

## RESEARCH ARTICLE

# Prognostic Significance of 14-3-3 $\gamma$ Overexpression in Advanced Non-Small Cell Lung Cancer

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### Abstract

The 14-3-3 protein has been shown to be involved in the cancer process. However, there is no understanding of the relationship between 14-3-3 $\gamma$  (14-3-3 gamma) expression and prognosis in advanced non-small cell lung cancer. In this study, we therefore investigated the association between protein levels by immunohistochemistry and clinicopathological features of advanced NSCLC patients. Survival curves were estimated using the Kaplan-Meier method and tested by log-rank. Multivariate analysis was conducted with the Cox's regression model to determine independence of factors. p values less than 0.05 were considered significant. A total 153 patients were studied, with 54.3% being stage III and 45.8% stage IV. Fifty-one cases (33.3%) were squamous cell carcinomas, and 98 cases (64.1%) were adenocarcinomas. High 14-3-3 $\gamma$  expression was seen in 59.5% and significantly correlated with lymph node metastasis (p=0.010) and distant metastasis (p=0.017). On Kaplan-Meier analysis, high 14-3-3 $\gamma$  expression was associated with poorer survival with a marginal trend toward significance (p=0.055). On multivariate analysis, age, treatment, and 14-3-3 $\gamma$  expression proved to be independent prognostic parameters. In vitro experiments indicated that 14-3-3 $\gamma$  overexpression also played a potential role in cancer invasion. In conclusion, our data suggest that 14-3-3 $\gamma$  overexpression is associated with invasion and a poor prognosis. Therefore, 14-3-3 $\gamma$  may be a potential prognostic marker of advanced non-small cell lung cancer.

**Keywords:** 14-3-3 $\gamma$  - immunohistochemistry - non-small cell lung cancer - invasion - metastasis

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### Introduction

Lung cancer is the most frequently diagnosed cancer and the leading cause of cancer death in males globally (Jemal et al., 2011). Approximately 85% of lung cancer cases are non-small cell carcinoma (NSCLC) (Herbst et al., 2008). Most lung cancer patients are of advanced stage at the time of diagnosis, resulting in a poor outcome with 5-year relative survival being less than 14% (Youlten et al., 2008). Despite advances in surgical treatment and chemotherapy, there has been little improvement in survival over the past 30 years (Siegel et al., 2012). Therefore, identification of a prognostic biomarker will help identify patients with poor outcome, who may need further treatments. In addition, the identified marker may be a potential target for drug development.

14-3-3 proteins are a family of highly conserved proteins, consisting of at least seven isoforms in mammalian cells, including  $\beta$ ,  $\epsilon$ ,  $\gamma$ ,  $\eta$ ,  $\sigma$ ,  $\tau/\theta$ , and  $\xi$ . These proteins are spontaneously self-assembled as homo-

heterodimers and function by binding to other various cellular proteins through phosphorylated serine residues (Xing et al., 2000). As a consequence, 14-3-3 proteins are involved in a wide variety of cellular processes (Morrison, 2009) including cell cycle progression (Umbricht et al., 2001; Yu et al., 2012), check point activation (Peng et al., 1997), apoptosis (Samuel et al., 2001), all of which are known to be important in tumorigenesis. Evidence is also accumulating to support the role of 14-3-3 proteins in tumor development and progression (Hermeking, 2003).

In lung cancer, four isoforms of 14-3-3 proteins including  $\beta$ ,  $\gamma$ ,  $\sigma$ , and  $\theta$  have been shown to be overexpressed in tumor tissue compared to normal mucosa (Qi et al., 2005). 14-3-3 $\gamma$  is encoded by the tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, gamma polypeptide (YWHAG) gene located on chromosome 7 (7q11.23) (Horie et al., 1999). It is comprised of 247 amino acids with a molecular weight of 28.2 kilodaltons. Normally, 14-3-3 $\gamma$  expression is relatively strong in brain, skeletal muscle, and heart, but

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weak in peripheral blood leukocytes (Horie et al., 1999). Overexpression of 14-3-3 $\gamma$  has been shown to contribute to lung tumorigenesis by promoting genomic instability (Qi et al., 2007) or by negative regulation of p53 (Jin et al., 2008; Radhakrishnan et al., 2011). This evidence raises the possibility that 14-3-3 $\gamma$  is involved in lung cancer development and progression. Although 14-3-3 $\gamma$  overexpression has been reported to be associated with poor prognosis in breast cancer (Song et al., 2006) and hepatocellular carcinoma (Ko et al., 2011), the clinical significance of 14-3-3 $\gamma$  expression in NSCLC has not been reported. In this study, we investigated the association of immunohistochemical expression of 14-3-3 $\gamma$  with 5-year survival in advanced NSCLC patients. In addition, *in vitro* experiments were performed on the effect of 14-3-3 $\gamma$  on the invasion of cancer cells, to explore the possible mechanisms for its role on tumor progression.

## Materials and Methods

### *Patients and tissue specimens*

One-hundred and fifty-three patients with stage III and IV of NSCLC who were diagnosed and treated at Songklanagarind Hospital from January 2006 to December 2008 were included. Clinicopathological data including gender, age, lymph node (LN) metastasis, distant metastasis, and treatment types were prospectively collected. Most patients were assessed with computed tomography (CT) to evaluate tumor extension and nodal involvement. Clinical staging was based on the TNM staging system of the International Union Against Cancer (7<sup>th</sup> Edition). The patients were followed-up until September 2012. Information on death was obtained from the provincial nationwide-linked register of deaths, where the law requires all deaths occurring in Thailand to be registered within 24 hours of occurrence.

Tissue was obtained at the time of diagnosis before any treatment was given. All histologic sections were reviewed by a pathologist (Thongsuksai P.) to confirm the histologic type of adenocarcinoma (ADC), squamous cell carcinoma (SCC) or large cell carcinoma according to the WHO classification of lung and pleural tumor (2004). In three cases, definite identification of type could not be made due to small tissue sample and were assigned as NSCLC. Cases with insufficient tissue for immunostaining were excluded. The study was approved by the Ethics Committee on Human Research, Faculty of Medicine, and Prince of Songkla University.

### *Immunohistochemical analysis*

Tissue samples were fixed in 10% buffered formalin and embedded in paraffin. Four-micrometer-thick sections were cut from paraffin-embedded blocks and were deparaffinized by xylene and followed by rehydration in ethanol. Antigen retrieval was achieved by heating the slides in 10 mM citrate buffer (pH 6.0) at 100°C for 10 min. The slides were immersed in 3% hydrogen peroxide in methanol to block endogenous peroxidase activity for 10 min and then incubated with 10% normal goat serum to block unwanted proteins at room temperature for 20 min. Subsequently, the slides were incubated overnight

at 4°C in a humidified chamber with 1:250 dilution of polyclonal antibody against 14-3-3 $\gamma$  (C-16, Santa Cruz Biotechnology, CA, USA). After washing, goat anti-rabbit IgG-B antibody (Santa Cruz Biotechnology) at a dilution of 1:300 was added. Colorimetric detection was performed with an ABC-peroxidase staining kit (Thermo Scientific, Rockford, USA) using diaminobenzidine solution (Merck, Darmstadt, Germany) as a substrate for 5 min. The slides were counterstained with hematoxylin (Santa Cruz Biotechnology). Normal bile duct tissue was used as positive control. Negative controls of same tissue without primary antibody were run in parallel. Slides were evaluated under light microscopy by two independent pathologists. The cases with discordant scores were resolved after a discussion to establish a consensus interpretation.

### *Evaluation of 14-3-3 $\gamma$ expression*

Immunostaining was evaluated quantitatively by percentage of positive cells and qualitatively by intensity of staining. The percentage of positive-stained cells were estimated from overall tumor cells in the slide and were scored as 0 ( $\leq 10\%$ ), 1 (11-30%), 2 (31-60%), and 3 ( $\geq 61\%$ ). The intensity of staining was scored as 0 (no staining), 1 (weak), 2 (moderate), and 3 (intense staining). A final score (0-9) was then obtained by multiplying both scores to finally determine the level of expression as negative, score 0; weak, score 1-3; moderate, score 4-6 and strong expression, score  $>6$ . Immunostaining was independently evaluated by two independent pathologists. In discordant cases, a consensus score was achieved by a discussion.

### *Cell culture*

A549 human lung cancer cell line was obtained from the American Type Culture Collection (Rockville, MD, USA). Culture media, fetal bovine serum (FBS), and antibiotics were purchased from Gibco (Grand Island, NY, USA). A549 cells were maintained in RPMI 1640 supplemented with 10% FBS, 100 U/mL penicillin, and 100 U/mL streptomycin in humidified atmosphere, 5% CO<sub>2</sub> at 37°C.

### *Small-interfering RNA (siRNA) transfection*

Non-Targeting control siRNA (control siRNA) and siRNA targeting 14-3-3 $\gamma$  (14-3-3 $\gamma$  siRNA) were purchased from Dharmacon (Thermo Fisher Scientific, MA, USA). Cells ( $2 \times 10^5$  cells/well) were seeded in antibiotic-free media in 6-well plates for 24h before siRNA transfection. Pre-plated cells were transfected with control siRNA or 14-3-3 $\gamma$  siRNA at 20 nM using Lipofectamine 2000 (Invitrogen, CA, USA). After transfection for 24h, the cells were harvested for invasion assay and efficiency of siRNA transfection was confirmed by western blot analysis.

### *Western blot analysis*

The siRNA transfected cells were lysed with lysis buffer containing cocktail protease inhibitors (Merck, Darmstadt, Germany). Total proteins were quantified using Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA). Proteins were separated by SDS-PAGE gel

(12%) and transferred to nitrocellulose membrane. The membranes were blocked for 1 h in phosphate buffered saline containing 0.1% Tween 20 and 5% skimmed milk, and incubated for 2h with primary antibodies against 14-3-3 $\gamma$  (C-16, Santa Cruz Biotechnology) and  $\beta$ -actin (Cell Signaling Technology, MA, USA) at a dilution of 1:500. After incubation with 1:5,000 of horseradish-peroxidase-conjugated secondary antibody (Cell Signaling Technology) for 1h. Protein levels were visualized using SuperSignal™ West Pico Chemiluminescent Substrate (Thermo Fisher Scientific). CL-XPosure™ film (Thermo Fisher Scientific) was used for X-ray film exposure. The film was developed in an automated processor. The signal was quantitated by scanning with the Molecular Imager Gel Doc XR+ system (Bio-Rad Laboratories) calibrated densitometer followed by analysis of digital image with Image Lab™ software. Relative protein levels of 14-3-3 $\gamma$  were calculated based on  $\beta$ -actin as the loading control.

#### Invasion assay

Cell invasion assay was performed in Transwell chambers attached with membrane filter (8  $\mu$ m pore size) (Costar, MA, USA). The filters were coated overnight at 37°C with 30  $\mu$ g of Matrigel (BD Bioscience, MA, USA) on the upper surface. The siRNA transfected cells were trypsinized and suspended in RPMI 1640 containing 10% FBS, and then seeded into the upper chamber at  $8 \times 10^4$  cells/200  $\mu$ L/well. The lower chamber was filled with same media (500  $\mu$ L/well). The chambers were incubated for 24 h, at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. The number of invaded cells attached at the lower surface of the filters was determined as previously described (Lirdprapamongkol et al., 2009). The cells on upper surface of the filters were wiped out by using a cotton swab, and then the filters were fixed with 25% methanol and stained with 0.5% of crystal violet solution. The stained cells remained on the lower surface of the filters were extracted with 0.1 N HCl in methanol, and absorbance was determined at 550 nm. Experiments were performed in duplicate wells. Number of invaded 14-3-3 $\gamma$  siRNA transfected cells was compared with number of invaded control siRNA transfected cells and expressed as percent invasion.

#### Statistical analysis

Statistical analysis was performed using Stata software version 12.1. The associations of between protein expression and clinicopathological variables were analyzed by chi-squared test. Survival time was calculated from date of diagnosis to date of death from any cause (end of event) or date of last follow-up (July 2012) if still alive (censored data). The survival probability was constructed by the Kaplan-Meier method and the difference among the survival curves of variable categories were tested using the log-rank test. Cox proportional hazards model was used to identify independent prognostic variables. In all analyses, 14-3-3 $\gamma$  expression were dichotomized to low expression (final score 0-3) and high expression (final score 4-9). For invasion assay, the data were presented as the mean  $\pm$  S.D. from two independent experiments. Statistical significant difference between control siRNA

and 14-3-3 $\gamma$  siRNA transfected cells was analyzed by the two-tailed Student's t-test. A p value less than 0.05 was considered to be statistically significant.

## Results

#### Expression of 14-3-3 $\gamma$ protein

A total of 153 patients with a mean age of 64 were included and clinicopathological variables of the patients are presented in Table 1. Immunostaining of 14-3-3 $\gamma$  protein was observed in the cytoplasm (Figure 1). In a few cases, nuclear staining was also observed. High expression was observed in 91 cases (59.48%) and low expression was observed in 62 cases (40.52%).

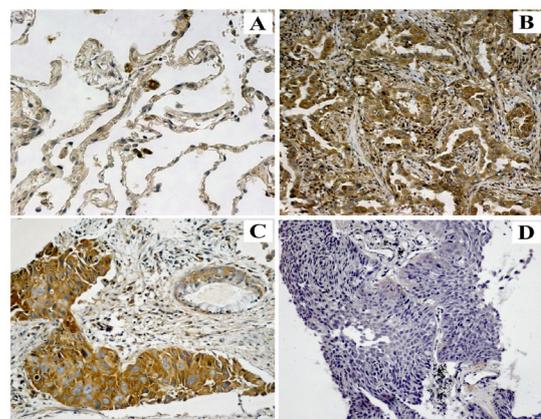
#### Correlation of 14-3-3 $\gamma$ with clinicopathological variables

The associations of clinicopathological variables with 14-3-3 $\gamma$  expressions are shown in Table 1. High expression (moderate to strong expression) was significantly

**Table 1. Correlation between Protein Expression Level and Clinicopathological Variables**

Variable		N	14-3-3 $\gamma$ expression (%)		p value
			Low expression	High expression	
Gender	Male	110	41 (37.27)	69 (62.73)	0.19
	Female	43	21 (48.84)	22 (51.16)	
Age	<60	54	24 (44.44)	30 (55.56)	0.466
	$\geq$ 60	99	38 (38.38)	61 (61.62)	
Histology	ADE	98	46 (46.94)	52 (53.06)	0.087
	SCC	51	15 (29.41)	36 (70.59)	
	NSCLC	4	1 (25.00)	3 (75.00)	
Clinical Stage	III	83	28 (33.73)	55 (66.27)	0.063
	IV	70	34 (48.57)	36 (51.43)	
LN metastasis	No	60	32 (53.33)	28 (46.67)	0.01
	Yes	93	30 (32.26)	63 (67.74)	
Distant metastasis	No	62	18 (29.03)	44 (70.97)	0.017
	Yes	91	44 (48.35)	47 (51.65)	
Treatment	No treatment	58	28 (48.28)	30 (51.72)	0.167
	Chemotherapy	44	19 (43.18)	25 (56.82)	
	Radiotherapy	19	4 (21.05)	15 (78.95)	
	Chemo-radiotherapy	32	11 (34.38)	21 (65.62)	

$\gamma$ , gamma; N, number; ADE, adenocarcinoma; SCC, squamous cell carcinoma; NSCLC, non-small cell lung cancer; LN, lymph node



**Figure 1. Immunohistochemical Staining of 14-3-3 $\gamma$  in Normal Lung Tissue. (no expression, A) and Corresponding Adenocarcinoma (Strong expression, B). Representative squamous cell carcinoma cases with weak expression (C) and strong expression (D). Original magnification; A, 400x and B-D, 200x**

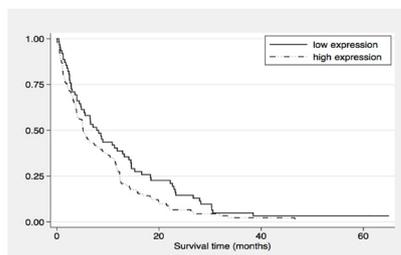
associated with lymph node metastasis (p=0.010) and distant metastasis (p=0.017). A moderate trend toward significance was shown in histology and clinical stage with p value 0.087 and 0.063, respectively. No significant associations between 14-3-3 $\gamma$  expression and other clinicopathological variables, namely gender, age, and treatment were observed.

*Association of 14-3-3 $\gamma$  expression with overall survival*

Median survival time was 5.7 months. A Kaplan-Meier curve showed that advanced NSCLC patients with high 14-3-3 $\gamma$  expression had a poorer overall survival compared to that of low expression with marginal significance (p=0.055) (Figure 2). In univariate analysis (Table 2), only treatment was significantly associated with overall survival (p<0.001). In addition, there was an association of 14-3-3 $\gamma$  expression with overall survival with a marginal trend toward significance (p=0.054) (Table 2). Multivariate analysis showed that age, treatment and 14-3-3 $\gamma$  expression were significant independent prognostic parameters (Table 3). High expression of 14-3-3 $\gamma$  was significantly associated with a poor survival with a risk ratio of 1.56 (95%CI, 1.11-2.20).

*Regulation of cell invasion by 14-3-3 $\gamma$  expression in NSCLC cells*

The significant association between high 14-3-3 $\gamma$  expression and metastasis was observed. Since cancer invasion is a critical step in metastatic process, we further



**Figure 2. Kaplan-Meier Estimates of Overall Survival in NSCLC Patients According to 14-3-3 $\gamma$  Expression**

**Table 2 Univariate Analysis of Clinicopathological Variables for Overall Survival**

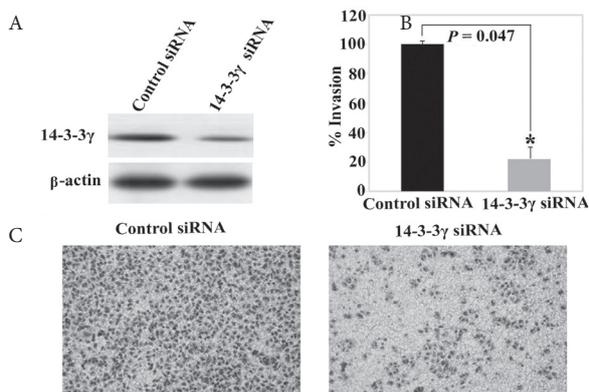
Variable		Risk ratio	95%CI	p value
Gender	Male	1		0.455
	Female	0.87	0.61-1.25	
Age	<60	1		0.512
	≥60	0.89	0.64-1.25	
Histology	ADE	1		0.172
	SCC	1.08	0.77-1.53	
	NSCLC	3.08	1.11-8.56	
Clinical stage	III	1		0.323
	IV	1.18	0.85-1.63	
LN metastasis	No	1		0.097
	Yes	0.75	0.54-1.05	
Treatment	No treatment	1		<0.001
	Chemotherapy	0.49	0.33-0.73	
	Radiotherapy	1.06	0.63-1.79	
	Chemo-radiotherapy	0.35	0.22-0.55	
14-3-3 $\gamma$ expression	Low	1		0.054
	High	1.38	0.99-1.92	

CI, confidence interval; ADE, adenocarcinoma; SCC, squamous cell carcinoma; NSCLC, non-small cell lung cancer; LN, lymph node; g, gamma

**Table 3. Multivariate Analysis of Clinicopathological Variables for Overall Survival**

Variable		Risk ratio	95%CI	p value
Age	<60	1		0.014
	≥60	0.63	0.45-0.91	
Treatment	No treatment	1		<0.001
	Chemotherapy	0.42	0.28-0.64	
	Radiotherapy	0.92	0.54-1.57	
	Chemo-radiotherapy	0.26	0.16-0.42	
14-3-3 $\gamma$ expression	Low	1		0.01
	High	1.56	1.11-2.20	

\*CI, confidence interval; g, gamma



**Figure 3. Effect of siRNA Down-Regulation of 14-3-3 $\gamma$  in A549 Cells, Showing.** Decreased expression of 14-3-3 $\gamma$  by immunoblotting (A); Significantly decreased invasion capability of A549 cells after 14-3-3 $\gamma$  siRNA treatment (B, C)

determined the role of 14-3-3 $\gamma$  on invasion of NSCLC cells. We used siRNA to knockdown 14-3-3 $\gamma$  in A549 human NSCLC cells. At 24 h after transfection, a 66% reduction of 14-3-3 $\gamma$  expression was observed in A549 cells transfected with 14-3-3 $\gamma$  siRNA at 20 nM (Figure 3A). The 14-3-3 $\gamma$  siRNA transfected cells showed a significant decrease in invasion capability by 78% (p=0.047) compared with control siRNA transfected cells (Figure 3B,C).

**Discussion**

Increasing evidence has shown the role of various isoforms of 14-3-3 proteins in tumorigenesis (Wang and Shakes, 1996; Fu et al., 2000; Aitken, 2006), but information on the role of 14-3-3 $\gamma$  is limited. We evaluated the immunohistochemical expression of 14-3-3 $\gamma$  in association with the overall survival of advanced NSCLC patients. A high proportion of cases (59.5%) showed increased expression of 14-3-3 $\gamma$ , which was significantly correlated with poor survival. In addition, in vitro experiments showed that 14-3-3 $\gamma$  affects cell invasion.

14-3-3 proteins have distinct tissue localization and play roles relating to tumorigenesis, which may be tumor and isoform specific. In cholangiocarcinoma, the 14-3-3  $\gamma$ ,  $\eta$ , and  $\theta$  proteins were increased in cholangiocarcinoma tissues compared to adjacent normal bile ducts (Wu et al., 2012). This result suggests that the overexpression of these proteins may contribute to bile duct tumorigenesis. In addition, previous study has demonstrated that 14-3-3  $\beta$ ,  $\gamma$ ,  $\sigma$  and  $\theta$  are overexpressed in lung cancer tissue compared to normal (Qi et al., 2005), indicating that these

four isoforms may be potentially interesting markers for lung cancer. In that study, 10 of 14 (71.4%) lung cancer tissues show overexpression of 14-3-3 $\gamma$  and that SCC (6/8, 75%) have slightly higher frequency of overexpression compared to ADC (4/6, 66.7%). These previous studies are supported by our results with a larger number of cases, which showed increased expression in all cases (60%), in SCC (70.6%) and in ADC (53.1%).

Metastasis is a main cause of mortality in cancer patients. The metastatic process is started in the primary tumor where cancer cells invade surrounding tissues and penetrate into lymphatic and blood circulations which bring them to distant sites. Our results showed that high expression of 14-3-3 $\gamma$  was significantly associated with metastasis to lymph nodes and distant sites, and poor survival in advanced NSCLC. Moreover, down-regulation of 14-3-3 $\gamma$  mediated by siRNA in human NSCLC cells resulted in reduction of invasion capability, suggesting that 14-3-3 $\gamma$  is involved in cancer invasion process. Our findings on the prognostic significance of 14-3-3 $\gamma$  in lung cancer are consistent with other studies in breast (Song et al., 2012) and hepatocellular carcinoma (Ko et al., 2011). In breast cancer, high expression of 14-3-3 $\gamma$  was found to be correlated with tumor size, tumor grade, and lower overall survival rate, while in hepatocellular carcinoma, increased expression of 14-3-3 $\gamma$  was predictive of extrahepatic metastasis and associated with lower survival. These observations suggest that 14-3-3 $\gamma$  contributes to tumor progression, probably via the process of tumor invasion and metastasis.

14-3-3 $\gamma$  has been proposed to be an oncogene with various functional pathways. Radhakrishnan & Martinez demonstrated the ability of 14-3-3 $\gamma$  transfected NIH3T3 mouse fibroblast cells to form tumors in SCID mice and that the transformation is via the activation of MAP kinase and PI3K signaling pathways (Radhakrishnan and Martinez, 2010). In hematopoietic cells, 14-3-3 $\gamma$  promotes cell survival and growth through the activation of PI3K and MAPK signaling cascades (Ajjappala et al., 2009). In human lung cancer cell line H322, 14-3-3 $\gamma$  overexpression results in abnormal DNA replication and polyploidization, indicating that 14-3-3 $\gamma$  may contribute to tumorigenesis by promoting genomic instability (Qi et al., 2007). Recent evidence also indicates that 14-3-3 $\gamma$  is a downstream protein acting as a negative p53 regulator (Radhakrishnan et al., 2011). Thus, previous evidence supports the role of 14-3-3 $\gamma$  on tumor cell survival and growth, but information on cell invasion is lacking. Our study, therefore suggests a potential role of 14-3-3 $\gamma$  on cancer invasion, which is an important step of metastasis. However, it still remains to be elucidated which invasion-related signaling pathway is involved.

In conclusion, our results demonstrated that 14-3-3 $\gamma$  overexpression is an independent prognostic marker among NSCLC patients with advanced stages. Our results indicate that 14-3-3 $\gamma$  may be a candidate marker for identifying patients who may benefit from adjunctive treatments. 14-3-3 $\gamma$  may also be a potential target for novel therapy. In addition, our result indicates that the protein may contribute to tumor progression or metastasis through the process of cancer invasion. Future studies are

required to determine the detailed mechanisms of 14-3-3 $\gamma$  on cancer invasion and metastatic processes.

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