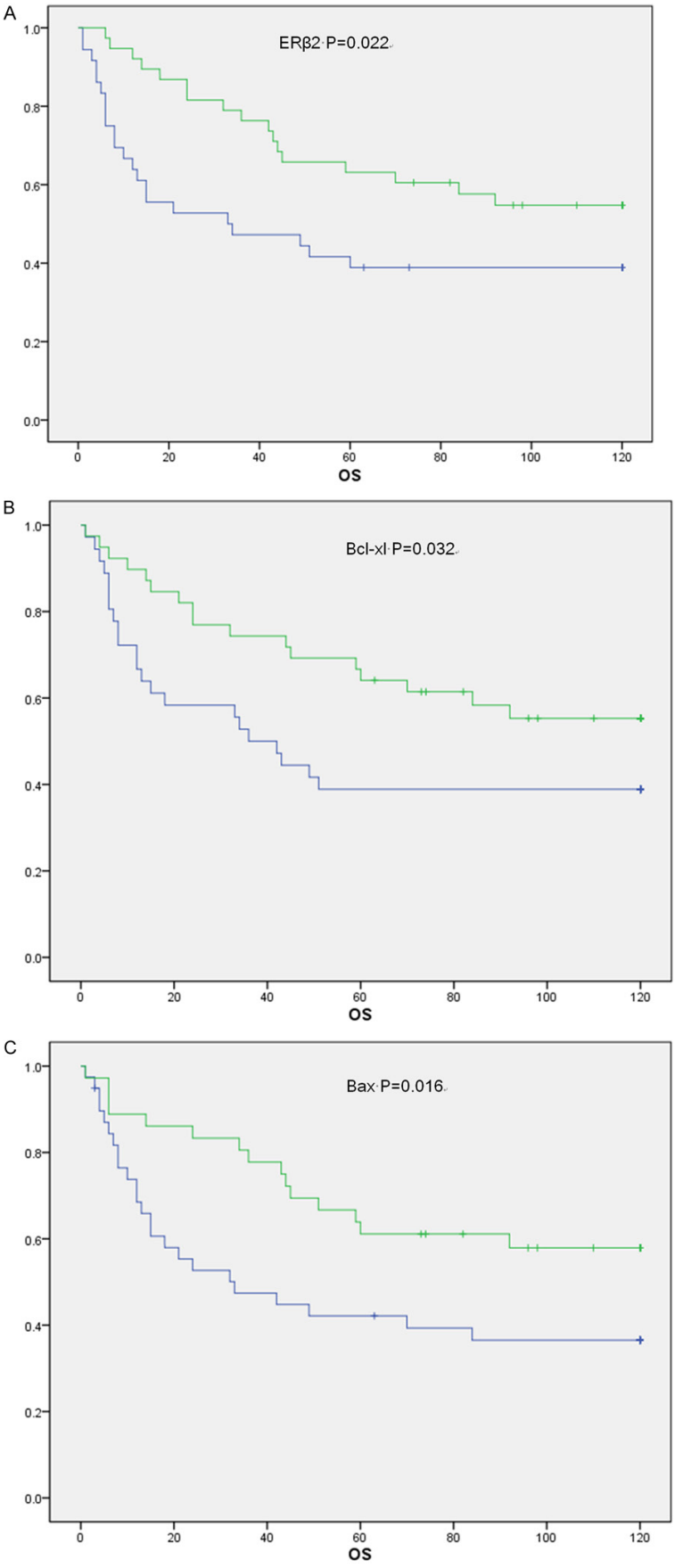

Figure 2. Evaluation of ERβ2, Bcl-xl and Bax as a predictor for OS by the Kaplan-Meier (KM) plot.

tivity as they have important and distinct prognostic implications.

Our data shows that ERβ2, bcl-xl and bax were expressed in NSCLC respectively. Compared with BPL tissues, ERβ2, bcl-xl and bax were over-expressed in NSCLC obviously through the immunohistochemistry. Further statistics by Spearman correlation method, we found there is significant association between ERβ2 and bcl-xl, bax respectively. This phenomenon suggests that ERβ2 not only is a prognostic marker, but also maybe involve in the progress of NSCLC by very complicated mechanisms. In our discovery, it was interesting that expression of ERβ2 was associated with both bcl-xl and bax, which were known as anti-apoptotic factors. It suggested that expression of ERβ2 may be associated with anti-apoptosis mechanism of cancer cells, but there was no more clear data about this finding now.

In recent reports, there were many findings about ERβ2 expressed in some organ tumors and BCL-xl and Bax expressed in lung cancers respectively [25, 26]. And there is no report about connection of them. On further analysis, we found the expression of ERβ2, BCL-xl and Bax were respectively correlated with TNM staging of patients with NSCLC, but negatively correlated with gender, age, smoking index, histological type and regional lymph node metastasis. Our data also showed that ERβ2, BCL-xl and Bax might be powerful

predictors of OS in NSCLC. They were significant associated with good OS.

In summary, we consider that expression ERβ2, BCL-xl and Bax can provide us help on the role and relationship with other makers of ERβ2. Our data shows that ERβ2, BCL-xl and Bax may be prognostic factors in NSCLC, though there still were many un-revealed questions, in particularity, the mechanism of them. These prognostic makers could play an important role in stratifying patients in clinical trials through analyzing more adequate cases.

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Disclosure of conflict of interest

None.

Address correspondence to: Zhaoguo Liu, Department of General Thoracic Surgery, The First Affiliated Hospital of Sun Yat-sen University, 58 Zhongshan 2nd Road, Guangzhou 510080, People's Republic of China. E-mail: 13570314163@163.com

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