Elevated pleural fluid RCAS1 is a diagnostic marker and outcome predictor in lung cancer patients

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Abstract. RCAS1, a type II membrane protein also secreted in soluble form, may be important in tumor cell evasion of immune surveillance and contribute to the aggressiveness of human tumors. We examined the implications of elevated pleural fluid RCAS1 at the onset of effusion in lung cancer patients. Of 102 patients presenting with pleural effusion, 59 proved to have a malignant effusion and 43, nonmalignant. Malignant effusions exhibited higher RCAS1 concentrations than nonmalignant effusions (mean ± SD; 36.3 ± 114 vs. 2.7 ± 1.8 U/ml; p=0.014). Lung cancer patients with pleural fluid RCAS1 concentrations below 15 U/ml had a longer mean survival than those with higher concentrations (4.7 vs. 1.7 months; p<0.05). By multivariate analysis, pleural fluid RCAS1 was an independent prognostic factor in lung cancer patients with effusion. In conclusion, RCAS1 determination at onset of pleural effusion is informative for both diagnosis and outcome prediction in lung cancer patients.

Introduction

Lung cancer, the most frequent cause of cancer death, also is a major cause of pleural effusion. Approximately 10% of lung cancer patients have pleural effusion at the time of initial diagnosis, while 30-40% develop pleural effusion later in the course of their disease (1). Differentiating malignant from nonmalignant pleural effusions is a critical clinical problem, and conventional methods have proven inadequate (2-5). Cytologic examination of pleural fluid fails to detect neoplastic cells in 40-50% of malignant effusions, and blindly obtained pleural needle biopsy specimens offer little additional sensitivity (6). Several investigators, therefore, have sought to improve diagnostic yield by measuring tumor markers in pleural fluid (7). A reliable clinical marker providing rapid and accurate diagnosis is greatly needed.

In addition to diagnostic issues, patients with malignant pleural effusion have a short life expectancy and are difficult to treat effectively (1). Pleural effusion in patients with lung cancer typically causes worsening symptoms including cough, pain, and dyspnea, which affect the quality of life, while increasing mortality. Development of a biologic marker that reliably reflects the state of pleural effusion would be useful in managing lung cancer patients with pleural effusion, facilitating timely and appropriate intervention.

A novel tumor-associated antigen termed receptor-binding cancer antigen expressed on SiSo cells (RCAS) 1, was first described in immunohistochemical studies of ovarian carcinoma (8). RCAS1 is a type II membrane protein thought to oligomerize through homotypic interactions between its C-terminal structures. RCAS1 acts as a ligand for a putative receptor present on normal cells including all peripheral lymphocytes (T, B, and NK cells). RCAS1 inhibits the growth of receptor-expressing cells and induces apoptotic cell death (9). In addition to its cell-membrane location, RCAS1 is secreted as a soluble protein (sRCAS1) detectable in fluids by enzyme-linked immunosorbent assay (ELISA) (10). We and other researchers have demonstrated that sRCAS1 might have value as a tumor marker (11-14). We suspected that this potential tumor marker had important clinical implications in lung cancer patients.

We presently studied 102 patients with pleural effusions of various etiologies to determine whether sRCAS1 in pleural fluid could serve as a diagnostic indicator of lung cancer and a predictor of survival time. We found that sRCAS1 is elevated in malignant pleural effusion caused by lung cancer, as well as being potentially predictive of survival outcome.

Materials and methods

Patient and pleural fluid characteristics. We studied 102 patients with pleural effusion admitted to the Sanyo National Hospital between April 2000 and August 2003. Signs and symptoms, demographic data, and radiologic results were recorded. The characteristics of these patients are summarized in Table I. This patient group included 74 men and 28 women, with a mean age of 69 years; 59 patients (58%) had malignant effusions, while 43 (42%) had nonmalignant effusions.

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Table I. Patient characteristics.

No. of patients	102
Male/female	74/28
Median age, years	69
Age range, years	22-95
Diagnosis	
Malignant disease	59
Lung cancer	45
Malignant mesothelioma	6
Breast cancer	4
Other cancers (prostate, thyroid, stomach, uterus)	4
Nonmalignant disease	43
Tuberculosis	21
Infection other than tuberculosis	10
Heart failure	4
Rheumatoid arthritis	3
Other	5

Table II. Biochemical and serologic characteristics of malignant and nonmalignant pleural effusions.

	Malignant (n=59)	Nonmalignant (n=43)	p-value
Total protein (g/dl)	4.60±0.88	4.28±1.33	p=0.704
Albumin (g/dl)	2.59±0.61	2.15±0.87	p=0.036
Total bilirubin (mg/dl)	0.87±1.17	0.65±0.70	p=0.246
LDH (IU/L)	871±859	978±837	p=0.358
Cholesterol (mg/dl)	92.9±34.0	77.1±36.4	p=0.056
Glucose (mg/dl)	114.0±46.7	116±62.6	p=0.743
CEA (ng/ml)	450±1296	2.82±6.41	p<0.0001

Values shown are mean ± standard deviation. LDH, lactate dehydrogenase; CEA, carcinoembryonic antigen.

Albumin and carcinoembryonic antigen (CEA) concentrations were significantly higher in malignant than in nonmalignant effusions. Total protein, total bilirubin, lactate dehydrogenase (LDH), cholesterol, and glucose concentrations in pleural fluid were similar between the two groups (Table II).

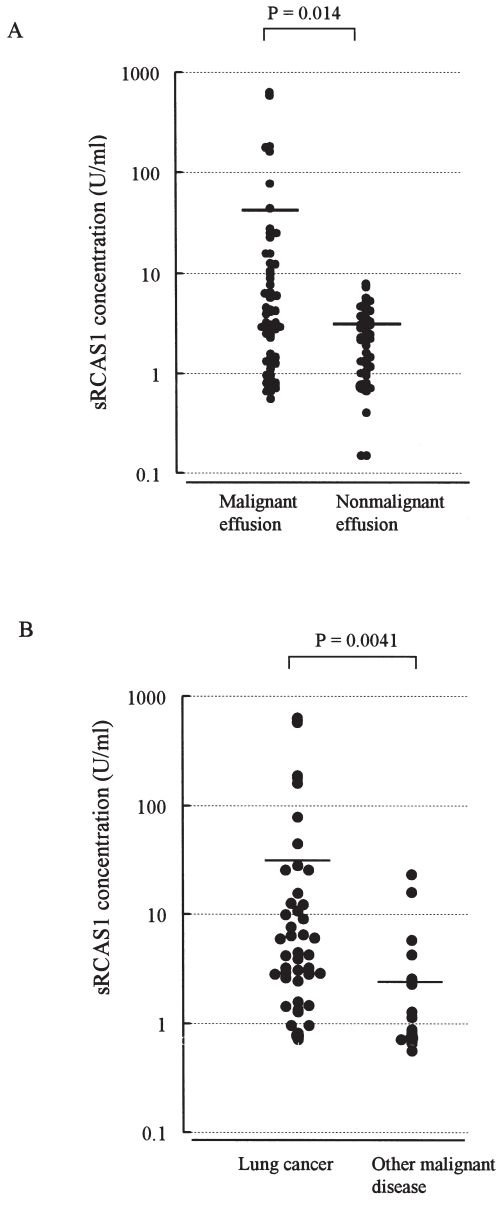

Figure 1. Concentrations of sRCAS1 in pleural effusions at onset. A, malignant effusions exhibited higher sRCAS1 concentrations than nonmalignant effusions (mean ± SD; 36.3±114 vs. 2.7±1.8 U/ml; p=0.014). B, pleural fluid sRCAS1 concentrations in lung cancer patients were higher than those in patients with other malignant diseases (mean±SD; 46.3±129 vs. 4.23±6.58 U/ml; p=0.041). Horizontal bars represent mean values.

Diagnosis of malignant pleural effusion. Malignant pleural effusion was diagnosed from either pleural fluid cytologic findings or malignant cells identified in a pleural biopsy specimen. Alternatively, when both of these microscopic assessments yield negative results, malignant effusion was diagnosed when a primary cancer was known to have disseminated and pleural fluid concentrations of established tumor markers were elevated.

Sample collection and determination of sRCAS1 concentration. Each sample of pleural fluid was collected in a syringe during thoracentesis after written informed consent. Samples were divided into two equal portions; one for cell collection and immunostaining (next paragraph) and another for sRCAS1

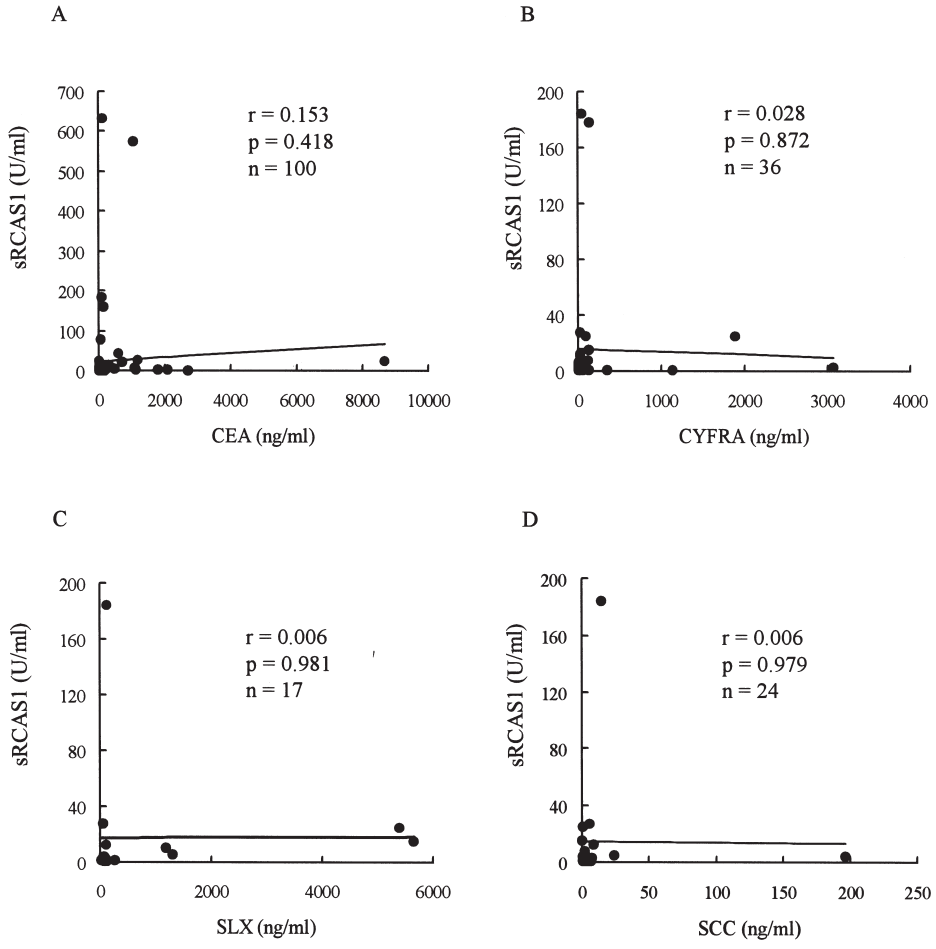

Figure 2. Correlation of pleural fluid sRCAS1 concentration with CEA (A), CYFRA (B), SLX (C), and ProGRP (D). No significant correlations were found between sRCAS1 and CEA (r=0.153, p=0.418), CYFRA (r=0.028, p=0.872), SLX (r=0.006, p=0.981), or ProGRP (r=0.101, p=0.829).

assay. The latter portion was centrifuged at 2000 rpm for 10 min, and the supernatant was frozen at -80˚C until assay for markers. Concentrations of sRCAS1 were measured by sandwich enzyme-linked immunosorbent assay (ELISA) using commercially available kits (Medical & Biological Laboratories, Nagoya, Japan).

Cell and tissue collection, and immunostaining. Lung cancer tissue specimens obtained from study patients who had undergone surgery at our hospital were fixed in formalin, dehydrated and then embedded in paraffin. Cells in pleural effusion were collected by centrifugation and then processed similarly. Sections of cells or tissues of 5 μm in thickness were cut from paraffin blocks and affixed to glass slides. After deparaffinization and rehydration, the cells or tissues were stained with monoclonal anti-RCAS1 antibody (Medical & Biological Laboratories, Tokyo, Japan), using a standard method reported previously (15). In brief, the cells were subjected to endogenous peroxide blockade with 3% hydrogen peroxide in methanol for 20 min, and then treated with an agent (Histofine SAB-PO kit: Nichirei, Tokyo, Japan) to block nonspecific binding of antibody. Sections then were incubated with the primary antibody at a dilution of 1:500 overnight at 4˚C, followed by incubation with biotinylated secondary antibody and visualization using the Dako Envision system (Dako, Carpinteria, CA). Hematoxylin was used for counterstaining. A tissue section of a squamous cell lung carcinoma previously demonstrated to express RCAS1 was used as a positive control. Nonimmunized mouse IgM was substituted for the primary antibody in negative controls.

Statistical analysis. Differences between independent groups were examined by the Mann-Whitney U test. Differences between more than two groups were determined by the Kruskal-Wallis test. Significance testing of correlations was evaluated using Spearman rank correlation analysis. A chi-squared test or trend test was used to analyze the relationships between pleural fluid sRCAS1 concentration and categorical variables. Probabilities of survival were estimated using the Kaplan-Meier method, and survival differences between two patient groups were determined by the log-rank test. Prognostic factors were analyzed using the Cox proportional hazard model. Values of $p<0.05$ were considered to indicate statistical significance.

Results

Diagnostic value of sRCAS1 in malignant pleural effusion. Malignant effusion exhibited higher RCAS1 concentrations than nonmalignant effusion (mean ± SD, 36.3±114 vs. 2.7±1.8 U/ml, p=0.014; Fig. 1A). Pleural fluid sRCAS1 concentrations in patients with lung cancer were higher than

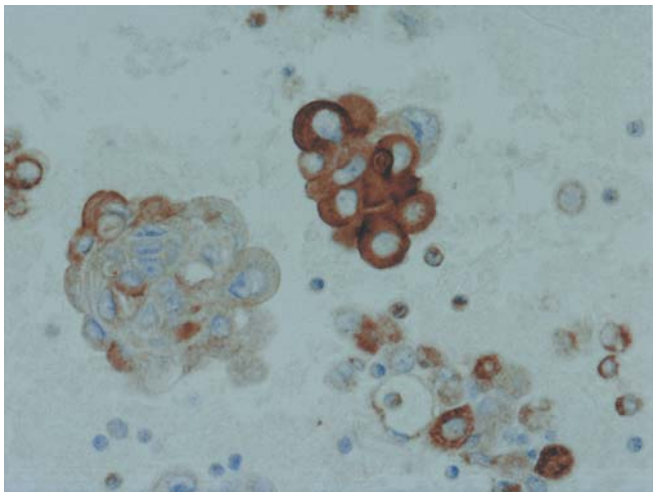

Figure 3. Representative photomicrograph of RCAS1 immunoreactivity in a cell block section prepared from pleural effusion in lung cancer patients (magnification, x400).

those in patients with other malignant diseases (mean ± SD, 46.3±129 vs. 4.23±6.58 U/ml, p=0.041; Fig. 1B).

Expression of RCAS1 in the cell block section derived from malignant pleural effusion. To confirm that RCAS1 is expressed by lung cancer cells in pleural effusion, we examined RCAS1 expression in immunohistochemically stained cell block sections prepared from malignant pleural effusion. In three of four such cases, lung cancer cell RCAS1 expression was confirmed in the effusion (Fig. 3). This finding strongly suggests that sRCAS1 in pleural fluid was secreted by lung cancer cells in the effusion.

Concentration of sRCAS1 in malignant pleural effusion associated with lung cancers. After we confirmed that the sRCAS1 concentration was elevated in malignant pleural fluid, we focused on the 45 lung cancer patients with malignant effusion. We first sought possible relationships between sRCAS1 and gender, age, histologic type of tumor, presence of distant metastases, Eastern Cooperative Oncology Group (ECOG) performance status (PS), serum LDH concentration, previous treatment, positive cytologic examination, serum total protein concentration, and location of pleural effusion. As shown in Table III, we found no significant correlation between sRCAS1 concentration and any of these clinicopathologic factors.

Table III. RCAS1 concentrations in pleural effusion of lung cancer patients.

Clinical variables		n	RCAS1, U/ml Mean ± SD	p-value
Gender	Male	31	46.6±120	
	Female	14	45.5±152	0.462
Age, years	≤65	16	25.5±56.2	
	>65	29	57.8±156	0.407
Histologic type	Adenocarcinoma	38	53.7±140	
	Small cell	3	10.7±12.3	
	Squamous	4	2.78±6.58	0.296
Metastasis	Yes	30	56.7±155	
	No	15	25.5±46.9	0.324
ECOG PS	0, 1	25	42.4±129	
	2, 3, 4	20	51.2±134	0.758
Serum LDH	<450	34	53.9±14.6	
	IU/l	≥450	1122.6±54.0	0.653
Previous treatment	Yes	22	43.2±137	
	No	23	49.3±124	0.666
Cytologic examination	Positive	28	56.9±159	
	Negative	17	2.7±55.5	0.419
Serum total protein, g/dl	<6.5	15	64.2±153	
	≥6.5	30	37.4±118	0.448
Location	Right	31	60.3±152	
	Left	14	15.4±41.5	0.286

n, number of patients; LDH, lactate dehydrogenase; ECOG, Eastern Cooperative Oncology Group; PS, performance status.

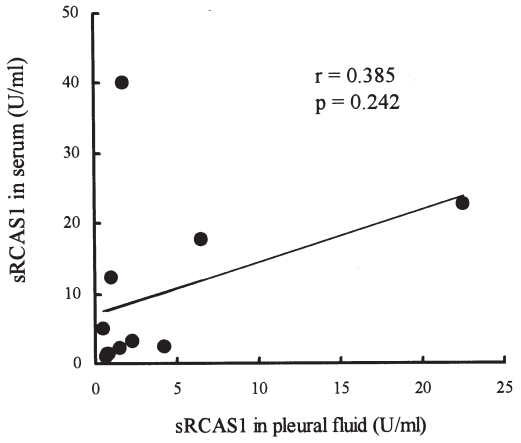

Figure 4. Lack of significant correlation between serum and pleural fluid concentrations of RCAS1 (r=0.385, p=0.242).

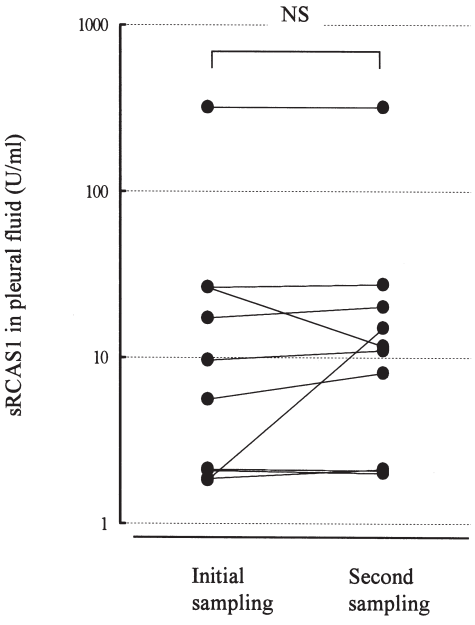

Figure 5. Lack of significant changes in pleural fluid RCAS1 concentrations during disease progression. The interval from first to second sampling ranged from 3 to 39 weeks.

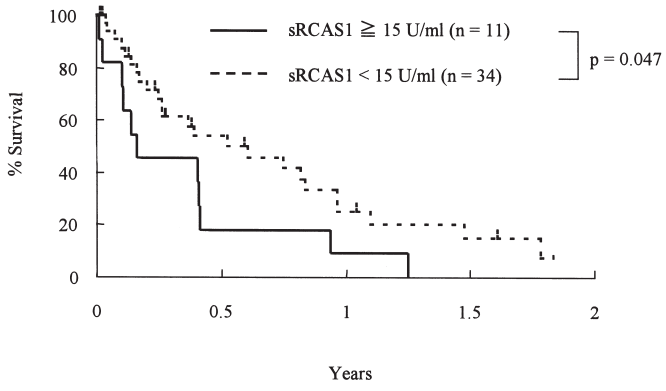

Figure 6. Relationship between pleural fluid RCAS1 concentrations at onset of pleural effusion and overall survival in lung cancer patients (Kaplan-Meier analysis). Survival of lung cancer patients with pleural fluid sRCAS1 concentrations of <15 U/ml (dotted line) was significantly longer than survival of patients with concentrations of >15 U/ml (solid line) ; (Median survival time, 4.7 vs. 1.7 months, p<0.05). Numbers in parentheses indicate numbers of patients studied.

We next analyzed correlations between sRCAS1 and CEA, cytokeratin 19 fragment (CYFRA), sialyl SSEA-1 (SLX), or squamous cell carcinoma antigen (SCC) in malignant pleural fluid. As shown in Fig. 2, no significant correlations were found between sRCAS1 and CEA (r=0.153, p=0.418), CYFRA (r=0.028, p=0.872), SLX (r=0.006, p=0.981), or SCC (r=0.006, p=0.979).

In 10 lung cancer patients, serum was collected at the time of pleural fluid sampling to assess the possible correlation between serum and pleural fluid concentrations of sRCAS1 (Fig. 4); no correlation was evident. Malignant pleural effusion recurred in 10 lung cancer patients, after a median interval of 10 weeks (range, 3 to 39) following the first thoracentesis. The mean sRCAS1 concentration in the second pleural fluid sample was 16.9 U/ml, showing no difference from initial samples (Fig. 5).

Only three specimens from the primary lung cancer site were available for immunohistochemical staining. In these cases, we found no relationship between degree of RCAS1 immunoreactivity at the primary site and pleural fluid sRCAS1 concentrations (Table IV).

Prognostic significance of pleural fluid sRCAS1 for lung cancer patients with malignant pleural effusion. The median survival time (MST) for all lung cancer patients in the current study was 4.8 months; the 1-year survival rate was 20.5%. Prognostic significance of pleural fluid sRCAS1 concentration and other factors in patients with malignant pleural effusion

Table IV. RCAS1 expression in tumors and RCAS1 concentrations in pleural effusion.

Patient no.	Age	Gender	Tumor	Histologic type	Primary tumor RCAS1 expression	Pleural fluid RCAS1 (U/ml)
1	42	Female	LC	Adenocarcinoma	P1	4.2
2	78	Male	LC	Adenocarcinoma	P2	0.8
3	74	Male	LC	Adenocarcinoma	P2	12.4

*According to the percentage of cells stained, our protocol divided specimens into four groups: no stained cells, P0; 1-5% of cells stained, P1; 6-80%, P2; ≥81%, P3. LC, lung cancer.

Table V. Prognostic factors in patients with malignant pleural effusion.

Prognostic factor		n	MST, months	1-year survival, %	p-value
Gender	Male	31	4.9	14.0	
	Female	14	3.1	31.0	0.517
Age, years	≤65	16	9.8	37.7	
	>65	29	2.8	9.7	0.022
sRCAS1, IU/ml	<15	34	4.7	25.0	
	≥15	11	1.7	9.1	0.047
Serum LDH, IU/l	<450	34	7.1	28.2	
	≥450	11	1.3	0.0	<0.001
ECOG PS	0, 1	25	7.1	26.6	
	2, 3, 4	20	1.9	15.0	0.017
Metastasis	Yes	30	6.2	23.3	
	No	15	2.4	19.3	0.264
Previous treatment	Yes	22	3.1	16.3	
	No	23	4.6	24.9	0.549
Location	Right	31	4.6	12.3	
	Left	14	3.1	39.8	0.032
Cytologic examination	Positive	28	3.1	20.2	
	Negative	17	5.0	19.7	0.755
Serum total protein, g/dl	<6.5	15	2.0	12.4	
	≥6.5	30	4.9	23.6	0.638

n, number of patients; MST, median survival time; LDH, lactate dehydrogenase; ECOG, Eastern Cooperative Oncology Group; PS, performance status.

was evaluated by univariate analysis (Table V). The cutoff value chosen for pleural fluid sRCAS1 concentration in lung cancer patients was 15 U/ml, twice the maximum value in nonmalignant effusion specimens. High pleural fluid sRCAS1 concentration, high serum LDH, poor PS, older age, and right-sided pleural effusion were factors associated with poor survival. Survival in lung cancer patients with pleural fluid sRCAS1 concentrations below 15 U/ml was significantly longer than in those with higher concentrations (MST, 4.7 vs. 1.7 months, $p<0.05$; Fig. 6).

To test the prognostic value of pleural fluid sRCAS1 concentration, we performed multivariate analysis of prognostic factors using the Cox proportional hazards model. We found that pleural fluid sRCAS1 concentration ($p=0.032$) and serum LDH ($p=0.021$) each had independent prognostic significance. ECOG PS ($p=0.408$), age ($p=0.151$), and sidedness of pleural effusion ($p=0.714$) lacked significant independent effects on survival (Table VI).

Discussion

In the current study, we found that profoundly elevated pleural fluid concentrations of sRCAS1 (>15 U/ml) measured at the onset of effusion correlated with shorter survival in lung cancer patients. Furthermore, multivariate analysis of prognostic factors identified pleural fluid sRCAS1 concentration as an independent prognostic factor for overall survival in lung cancer. These results suggest that sRCAS1 measured at the time of initial presentation with pleural effusion is an indicator not only for presence of lung cancer but also subsequent survival outcome.

Cytotoxic T lymphocytes recognizing tumor-specific antigens and NK cells are important in tumor elimination at early stages of cancer progression or metastasis (16). Because RCAS1-receptor expression is upregulated by activation of lymphocytes, RCAS1 helps to induce cell arrest and apoptosis in activated T and NK cells. These findings indicate that RCAS1 on tumor cells may convey an important advantage in avoiding host immune surveillance. RCAS1 is expressed in various cancers including those of the lung (15,17), breast (18), esophagus (19), pancreas (20), liver (21), stomach (22,23), gallbladder (24), and skin (25). RCAS1 expression also has been associated with aggressive tumor phenotypes, such as poor differentiation and advanced stage (18,21,22). Furthermore, RCAS1 expression was reported to be significantly and negatively related to overall survival of patients with cancers of the lung (15,17), esophagus (19), stomach (22), and gallbladder (24). Thus, RCAS1 facilitates the aggressive

Table VI. Multivariate analysis of prognostic factors by the Cox proportional hazard model.

	HR	95% CI	p-value
Serum LDH			
≥450	1		
<450	0.246	0.075-0.810	0.021
ECOG PS			
2, 3, or 4	1		
0 or 1	0.645	0.228-1.823	0.408
Age			
≥65	1		
<65	1.883	0.793-4.467	0.151
Location			
Right	1		
Left	0.844	0.341-2.091	0.714
sRCAS1, U/ml			
≥15	1		
<15	0.404	0.177-0.924	0.032

HR, hazard ratio; CI, confidence interval; LDH, lactate dehydrogenase; ECOG, Eastern Cooperative Oncology Group; PS, performance status.

behavior of human tumors. In its secreted form, RCAS1 strongly suppresses immune cell proliferation and induces apoptosis in RCAS1 receptor-expressing cells, as does the transmembrane form (9). These results suggest importance for sRCAS1 in immune evasion by tumors. One inference from this is that sRCAS1 in effusion could be a good indicator for follow-up of lung cancer patients, as was supported by our results.

Differentiating malignant from nonmalignant pleural effusions is a critical clinical problem, and conventional methods have proven inadequate (26). Cytologic examination of pleural fluid fails to detect neoplastic cells in 40-50% of malignant effusions, and blindly obtained pleural needle biopsy specimens have shown little advantage in this respect (27). A reliable clinical marker for rapid and accurate diagnosis of malignant effusion is greatly needed. We previously reported preliminary studies suggesting that sRCAS1 could serve as a tumor marker for diagnosis of malignant pleural effusion (11). The current extended study including a larger number of patients confirmed our preliminary result, and suggested that RCAS1 determination in malignant pleural effusion at its onset should be diagnostically informative. In addition, we found no significant correlation between sRCAS1 and CEA, CYFRA, SLX, or SCC; potential markers in pleural fluid that have shown limited reliability when considered separately (7). Thus, our results suggest a role for sRCAS1 in a panel of diagnostic markers for malignant pleural effusion. Further studies of this specific possibility are needed.

In conclusion, sRCAS1 concentrations in malignant pleural effusion associated with lung carcinoma are significantly higher than those in nonmalignant pleural fluid. Determination of sRCAS1 at the onset of pleural effusion is diagnostically informative, while sRCAS1 concentration is an independent prognostic factor that shows promise in follow-up of lung cancer patients who develop effusion.

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