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Quantitative detection of survivin in malignant pleural effusion for the diagnosis and prognosis of lung cancer

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ABSTRACT

In the present study, pleural effusions are the first time to be used as the specimens for detection of survivin expression in lung cancer patients. We demonstrated that by quantifying survivin expression with enzyme-linked immunosorbent assay (ELISA) in the 80 effusion samples exhibited a diagnostic power of 85% and 75% in sensitivity and specificity, respectively. A multivariate analysis with the Cox regression model revealed that both high survivin expression and cancer cells of stage IV were the indicators for poor prognosis of lung cancer. In conclusion, quantitative assay of survivin in pleural effusion could be useful both in diagnosis and prognosis for lung cancer.

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1. Introduction

Pleural effusions are common complications in lung cell malignancy. Clinical diagnosis of malignance in effusions can be troublesome due to the various cellular compositions of effusions. The differentiation between malignant and nonmalignant effusions by the conventional diagnostic methods are sometimes difficult, and only about 50–70% of the patients with pleural malignancies can be diagnosed by the cytological examinations of the pleural fluids [1]. Adjunct methods such as invasively percutaneous pleural biopsy, enzyme-linked immunosorbent assay to assess the mucin concentration, and detection of telomerase activity in effusion cells have been reported to improve the sensitivity and accuracy of the diagnosis of malignant pleural effusions (MPE) [2–4]. Despite all these various diagnostic approaches, definite diagnosis is still unclear in some patients and the more invasive procedure, such as thoracoscopy or thoracotomy, would be needed if a proven diagnosis is essential.

The prediction of prognosis for lung cancer is usually difficult, and an alternative approach with a higher sensitivity and specificity, lower risk, and comfort is therefore desirable. Usually, the ideal tumor marker should be absent from normal or benign tissue but should be expressed in all cancer cells. Furthermore, it should be detected in a readily available body fluid such as serum or urine thus obviating the need for an invasive procedure [5]. Survivin is a member of the inhibitor of apoptosis proteins (IAP) family of molecules [6] and a number of *in vitro* and *in vivo* studies have shown that survivin inhibits apoptosis [7,8]. Considerable evidence exists that survivin also plays a role in regulating mitotic progression [9,10] and angiogenesis [11]. Up-regulation of survivin is found in most cancers and highly over-expressed in common human malignant tumors but was rarely detectable in normal differentiated adult tissue [12].

Survivin is an attractive cancer marker as it is almost universally up-regulated in malignancy. Monzo et al. reported that the non-small-cell lung cancer (NSCLC)

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patients without survivin expression had significantly better overall survival than those with survivin expression [13]. Traditionally, prognostic factors for cancer include parameters such as tumor size, tumor grade and presence or absence of local lymph node metastasis have been the most important determinant of prognosis in NSCLC. Several abnormalities of dominant oncogenes have been described as potential prognostic markers in operable NSCLC, including k-*ras* mutations, and tumor suppressor genes [14,15]. However, few cellular genes have been identified whose altered regulation correlates with prognosis in patients with advanced NSCLC (stage IIIB or IV). As described above, survivin may be a novel predictive/prognostic marker of the malignant disease.

Up-to-date, most of the studies that have investigated a potential diagnostic role for survivin have focused on bladder cancer [16–18] and has not yet been quantified in pleural effusion for lung cancer. In the present study, we investigate the survivin expression in pleural effusion cells from patients with lung cancer by an enzyme-linked immunosorbent assay (ELISA) analysis. The aims of this study are to investigate the diagnostic and prognostic power of quantitative survivin expression as compared to cytology in pleural effusion using ELISA assays and to assess its potential diagnostic and prognostic use.

2. Materials and methods

2.1. Patients and effusion samples

Eighty pleural effusion specimens were collected from patients at the pulmonary and critical care department of Chang Gang Memorial hospital from April 2003 to May 2005, which was collected undergoing a diagnostic cytological examination and survivin expression. Effusion samples are obtained under ultrasound guiding through a heparinized syringe, and then stored at −80 °C until analysis. Sixty patients with newly diagnosed lung cancers were enrolled in this study and 20 patients with benign lung diseases as a control group. Patients will take the same diagnostic algorithm, and pleural biopsy or thoracoscopy will be performed for patients with an exudate of unknown origin. During the course of this study, clinical diagnosis and survivin assay were performed independently by physicians and laboratory technicians, respectively. The histological features of the surgical specimens were classified into 43 (72%) adenocarcinomas (including bronchioalveolar carcinomas), 11 (18%) squamous cell carcinomas, and 6 (10%) small-cell carcinomas.

Diagnosis is confirmed by either cytological examination of pleural fluid or by pathological examination of parietal pleural biopsy samples obtained by a percutaneous biopsy or thoracotomy. All specimens were examined by an experienced pathologist. Histopathological diagnosis was carried out according to the Mountain classification of lung tumors [19]. According to the final diagnosis, the patients will be classified into lung cancer with malignant pleural effusion (MPE), para-MPE, and nonneoplastic diseases with nonmalignant pleural effusion (non-MPE). The para-MPE is defined as caner with negative diagnostic results both in cytology and biopsy. These patients should not have coexisting or previous cancer disease. Patients in this group will undergo periodic clinical and radiological follow-up over a 6-month period to exclude the possibility of occult malignancy. Overall survival was calculated from the date of diagnosis to death.

2.2. Preparation of recombinant survivin and generation of survivin antibodies

The expression clone of survivin was generated by transformation using a host expression strain of *Escherichia coli* BL21(DE3) pLysS. Specifically, the plasmid pET14b-survivin was created by amplified the cDNA of survivin from SKGIIIa cell and cloned into a pET14b (Novagen) expression vector. Sequencing analysis, using T7 promoter probes, was performed to confirm the gene sequence of survivin. Isopropyl β-D-thiogalactopyranoside was used to induce protein expression. The cells were harvested by centrifugation and then disrupted by sonication in lysis buffer, containing 0.2 M Tris–HCl, pH 7.5, 20% (v/v) glycerol, and 1 mM phenylmethylsulfonyl fluoride, 4 °C. Expression of the recombinant protein was confirmed by immunoblotting using a rabbit polyclonal antibody to survivin (Novus Biologicals). Recombinant survivin was purified by using a HiTrap Chelating HP column (GE Healthcare, Sweden) according to the protocol provided by the manufacturer.

Monoclonal antibody (Mab) reacting with survivin was obtained from hybridomas generated from mice that had not been immunized exogenously according the standard method. Briefly, lymphocytes extracted from the spleens of two mice were fused with myeloma cell line. Hybridoma supernatants were screened for binding to survivin. Selected hybridomas were cloned by limiting dilution, and cells were infected intraperitoneally into Pristane primed BALB/C mice to produce ascites fluid. After cloning, all MABs were characterized and purified from ascites by affinity chromatography on protein A-Sepharose CL-4B as specified by the manufacturer (Pharmacia Biotech, Uppsala, Sweden). Survivin polyclonal antibody was obtained from antisera of rabbits that had been immunized with purified survivin. The primary dose of antigen was infected subcutaneously with complete Freund's adjuvant. Booster in incomplete Freund's adjuvant was given at 2-week intervals. Antiserum was obtained 2 weeks after the last booster. Antibodies was precipitated with ammonium sulfate and extensively dialyzed against PBS and stored at −20 °C until use.

2.3. Enzyme-linked immunosorbent assay (ELISA)

A double-antibody (ELISA) was set up according to the standard method. Briefly, microtiter plate (96 wells; Maxisorb; Nunc) were coated with monoclonal anti-survivin antibody in 50 mM sodium carbonate buffer, pH 9.6. Free binding sites were blocked with 2.5% (wt./vol.) bovine serum albumin. Pleural effusions were tested in triplicate, and survivin was detected with a horseradish peroxidase-conjugated polyclonal anti-survivin antibody. Recombinant survivin was used as a standard for the calibration. Optical

absorption was read on a VERSAmax microplate reader (Molecular Devices). In order to normalize the survivin expression in the pleural effusion cells, the specific survivin expression is defined as the survivin amount (ng) dividing the total protein amount (mg) of the effusion cell lysates. Protein amount was determined by Lowry assay (DC protein assay, Bio-Rad, Hercules, CA, USA).

2.4. Statistical analysis

Statistical analysis was performed using the SPSS for windows release 12.0 package program (SPSS Inc., Chicago, IL, USA). Descriptive statistics are expressed as means ± SD. χ^2-test was applied to assess categorical data associated with comparison of data between the groups. According to the final diagnosis, the following calculations will be made: sensitivity; specificity; positive predictive value; negative predictive value; and likelihood ratios. The association between survivin expression and various clinicopathological variables and disease are examined by Student's *t*-test and analysis of variance (ANOVA) test with the Tukey test post hoc for numerical values. Discriminative power (i.e., the model's ability to differentiate between survivin expression which positive and negative) was assessed using the area under a receiver operating characteristic (AUROC) curve. Cutoff value was determined by obtaining the optimal Youden's index (sensitivity + specificity − 1). Survival analyses will be conducted according to the Kaplan–Meier method and survival characteristics are compared using the log rank test. The Cox proportional hazard regression model is used to compare the relative influences of different prognostic factors. A value of $p < 0.05$ was accepted as statistically significant.

3. Results

3.1. Detection of survivin expression in pleural effusion

Among the recruited patients, twenty are nonmalignancy patients which include nine with tuberculosis (TB) pleurisy, one with parapneumonic effusion, three with liver cirrhosis, three with end stage renal disease, and four with congestive heart failure as the negative control (Table 1). The other 60 subjects are histologically or cytology verified lung cancer patients which contained some inoperable NSCLC cases (3.8% stage IIIA, 6.3% stage IIIB and 65% stage IV) received the treatments at the CGMH by different protocols. To quantify the expression of survivin in the pleural effusions the survivin ELISA assay was employed, and a normalized survivin expression value was defined by dividing the survivin amount (ng) with total protein amount (mg) in each cell lysate sample. The normalized survivin values were ranged from 0 to 9.235 ng/mg in the 80 pleural effusion samples. When taking a normalized survivin expression value 0.0062 ng/mg as a cutoff point in survivin ELISA assay resulted in the optimal Youden's index 0.545. Youden's index is the measure of the probability of correct classifications that is invariant of prevalence. Fig. 1 showed the ROC curve and the AUROC that confirmed the good discriminatory power of the specific survivin expression (AUROC = 0.802; 95% CI: 0.701–0.903, $p < 0.0001$).

The normalized survivin expression was defined as positive when the value is greater than 0.0062, and the result showed that there were positive expressions of survivin in 51 of 60 (85%) lung cancer samples and in 5 of 20 (25%) histologically nonmalignance samples (Table 1). All the five positive survivin tests in nonmalignance samples were collected from TB pleurisy patients. Among the 51 samples with positive survivin expressions, 6 samples were para-MPE and 45 samples were MPE. Table 2 shows that pleural effusion survivin was detected in 14 patients with initial negative cytological findings and finally proved as cancer by pleural biopsy or thoracoscopy. They are 12 cancer patients without proving malignancy in effusion (para-MPE) during their all clinical courses. Among them, six patients showed no survivin expression, and another six patients showed positive survivin expressions. In comparison of diagnostic methods in pleural effusion, data in table 2 indicates that survivin expression and conventional cytology methods provide the sensitivity of 85% and of 57%, respectively.

Table 1
Survivin detection in 80 pleural effusion specimens using a quantitative analysis

	Total no. of patients	No. of patients survivin-negative	No. of patients survivin-positive
Lung cancers	60		
With MPE	48	3	45
With para-MPE	12	6	6
Nonneoplastic disease	20		
Tuberculosis	9	4	5
Congestive heart failure	4	4	0
End stage renal disease	3	3	0
Liver cirrhosis	3	3	0
Parapneumic effusion	1	1	0
Total	80	24	56

3.2. Prognostic implications of survivin expression

The results of all analyses of survivin expression and the clinicopathological features of the 60 patients are summarized in Table 3. There were no correlations between positive survivin expression and age, sex, disease, histologic subtype, performance status (Eastern Cooperative Oncology Group, ECOG) and tumor stage (TNM) system [19], and MPE is found to be correlated with high expression of survivin. A multivariate analysis was also performed according to the Cox regression model, and no difference in survival was detected when the data was analyzed by patients' age, sex tumor histology or lymph node status. Only the high survivin expression and stage IV were found to be independent poor prognostic indicator for overall survival (Table 4). For the analysis of the association between expression of survivin and survival, we divided the 60 lung

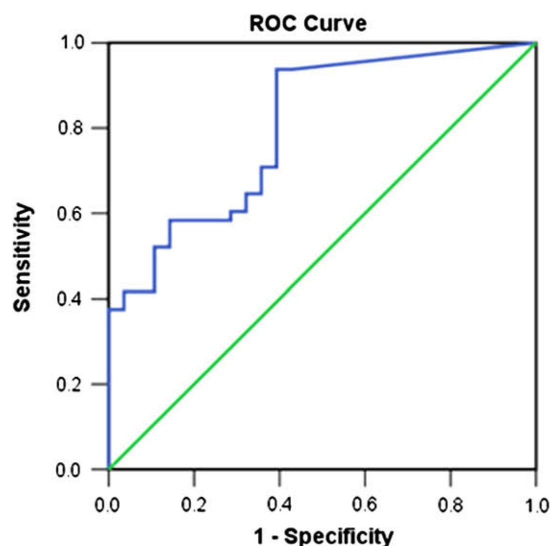

Fig. 1. ROC curves for survivin expression classification (AUROC curve is 0.802, $p < 0.001$). The plot was constructed by computing the sensitivity vs. (1 − specificity) for the different possible cutoff points of the survivin ELISA assay.

Table 2
Survivin and cytological detection in 60 lung cancer patients with pleural effusion

	Total no. of patients	No. of patients with para-MPE	No. of patients with MPE
Survivin (+), cytology (+)	31	0	31
Survivin (+), cytology (−)	20	6	14
Survivin (−), cytology (+)	3	0	3
Survivin (−), cytology (−)	6	6	0
Total	60	12	48

Table 3
Correlation between pleural effusion survivin and selected univariate parameters

	No. of cases tested	Mean (SD) survivin	*p*-Value
Age			0.094
>65 year	33	1.36 ± 2.37	
<65 year	27	0.81 ± 1.54	
Sex			0.602
Male	35	1.14 ± 2.27	
Female	25	1.09 ± 1.71	
Histology			0.303
Adenocarcinoma	43	1.19 ± 2.03	
Squamous cell cancer	11	0.19 ± 0.26	
Small-cell cancer	6	1.83 ± 3.15	
Malignant pleural effusion			<0.001
Positive	48	1.38 ± 2.22	
Para-	12	0.80 ± 0.11	
Performance status			0.519
ECOG: 1	2	0.27 ± 1.37	
2	7	0.36 ± 0.69	
3	51	1.24 ± 2.17	
Node stage			0.307
N1	15	0.81 ± 1.65	
N2	22	0.77 ± 1.92	
N3	23	1.63 ± 2.34	
Stage			0.519
Stage IIIa	2	0.27 ± 0.14	
Stage IIIb	7	0.36 ± 0.69	
Stage IV	51	1.24 ± 2.17	

Abbreviation: ECOG, eastern cooperative oncology group.

Table 4
Multivariate analysis related to survival using Cox regression

	p-Value	Hazard ratio	95% CI
Sex	0.457	0.573	0.132–2.481
Age	0.326	0.429	0.079–2.320
Node stage	0.325		
N2	0.287	2.600	0.447–15.119
N3	0.438	0.497	0.085–2.904
Performance status	0.691		
ECOG: 1	0.771	0.745	0.102–5.411
ECOG: 2	0.575	1.930	0.193–19.269
Survivin expression	0.025	13.636	1.395–133.262
Stage IV	0.031	15.253	1.435–123.273

Abbreviation: ECOG, eastern cooperative oncology group.

cancer patients into two groups using its mean value of normalized survivin expression (0.17 ng/mg) as a cutoff point. The mean survival period of patients was determined as 9.83 ± 8.62 months in lung cancer patients.

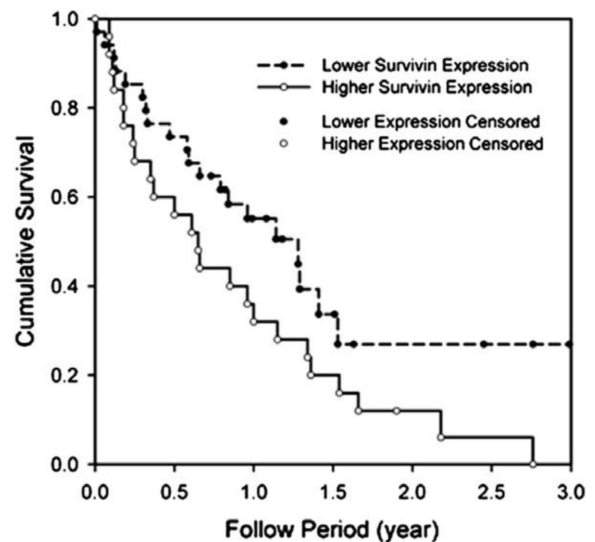

Fig. 2. Prognostic importance of survivin expression on lung cancer patients with malignant pleural effusion. Kaplan–Meier curve shown patient survival according to survivin expression. There is a significant correlation between survivin expression and short survival ($p = 0.04$).

Thirty patients (50%) were classified into a high survivin expression group. There was significant correlation between the high-expression survivin and poor survival (Fig. 2). The low-expression survivin patients had significantly longer overall survival period ($p = 0.025$; relative risk, 13.636; 95% CI: 1.395–133.262) compared with high-expression survivin patients. Mean survival period was 7.82 ± 8.89 months in patients with high survivin expression, whereas 11.91 ± 8.23 months in patients with low survivin expression.

4. Discussion

To the best of our knowledge, this is the first study of pleural effusions in which conventional cytology and a quantitative survivin ELISA were compared with for the detection of malignancy. We demonstrated that by quantifying the expression of survivin in malignant pleural effusion is much more sensitive (85%) than routine cytological method (57%) for the diagnosis of lung cancer. Although cytology remains the gold standard for the routine analysis of pleural effusions, survivin expression could be used as the second diagnostic step in certain cases with negative or ambiguous cytology.

Weikert et al. detected survivin mRNA with bladder cancer [20]. None of the urine samples from 11 healthy subjects or 22 patients with benign genitourinary disease had detectable survivin levels. In their study urinary survivin yielded a sensitivity of 68.6% and a specificity of 100% for non-invasive bladder cancer, while voided urine cytology only gave a sensitivity of 31.4% and a specificity of 97.1%. At our study for diagnosis of malignant pleural effusions, we noticed that a lower specificity (75%) was caused by detecting the positive survivin expression in 5 of 9 of tuberculosis patients' pleural effusions. Tuberculosis effusions are thought to result from a delayed hypersensitivity reaction to mycobacterial antigens in the pleural space [21], and the proportion of T-lymphocytes is higher in pleural fluid than in blood. Those pleural helper T cells with a ‘‘memory" phenotype are the cells that

proliferate and produce gamma interferon [22]. Survivin mRNA has been reported in normal peripheral lymphocytes and in *ex vivo* activated T-lymphocytes [23,24]. Sharief and Semra also demonstrated significant up-regulation of survivin in some unstimulated intrathecal lymphocytes from the patients with multiple sclerosis [25]. These reports provide evidence that survivin may become up-regulated during the proliferation of nonmalignant cells, including T cell. Therefore, the present study implied if the TB infected patients can be diagnosed and distinguished from the lung cancer patients, the specificity of the lung cancer diagnosis by survivin detection in the pleural effusion can reach 100%.

The rational for investigating survivin as a prognostic marker in malignancy is based on its ability to inhibit apoptosis, promote proliferation and enhance angiogenesis. Therefore, survivin is likely to be involved in tumor progression, and consequently increased its expression would be expected to predict aggressive disease. A number of reports have shown that high levels of survivin in tumor are associated with adverse outcome in patients with different types of cancer [13,26–28]. It should be pointed out however, that most of these studies contained relatively small numbers of patients and were retrospective in design. Span et al. reported that survivin mRNA was not correlated with patient age, nodal status, tumor size, histological grade, and hormone receptor status in a breath cancer study [29]. Besides, Ryan et al. showed that survivin mRNA level was an independent prognostic factor in breast cancer [30]. In the present study, we found that the survivin expression was also not correlated with cancer patients' age, tumor size, lymph node status, hormone receptor status. However, we demonstrated that by using multivariate analysis with overall survival as endpoint, nodal status and survivin expression showed to be the independent prognostic factor for lung cancer.

In conclusion, our data suggest that the lung cancer patients with the low survivin expression in pleural effusion had longer survival rate. Quantitative assay of survivin in pleural effusion could be both a useful diagnostic marker and an important prognostic factor for lung cancer.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.canlet.2008.08.023.

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