

## Overexpression of 14–3–3 $\gamma$ Induces the Migration and Invasion of Human Lung Adenocarcinoma A549 Cells

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Received 10 March 2020 • Revised 25 October 2020 • Accepted 25 October 2020 • Published online 1 April 2021

### Abstract:

**Objective:** 14–3–3 gamma ( $\gamma$ ) is known to modulate the development and progression of many cancers. However, the evidence in lung cancer is still unclear. In this study, effects of 14–3–3 $\gamma$  on tumor cell migration and invasion were investigated.

**Material and Methods:** A 14–3–3 $\gamma$  expression vector was made and transfected into A549 cells. In-vitro scratch assay and transwell assay were applied to assess migration and invasion, respectively. Western blotting was used to detect expression of proteins related to epithelial–mesenchymal transition.

**Results:** Closing rate of scratch wounds, both in classical and non-classical scratch assay, was significantly increased in 14–3–3 $\gamma$ -overexpressing cells in comparison to the controls. Similarly, by transwell assay, a significant increase in the invasion and migration was shown in the 14–3–3 $\gamma$ -overexpressing cells in comparison to the null vector cells, by approximately 79.2% (p-value=0.002) and 131.2% (p-value<0.001), respectively. In addition, increased 14–3–3 $\gamma$  expression resulted in a significant increase of  $\beta$ -catenin and Snail but not for E-cadherin and vimentin.

**Conclusion:** The study demonstrates the role of 14–3–3 $\gamma$  protein on lung cancer progression via migration and invasion processes, possibly providing a new targeted therapy for non-small cell lung cancer.

**Keywords:** 14–3–3 $\gamma$ , A549 cells, epithelial–mesenchymal transition, invasion, migration

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J Health Sci Med Res .....  
doi: 10.31584/jhsmr.2021796  
www.jhsmr.org

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## Introduction

Lung cancer is the most common malignant tumors, with an incidence (11.6% of the total cases) and mortality rate (18.4% of the total cancer deaths).<sup>1</sup> It is estimated that metastasis, wherein cancer cells spread throughout the body and establish new colonies in a distant organ, remains the cause of 90.1% of deaths.<sup>2</sup> During tumor progression, acquisition of the migration and invasion abilities allows cancer cells to adjacent tissues and distant organ contributing to metastasis.<sup>3</sup> Tumor metastasis is a process involving with several strategies, including switch in cell property from epithelial to mesenchymal phenotype or enhancing the ability of mesenchymal movement.<sup>4,5</sup> Therefore, to control the metastasis, investigation of the key proteins regulating these processes is important for the development new therapeutic targets, so as to reduce mortality.

14-3-3 protein is a group of proteins consisting of seven distinct isoforms in human ( $\beta$ ,  $\gamma$ ,  $\epsilon$ ,  $\eta$ ,  $\sigma$ ,  $\tau$ ,  $\zeta$ ).<sup>6</sup> The 14-3-3 proteins directly bind to phosphor-serine/threonine motifs of various protein partners.<sup>7</sup> These proteins play crucial roles in the regulation of several biological processes; such as: apoptosis, cell signaling, protein trafficking, and cell adhesion and motility.<sup>8</sup> Among 14-3-3 proteins, 14-3-3 gamma ( $\gamma$ ) is one of the isoforms that relates to tumorigenesis. Increased expression of 14-3-3 $\gamma$  has been reported to be associated with poor survival and metastasis in breast<sup>9</sup> and liver cancer.<sup>10</sup> In lung cancer, our previous report has shown that 14-3-3 $\gamma$  overexpression is related to nodal and distant metastasis in advanced non-small cell lung cancer (NSCLC) patients.<sup>11</sup> In addition, we have also revealed that an decreased 14-3-3 $\gamma$  expression reduced invasion and migration abilities of NSCLC cells, by suppressing metalloproteinase enzyme through regulation of the epithelial-mesenchymal transition (EMT).<sup>12</sup> In this study, we once again confirmed our previous results, by further exploration as to whether overexpression of 14-3-3 $\gamma$  promotes the invasion and migration of A549 cells.

## Material and Methods

### Cell culture

Human lung carcinoma cells (A549) were purchased from American Type Culture Collection [ATCC, Rockville, The United States of America (USA)]. Cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 media (Invitrogen, Carlsbad, USA) supplementing with 10.0% fetal bovine serum albumin (FBS), and 1.0% penicillin/streptomycin (Gibco, Grand Island, USA), then the cells were incubated in a humidified 5.0% CO<sub>2</sub> incubator at 37 °C.

### Construction of recombinant deoxyribonucleic acid (DNA)

Total ribonucleic acid (RNA) was extracted using Trizol reagent (Invitrogen). Complementary DNA (cDNA) was reverse-transcribed by Super Script pre-amplification kit (Bio-Rad Laboratories, Hercules, USA). A pair of specific primers, containing EcoRI and XbaI restriction enzyme sites, were designed according the sequence of 14-3-3 $\gamma$  cDNA obtained from GenBank (Accession Number NM\_012479). 14-3-3 $\gamma$  cDNA was amplified by Polymerase Chain Reaction (PCR) using Thermal Cycler (Bio-Rad Laboratories). The amplified 14-3-3 $\gamma$  cDNA was ligated into the pTracer-CMV2 mammalian expression vector (Thermo Fisher Scientific, Waltham, USA). Inserted 14-3-3 $\gamma$  cDNA was confirmed by double restriction enzyme digestion at EcoRI and XbaI sites (New England Biolabs, Ipswich, USA).

### Bacterial transformation

Competent TOP10F' E. coli (Invitrogen) were prepared by the calcium chloride method. The recombinant plasmid pTracer-CMV2-14-3-3 $\gamma$ , and empty vector were transformed to competent cells by heat-shock method. Transformant cells were streaked in Luria-Bertani (LB) agar, containing 100  $\mu$ g/ml of ampicillin (Invitrogen, Carlsbad, USA) and incubated at 37 °C for 16-20 hours (hrs). Single colonies were randomly selected and cultured in LB broth

containing 100  $\mu\text{g/ml}$  of ampicillin at 37 °C and agitated for 12 hrs. Plasmids were extracted and purified by S.N.A.P™ plasmid DNA MiniPrep kit (Invitrogen). Positive plasmids were identified by DNA sequencing.

### Transient transfection

A549 cells were suspended in antibiotic-free media containing 10.0% FBS and seeded into 24-well plates ( $2 \times 10^5$  cells/well) overnight. When the cells were approximately 80.0% confluent, they were then transfected with 20  $\mu\text{g}$  of the recombinant plasmid (Over-14-3-3 $\gamma$ ) by 2  $\mu\text{l/well}$  of FuGENE® HD transfection reagent (Promega, Madison, USA). The cells were then grown at 37 °C in an atmosphere with 5.0% CO<sub>2</sub>, for 48 hrs. The protein expression of 14-3-3 $\gamma$  of the transfected cells was determined by western blotting. Non-transfected cells (NT) and transfected cells with empty vector (Null vector) were assigned as the control.

### 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay

After cell transfection, the A549 cells were re-seeded into 96-well plates at a density of  $0.5 \times 10^5$  cells/well. Then, the cells were cultured and treated with MTT solution as previously described.<sup>12</sup> Mean absorbance value for the NT group was considered as 100% viability, while other groups were evaluated as a percentage of cell viability relative to the NT group.

### Western blotting

Primary antibodies against 14-3-3 $\gamma$  (Santa Cruz Biotechnology, California, USA), E-cadherin, Vimentin,  $\beta$ -catenin, Snail,  $\beta$ -actin, and a horseradish peroxidase-conjugated, secondary antibody (Cell signaling technology, Danvers, USA) were used. Protein preparation and separation were performed as previously described.<sup>12</sup> The membranes were probed with each primary antibody at

a dilution of 1:500 overnight at room temperature, then further incubated with a secondary antibody (dilution of 1:2,000). Level of proteins was visualized using SuperSignal™ West Dura extended duration chemiluminescent substrate (Thermo Fisher Scientific). Images were captured by a couple-charged device (CCD) camera (Vilber Lourmat, Eberhardzell, Germany). Band intensity was quantitated by Image J program version 1.51 (23 April 2018). Relative level of all proteins was calculated based on  $\beta$ -actin as an internal control.

### In vitro scratch assay

Migration of tumor cells was investigated by scratch assay in a 96-well plate. The wells were pre-coated with 4.8  $\mu\text{g/well}$  of Matrigel™ basement membrane matrix (BD Bioscience, Bedford, Massachusetts, USA) at 37 °C for 30 minutes. Following transfection for 48 hrs, cells were harvested and re-suspended in 100  $\mu\text{l}$  of antibiotic-free media containing 10.0% FBS at a density of  $0.8 \times 10^5$  cells/well. Then, the cells were incubated until the cells reached 90.0% confluence. A straight scratch was made by a pipette tip. Antibiotic-free media containing 1.0% FBS was added and cultured for another 24 hrs. The same protocol was used for the classical scratch assay, except that the wells were not pre-coated with the Matrigel. Ability of cell migration was photographed at 0, 6, and 24 hrs using an inverted microscope equipped with a digital camera (Olympus, Tokyo, Japan). Width of wound field was measured as well as which percentage of wound closer was calculated by the following formula:

$$\% \text{ of wound closer} = \left[ \frac{W_{t=0h} - W_{t=\Delta h}}{W_{t=0h}} \right] \times 100$$

Wherein,  $W_{t=0h}$  was the width of wound field immediately after scratching, and  $W_{t=\Delta h}$  was the width of wound field measured at 6 or 24 hrs.

### Transwell migration and invasion assay

Abilities of cell migration and invasion were determined using a transwell insert attaching with 8.0- $\mu$ m pore membranes (Corning Inc., Corning, New York, USA) according to our previous study.<sup>12</sup> A549 cells at a density of  $0.5 \times 10^5$  cells were suspended in an antibiotic-free media containing 1.0% FBS (200  $\mu$ l) and loaded into the insert, upon which 500  $\mu$ l of antibiotic-free media containing 10.0% FBS as a chemoattractant was added to the lower chamber. After 48 hrs, the cells that migrated and invaded into the lower membrane surface were stained with crystal violet solution. The number of stained cells was counted from 10 random fields of each insert.

### Statistical analysis

Mean ( $\pm$ standard deviation, S.D.) was calculated from three independent experiments, each performed in triplicate. Differences between experiment groups were tested by unpaired t-test using GraphPad Prism software (GraphPad Prism, San Diego, California, USA). P-values  $<0.050$  was considered as statistical significance.

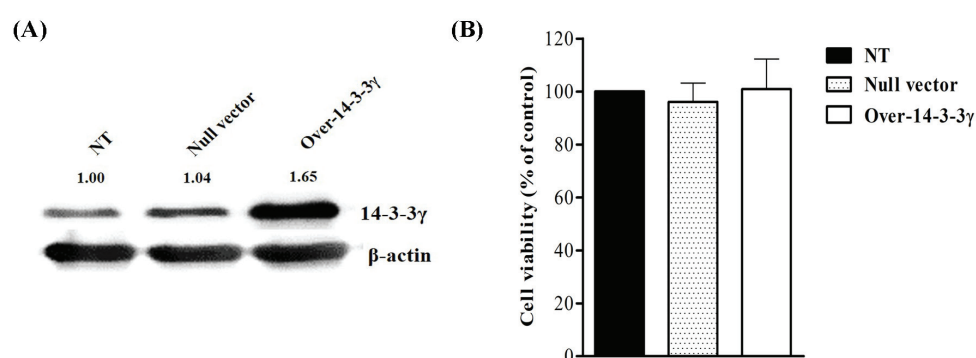
## Results

After transient transfection, the results revealed an increase of 14-3-3 $\gamma$  expression by 65.3% and 61.1% in transfected cells, compared to NT and Null vector groups, respectively (Figure 1A, all p-value $<0.001$ ). Subsequently, the cell viability of the A549 cells was measured by the MTT assay and there was no effect on viability in 14-3-3 $\gamma$ -overexpressing cells (Figure 1B).

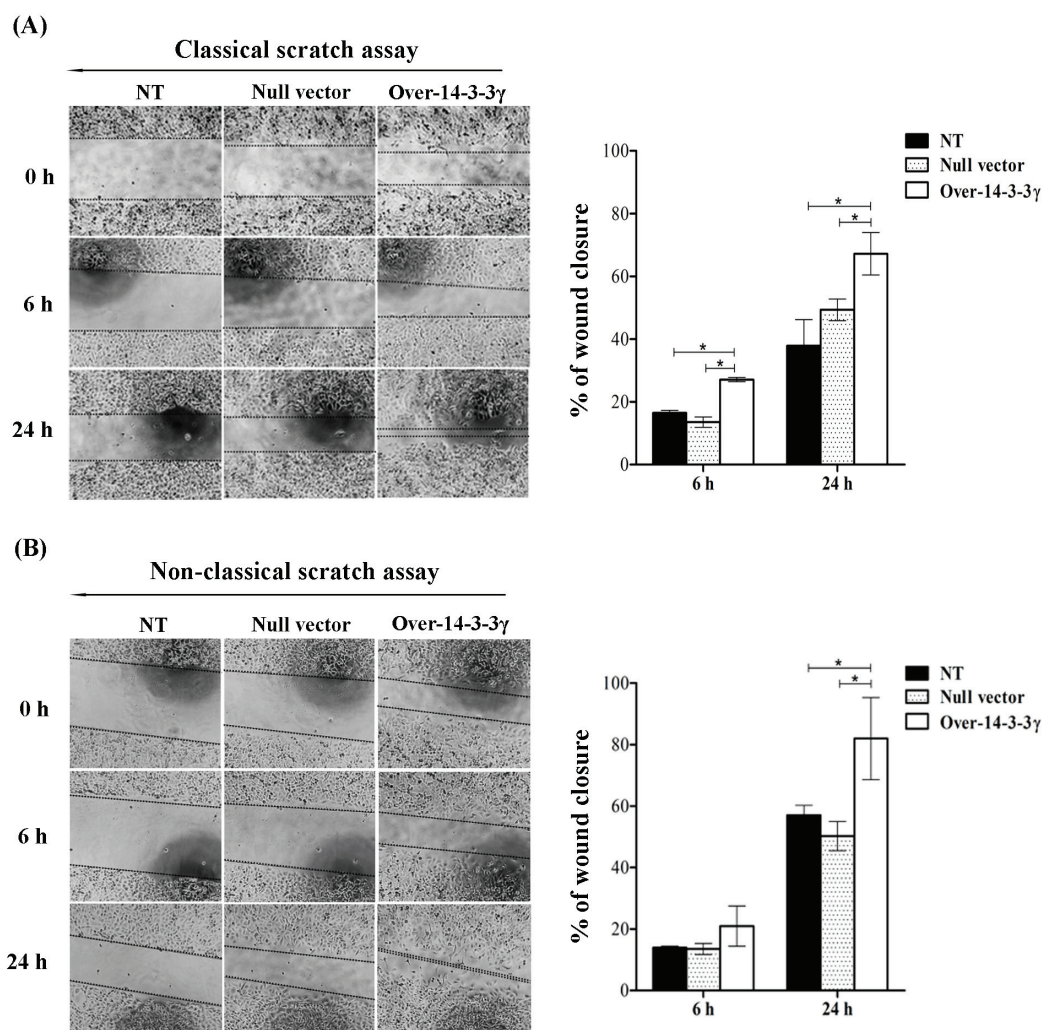
### Effect of 14-3-3 $\gamma$ on wound healing of A549 cells

Analysis of the motility behavior of A549 cells showed that: in classical scratch assay, 14-3-3 $\gamma$ -overexpressing cells exhibited significant increase of migration ability by 33.3% (p-value $<0.001$ ), 36.4% (p-value $<0.001$ ) at 6 hrs, 29.2% (p-value=0.009) and 18.6% (p-value=0.015) at 24 hrs, compared to the NT and Null vector groups, respectively (Figure 2A).

In addition, to the given normal physiological condition of cell migration, the non-classical scratch assay, in which plates were pre-coated with Matrigel<sup>TM</sup>, was also



**Figure 1** Expression of 14-3-3 $\gamma$  protein and cell viability in A549 cell, after overexpression of 14-3-3 $\gamma$  (Over-14-3-3 $\gamma$ ). (A) Western blot analysis of 14-3-3 $\gamma$  protein in A549 cells, after transient transfection, with 14-3-3 $\gamma$  plasmid.  $\beta$ -actin served as a loading control. (B) Effect of overexpression of 14-3-3 $\gamma$  on cell viability determined by the MTT assay. Data are expressed as the mean $\pm$ standard deviation from three, independent experiments. Non-transfected cells (NT), Empty vector-transfected cells (Null vector).



**Figure 2** Effect of 14-3-3 $\gamma$  overexpression (Over-14-3-3 $\gamma$ ) on (A) Classical scratch and (B) Non-classical scratch assay of A549 cells at 6 and 24 hour. Straight scratch was photographed under microscope. Original magnification, 20 $\times$ . Data are expressed as the mean $\pm$ standard deviation from three-independent experiments.

\*Significantly different from non-transfected cells (NT), or empty vector-transfected cells (Null vector) at p-value< 0.050.

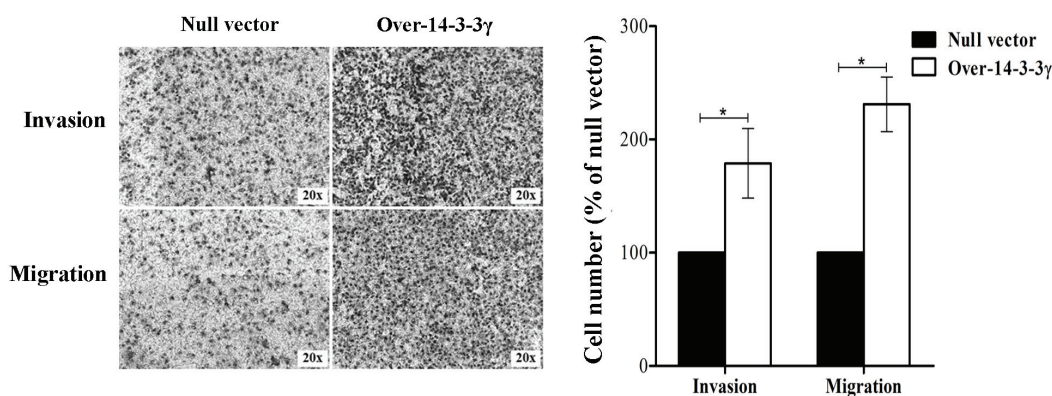
performed. The result showed that the migration ability was obviously increased after overexpression of 14-3-3 $\gamma$  by 25.5% (p-value=0.048) and 32.1% (p-value=0.002), in comparison to the NT and Null vector groups, respectively at 24 hrs (Figure 2B).

#### Effect of 14-3-3 $\gamma$ on transwell migration and invasion abilities of A549 cells

Ability of invasion coupled with migration after 14-3-3 $\gamma$  transfection was determined by transwell assay. The results revealed that: 14-3-3 $\gamma$  displayed significant

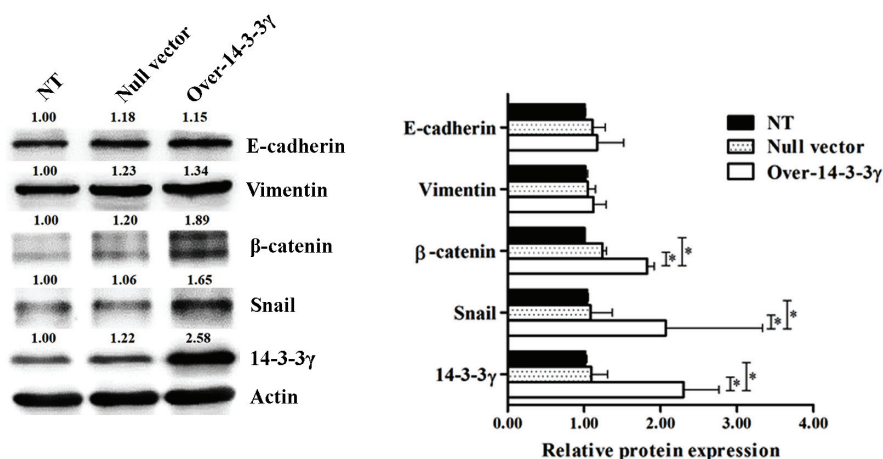
increase in both invasion of A549 cells (79.5% increase in comparison to the Null vector group,  $p$ -value=0.002) in

addition to migration (131.0% increase in comparison to the Null vector group,  $p$ -value<0.001) (Figure 3).



**Figure 3** Effect of 14-3-3 $\gamma$  overexpression (Over-14-3-3 $\gamma$ ) on invasion and migration using A549 cells, as determined by transwell assay. The invading and migrating cells on the lower surface of the membranes were stained with crystal violet. Original magnification, 20 $\times$ . The number of cells was determined under a microscope. Data are expressed as the mean $\pm$ standard deviation from three, independent experiments.

\*Significantly different from empty vector-transfected cells (Null vector) at  $p$ -value<0.050.



**Figure 4** Effect of 14-3-3 $\gamma$  overexpression (Over-14-3-3 $\gamma$ ) on epithelial-mesenchymal transition-associated proteins in A549 cells, as demonstrated by western blot analysis.  $\beta$ -Actin was used as a loading control. Data are expressed as the mean $\pm$ standard deviation from three, independent experiments.

\*Significantly different from non-transfected cells (NT), or empty vector-transfected cells (Null vector) at  $p$ -value<0.050.

### Effect of 14-3-3 $\gamma$ on the expression of EMT-associated proteins in A549 cells

To further understand the molecular mechanism that regulates the cell phenotype, we evaluated the change of EMT-related proteins after overexpression of 14-3-3 $\gamma$  by western blotting. We found no significant alteration of expression level of E-cadherin and vimentin. However, a significant increase of  $\beta$ -catenin was found in 14-3-3 $\gamma$ -overexpressing cells at 81.2% (p-value=0.007) and 47.7% (p-value=0.017), in comparison to the NT and Null vector groups, respectively. Similarly, an elevated expression of 14-3-3 $\gamma$  in A549 cells remarkably increased Snail expression by 44.3% (p-value=0.025) and 52.6% (p-value=0.033), in comparison to NT and Null vector groups, respectively (Figure 4).

## Discussion

In the present study, we demonstrated that overexpression of 14-3-3 $\gamma$  significantly promoted the invasion as well as migration of A549 cells, by enhancing the EMT-associated proteins; such as,  $\beta$ -catenin and Snail, but not E-cadherin and vimentin. It has been revealed that the enhanced expression of 14-3-3 $\gamma$  is significantly associated with tumorigenesis of various cancers. For example, 14-3-3 $\gamma$  is reported to be differentially expressed in head and neck squamous cell carcinoma<sup>13</sup> and osteosarcoma.<sup>14</sup> A study of Ajappala et al. revealed that: IL-3 induces expression of 14-3-3 $\gamma$ , with eventually activates the phosphoinositide 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK), which in turn signals pathways to promote survival and growth of hematopoietic Ba/F3 cells.<sup>15</sup> Similarly in NIH3T3 mouse fibroblast cells, ectopic expression of 14-3-3 $\gamma$  initiates tumor formation in SCID mice, via the activation of PI3K and MAPK pathways.<sup>16</sup> Furthermore, increased expression of 14-3-3 $\gamma$  is shown in hepatocellular carcinoma<sup>10,17</sup> and breast cancer<sup>9</sup>, with a significant association of worse progression-free and overall survival.<sup>9,10</sup>

For lung cancer, several studies have revealed that: the 14-3-3 $\gamma$  is involved with tumorigenesis. Qi et al. (2005) have shown that: 14-3-3 $\gamma$  is one of 14-3-3 isoforms existing only in tumor tissues and NSCLC cell lines (A549 and H358, except in H322), with high abundance.<sup>18</sup> Ectopic expression of 14-3-3 $\gamma$  in H322 cells causes polyploidy promoting cell cycle progression.<sup>19,20</sup> We previously found that 14-3-3 $\gamma$  overexpression in tumor is related to nodal and distant metastasis in advanced NSCLC.<sup>11</sup> In the current study, we confirmed these results in that: 14-3-3 $\gamma$  overexpression significantly enhanced the ability of cell migration and invasion of lung cancer, indicating in the oncogenic role of 14-3-3 $\gamma$ .

Cell migration and invasion are key steps leading to the metastasis of tumors. More recently, Hiraoka et al. have shown that pseudopodial protrusion, which is an associated process with tumor cell migration and invasion, are induced in 14-3-3 $\gamma$ -overexpressing breast cancer cells.<sup>21</sup> In our previous study, we had shown a significant reduction of invasion and migration in 14-3-3 $\gamma$ -suppressing A549 and H358 cells, which is involved with inhibition of matrix metalloproteinase (MMP)-2 and MMP-9, through the regulation of EMT.<sup>12</sup> Over the past several decades, there has been a dramatic increase in the studies showing the association of 14-3-3 protein with EMT-related proteins. By sequence scanning, Hou et al. revealed that Snail, a prominent inducer of EMT by repressing E-cadherin expression, binds directly to 14-3-3 proteins and this interaction significantly induces Snail-mediated repression and EMT.<sup>22</sup> Accordingly, synergistic effect of 14-3-3 $\zeta$ , and atypical protein kinase C leads to Snail expression during the EMT of A549 cells, via the NF- $\kappa$ B pathway<sup>23</sup> and cholangiocarcinoma cells via the GSK-3 $\beta$  pathway.<sup>24</sup> Furthermore, phosphorylation of  $\beta$ -catenin, resulting in the  $\beta$ -catenin/ 14-3-3 $\zeta$  complex formation, promotes cell invasiveness through activation of EMT.<sup>25</sup> A study by Lai et al. (2014) also demonstrated that 14-3-3 $\gamma$  binds directly to  $\beta$ -catenin.<sup>26</sup> Since 14-3-3 proteins function through

binding to various proteins<sup>8,27</sup>, an increase in  $\beta$ -catenin and Snail because of, an increased expression of 14-3-3 $\gamma$  contributes to induction of metastatic phenotype of A549 cells in our study.

Although, substantial alteration of the migration and invasion abilities and EMT-associated proteins was observed, transient expression of 14-3-3 $\gamma$  did not alter the EMT markers, E-cadherin and vimentin in the present study. This result is in contrast to our previous report showing that knockdown of 14-3-3 $\gamma$  contributed to reduction of the expression of vimentin in A549 and H358 cells.<sup>12</sup> It may due to the fact that 14-3-3 $\gamma$  expression level might be not enough to affect the EMT markers. In addition, there is evidence showing that the status of epithelial and mesenchymal markers is not always an indicator for EMT. Hollestelle et al. have found that not all human breast cancer cell lines that have undergone EMT have concomitantly lost E-cadherin, and gained N-cadherin and vimentin.<sup>28</sup> This may indicate that, in our study, a significant alteration of E-cadherin and vimentin is not a necessity for the EMT and other regulating pathways may be involved.

## Conclusion

The results of our study indicate that 14-3-3 $\gamma$  could promote migration and invasion in A549 human lung carcinoma cells. Therefore, 14-3-3 $\gamma$  may be a promising anti-metastasis candidate gene for NSCLC.

## Acknowledgement

This study was funded by Faculty of Medicine, Prince of Songkla University, Songkhla, Thailand (grant no. 570230042). The Research center for cancer control in Thailand are acknowledged for their research facilities.

## Conflict of interest

The authors declare that they have no competing interests.

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