Methylation of 14-3-3σ gene and prognostic significance of 14-3-3σ expression in non-small cell lung cancer

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Abstract. Loss of 14-3-3σ expression through DNA methylation has been associated with carcinogenesis and the prognosis for various cancer types. Detection of methylation of the gene in serum may be useful for diagnostic utility. The present study aimed to investigate the correlation between 14-3-3σ methylation level in 36 paired tumor tissues of non-small cell lung cancer (NSCLC) and matched serum using methylation-specific polymerase chain reaction. The prognostic significance of 14-3-3σ expression in 167 NSCLC was also evaluated using immunohistochemistry. Methylation of the 14-3-3σ gene was identified in all samples. The methylation level in the serum (mean 87.7%, range 64.6-100%) was higher compared with tumor (mean 46.7%, range 25.3-56.3%). However, no significant correlation between methylation levels in tissues and serums was observed (Spearman's correlation, -0.036; P=0.837). In the 167 tumor tissues, the majority of the cases (83.8%) exhibited negative expression. Adenocarcinoma is more likely to exhibit negative expression (91.4%) compared with squamous cell carcinoma (70.2%). No significant difference was identified in the overall survival according to 14-3-3σ expression status and 14-3-3σ expression did not demonstrated independent prognostic significance. In conclusion, NSCLC harbors certain levels of 14-3-3σ methylation in the tumor and the sera of patients. The clinical value of serum 14-3-3σ methylation should be further elucidated. Immunohistochemical expression 14-3-3σ protein has limited value on prognostic significance.

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Abbreviations: NSCLC, non-small cell lung cancer; LN, lymph node; ADC, adenocarcinoma; SCC, squamous cell carcinoma; MSP, methylation-specific polymerase chain reaction

Key words: 14-3-3σ, methylation, prognosis, non-small cell lung cancer, survival, immunohistochemistry

Introduction

Lung cancer is the most common type of cancer among men worldwide, accounting for ~16.7% of all estimated new cancer cases (1). In Thailand, lung cancer is the second leading cancer in men with an age-standardized incidence rate of 27.1/100,000 (2). Approximately 85% of patients with lung cancer are diagnosed with non-small cell lung cancer (NSCLC). The survival of patients with lung cancer primarily depends on the stage of disease at the time of diagnosis. The 5-year survival rate of patients with early stage NSCLC is between 25 and 52%, while the rate is <4% for those with advanced stages (3). Although several advanced therapeutic modalities are available, the mortality rate remains high. Thus, identifying biological markers that are able to detect the disease at the early stage, or predict treatment response or prognosis is important for improving patient survival.

14-3-3 proteins are small acidic polypeptides that are 28-33 kDa in size, and consist of at least seven isoforms, β, ε, γ, η, σ, τ/θ, and ξ, in mammalian cells. The proteins are spontaneously self-assembled to form homodimers or heterodimers and bind to various cellular proteins (4). The interaction between 14-3-3 proteins and other proteins has been demonstrated in a number of signaling pathways, including cell cycle progression, signal transduction, and apoptosis (5,6). Among the various isoforms, 14-3-3σ is the most common isoform reported to be involved in carcinogenesis via a tumor suppression manner (7). Loss of 14-3-3σ expression has been demonstrated in a variety of cancer types, particularly in adenocarcinoma-type tumors, including breast carcinoma (8) and gastric carcinoma (9). Subsequently, a number of these studies have demonstrated that epigenetic silencing through CpG methylation is responsible for the loss or reduction of 14-3-4σ expression (10). With regards to prognosis, loss of expression of this protein has been reported to be associated with poor prognosis in ovarian and nasopharyngeal carcinoma (11,12). By contrast, poor overall survival in patients with high expression of 14-3-3σ has been revealed in colorectal cancer, oral squamous cell carcinoma and gastric cancer (13-15).

In lung cancer, 14-3-3σ expression has been demonstrated to be abundantly expressed in cancerous tissue samples compared with normal lung tissues (16). In contrast with the results identified in breast cancer (8), it has been reported that 14-3-3σ expression is observed in the majority of lung adenocarcinoma (17) or NSCLC (18) tissues, and methylation is more frequently observed in small cell lung cancer compared with NSCLC (18). However, these findings have been reported in a few studies with a limited number of cases. In addition, there is little evidence to suggest that peripheral DNA sources reflect 14-3-3σ methylation in NSCLC tissue. In the present study, the association of 14-3-3σ methylation between tumor tissues and matched serum was investigated, and the prognostic value of 14-3-3σ expression was evaluated.

Materials and methods

Patients and specimens. Tissues and matched serum samples were obtained from 36 NSCLC patients who had not received any previous treatment. The obtained tissues were frozen immediately at -80˚C for DNA extraction. For the matched serum samples, 10 ml peripheral blood was collected from the fore arm vein and kept at room temperature for 2 h. Serum was separated by double centrifugation at 1,600 x g for 10 min and kept at -80˚C until analysis. All patients were diagnosed at Songklanagarind Hospital (Hat-Yai, Thailand), a university hospital in Southern Thailand, between May 2012 and April 2013. Fresh tumor tissue for methylation analysis was obtained via bronchial biopsy through bronchoscopy simultaneously when tissue was obtained for pathological diagnosis. Normal serum samples (n=7) were collected from healthy blood donors. Written informed consent was obtained from all patients. The mean age was 61 years (range, 32-83 years). Twenty-one patients were males and 15 were females. Seventeen cases were adenocarcinoma (ADC) and 15 cases were squamous cell carcinoma (SCC). The specific subtype was not specified in 4 cases, which were recorded as NSCLC-unclassified.

For the evaluation of prognostic significance of 14-3-3σ expression, 167 patients with stage I-IV of NSCLC who were diagnosed, and treated at Songklanagarind Hospital between January 2006 and December 2008 were included. The clinicopathological data, including the clinical stage were retrieved from the hospital registry data. Clinical staging was based on the Tumor Node Metastasis staging system of the International Union against Cancer (7th Edition) (19). The histological diagnosis was performed according to the WHO classification of lung and pleural tumors (2004) (20). The patients were followed up until September 2012. Data associated with the mortality of patients was obtained from the provincial nationwide-linked register of mortalities, where the law requires all mortalities that have occurred in Thailand to be registered within 24 h of occurrence. The present study was approved by the Ethics Committee on Human Research, Faculty of Medicine, and Prince of Songkla University (EC, 54-273-04-2-3 and 55-020-04-1-2).

Methylation-specific polymerase chain reaction (MSP). Genomic DNA was extracted from frozen tissue and serum samples using standard proteinase K/phenol/chloroform methods (21). The structural integrity of DNA was confirmed using 1% agarose gel electrophoresis and quantified with a spectrophotometer. The genomic DNA (1 μg) was subjected to sodium bisulfite modification using the EZ DNA Methylation-Gold kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's protocol. Modified DNA was resuspended in 15 μl of nuclease-free water, quantified using a spectrophotometer and stored at -70˚C. For MSP analysis, the modified DNA (50 ng) was amplified using methylation or unmethylation primers spanning the region between CpG dinucleotides 3 and 9 of the 14-3-3σ gene. The primers were designed according to a previous report by Ferguson *et al* (8). Primers sequence were as follows: Methylation forward, 5'-GATATGGTAGTTTTTATGAAA GGCGTCG-3' and reverse, 5'-CCTCTAACCGCCCACCAC G-3'; unmethylation forward, 5'-GATATGGTAGTTTTT ATGAAAGGTGTTGTG-3' and reverse, 5'-CCCTCTAAC CACCCACCACA-3'. The MSP conditions maintained were as follows: 1 cycle at 94˚C for 3 min; 35 cycles at 94˚C for 30 sec, 64˚C (methylated reaction) or 59˚C (unmethylated reaction) for 30 sec, 72˚C for 45 sec; and 1 cycle at 72˚C 10 min. The MSP products were 108 and 109 bp for methylation, and unmethylation primers, respectively. Universal human methylated and unmethylated DNA strands (Zymo Research) were used as a positive control for each primer. Following amplification, the MSP products were separated on a 10% polyacrylamide gel, stained with ethidium bromide for 10 min at room temperature, visualized as bands under ultraviolet illumination and imaged using Gel Doc™ XR (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The density of bands was measured using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The relative density of each methylated and unmethylated products were obtained by dividing their values by the density of the corresponding positive control. The 14-3-3σ methylation level percentage was calculated as follows: Relative density of methylated products/(relative density of methylated products + relative density of unmethylated products).

Immunohistochemistry. Sections 4 μm thick were cut from a paraffin-embedded block, deparaffinized with xylene and rehydrated with ethanol. Antigen retrieval was enhanced by rapid heating in a microwave in a citrate buffer (10 mM, pH 6.0) for 10 min. Endogenous peroxidase activity was blocked at room temperature by incubation with 3% hydrogen peroxide in methanol for 10 min. The slides were then incubated with 10% normal goat serum (Santa Cruz Biotechnology, Dallas, TX, USA) at room temperature for 20 min and incubated with monoclonal antibody against 14-3-3σ (5D7, sc-100,638; Santa Cruz Biotechnology) at a dilution of 1:800 overnight at 4˚C in a humidified chamber. After washing with PBS (pH 7.4), the slides were incubated with a biotinylated goat anti-mouse IgG-B (sc-2039; Santa Cruz Biotechnology) at a dilution of 1:300 for 40 min at room temperature. Antigen-antibody complexes were detected using the avidin-biotin complex staining kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and a diaminobenzidine solution (Merck KGaA, Darmstadt, Germany) as a substrate for 5 min at room temperature. Finally, the slides were counterstained with hematoxylin for 5 min at room temperature (Santa Cruz Biotechnology),

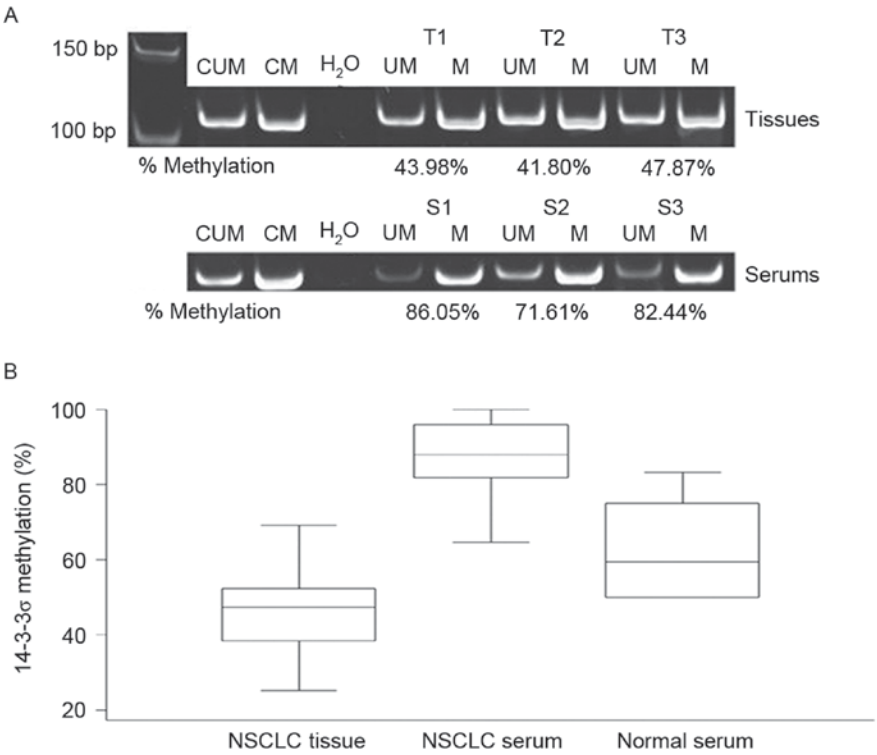

Figure 1. Methylation-specific polymerase chain reaction analysis of the 14-3-3σ gene in tumor tissue and serum. (A) Methylated and unmethylated products as well as controls (CM, CUM, and H_2O) on a 10% polyacrylamide gel. (B) Boxplot presenting the relative methylation levels of various sample groups. T, tumor tissue; S, serum; M, methylated; UM, unmethylated; CM, control methylated; CUM, control unmethylated.

cover slipped and examined under a light microscope at x200. Oral squamous carcinoma tissue from a patient with oral cancer was used as a positive control. Negative controls using the same tissue without primary antibody were run in parallel.

Evaluation of immunohistochemical staining. Immunoreactivity was qualitatively and quantitatively evaluated in terms of intensity, and percentage of positively stained cells, respectively. The intensity was scored as follows: 0, no staining; 1, weak; 2, moderate; and 3, intense. The percentage of positive cells was scored as follows: 0, ≤10%; 1, 11-30%; 2, 31-60%; and 3, ≥61%. Final scores (0-9) were then obtained through multiplication of both scores. Four expression groups were assigned as follows: No expression, final score 0; weak expression, final score 1-3; moderate expression, final score 4-6; and strong expression, final score 7-9. The expression of 14-3-3σ was dichotomized to give negative expression (final score 0) and positive expression (final score 1-9). Immunostaining was evaluated by two independent pathologists, and discordant cases was reevaluated and scored on the basis of consensus interpretation.

Statistical analysis. Methylation levels are presented as the mean ± standard deviation. The differences and correlation of methylation level between tumor, and matched serum were analyzed using a paired t-test and the Spearman correlation, respectively. The associations between 14-3-3σ expression and clinicopathological variables were analyzed using the chi-squared test. The survival rates according to 14-3-3σ expression status and other variables were examined using Kaplan-Meier analysis, and compared using the log-rank test. Cancer-associated mortality was considered to be the end event. The Cox multivariate proportional hazards model was used to identify independent prognostic variables. $P<0.05$ was considered to indicate a statistically significant difference. Statistical analysis was performed using STATA software version 12.1 (StataCorp LP, College Station, TX, USA).

Results

14-3-3σ methylation in tumor and serum. Methylation of 14-3-3σ gene was identified in all samples. Representative methylated and unmethylated products of the samples run on the 10% polyacrylamide gel are presented in Fig. 1. The mean methylation level across all tumor tissues was 46.7% (range, 25.3-69.2%). The methylation level in ADC (mean, 43.6%; range, 25.3-56.3%) and SCC (mean, 48.6; range, 32.7-68.6%) samples were comparable. The mean methylation level in patient sera was ~2 times higher compared with that of the primary tumors with a mean value of 87.7% (range, 64.6-100%). However, the methylation levels in tissues and serums were not linearly correlated [Spearman's correlation (r), -0.036; P=0.837; Fig. 2]. The methylation level in normal serum (mean, 60.2%; range, 50.0-75.0%) was lower compared with in patient sera.

Correlation between 14-3-3σ methylation and protein expression. The correlation between 14-3-3σ methylation and immunohistochemical protein expression was evaluated in 32 cases. The 14-3-3σ protein was primarily observed in the cytoplasm (Fig. 3) and 18 cases (56.2%) exhibited no expression. The remaining cases exhibited weak expression (7 cases,

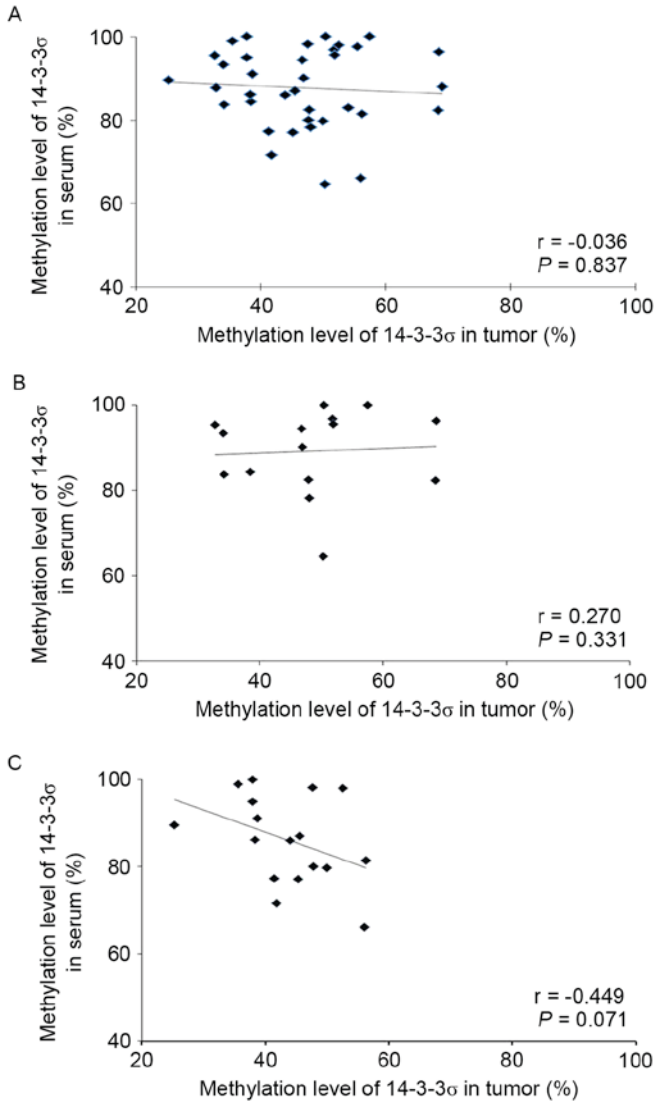

Figure 2. Scattered plots with regression line of methylation levels between tissues and matched sera in (A) all tumors, (B) squamous cell carcinoma, and (C) adenocarcinoma of patients with non-small cell lung cancer.

21.9%) and moderate to strong expression (7 cases, 21.9%). No significant correlation was observed between immunohistochemical expression and the methylation level (r, 0.153; P=0.402).

Association between 14-3-3σ expression and clinicopathological variables. The immunohistochemical expression of 14-3-3σ protein in relation to clinicopathological characteristics and prognosis was evaluated in 167 patients. The patients had a mean age of 64 years (range, 37-93 years; Table I). The majority of patients exhibited the advanced stages of the disease (89.8%). The majority of the cases (140 cases, 83.8%) revealed no expression, whereas 19 (11.4%) and 8 (4.8%) cases demonstrated weak expression, and moderate/strong expression, respectively. Patients in the ADC group had a significantly higher frequency of no expression (91.4%) compared with SCC (70.20) (P=0.002). In the further analysis, the weak to strong expression samples were grouped as positive expression. Sex and histological type were identified to be significantly associated with the expression status. In addition, tumors in males

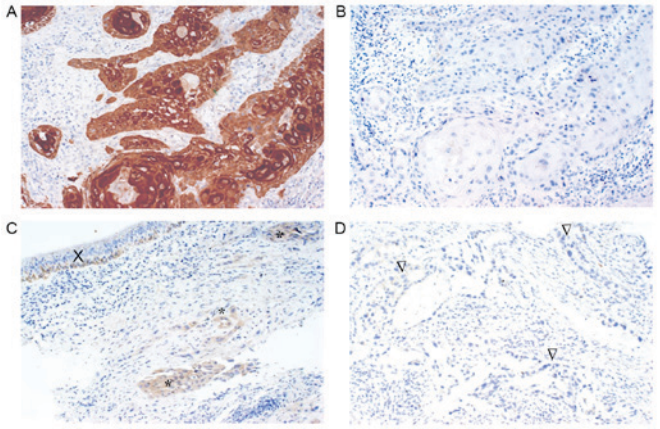

Figure 3. Immunohistochemical staining of 14-3-3σ. (A) Representative case of oral squamous cell carcinoma showing strong expression as a positive control and (B) no expression as a negative control. (C) (*) Lung squamous cell carcinoma with moderate expression and (X) positive staining of normal bronchial epithelial cells. (D) (V) Lung adenocarcinoma representing no expression. Original magnification, 200x.

had a significantly higher frequency of positive expression compared with that of females (Table I).

Prognostic significance of 14-3-3σ expression. The patients had a median survival time of 5.7 months. The Kaplan-Meier estimates revealed no significant difference in overall survival according to 14-3-3σ expression status. Furthermore, no significant difference was identified in survival for ADC and SCC groups with P=0.13 and P=0.60, respectively (data not shown). Clinical stage, surgery, chemotherapy and histological type were associated with survival rates in the univariate analysis, but only age, and treatments were significant independent prognostic parameters in the multivariate analysis (Table II). 14-3-3σ expression did not exhibit prognostic significance.

Discussion

In recent years, the aberrant expression levels of the 14-3-3 protein family have been reported in various cancer types and as potential novel biological markers (4). Among various isoforms, 14-3-3σ is the most common isoform reported to be involved in carcinogenesis via a tumor suppressive manner (7). Loss of 14-3-3σ expression has been reported in various types of epithelial cancer and is reported to be associated with hypermethylation of the promoter of the gene (8-10). In the present study, the methylation status of the NSCLC tissue in relation to protein expression as well as in relation to the methylation level in their match serum was evaluated. The results revealed that all tumors harbored certain levels of methylation; however, it was not correlated with the level of protein expression. In addition, it was demonstrated that methylation level in serum was significantly higher compared with in primary tumor samples.

Hypermethylation of CpG islands is a well-known epigenetic mechanism for inactivating tumor suppressor genes, thus contributing significantly to tumor development (10,22). Methylation in the promoter region of the 14-3-3σ gene has been demonstrated in a high proportion of breast (90%) (23),

Table I. Correlation between 14-3-3σ expression and clinicopathological variables.

Variable	No. of cases	14-3-3σ expression (%)		P-value
		Negative	Positive	
Sex				0.03
Male	124	99 (79.8)	25 (20.2)	
Female	43	41 (95.3)	2 (4.7)	
Age, years				0.46
<60	63	55 (87.3)	8 (12.7)	
≥60	104	85 (81.7)	19 (18.3)	
Histological type				0.002
ADC	105	96 (91.4)	9 (8.6)	
SCC	57	40 (70.2)	17 (29.8)	
NSCLC-UC	5	4 (80.0)	1 (20.0)	
Clinical stage				0.38
I	10	8 (80)	2 (20)	
II	5	3 (60)	2 (40)	
III	59	51 (86.4)	8 (13.6)	
IV	9	77 (84.6)	14 (15.4)	
Unknown	2	1 (50)	1 (50)	
LN metastasis				0.24
No	72	64 (88.9)	8 (11.1)	
Yes	95	76 (80)	19 (20)	
Distant metastasis				0.93
No	76	63 (82.9)	13 (17.1)	
Yes	91	77 (84.6)	14 (15.4)	
Surgery				0.92
No	157	132 (84.1)	25 (15.9)	
Yes	10	8 (80)	2 (20)	
Chemotherapy				0.22
No	87	70 (80.5)	17 (19.5)	
Yes	80	70 (87.5)	10 (12.5)	
Radiotherapy				0.14
No	116	101 (87.1)	15 (12.9)	
Yes	51	39 (76.5)	12 (23.5)	

ADC, adenocarcinoma; SCC, squamous cell carcinoma; NSCLC-UC, non-small cell lung cancer-unclassified; LN, lymph node.

nasopharynx (84%) (24), ovary, endometrium and prostate (11) carcinoma. The results of the present study demonstrated that all NSCLC tumor samples harbored methylation in the promoter of 14-3-3σ gene (relative methylation level, 25.3-69.2%). The methylation status may be reported as partial methylation as methylated and unmethylated products were identified. These results are consistent with that of Shiba-Ishii and Noguchi (25) where invasive adenocarcinoma harbored partial methylation. SCC, in the present study, also revealed a comparable methylation level with ADC. However, these results were inconsistent with the study of Osada *et al* (18), whereby hypermethylation was identified to be frequent in small cell carcinoma cell lines, but rare in NSCLC cell lines.

It is well known that circulating cell-free DNA is released into the blood of patients with cancer, with increasing levels compared with normal healthy individuals (26), thus allowing for the detection of gene alternation of the primary tumor. Detection of hypermethylation in the promoter regions of certain tumor suppressor genes in the serum of patients with NSCLC was first reported by Esteller *et al* (27). Later, Ramirez *et al* (28) detected methylation in the sera of one-third of 115 advanced-stage patients with NSCLC. In the present study, a higher methylation level (mean, 87.7%) was observed in the serum of the patients compared with normal serum (mean, 60.2%). In addition, the serum methylation was level was two to three times higher compared with the matched primary tumor samples and was not linearly correlated. The possible explanation is that the circulating DNA is contaminated by other sources, including inflammatory cells reacting to the tumor. The inflammatory process

Table II. Univariate and multivariate analysis of clinicopathological variables for overall survival.

	Univariate analysis		Multivariable analysis	
Variable	Risk ratio (95% CI)	P-value	Risk ratio (95% CI)	P-value
Sex		0.339		
Male	1			
Female	0.85 (0.61-1.19)			
Age, years		0.609		0.031
<60	1			
≥60	1.08 (0.79-1.48)		0.69 (0.50-0.97)	
Histological type		0.022		
ADC	1			
SCC	1.27 (0.93-1.74)			
NSCLC-UC	3.9 (1.56-9.76)			
Clinical stage		<0.001		
I	1			
II	6.19 (1.47-26.11)			
III	9.02 (2.79-29.10)			
IV	9.62 (3.00-30.87)			
Unknown	52.6 (10.17-272.16)			
Surgery		<0.001		<0.001
No	1			
Yes	0.09 (0.03-0.28)		0.06 (0.02-0.20)	
Chemotherapy		<0.001		<0.001
No	1			
Yes	0.5 (0.37-0.68)		0.47 (0.33-0.66)	
Radiotherapy		0.069		0.015
No	1			
Yes	0.75 (0.55-1.03)		0.66 (0.47-0.92)	
14-3-3σ expression		0.248		
Negative	1			
Positive	1.44 (0.95-2.19)			

CI, confidence interval; ADC, adenocarcinoma; SCC, squamous cell carcinoma; NSCLC-UC, non-small cell lung cancer-unclassified.

has been demonstrated to serve a role in the pathogenesis of NSCLC and the majority of lung cancer cases coexist with inflammatory reactions (29). In addition, lysis of peripheral blood lymphocytes during serum separation may cause an artificial increase in DNA (30). However, this risk was minimized by performing centrifugation of the collected serum within 2 h.

Methylation of the promoter region of genes is typically associated with decreased or a loss of protein expression. Shiba-Ishii and Noguchi (25) identified an inverse correlation between the level of the 14-3-3σ transcript and methylation level in lung adenocarcinoma tissue. By contrast, no significant correlation was identified in present study. Similarly, no significant correlation between the methylation of the 14-3-3σ gene in the tumor and protein expression was noted in the study of Osada *et al* (18). The authors demonstrated that certain SCLC tissues exhibited almost complete unmethylation of the 14-3-3σ gene as indicated by the loss of protein expression. This may indicate that 14-3-3σ protein expression is affected by additional mechanisms. Furthermore, clinical tissue specimen may be contaminated by other cells/tissue, including stromal cells or inflammatory cells as reported by Osada *et al* (18), whereby it was demonstrated that microdissected stromal tissue also harbored 14-3-3σ hypermethylation.

Previous studies regarding the expression of 14-3-3σ in NSCLC are conflicting. Osada *et al* (18) reported immunohistochemical expression of 14-3-3σ in 21/22 NSCLC specimens and Shiba-Ishii *et al* (17) observed immunopositive staining in 95% of ADC. By contrast, Liu *et al* (31) observed the down-regulation of 14-3-3σ in NSCLC cell lines. The present study demonstrated that the majority of NSCLC (84%) demonstrated no expression of 14-3-3σ protein following immunohistochemistry, which is consistent with the results of studies on other cancer types, in particular breast (8) and prostate (32) cancer. The number of specimens examined may contribute to the contradictory results in lung cancer.

Regarding the prognostic role, the decreased expression of 14-3-3σ has been reported to be correlated with a short survival rate in esophageal squamous cell carcinoma (33) and ovarian cancer (33,34), and a good survival rate in gastric cancer (35). However, the present study did not identify prognostic significance of 14-3-3σ expression in NSCLC. This may possibly be due to the small numbers of patients with a positive expression. In addition, the majority of the patients had stage III-IV cancer, thus the insignificance may also be due to the homogeneity of cases regarding of stage of disease.

In conclusion, the results of the present study have demonstrated that NSCLC harbored partial 14-3-3σ methylation and may, in part, contribute to the loss of protein expression in the tumor. The serum of patients with advanced NSCLC exhibited a high level of 14-3-3σ methylation, but its clinical value remains to be elucidated. The prognostic significance of immunohistochemical expression of the protein was not demonstrated, possibly due to the small number of cases with positive expression and homogeneity of advanced cases.

Acknowledgements

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