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Original Article

Altered expression profile of apoptosis-related molecules correlated with clinicopathological factors in non-small-cell lung cancer

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Abstract: Apoptosis-related molecules can be abnormally expressed in cancers and underscore the hallmark of resisting cell death in cancer cells. This study was aimed to observe the expression patterns of apoptosis-related molecules in lung cancer and paired non-cancerous tissues, and to observe if there is a correlation between the expression of these apoptotic molecules and clinicopathologic parameters. Immunohistochemistry (IHC) was performed to analyze the expression level of CASP3, CASP8, CASP9, PARP1, Cleaved CASP3 (C-CASP3), Cleaved PARP1 (C-PARP1), XIAP, BIRC5 (Survivin) and BCL2 in lung cancer and paired non-cancerous tissues. We found that apoptosis-related molecules CASP3, CASP9, BCL2, BIRC5 and PARP1 are abnormally expressed in lung cancer cells and their expression were correlated with histology. BCL2, BIRC5 and PARP1 are expressed at higher levels in SCC than in non-SCC. C-PARP1 expression might be an independent prognostic factor for NSCLC.

Keywords: Apoptosis, PARP1, caspase 3, BCL2, lung cancer

Introduction

Lung cancer is the leading cause of mortality in China and the rest of the world [1]. Chemotherapy is the most important strategy for the treatment of advanced lung cancers. Recently, specific targeting of driver genes like the epidermal growth factor receptor (EGFR) and anaplastic lymphoma kinase (ALK) has also been successful [2]. However, most cases eventually develop resistance to both chemotherapy and targeted therapy. New strategies should be developed and targeting pro-apoptotic proteins may be an ideal one.

"Resisting cell death" is one of the hallmarks of cancer [3, 4]. Apoptosis related signaling molecules play a key role in maintaining physiological homeostasis but they can be abnormally expressed during the development and evolution of a tumor. They are generally categorized into regulatory molecules (regulator) and effector molecules (effector) [5]. Recently, research on apoptosis pathways and the resistance of cancer cells to apoptosis has made some progress with publications on inhibitors of TARP [6, 7] and Bcl-2 [8, 9] and CASP3 activators [10]. Activation of pro-apoptotic genes can be a promising strategy for cancer treatment and holds promise for translation into clinical practice.

In this study we observed the expression pattern of apoptosis related molecules in lung cancer and adjacent non-cancerous tissues. Furthermore we analyzed the relationship between the expression level of apoptotic molecules and clinical prognostic parameters.

Materials and methods

Patient selection and tumor specimens

Formalin-fixed paraffin-embedded tissues of 139 cases of non-small-cell lung carcinoma (NSCLC) were collected from 2003 to 2006. None of the patients received chemotherapy or other antitumor therapy before operation, and clinical and pathological data were collected. Patients were followed-up until August 1, 2013 or until after operation. Cancer tissue and matching adjacent non-cancerous tissue paraf-

Table 1. Results of IHC analysis of apoptotic molecules in paired and unpaired lung cancer versus adjacent non-cancerous tissues

	IHC scores								
	C-CASP3	CASP3	CASP9	PARP1	BIRC5	BCL2	XIAP	CASP8	C-PARP1
Paired									
Cancer (n=25)	2.88±3.93	6.72±3.45	8.48±2.60	8.92±3.11	7.08±4.24	4.50±4.24	11.2±2.53	0.96±2.09	0.00±0.00
Adjacent (n=25)	0.00±0.00	0.76±0.44	1.00±0.00	0.88±0.33	0.12±0.44	0.58±0.50	1.00±0.00	2.32±3.17	0.00±0.00
t	3.664	8.969	14.385	13.130	8.156	4.850	20.176	-1.557	/
P	0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.133	/
Unpaired									
Cancer (n=139)	8.11±3.50	2.62±3.59	1.09±2.22	8.72±2.97	7.71±4.201	4.16±3.92	11.83±1.12	9.68±2.87	0.30±1.31
Adjacent (n=25)	0.76±0.44	0.00±0.00	2.32±3.17	1.00±0.00	0.12±0.440	0.56±0.51	1.00±0.00	0.88±0.33	0.00±0.00
Wilcoxon W	435.000	1250.000	10941.500	350.000	446.500	1241.500	350.000	372.000	1950.000
P	<0.001	<0.001	0.027	<0.001	<0.001	<0.001	<0.001	<0.001	0.219

fin specimens of 26 patients were used as controls. Adjacent non-cancerous tissue specimens were obtained 5-7 cm away from the edge of the lung tissue. The tissue microarray (TMA) preparation instrument (Beecher Instruments) was used for preparation of the tissue chips.

Main reagents and instruments

Rabbit anti-Human CASP8 (Caspase 8), CASP9 (Caspase 9), XIAP, BCL-2, PARP1, BIRC5/Survivin antibodies were purchased from Abcam; CASP3 (Caspase 3), C-CASP3 (cleaved-Caspase3), C-PARP1 (Cleaved-PARP1) were purchased from Cell Signaling Technology Inc. The Dako REAL™ EnVision™ Detection System, Peroxidase/DAB+, Rabbit/Mouse kit was purchased from Jinqiao Company. The major instruments used were a Leica RM 2106 microtome, a ZMN-6802 pathological tissue bleaching and drying apparatus (Changzhou City Beverly Electronics Limited Company) and an optical microscope (Leica).

Preparation of the tissue microarray

Formalin-fixed and paraffin embedded samples were routinely prepared. For each tissue block, Hematoxyline-eosin (HE) staining was performed to make sure there was sufficient tumor content for TM preparation. A core of the specimen from each donor tissue block was taken by puncture and arranged on a recipient paraffin block with predefined coordinates. The TMA slides were subjected to immunohistochemistry (IHC). By staining one or two master slides, an entire cohort of cases could been analyzed, while maintaining the complete demographic and outcome information for each case.

Detecting the expression of antibodies by IHC

Sections of 4 μm were sliced from the TMA. Then sections were baked, dewaxed, rehydrated, autoclaved for antigen retrieval and incubated with H_2O_2 for 10 minutes to block internal biotin. After PBS rinsing. sheep serum was added and incubated for 10 minutes. Then CASP3, CASP8, CASP9, PARP1, C-CASP3 (Cleaved-CASP3), C-PARP1 (Cleaved-PARP1), XIAP, BCL2 or BIRC5 first antibodies were added respectively for 1 h at room temperature or overnight at 4°C followed by the addition of a second antibody for 15-30 minutes at room temperature. Hematoxylin counterstain was added for 1 minute and the sections were then subjected to gradient alcohol dehydration followed by xylene treatment and mounted with neutral resin for observation under a light microscope.

Interpretation of IHC results

In each sample the staining intensity (negative=0, modest=1, intermediate=2, strong=3) and positive cell proportion (0%=0, 1=1-10%, 11-50%=2, 51=3-80%, 81-100%=4) were recorded according to Koo et al. [15]. IHC score for each sample was calculated by multiplying the intensity score with the proportion score, yielding a score with a range from 0 to 12. The median value of all scores was used as a cut-off value to separate high-expression from low-expression groups.

Statistical analysis

SPSS17.0 statistical software was used for analysis of the experimental results. We used the original score to analyze the differential

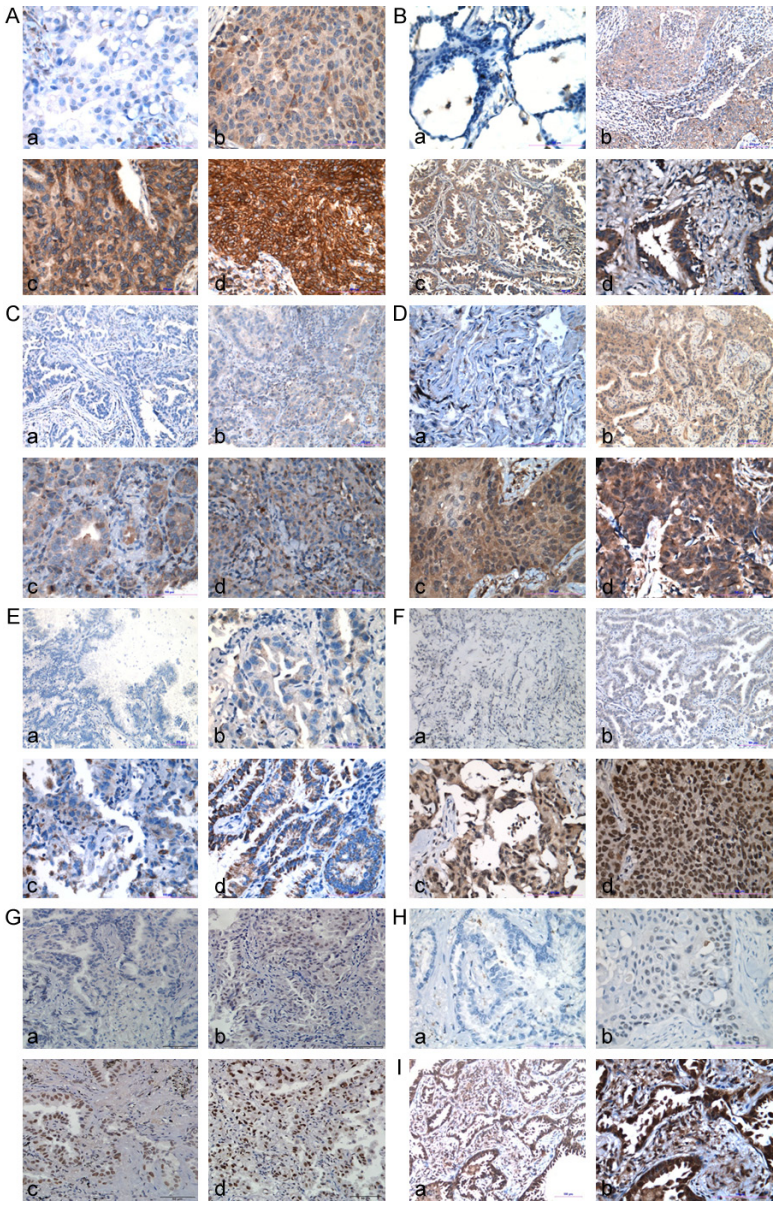

Figure 1. (A) Representative photographs of BCL2 staining by IHC as negative (a), modest (b), intermediate (c) and strong (d). (B) Representative photographs of CASP3 staining by IHC as negative (a), modest (b), intermediate (c) and strong (d) in TMA. (C) Representative photographs of CASP8 staining by IHC as negative (a), modest (b), intermediate (c) and strong (d) in TMA. (D) Representative photographs of CASP9 staining by IHC as -(a), +(b), ++(c), +++(d) in TMA. (E) Representative photographs of C-CASP3 staining by IHC as negative (a), modest (b), intermediate (c) and strong (d) in TMA. (F) Representative photographs of PARP1 staining by IHC as negative (a), modest (b), intermediate (c) and strong (d) in TMA. (G) Representative photographs of BIRC5 (survivin) staining by IHC as negative (a), modest (b), intermediate (c) and strong (d) in TMA. (H) Representative photographs of C-PARP1 staining by IHC as -(a), +(b) in TMA. (I) Representative photographs of PARP1 staining by IHC as ++(a), +++(b).

expression of proteins in paired samples. A paired t test was used if the data was obeying normal distribution, for paired samples a Wilcoxon signed rank sum test was used. For non-paired samples the two independent samples t test was used after checking for homogeneity of variance, or the Wilcoxon signed rank sum test used. For the correlation of two variables a Pearson linear correlation analysis method was used. A non-parametric Mann-Whiteney U test or Kruskal-Wallis tests was used to analyze the association of protein expression and clinical pathological data. The survival was estimated by a Kaplan- Meier curve and a log-rank test was used to compare the difference between groups. A Cox regression (Forward Stepwise Likelihood Ratio) model was used for multivariate analysis of survival associated factors. A P value<0.05 was deemed as statistically significant.

Results

Expression levels of apoptosis related molecules in paired cancer versus adjacent tissues samples

In the 26 control cases of paired cancer versus adjacent tissue samples, a paired sample t test showed that molecules CASP3, CASP9, PARP1, C-CASP3, XIAP and BCL2 were all expressed in cancer tissue at a higher level than in adjacent tissues (P<0.01). C-PARP1 was not detected in any of the samples and CASP8 was not significantly differentially expressed between cancer and adjacent tissue (P>0.05, **Table 1**).

Bivariate analysis revealed that the following apoptotic molecules showed statistically significant correlation: adjacent tissue BCL2 and adjacent tissue CASP8 (r=0.490, P=0.015),

Table 2. Correlation of expression levels of apoptotic molecules with clinicopathologic factors

Parameter	No. (%)	BCL2		CASP3		CASP8		CASP9		C-CASP3		C-PARP1		XIAP		PARP1		BIRC5	
		Mean	P	Mean	P	Mean	P	Mean	P	Mean	P	Mean	P	Mean	P	Mean	P	Mean	P
Age																			
≤61 y	56 (50)	4.00	0.332	8.09	0.600	1.39	0.048	9.14	0.187	3.11	0.440	0.36	0.506	11.75	0.550	9.29	0.512	7.27	0.238
>61 y	56 (50)	4.73		8.52		0.71		8.43		2.29		0.39		11.95		9.86		8.23	
Sex																			
Male	74 (66.07)	4.66	0.149	8.38	0.708	0.85	0.401	8.80	0.984	2.69	0.965	0.69	0.191	11.96	0.232	10.08	0.026	8.93	0.000
Female	38 (33.93)	3.79		8.16		1.45		8.78		2.71		0.16		11.63		8.58		5.45	
Smoking status																			
Never smoker	57 (50.89)	3.82	0.111	8.02	0.375	1.35	0.275	8.86	0.876	2.77	0.820	0.11	0.026	11.70	0.083	9.05	0.077	6.00	0.000
Current or ex-smoker	55 (49.11)	4.93		8.60		0.75		8.71		2.62		0.65		12.00		10.11		9.56	
Histology																			
SCC	33 (29.46)	5.85	0.010	8.27	0.926	0.12	0.002	8.76	0.935	1.09	0.003	0.73	0.038	11.91	0.890	10.21	0.076	9.91	0.000
Non-SCC	79 (70.53)	3.75		8.32		1.44		8.80		3.37		0.23		11.82		9.30		6.85	
Degree of differentiation																			
Poorly	17 (15.17)	6.24	0.044	8.00	0.627	1.06	0.920	8.12	0.249	2.76	0.898	0.47	0.446	12.00	0.457	10.00	0.481	8.82	0.249
Moderately/Well	95 (84.82)	5.03		8.36		1.05		8.91		2.68		0.36		11.82		9.49		7.56	
Pathol. Stage																			
I-II	85 (75.89)	4.51	0.794	8.52	0.187	1.09	0.304	8.54	0.172	2.61	0.826	0.22	0.125	11.96	0.010	9.35	0.259	7.60	0.896
III-IV	27 (24.10)	3.93		7.63		0.93		9.56		2.96		0.85		11.46		10.26		8.22	
PS score																			
0	63 (56.25)	3.76	0.042	8.48	0.562	1.25	0.330	8.44	0.182	3.02	0.249	0.00	0.001	11.81	0.440	9.40	329	7.81	0.954
1	49 (43.75)	5.14		8.08		0.80		9.02		2.22		0.86		11.90		9.80		7.67	
TP53																			
0-1	88 (78.57)	4.45	0.406	8.33	0.918	1.02	0.430	8.76	0.710	2.85	0.394	0.48	0.127	11.97	0.048	9.42	0.424	8.20	0.041
2-3	24 (21.43)	4.04		8.21		1.17		8.88		2.13		0.00		11.39		10.12		6.08	

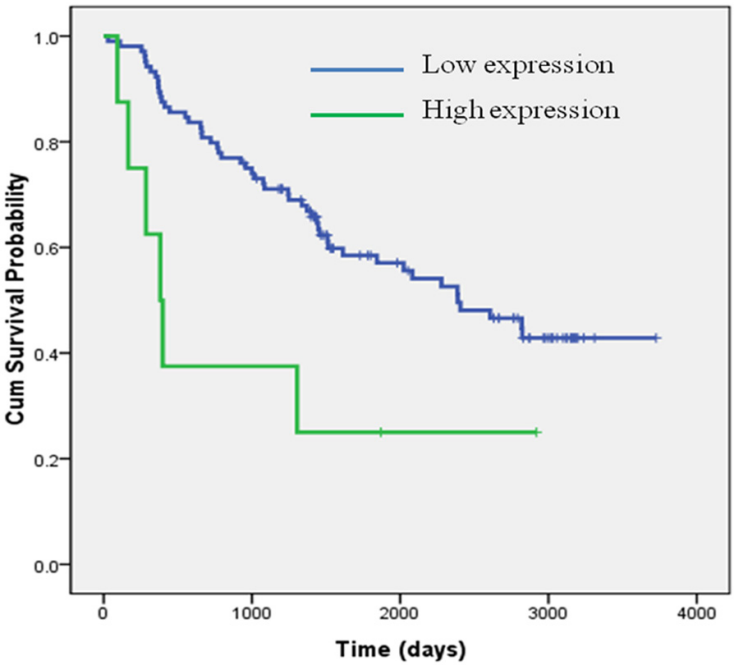

Figure 2. Kaplan-Meir curve analysis showing the relationship between C-PARP1 and overall survival in non-small cell lung cancer population.

cancer tissue CASP3 and CASP9 (r=0.575, *P*=0.003), cancer tissue BCL2 and C-CASP3 (r=-0.478, *P*=0.018), cancer tissue BIRC5 and adjacent tissues BCL2 (r=-0.407, *P*=0.048), cancer tissue BIRC5 and cancer tissue CASP3 (r=-0.449, *P*=0.024), cancer tissue BIRC5 and adjacent tissue CASP3 (r=-0.416, *P*=0.038).

Expression levels of apoptosis related molecules in unpaired cancer versus adjacent tissues samples

In the non-paired cancers (n=139) versus adjacent (n=26) tissues samples, CASP3, CASP9, PARP1, C-CASP3, XIAP, BIRC5 and BCL2 were all expressed in cancer tissue at a higher level than in the adjacent tissues (*P*<0.01). A two independent sample Wilcoxon non-parametric rank test for C-PARP1 showed no significant statistical difference for expression in cancer or adjacent tissues samples (*P*>0.05), CASP8 molecular expression in carcinomas was significantly lower than in the adjacent tissues samples (*P*<0.05, **Table 1**).

Bivariate analysis revealed that the expression levels of the following apoptotic molecules in carcinoma showed statistically significant correlation: BCL2 and CASP3 (r=0.267, P=0.001), BCL2 and CASP9 (r=0.319, *P*<0.001), BCL2 and C-PARP1 (r=0.281, *P*<0.001), BCL2 and XIAP (r=0.353, *P*<0.001), BCL2 and PARP1 (r=0.355, *P*<0.001), CASP3 and CASP9 (r=0.669, *P*<0.001), CASP3 and C-CASP3 (r=0.311, *P*<0.001), CASP3 and XIAP (r=0.611, *P*<0.001), CASP3 and PARP1 (r=0.532, *P*<0.001), CASP8 and XIAP (r=-0.166, P=0.035), CASP9 and C-CASP3 (r=0.208, *P*=0.008), CASP9 and XIAP (r=0.701, *P*<0.001), CASP9 and PARP1 (r=0.610, *P*<0.001), C-CASP3 and XIAP (r=0.290, *P*<0.001), PARP1 and XIAP (r=0.750, *P*<0.001), BIRC5 and BCL2 (r=0.399, *P*<0.001), BIRC5 and CASP3 (r=0.561, *P*<0.001), BIRC5 and CASP9 (r=0.537, *P*<0.001) , BIRC5 and C-PARP1 (r=0.219, *P*<0.005), BIRC5 and XIAP (r=0.587, *P*<0.001) and BIRC5 and PARP1 (r=0.651, *P*<0.001).

Location of apoptotic molecules in the cell

In lung cancer tissues, IHC staining revealed that BCL2, XIAP, CASP3, CASP8, CASP9, PARP1, C-CASP3, C-PARP1 and BIRC5 were expressed with a clear location specificity, i.e., BCL2, XIAP, CASP3, CASP8, CASP9 and C-CASP3 in the cytoplasm and PARP1 and C-PARP1 mainly in the nuclei. Representative pictures of the expression of each molecule are shown in **Figure 1A-I**.

Expression levels of apoptotic molecules in relation to clinicopathologic factors

The following cases with complete clinical information and IHC results were included to study the expression levels of apoptotic molecules in relation to clinicopathological factors: 68 stage I, 17 stage II, 24 stage III and 3 stage IV cases (total n=112). Nonparametric statistical analysis of each group showed that the expression levels of C-CASP3 and CASP8 was higher in non-squamous cell carcinoma (non-SCC) than in squamous cell carcinoma (SCC) (*P*<0.05); The expression levels of BCL2 and PARP1 in SCC was higher than in non-SCC (*P*<0.05); PARP1 was also associated with male smokers (*P*<0.05); The expression of BIRC5 (survivin) in the male, non-smoking group was higher than

Table 3. Analysis of the expression of apoptotic molecules and clinical factors with OS by Kaplan-Meir survival curve

	PS		Disease Stage				N			C-PARP1		PARP1		CASP3	
	0	1	I	II	III	IV	0	1	2	Low	High	Low	High	Low	High
I-IV															
mOS	/	1455±289.9	/	1076±366.7	957±292.1	382±103.7	/	1014±345.4	1084±229.2	/	382±79.2	/	/	/	/
χ^2	2.908			36.493			21.903			7.347					
P	0.048			<0.001			<0.001			0.007					
III-IV															
mOS	/	/	/	/	/	/	/	/	/	1084±229.2	286±144.5	/	/	/	/
χ^2										13.585					
P										<0.001					

Table 4. Association of apoptotic molecules and clinical factors with OS by Cox multivariate regression model

Variables	Hazard Ratio	95% CI	*P* value
Overall survival of all stages			
Disease stage[a]	2.47	1.76-3.47	0.000
Histology[b]	0.50	0.26-0.95	0.035
C-PARP1[c]	1.20	1.03-1.38	0.019
Overall survival in stage III patients			
C-PARP1[c]	1.38	1.10-1.73	0.006

Label: a, Compared with stage I patients; b, Compared with SCC; c, High expression of C-PARP1 compared with low expression.

in the female, non-smoking group ($P<0.01$); BIRC5 (Survivin) expression in the SCC was higher than in non-SCC ($P<0.01$); The relationship between BIRC5 (Survivin) and TP53 was negatively correlated ($P=0.041$) (**Table 2**).

Expression levels of C-PARP and survival

A Kaplan Meir survival curve showed that patients with a low expression level of C-PARP1 had a relatively longer OS than patients with a high expression (**Figure 2**), suggesting that C-PARP1 may be an independent prognostic molecular marker of overall survival (OS). Other factors in relation to OS were personal status score, disease stage and N stage as listed in **Table 3**.

Multivariate Cox regression analysis for survival-associated apoptotic molecules

Multivariate Cox regression was performed on 112 cases with complete clinical information and IHC results. Variables age, PS score, histological type, stage of disease and the molecular expression levels were entered into the Cox proportional hazard regression model. These variables had a *P* value<0.05 by univariate analysis and were included in the multivariable Cox regression model, a forward stepwise (likelihood ratio) method was used. The multivariable Cox regression model analysis showed that except for disease staging and histological type, C-PARP1 was associated with OS in NSCLC. Therefore, the expression level of C-PARP1 might be an independent prognostic factor for OS (**Table 4**).

Discussion

The most encouraging phenomenon in the development of modern medicine in the 21st century is that by studying the molecules, receptors and signal transduction pathways that have an effect on tumors, the etiology, prevention and treatment of tumors can be brought together very well. With the in-depth understanding of the development and evolution of tumors, especially with the understanding of the mechanisms of action of key genes, regulatory molecules and receptors for treatment, targeted inhibitors for EGFR [11, 12], VEGFR [13], mTOR [14, 15], EML4-ALK fusion [16, 17] and ROS1 fusion [18] have been applied in the clinical treatment of common tumors.

This study began with analyzing the expression levels of apoptosis signal transduction pathway related molecules in NSCLC. This was done to see if the expression levels correlated with clinical and pathological factors, and to see if these molecules could be of potential prognostic significance. Apoptotic molecules like BCL2 and survivin may be a suitable and effective target for treatment. In this study, by using a TMA technique combined with IHC, we systematically tested the expression levels of important apoptosis-related molecules in cancer tissues from patients with lung cancer. These molecules from the apoptosis signal pathways were mainly upstream regulating molecules and downstream effectors [19], including CASP3, CASP8, CASP9, PARP1, C-CASP3 (activated form of cleaved CASP3), C-PARP1 (PARP1 fragment product CASP3/7 after cleavage), XIAP, BCL2 and BIRC5.

The results found in the TMAIHC test containing 139 cases (including paired paraffin specimens of 26 patients with cancer and adjacent non-cancerous tissue), showed that whether in paired, or unpaired cancer versus non-cancerous adjacent tissues, CASP3, PARP1, CARP9, XIAP, BCL2 and BIRC5 apoptosis-related molecules were expressed significantly higher in cancer tissues. CASP3 is an important pivotal signaling molecule in apoptosis and this suggests that CASP3, BCL2, PARP1, BIRC5 may be molecular markers and targets for drug intervention.

Bivariate analysis revealed that the expression levels of several apoptotic molecules had statistically significant correlation in cancer tis-

sues. These significant correlations confirmed the internal interactions of these apoptotic proteins. Although statistical analysis does not necessarily represent a causal relationship, it cannot be denied that the possible functional interactions between the molecules and the specific mechanisms of these concurrent changes of apoptotic proteins needs further study.

This study also found that the expression levels of C-CASP3 and CASP8 were higher in non-SCC than in SCC whereas BCL2 and PARP1 expression levels in SCC were higher than in non-SCC. The expression of BIRC5 (survivin) in non-smoking male patients was higher than in non-smoking female patients; BIRC5expression in the SCC was higher than in non-SCC. PARP1 and BIRC5 were associated with factors as smoking and SCC, suggesting that BCL2, PARP1 and BIRC5 might be important targets for the treatment of SCC lung cancer in smokers and other subsets of patients. BIRC5 and TP53 showed a negative relationship. It is known that wild type P53 inhibits the expression of survivin at mRNA and protein levels [20, 21]. When P53 is mutated, it cannot form P53/HDAC with repressor protein/deacetylation of histone (histone deacecylases, HDAC) complexes to bind to survivin promoter, modify its chromosome configuration, and lead to transcription factor E2F2 void of being transformed into E2F/Rb complexes, resulting in the abnormal expression of survivin [22, 23]. High expression of survivin can also trigger the anti-apoptotic mechanisms leading to cell proliferation and promotion of tumor formation [24, 25].

A Kaplan-Meir survival curve analysis and a multivariate Cox regression model analysis showed that C-PARP1 is associated with OS in NSCLC. This is consisted with a previous report that showed that high expression of C-PARP1 molecules is associated with a shorter median OS in patients with lung cancer [26]. Analysis suggested that C-PARP1 might be the primary independent adverse prognostic factor in lung cancer. However, its prognostic potential biological function was not clear and needs further study. Because the number of lung cancer cases in this study is limited, there is an urgent need to expand the sample size to verify the prognostic significance of the positive expression of C-PARP1 in lung cancer in future studies. Though a relation between CASP3, PARP1, apoptosis and prognosis of lung cancer have been reported in the literature, the molecular mechanisms of CASP3 and PARP1 affecting prognosis in different genotypes of lung cancer still needs further investigation. Other shortcomings of this study are limited sample size, the use of a retrospective study.

In summary, apoptosis-related molecules CASP3, CASP9, BCL2, BIRC5 and PARP1 are abnormally expressed at high levels and correlate with histology in lung cancer. BCL2, BIRC5 and PARP1 are expressed at higher levels in SCC then in non-SCC. C-PARP1 expression might be an independent prognostic factor for NSCLC. The effects of targeting these altered apoptotic proteins needs further intensive study as they could be a good target in the treatment of lung cancer.

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Disclosure of conflict of interest

None.

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