

• Basic Research •

Subcellular localization of survivin in non-small cell lung cancer

Lang He,^{1,2} Mei Hou,¹ Jie Zhang,¹ Nie Xu¹ and Ping Chen¹

1. Department of Medicine,
Cancer Center,
West China Hospital,
Sichuan University,
Chengdu, 610041,
P. R. China
2. Cancer Center,
Nanchong Central Hospital,
Sichuan, Nanchong, 637000,
P. R. China

Correspondence to: Mei Hou

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[Abstract] **Background and Objective:** Survivin, a member of inhibitors of apoptosis protein (IAP) family, is expressed in most tumors as well as in different subcellular units of tumors. This study was to investigate the clinical significance of survivin in different subcellular units in non-small cell lung cancer (NSCLC). **Methods:** The protein expression of survivin was detected by immunohistochemistry (IHC) in the specimens from 51 cases of NSCLC and 21 cases of paracancerous tissues. The relationship between survivin expression and clinical characteristics of patients was analyzed using SPSS 13.0 software. **Results:** Positive expression of survivin was mainly detected in cytoplasm and / or nucleus of NSCLC tissues, and the positive rates were 49.0% (25/51), 72.5% (37/51), 3.9% (2/51), 27.5% (14/51) and 23.5% (12/51) in cytoplasm only, in cytoplasm, in nucleus only, in nucleus, and both in cytoplasm and nucleus, respectively. The positive expression rate of survivin was significantly higher in NSCLC tissues (76.5%, 39/51) than in paracancerous tissues (19.0%, 4/21) ($P=0.000$). The expression of survivin in cytoplasm was correlated with differentiation of tumors ($P=0.007$). Positive staining of survivin in nucleus, both in cytoplasm and nucleus were significantly related to clinical stage and N stage of NSCLC ($P<0.05$). The positive rate of survivin was higher in III + IV or N1 + N2 stage patients than in I + II or N0 stage patients, respectively ($P<0.05$). The five-year survival rate was lower in patients with positive expression of survivin in nucleus than in those with negative expression in nucleus ($P<0.05$). The clinical stage and status of recurrence or metastasis were two independent prognostic factors for the survival of NSCLC patients. **Conclusions:** Expression of survivin might be related to the origin and development of NSCLC. The positive expression of survivin in nucleus might be associated with invasion, progression and poor prognosis of NSCLC.

Key words: non-small cell lung cancer, BIRC5 protein, subcellular fractions, prognosis, immunohistochemistry

To date, lung cancer has become a malignant tumor with the greatest threat to human health. It is regarded as the primary killer among all cancers and there are over 500,000 emerging cases annually.¹ Non-small cell lung cancer (NSCLC) accounts for 80% of all cases of lung cancer and no breakthrough has been made in the treatment and prognosis for NSCLC. The prognosis of NSCLC is poor and the five-year survival rate is only 13% even for stage IIIA patients with surgery possibility.² Seeking a sensitive and effective tumor marker for the early detection of NSCLC has been a

hot spot.

Survivin is a member of the inhibitor of apoptosis of the protein (IAP) family and is one of the most powerful inhibitors of apoptosis. Most studies regard survivin as a double-function protein, which is closely correlated with tumor prognosis. Some studies have reported cytoplasmic and nuclear expression of survivin in tumor cells,³ but the prognostic value of surviving is unclear. The current research assessed the expression of survivin in NSCLC cells using the immunohistochemistry (IHC) assay and primarily evaluated the clinical significance of cellular localization of survivin in tumor cells.

Materials and Methods

Clinical samples. Fifty-one samples of cancer tissues and 21 samples of paracancerous tissues from 51 NSCLC patients (paracancerous tissues were sampled 5 cm away from the tumors visible edge). All 51 NSCLC patients had an ECOG (Eastern Cooperative Oncology Group) score <2. Their weight loss from the onset of symptoms to surgery was less than 10%. None of the patients underwent chemotherapy and favorable conditions were observed for them after surgery. Definitive diagnosis was confirmed through pathological examination. There were 40 males and 11 females, aged from 37 to 82 years old with a median age of 60 years old. Twenty-four patients were less than 60 years old, while the other 27 were no less than 60. There were 33 smokers and 18 non-smokers. According to the WHO classification of lung cancer (2003 Version), there were 21 cases of squamous cancer, 24 cases of adenocarcinoma and six cases of adenosquamous carcinoma, of which 25 were highly/ moderately differentiated and 26 cases were lowly differentiated. According to the International Union Against 1997 Cancer (UICC) tumor-node-metastasis (TNM) staging system, there were 28 cases of stage I/II NSCLC and 23 cases of stage III/IV NSCLC, including 18 cases of stage III lung cancer (four cases of stage IIIB lung cancer after surgery, or stage IIIA cancer before surgery) and five cases of stage IV lung cancer (two cases of stage T3N2M1 lung

cancer and one case of stage T2N2M1 lung cancer with local brain metastasis; and the other two cases with initial diagnosis of stage IIIA lung cancer were later confirmed as stage IV lung cancer in surgery when a single metastatic loci was observed in the other lung). There were 32 cases of stage T1/T2 lung cancer, 19 cases of stage T3/T4 cancer, 26 cases of stage N0 cancer, and 25 cases of stage N1/2 cancer. Fifty-one cases of NSCLC included 37 cases of peripheral-type lung cancer and 14 cases of central-type lung cancer. For platinum-based chemotherapy regimens, 16 patients underwent less than twice, and 35 patients underwent twice or more than twice. Medical records of NSCLC patients were retrieved from the Department of Medical Records, West China Hospital, Sichuan University (Chengdu, Sichuan, China) and patients survival was followed up through telephone. The telephone follow-up period started from definitive diagnosis by pathological examination to the death of the patient or until June 1st, 2007. The follow-up rate was 100%.

Main antibodies and reagents. The rabbit anti-human survivin polyclonal antibody (Batch no: ZA-0458) and the PV-6000 IHC kit were purchased from Zhongshan Goldenbridge Biotechnology Co., Ltd (Beijing, China).

Experiment methods. Breast cancer tissues with puffy cytoplasmic granules were regarded as the positive control in the research. A pilot experiment was administered first to identify the positive control and the blank control with PBS replacing the primary antibody. Optimum conditions for the IHC assay for detection of survivin was confirmed as follows: a two-step approach (using the ENVISION and LSAB + AP detection systems), repair with EDTA in autoclave, and a working concentration of 1:100. The tissues were sectioned, inactivated with endogenous peroxidase and repaired with antibodies. The sections were incubated with the primary antibody at 37°C and then stored in the fridge overnight. On the following day, the sections were incubated with antibody (1:1 for dilution) at 37°C. The sections were colored, restained, dehydrated, cleared and blocked consecutively.

Assessment of results. Survivin could be positively stained in cytoplasm and/or nucleus. It was defined as follows: number of cases with positive cytoplasmic staining = number of cases with positive cytoplasmic and nuclear staining + number of cases with single positive cytoplasmic staining; and number of cases with positive nuclear staining = number of cases with positive cytoplasmic and nuclear staining + number of cases with single positive nuclear staining. The scoring method for survivin was modified from the method specified in the research by Tanaka et al.⁴ The mean percentage (N) of positive tumor cells was determined in five high-power areas and one of the following five categories was assigned: 0, $\leq 5\%$; 1, 5-25% (25% included); 2, 25-50% (50% included); 3, 50-75%; and 4, $\geq 75\%$. ($= 25\%$ is classified to category 1, $= 50\%$ is classified to category 3). The intensity of survivin staining was scored as follows: 1, weak (+); 2, moderate (++); and 3 (+++), intense. The score of the percentage of positive tumor cells and staining intensity were multiplied to produce a weighted score for each case. Cases with weighted scores <1 were defined as negative, otherwise positive.

Statistical analysis. All analyses were performed using SPSS version 13.0. The comparison of rates was done via the chi-square test. The correlation between research factors and post-surgery survival was evaluated using the Cox proportional regression model. The correlation between different survival curves regarding different research factors was evaluated with the Kaplan-Meier method. A statistically significant difference was considered if a p value was <0.05 .

Results

Localization of survivin in NSCLC tissues. Positively stained survivin was mainly distributed in cytoplasm and/or nucleus in the shape of buffy granules. The overall positive rate of survivin in 51 NSCLC tissues was 76.5 % (39/51) and the overall negative rate was 23.5 % (12/51). The positive rate of cytoplasmic and nuclear survivin was 23.5 % (12/51). The positive rate of cytoplasmic survivin alone was 49.0 %

(25/51) , while the positive rate of cytoplasmic survivin was 72.5 % (37/51). The positive rate of nuclear survivin alone was 3.9 % (2/51) , whereas the positive rate of nuclear survivin was 27.5 % (14/51) (Fig. 1).

Overall expression of survivin in NSCLC and paracancerous tissues. The positive rates of survivin were 76.5 % (39/51) and 19.0 % (4/21) in NSCLC and paracancerous tissues, respectively. A statistically significant difference was noted regarding the positive rate of survivin between the two groups ($P=0.000$).

Correlation between subcellular localization of survivin and clinical parameters. Correlation between cytoplasmic expression of survivin and clinical parameters. It was found that the positive cytoplasmic expression of survivin was significantly correlated with the differentiation degree of NSCLC (Fig. 1). The positive rate of cytoplasmic survivin in the moderately differentiated group was higher than that in the lowly differentiated group.

Correlation between nuclear expression of survivin and clinical parameters. The positive nuclear expression of survivin was apparently correlated with age, clinical staging and N staging (Table 2).

Correlation between cytoplasmic and nuclear expression of survivin and clinical parameters. The positive cytoplasmic and nuclear expression of survivin was apparently correlated with clinical staging and N staging (Table 3).

Correlation between subcellular localization of survivin and prognosis. Correlations between positive cytoplasmic expression of survivin, positive nuclear expression of survivin, positive cytoplasmic and nuclear expression of survivin and the five-year survival are listed in Tables 4, 5 and 6. Statistical analysis demonstrated that the positive nuclear expression of survivin was correlated with the five-year survival ($p<0.05$). The survival rate of patients with positive nuclear expression of survivin was decreased compared with that of those with negative nuclear expression.

Comparison of survival curves of positive groups of survivin in cytoplasm, nucleus, and both cytoplasm and nucleus. The positive rates

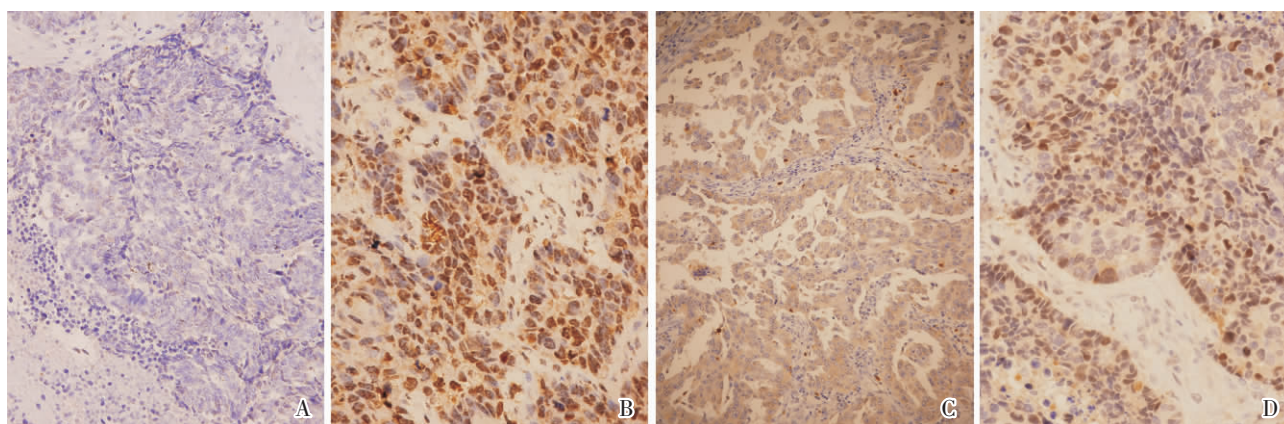


Figure 1 Expression of survivin in non-small cell lung cancer

A: Negative expression of survivin in NSCLC (EnVision, $\times 400$)

B: Positive expression of survivin both in cytoplasm and nucleus of moderately differentiated squamous cell carcinoma of lung (EnVision, $\times 400$)

C: Positive expression of survivin in cytoplasm of poorly differentiated adenocarcinoma of lung (EnVision, $\times 200$)

D: Positive expression of survivin in nucleus of poorly differentiated adenocarcinoma of lung (EnVision, $\times 400$)

Table 1 Relationship between expression of survivin in cytoplasm and differentiation of non-small cell lung cancer (NSCLC)

Differentiation Cases	Expression of survivin in cytoplasm of NSCLC				χ^2	<i>P</i>
	Positive (cases)	Negative (cases)	Positive rate (%)			
Poor	26	14	12	53.8	9.316	0.002
Moderate+Well	25	23	2	92.0		

χ^2 poor-moderate=7.923; $P_{\text{poor-moderate}}=0.005$; $P_{\text{moderate-well}}=1.000$; $P_{\text{poor-well}}=0.246$ ($\alpha=0.0125$).

of cytoplasmic survivin, nuclear survivin, and both cytoplasmic and nuclear survivin were 49.0 % (25/51), 3.9 % (2/51) and 23.5 % (12/51), respectively. The five-year survival rates 52.0% (13/25), 0 and 25.0 % (3/12). The five-year survival rate for the patients with positive cytoplasmic expression was statistically significantly higher than those with positive nuclear expression, or those with both positive cytoplasmic and positive nuclear expression, but no statistically significant difference was noted between survival curves for different staining areas ($P > 0.05$) (Fig.2).

Factors affecting prognosis of NSCLC in the Cox regression model. The pathological type, the differentiation degree, clinical staging, T staging, lymph node metastasis, tumor location, gender, age, smoking history,

Table 2 Relationship between expression of survivin in nucleus and clinical parameters of non-small cell lung cancer (NSCLC)

Parameter	Cases	Expression of survivin in nucleus of NSCLC				χ^2	<i>P</i>
		Positive (cases)	Negative (cases)	Positive rate (%)			
<60 years	24	10	14	41.7	4.600	0.032	
≥ 60 years	27	4	23	14.8			
I + II stages	28	4	24	14.3	5.403	0.020	
III + IV stages	23	10	13	43.5			
N0 stage	26	3	23	11.5	6.744	0.009	
N1-2 stages	25	11	14	44.0			

expression of survivin, and recurrence/metastasis were introduced into the Cox regression mode for multivariate analysis. The clinical staging and recurrence/metastasis were regarded as significant factors in the model. The death risk of patients with stage III+IV NSCLC was 2.600 times higher than that of those with stage I+II NSCLC, while the death risk for patients without recurrence/metastasis was 0.211 times of that of those with recurrence/metastasis.

Discussion

Numerous studies have indicated that survivin is expressed in most tumor tissues, but lowly or even not expressed in normal tissues of

Table 3 Relationship between survivin expression both in cytoplasm and nucleus and clinical parameters of non-small cell lung cancer (NSCLC)

Parameter	Cases	Expression of survivin both in cytoplasm and nucleus				χ^2	P
		Positive	Negative	Positive			
		(cases)	(cases)	rate (%)			
I + II stages	28	3	25	10.7	5.667	0.017	
III + IV stages	23	9	14	39.1			
N0 stage	26	3	23	11.5	4.238	0.040	
N1-2 stages	25	9	16	36.0			

Table 4 Relationship between expression of survivin both in cytoplasm and nucleus and 5-year survival rate of in non-small cell lung cancer (NSCLC)

Group	Cases	Positive (cases)	Negative both in cytoplasm and nucleus (cases)	Positive rate (%)	χ^2	P
Survival<5 years	28	9	19	32.1	2.560	0.110
Survival≥5 years	23	3	20	13.0		

Table 5 Relationship between expression of survivin in cytoplasm and 5-year survival rate in non-small cell lung cancer (NSCLC)

Group	Cases	Positive	Negative in cytoplasm	Positive rate (%)	χ^2	P
Survival <5 years	28	21	7	75.0	0.187	0.665
Survival ≥5 years	23	16	7	69.6		

Note:positive rate in cytoplasm=(positive cases both in cytoplasm and nucleus+positive cases in cytoplasm only)/total cases ×100%.

Table 6 Relationship between expression of survivin in nucleus and 5-year survival rate in non-small cell lung cancer (NSCLC)

Group	Cases	Positive (cases)	Negative in nucleus (cases)	Positive rate (%)	χ^2	P
Survival<5 years	28	11	17	39.3	4.366	0.037
Survival ≥5 years	23	3	20	13.0		

Note:positive rate in nucleus=(positive cases both in cytoplasm and nucleus+ positive cases in nucleus only)/total cases ×100%.

adults.^{5,6} Some experts reported that survivin is an early molecular event in the occurrence of tumor and that positive expression of survivin implies possibility of canceration in histologically normal tissues.⁷ Similar findings were obtained in

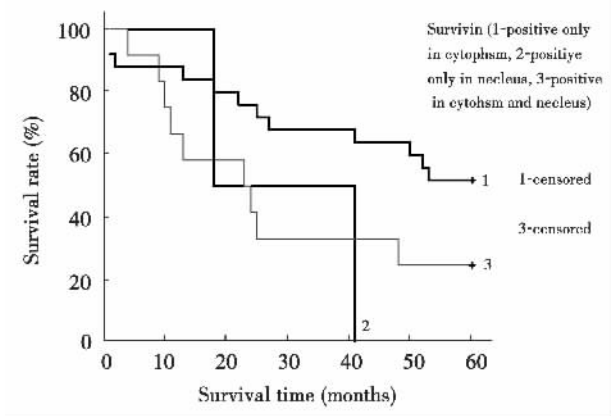


Figure 4 Survival curves of positive groups of survivin in cytoplasm only , in nucleus only and both in cytoplasm and nucleus in non-small cell lung cancer ($\chi^2=4.750,P=0.093$)

the current research that the positive expression rate of survivin was as high as 76.5 % (39/51), which was consistent with the result from Chen et al.⁸ on NSCLC using the RT-PCR assay and from Asanuma et al.⁹ on breast cancer using the IHC assay, but was higher than that obtained by Akyü rek et al.¹⁰ on NSCLC using the IHC assay. The statistical analysis also indicated that there was a statistically significant difference in the positive expression rate of survivin in tumor tissues and paracancerous tissues ($p<0.001$), implying that survivin is possibly correlated with occurrence of NSCLC.

In the current research, survivin was found to be expressed in both nucleus and cytoplasm of NSCLC cells, which is consistent with other findings.¹¹ The nuclear expression of survivin is possibly correlated with cell proliferation, while the cytoplasmic expression of survivin may play a role in regulating cell survival without affecting cell proliferation. Some research reported that wild-type survivin and surviving 2B were expressed in cytoplasm, while survivin Δ Ex3 was expressed in nucleus, each with varied functions.¹² A majority of reports indicated that survivin is mainly positively expressed in cytoplasm and correlated with tumor prognosis, which was supported by Als et al.¹³ Some studies found positive nuclear expression of survivin. Sohn et al.¹⁴ found the positive expression rates of survivin in nucleus, cytoplasm, and both nucleus and cytoplasm were 11.3%, 31.3% and 22.5%, respectively, in samples of breast cancer,

indicating that positive cytoplasmic expression is correlated with clinical staging, the differentiation degree and the lymph node metastasis. Ferrandina et al.¹⁵ reported that the positive cytoplasmic and nuclear expression rates of survivin in samples of ovarian cancer were respectively 84.5% and 29.1% , without a correlation between the positive expression rate and clinical features. In the current research, results also indicated that the positive NSCLC expression rates of survivin in both cytoplasm and nucleus, cytoplasm, and nucleus were similar to those in the research by Ferrandina et al.¹⁵ The positive cytoplasmic and nuclear expression rates were similar to the findings by Sohn.¹⁴ In the current research, the positive cytoplasmic expression rate of survivin was significantly correlated with the differentiation degree ($p=0.002$), and the positive expression rate of survivin in the moderately differentiated group was higher than that in the poorly differentiated group ($p=0.005$). The positive nuclear expression rate and the positive nuclear and cytoplasmic expression rate were apparently correlated with clinical staging and N staging. The positive nuclear expression rate of survivin for patients with stage III+IV NSCLC was higher than those with stage I+II NSCLC ($p=0.020$) and the positive nuclear expression rate of survivin for patients with stage N0 NSCLC was higher than those with stage N0 NSCLC ($p=0.009$), implying that the positive nuclear expression may indicate invasion and progression of tumors. Ferrandina et al.¹⁵ proposed that subcellular localization of survivin was not correlated with tumor prognosis, but other studies supported the correlation between the positive nuclear expression of survivin and prognosis. However, Lu et al.¹⁸ suggested that positive nuclear expression of survivin was correlated with the process of invasion. The current research found that the five-year survival for patients with positive nuclear expression was significantly higher than those with negative expression ($p<0.05$) and the survival for patients

with positive nuclear expression was shorter, which was consistent with the findings by Lu et al.,¹⁸ indicating that survivin is valuable for evaluating biological behaviors and prognosis, as compared with positive cytoplasmic expression. The nuclear expression may affect biological activities of lung cancer, promote the invasion capacity, and enable invasion and metastasis, leading to poor prognosis for lung cancer. The research also found that the five-year survival rate of the group with positive cytoplasmic expression was much higher than both the group with positive nuclear expression and the group with positive cytoplasmic and nuclear expression. Though the group with positive nuclear expression had the survival advantages, no statistically significant differences were noted in survival curves among these three groups ($p>0.05$). The sampled cases in the current research may not be enough to verify advantages in the survival for the group with positive nuclear expression. Additionally, the Cox regression model for multivariate analysis demonstrated that clinical staging and recurrence/metastasis were factors affecting the survival of NSCLC and that the overall survival for stage I/II cancer and non-metastasis/recurrence cases was prolonged comparatively. The death risk of patients with stage III+IV NSCLC was 2.600 times higher than the risk for those with stage I+II NSCLC, while the death risk for patients with recurrence/metastasis was 4.740 times higher than the risk for those without recurrence/metastasis.

The current research indicates that survivin is possibly correlated with the development and progression of NSCLC, and that positive nuclear expression of survivin is possibly correlated with prognosis of NSCLC. However, no consensus has been reached on the in-depth correlation between survivin and NSCLC. The prospective research using a large sample is required to identify and verify physiological and clinical values of subcellular localization of survivin.

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