A multiple marker analysis of apoptosis-associated protein expression in non-small cell lung cancer in a Chinese population

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Abstract: A failure to undergo apoptosis is widely thought to be an important event in cancer formation and progression. Although there have been many studies in vitro that provide evidence for this suggestion, the roles of apoptosis-associated proteins in cancer tissues in vivo are not as yet fully understood. Moreover, multiple marker analyses of apoptosis-associated protein expression in non-small cell lung cancer (NSCLC) tissues are scarce. In the present study, we investigate the expression of a group of apoptosis-associated proteins including bcl-2, caspase-3, fas, fas ligand (fasL) and survivin, and its clinical significance in NSCLC tissues using immunohistochemistry (IHC). Bcl-2 staining in cancer tissue cells was found in cytoplasm and the positive rate was 38.2% (29/76). Caspase-3 staining was mainly seen in cytoplasm of cancer tissue cells (53.9% [41/76]) with a few cases of nuclear staining (6.6% [5/76]). Fas staining was seen in cytomembrane (15.8% [12/76]) and cytoplasm (42.1% [32/76]) of cancer tissue cells. Likewise, fasL also showed staining in cytoplasm (55.3% [42/76]) and cytomembrane (44.7% [34/76]) of cancer tissue cells. Survivin staining was seen in cytoplasm but not nuclear of cancer tissue cells and the positive rate was 48.7% (37/76). Higher cytoplasm expression of bcl-2 was associated with large tumor size (≥3cm) in NSCLC (p < 0.05). Decreased cytoplasm expression of fas was associated with poor grade in NSCLC (p < 0.05). A negative correlation was found between bcl-2 and cytoplasm caspase-3 expression in NSCLC (p < 0.001). No separate expression of the apoptosis-associated proteins in NSCLC was linked to overall survival of patients (p > 0.05). Multiple marker analyses revealed caspase-3+/cytomembrane fasL− to be linked to better survival of patients with NSCLC (p < 0.05). These results indicate that apoptosis-associated proteins may impact a variety of clinicopathological features of NSCLC and may co-operatively influence the prognosis of patients with this malignant tumor. (Folia Histochemica et Cytobiologica 2011, Vol. 49, No. 2, 231–239)

Key words: apoptosis, bcl-2, caspase-3, fas, fas ligand, IHC, NSCLC, survivin

Introduction

Dysregulation of apoptosis plays an important role in the development of a variety of human patholo-
cancer cells via activation of the apoptotic pathways. But in fact, there are more complicated contexts of cancer cells in cancer tissues compared to cell lines in vitro. The clinical significance of separate apoptotic regulatory proteins’ expression in lung cancer has been widely described. But there is little data of multiple marker analysis concerning immunoreactivity of apoptosis-associated proteins regarding different pathways of apoptosis in relation to clinicopathological parameters and patients’ prognosis. Here, we present a study investigating the expression of a group of apoptosis-associated proteins in non-small cell lung cancer (NSCLC) tissues, and its clinical significance.

**Material and methods**

**Tissue samples.** Formalin-fixed, paraffin-embedded blocks of NSCLC tissues (76 cases, from 1998 to 2005) were randomly drawn from the Department of Pathology of the First Affiliated Hospital of China Medical University, Shenyang, China, after approval by the Institutional Review Board. All the cases had complete follow-up records. Informed consent was obtained from all enrolled patients prior to surgery. None of the patients had received chemotherapy or radiotherapy before tumor excision. Of the patients, 46 were male and 30 were female, a 1.53:1 ratio of male to female. Patients’ ages at the time of surgery ranged from 26 to 78 years, average 57.1. The tumors were classified according to the TNM stage revised by the International Union Against Cancer (UICC) in 2002 [15] and consisted of 38 (50.0%) stage I, 12 (15.8%) stage II, 25 (32.9%) stage III and one (1.3%) stage IV tumors. Histological tumor subtypes were assessed according to the criteria for classification of lung cancer by the World Health Organization (WHO) [16] by at least two pathologists. There were 34 (44.7%) squamous cell carcinomas (SCCs), 39 (51.3%) adenocarcinomas, two (2.6%) large cell carcinomas, and one (1.3%) adenosquamous carcinoma.

**Immunohistochemical staining.** Immunohistochemical (IHC) assay for bcl-2, caspase-3, fas, fas ligand (fasL) and survivin expression was performed on the sequential paraffin-embedded tissue sections using the peroxidase labeled streptavidin-biotin method. Commercially available antibodies for bcl-2 (MS-597), caspase-3 (MS-1121), survivin (RB-1629) (Thermo Fisher Lab Vision, Fremont, CA, USA), dilution: bcl-2: 1:50; caspase-3: 1:50; survivin: 1:100), fas (SC-715) and fasL (SC-834) (Santa Cruz Technology, Santa Cruz, CA, USA), dilution: fas: 1:100, fasL: 1:100 were used to recognize these proteins. Immunohistochemical staining was performed using UltraSensitive™ S-P kits (Maixin Biotechnology, Fuzhou, Fujian, China) according to the manufacturer’s instructions. Sequential slides with 4 μm-thick tissue sections were deparaffinized and hydrated in sequential treatment of xylene, ethanol and water. Citrate buffer (0.01 M citric acid, pH 6.0) was used to retrieve antigens in a heated pressure cooker. Endogenous peroxidase activity and non-specific binding were blocked with 3% H2O2 and non-immune sera, respectively. Sections were then incubated with primary antibodies overnight at 4°C. The following day, biotinylated secondary antibody and streptavidin-horseradish peroxidase (Maixin Biotechnology) were added. The peroxidase reaction was developed with 3,3′-diaminobenzidine tetrahydrochloride (Maixin Biotechnology). Counterstaining was done lightly with hematoxylin, and the sections were dehydrated in alcohol before mounting. For the negative control, phosphate-buffered saline (PBS) was used in place of the primary antibodies. The positive control was as follows: bcl-2: lymph tissues of tonsil; caspase-3: appendix mucosa; fas: hepatocarcinoma; fasL: liver epithelium; survivin: stomach mucosa epithelium.

**Immunohistochemical evaluation.** The expression pattern of the proteins was evaluated by at least two investigators who did not know the antibodies used and had no knowledge of the patients’ clinical status. Cases with discrepancies were jointly re-evaluated by the investigators, and a consensus was obtained. Scoring of immunohistochemistry was based on two parameters: the proportion of immunopositive cells and their intensity of immunoreactivity. The proportion of immunopositive cells was categorized as follows: 0: < 10%; 1: ≥ 10% to < 25%; 2: ≥ 25% to < 50%; 3: ≥ 50% to < 75% and 4: ≥ 75%. The staining intensity was categorized by relative intensity as follows: 0: no positivity; 1: weak; 2: moderate and 3: strong. A final immunoreactivity score of each sample was obtained by multiplying the two individual scores. To obtain final statistical results, a final score of less than 2 was considered to be negative (also represented by ‘−’), while scores of 2 or more were considered to be positive (also represented by ‘+’).

**Statistical analysis.** All statistical analyses were performed using SPSS 13.0 for Windows (SPSS Inc., Chicago, IL, USA). The Chi-Square test was used to analyze the relationship between expression of apoptosis-associated proteins and clinicopathological factors. Spearman’s correlation coefficient (r) was used to find the correlation between expressions of the proteins. The probabilities of overall survival were calculated using the Kaplan–Meier method and were compared using the log-rank test. For determining factors related to overall survival, a Cox proportional hazard model was utilized and p values of less than 0.05 were considered statistically significant.
Results

Expression of five apoptosis-associated proteins in NSCLC tissues

Staining of five apoptosis-associated proteins in NSCLC tissues was shown in Figure 1. Generally, weak bcl-2 staining in cancer tissue cells was found in cytoplasm. The positive rate of bcl-2 expression in NSCLC was 38.2% (29/76). Caspase-3 staining was mainly seen in cytoplasm of cancer tissue cells (53.9% [41/76]). A few cases of nuclear staining were also found (6.6% [5/76]), while there was only one case with sole nuclear staining. Fas staining was seen in cytomembrane (15.8% [12/76]) and cytoplasm (42.1% [32/76]) of cancer cells with a total positive rate of 48.7% (37/76). Its staining is generally very weak in NSCLC with a mean staining score of 2.16 in membrane and 2.69 in cytoplasm fas positive cases. FasL showed staining in cytoplasm (55.3% [42/76]) and cytomembrane (44.7% [34/76]) of NSCLC tissue cells with a total positive rate of 63.2% (48/76). Survivin staining was seen in cytoplasm but not nuclear of NSCLC tissue cells and the positive rate was 48.7% (37/76).

Relationship between expression of apoptosis-associated proteins and clinicopathological factors in NSCLC

The relationship between expression of five apoptosis-associated proteins and different clinicopathological factors in NSCLC is shown in Table 1. No significant correlation was found between the expression of caspase-3, fasL, or survivin in NSCLC and any of the clinicopathological factors (p > 0.05). Higher cytoplasm expression of bcl-2 was associated with large tumor size (≥ 3 cm) in NSCLC (p < 0.05). Decreased cytoplasm expression of fas was associated with poor grade in NSCLC (p < 0.05). There was a tendency for an association between membrane fasL expression and advanced TNM stages in NSCLC for a close p value (p = 0.082).

Kaplan–Meier analysis

The median follow-up period of patients with NSCLC was 45.6 months, with a range from three to 111 months. The overall mean survival time was 46.11 ± 3.39 months. The overall Kaplan–Meier survival curves for expression of five apoptosis-associated proteins revealed no separate expression of the five apoptosis-associated proteins in NSCLC to be linked to overall survival (p > 0.05). We also analyzed the prognostic significance of separate expression of these proteins in NSCLC with advanced TNM stages (III and IV stages), but no significant result was obtained (p < 0.05). To further evaluate prognostic factors for patients with NSCLC, a multivariate Cox regression analysis was carried out. As shown in Table 3, in an analysis of 76 patients, lymph node metastasis, tumor size and TNM stage were independent factors that impacted lung cancer patients’ prognosis (p < 0.05). But sex, age, histological type, differentiation and separate expression of the five proteins had no statistical correlation to prognosis (p > 0.05). The log-rank test for multiple marker analyses for patients’ survival revealed a difference in the survival time of patients based on the presence or absence of caspase-3 and cytomembrane fasL expression. The survival time of patients with caspase-3+/cytomembrane fasL+ tumors (60.81 ± 4.92 months) was significantly longer than the other three groups (p < 0.001) (Figure 3). Patients with caspase-3+/cytomembrane fasL– tumors showed shorter survival time (29.49 ± 4.37 months) than caspase-3+/cytomembrane fasL– tumors (42.07 ± 3.88 months) (p < 0.05). No significant difference was found between the caspase-3+/cytomembrane fasL+ group (33.70 ± 5.25 months) and the above two groups (p > 0.05). No significant difference was found in patients’ prognosis based on expressions of other proteins (p > 0.05).

Discussion

Apoptosis, the commonest and best-defined form of programmed cell death, is a physiological process of cell elimination, which is important for the maintenance of embryonic development and cell homeostasis [17–20]. It is generally agreed that cell populations are tightly regulated by their rates of proliferation, differentiation and death. Dysfunction of any one of these processes can result in either uncontrolled cell...
Figure 1. Expression of apoptosis-associated proteins in NSCLC tissues using IHC. Bcl-2 staining in cytoplasm in SCC (A) and adenocarcinoma (B). Lymphocytes were also stained in nuclear as an intrinsic control in caspase-3 expression in cytoplasm and nuclears in SCC (C) and adenocarcinoma (D). Weak and diffuse cytoplasm and weak cytomembrane staining of fas in SCC (E) and adenocarcinoma (F). Cytoplasm and membrane staining of fasL in SCC (G) and adenocarcinoma (H). Cytoplasm staining of survivin in SCC (I) and adenocarcinoma (J) (× 400)
growth or uncontrolled cell death [1–5, 21–23]. But it remains unclear as to what degree cell proliferation and/or cell death must occur to initiate tumor formation. The analysis of apoptosis-associated proteins is important because of the key role those processes play in carcinogenesis.

We investigated five apoptosis-associated proteins in NSCLC tissues, namely bcl-2, caspase-3, fas, fasL, and survivin. According to data updates, these proteins may be important for the balance of cell proliferation and cell death to maintain cellular homeostasis in normal lung tissues [24–28]. But their actual roles in can-
cancer tissues including NSCLC are less clear. Though many studies have found heterogeneity of expression of apoptosis-associated proteins in NSCLC, we don’t yet know to what degree they influence apoptosis of the cancer cells in NSCLC tissues. In addition, it remains controversial as to how they influence the biological features of cancer cells in vivo.

In the current study, we found bcl-2, which was originally cloned from the t(14;18) translocation breakpoint found in follicular B-cell lymphomas, to be related to large tumor size in NSCLC. Bcl-2 is a prototype of the proteins involved in anti-apoptotic regulatory pathway, and its overexpression may result in cell proliferation, as cells normally scheduled for death may undergo further mutations [29, 30]. Overexpression of bcl-2 may be involved in stress response against hypoxia which is frequently seen in a solid tumor as a result of tumor expansion to prevent cancer cells from apoptosis. Despite the bcl-2 negative influence on apoptosis, the results of published studies indicate positive or no prognostic value of bcl-2 in lung cancer [31–35], though the reason why is unclear and conflicts still exist. It is possible that bcl-2 expression may be associated with other features of tumors that define a more favorable prognosis function besides its function to promote tumor growth. In

**Figure 2.** Bcl-2 and caspase-3 expression in sequential sections showing a negative correlation. Weak staining (score < 2) of bcl-2 in SCC (A) and concurrently positive staining (score > 2) in adenocarcinoma (B). Positive staining of caspase-3 in cytoplasm (score > 2) in SCC (C) and concurrently negative staining (score = 0) in adenocarcinoma (× 400)

**Table 3.** Multivariate Cox proportional hazard analysis for overall survival of 76 patients with non-small cell lung cancer

<table>
<thead>
<tr>
<th>Factors</th>
<th>$\beta$</th>
<th>SE</th>
<th>p</th>
<th>Exp ($\beta$)</th>
<th>95% CI for Exp ($\beta$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNM stage*</td>
<td>0.512</td>
<td>0.295</td>
<td>0.003</td>
<td>1.209</td>
<td>0.962–2.592</td>
</tr>
<tr>
<td>Tumor size*</td>
<td>0.618</td>
<td>0.392</td>
<td>0.045</td>
<td>2.314</td>
<td>1.174–2.833</td>
</tr>
<tr>
<td>Lymph node metastasis*</td>
<td>1.327</td>
<td>0.501</td>
<td>0.002</td>
<td>4.372</td>
<td>1.032–9.540</td>
</tr>
<tr>
<td>bcl-2 expression</td>
<td>0.276</td>
<td>0.183</td>
<td>0.074</td>
<td>2.013</td>
<td>1.282–4.257</td>
</tr>
<tr>
<td>caspase-3 expression</td>
<td>0.325</td>
<td>0.279</td>
<td>0.083</td>
<td>1.784</td>
<td>1.192–3.490</td>
</tr>
<tr>
<td>fas expression</td>
<td>0.309</td>
<td>0.326</td>
<td>0.102</td>
<td>1.934</td>
<td>1.097–3.052</td>
</tr>
<tr>
<td>fasL expression</td>
<td>0.418</td>
<td>0.187</td>
<td>0.095</td>
<td>1.046</td>
<td>0.992–2.843</td>
</tr>
<tr>
<td>survivin expression</td>
<td>0.653</td>
<td>0.254</td>
<td>0.189</td>
<td>2.308</td>
<td>1.347–4.016</td>
</tr>
</tbody>
</table>

bcl-2, caspase-3, fas and survivin: cytoplasm staining; fasL: cytomembrane staining; *independent factors (p < 0.05)
Apoptosis-associated proteins in non-small cell lung cancer

our study, we did not find bcl-2 expression in NSCLC to be associated with patients’ survival. However, we can’t deny its possible functions in this malignant tumor, because the study was limited in that the cohort we studied was limited and no post-surgical treatment information was available for the patients included in the database, which may have contributed to differences in survival.

Caspase-3 is an important downstream effector cysteine protease in the apoptotic pathway. Either a positive or a negative correlation between expression of caspase-3 and malignancy of the tumor has been reported [36, 37]. Our study did not find it was associated with any of the clinicopathological factors in NSCLC. As the antibody we used can recognize both pro- and activated caspase-3, the activity of caspase-3 in NSCLC tissues was not clear. It may be necessary that this activity is taken into account to further understand its influence on the biology of NSCLC. But, as we will discuss later, despite this, the expression of caspase-3 does have an influence on patients’ survival, though not separately.

Apoptosis pathway through fas and its ligand fasL is involved in tissue homeostasis and the elimination of target cells by cytotoxic T cells. Loss of fas or the function of fas has been implicated in tumor progression of several cancers [38, 39]. Decreased cell surface fas expression in lung tumors has also been observed in some studies [40–42]. Consistent with these studies, we also found a very low level of membrane fas in NSCLC, although the positive rate of cytoplasm fas expression in our study was relatively higher than those studies. This may be due to the different assessment methods used. But scores of fas expression in cytoplasm in NSCLC in our study were generally very small, ranging from 0 to 6, with a median of 2.69. Our study didn’t find fas membrane expression to be associated with any pathological features of NSCLC and patients’ outcome. We cannot rule out its possible role in NSCLC, because the small number of positive cases made it difficult to reach a conclusion. A cohort including more cases would seem to be important for analysis of this protein. In addition, the distinct pattern of fas expression in tumor stroma should also be taken into account to further investigate its function in NSCLC, which is absent from our study. Interestingly, we found that decreased cytoplasm expression of fas was associated with poor grade in NSCLC, which was not mentioned in other studies. But the reason for this was not made clear in our study. It seems that in better-differentiated tumors, abnormal cellular localization of fas is more frequent, while in poorly-differentiated tumors, fas expression was entirely reduced. On the other hand, membrane fasL expression was found to be increased in many studies [41, 43] and likewise in our study. Increased fasL expression was considered to participate in tumor development and immune escape [38, 40]. We did not find fasL alone to have any association with clinicopathological factors and patients’ survival, but it is possible that fasL may have an impact on the clinical stage of NSCLC. In addition, fasL in conjunction with caspase-3 may have an influence on the outcome of patients with NSCLC.

Survivin is a member of the family of inhibitors of apoptosis proteins. It functions as a key regulator of programmed cell death. The prognostic value of survivin for survival of patients with NSCLC remains controversial. In some reports, survivin-positive expression correlated with more aggressive behavior and poorer prognosis [44, 45]. Recent studies found nuclear survivin to be more important compared to cytoplasm survivin as a promising prognostic marker [46, 47]. In our study, we did not find any association between survivin expression and clinicopathological factors and patients’ survival. As the antibody we used in our study can recognize only cytoplasm survivin, as the manufacturer’s instructions state, we cannot deny the possible role of nuclear survivin in NSCLC. But our study does not support the prognostic significance of cytoplasm survivin in NSCLC.

The proteins studied here respectively play roles in the two fundamental pathways in apoptosis: the death receptor pathway and the mitochondrial pathway. In in vitro studies, these proteins are proved to be concurrently involved in cell apoptosis and have interactions with each other. But in our study, we only found bcl-2 and caspase-3 to have correlation in NSCLC tissues. It seems that the apoptosis pathways in cancer tissues are more complicated than that in
non-tumor or tumor in vitro cell lines. The negative correlation between bcl-2 and caspase-3 in our study is not simple to explain for a complicated context in cancer tissues. But this finding seems to be consistent with the suggestion that bcl-2 may regulate cell death by controlling mitochondrial membrane permeability and inhibiting caspase activity. We can infer from this result that the apoptosis-associated proteins may actually directly or indirectly interact with each other, but perhaps in different ways from those in cell lines.

The understanding of apoptosis has provided the basis for novel targeted therapies that can induce death in cancer cells or sensitize them to established cytotoxic agents and radiation therapy. However, to date, despite the great excitement over potential benefits of targeting the apoptosis pathways, the likelihood of achieving long-lasting therapeutic benefits for patients with malignant tumors, including NSCLC, remains uncertain. Our study did not find any of the proteins studied here to have independent significance for patients’ prognosis. We can’t deny the possible influence of these proteins on patients’ prognosis, but we bear in mind the importance for any of the proteins to be reconsidered in the context of levels of other apoptotic or non-apoptotic proteins to show its prognostic importance.

We found caspase-3/cytomembrane fasL predicts a better survival of patients with NSCLC compared to the other groups. Though caspase-3 is one of the most important molecules in the apoptosis cascade, studies of it in terms of lung cancer outcome have been limited. Several studies of caspase-3 expression have shown conflicting effects on survival [48–50]. Our study indicates that the total amount of caspase-3 expression was important for its function in NSCLC tissues, although the activity of the protein remains unclear. In addition, our finding indicates the two apoptosis-associated proteins may co-operatively affect patients’ survival, rather than only one protein acting alone. As for another apoptosis-associated protein we studied, bcl-2, some studies suggest that the combination of decreased bcl-2 expression and increased p53 expression predicts the poorest survival [32, 34]. There is growing interest in combining the results of two or more markers to provide prognostic information, although data is still scarce. It seems that the overall status of the cell death machinery, rather than just the expression levels of the individual proteins, might explain how it affects tumorigenesis. Combined therapies simultaneously targeting apoptosis and survival signaling defects might shift the balance from tumor growth stasis to cytotoxic therapeutic responses that might be associated with greater therapeutic benefits.

In conclusion, our study indicates a marked heterogeneity of apoptosis-associated proteins including bcl-2, caspase-3, fas, fas-L, and survivin in NSCLC tissues, the possible interaction of bcl-2 and fas with the tumor’s clinicopathological features, the possible interaction of bcl-2 and caspase-3 with each other, and the potential influence of caspase-3 and fasL coexpression on patients’ prognosis. To date, information about the function of apoptosis-associated proteins in NSCLC is still limited. More studies are needed to understand the roles of apoptosis-associated proteins in the carcinogenesis and progress of this malignant tumor. Further understanding of the molecular defects and the regulation of the apoptosis pathways in tumors will help with the discovery of novel targeted agents and the design of clinical trials that are based on the specific molecular defects.

References


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