

Expression profile of RhoGDI2 in lung cancers and role of RhoGDI2 in lung cancer metastasis

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Abstract. Rho GDP dissociation inhibitors (RhoGDIs) are important regulators of the GTP hydrolase activity and biological functions of Rho GTPases. RhoGDI2 has been shown to be a metastasis suppressor in bladder cancer and several other cancers. However, the underlying mechanism, effector targets, and the cognate biological functions of RhoGDI2 are not fully understood. To investigate the possible role of RhoGDI2 in lung cancer tumorigenesis and metastasis, the expression pattern of RhoGDI2 in various lung cancer tissue samples and lung cancer-derived cell lines were profiled at both mRNA and protein levels. Furthermore, possible interplay between PI3K/Akt/mTOR pathway activation/inhibition and RhoGDI2 signalling is examined in lung cancer-related cell lines. Our results suggest that RhoGDI2 is likely to be involved in lung tumor malignancy and metastasis.

Introduction

Lung cancer is both the leading cause of cancer-related deaths in men and one of the most common forms of cancer in women, killing more than one million people annually worldwide (<http://www.who.int/mediacentre/factsheets/fs297/en/>). Clinically, lung cancer remains a disease with dismal prognosis. This has been attributed mainly to the low cost-effectiveness of early detection methods and the lack of successful therapeutic options for the highly metastatic forms of lung cancer (1).

Metastasis, recognized as one of the hallmarks of malignancy, is a complex, multi-step process in which cancer cells migrate from the original tumor site to other locations in the body. During metastasis, tumor cells usually exhibit some

characteristics that normal and non-metastatic cancer cells lack. On one hand, these acquired characteristics enhance the invasive and migrant nature of metastatic tumor cells. On the other hand, the expression of certain molecules in normal and benign cancer cells is found to suppress the metastasis process and these molecules are often lost in metastasizing cancer cells, whereas some molecules are found specifically up-regulated in metastatic tumors.

An antithetical group of genes encode molecules that specifically regulate the spread of cancer cells but not the tumorigenicity by targeting multiple steps of the metastatic process (2). One of these molecules, RhoGDI2, was initially identified as metastasis suppressor in bladder cancer and later found to be lost in many metastatic human tumors (3).

RhoGDI2 belongs to a small family of proteins that also include RhoGDI1 and RhoGDI3. RhoGDI2 is primarily expressed in hematopoietic and endothelial cells (4) and is also found differentially expressed in some human cancers. For example, RhoGDI2 suppresses invasion and metastasis in bladder cancer (3,5) and is a positive prognostic factor (6). Moreover, RhoGDI2 is selectively down-regulated in Hodgkin lymphoma cells compared with non-Hodgkin lymphoma (7). In contrast, RhoGDI2 is thought to be involved in the growth and invasive behavior in ovarian, breast and gastric cancers (8-10). Thus, the actual roles of RhoGDI2 protein in tumor progression remain ununified, suggesting that different regulatory pathways may be involved in different tumors.

One of the key functions of Rho GTPase proteins is to remodel cytoskeleton components thus influence cell mobility. Therefore, it is not surprising that regulation of Rho family proteins can have profound impact on metastasis, a process that involves cancer cells migrating from one location to another and invading the destination tissue/organ. While RhoGDI2 was reported to be involved in the regulation of metastasis of several cancer types, the downstream signalling events have not been fully delineated. In bladder, endothelin and neuromedin were identified to be down-regulated by RhoGDI2 through expression profiling (11,12), whereas Rac-1 was found to be bound and activated by RhoGDI2 (13). Interestingly, Src kinase seems to phosphorylate RhoGDI2 at Tyr153, which stimulates the metastasis suppressor activity of RhoGDI2 (14).

Parallel to Rho proteins, there are other signalling pathways that also regulate cytoskeleton dynamics in the cell, e.g., the Akt signalling pathway. Akt/protein kinase B is a major downstream effector of growth factor receptor tyrosine kinases

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that signal via phosphatidylinositol-3 kinase (PI3K) (15). Receptor-mediated activation of PI3K or loss of expression of phosphatase PTEN, one of the most frequently mutated tumor suppressors, often results in elevated Akt activity. The PI3K/Akt pathway plays a major role in several key cellular processes, including cell survival and apoptosis, glucose metabolism, cell cycle progression, and gene expression (16). Furthermore, PI3K/Akt pathway is one of the major up-regulated signalling events during the development of many cancers.

Herein, we explored possible roles of RhoGDI2 in lung cancer, and probed a potential interplay between RhoGDI2 expression and Akt signalling pathway.

Materials and methods

Clinical samples and cell lines. Paraffin-embedded specimens (112) were obtained from the Shengjing Hospital of China Medical University and 20 frozen specimens were obtained from Liaoning Provincial Tumor Hospital; all with consent from patients. Fresh cancerous specimens and corresponding normal tissue samples were plunged into liquid nitrogen immediately after resection and stored at -80°C until the extraction of RNA and protein.

Lung cancer cell lines A549, SPC-A-1, 95D and NCI-H446 were purchased from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. HeLa cell line is a gift of the Department of Cell Biology, China Medical University.

Reagents and antibodies. Recombinant human HGF (rhHGF) was purchased from R&D Systems (Minneapolis, MN). LY294002 and rapamycin were purchased from Sigma Chemical (Sigma, St. Louis, MO). Anti-RhoGDI2 was purchased from Abcam Inc. (Cambridge, MA). Anti-GAPDH were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Immunohistochemistry. Immunostaining was performed using the immunohistochemical SP (streptavidin-peroxidase) method. Sections were deparaffinized in xylene, and rehydrated in gradients of alcohol and water. Tissues were immunohistochemically stained with diluted primary antibody against RhoGDI2 (1:100) overnight at 4°C using a Histostain-Plus kit (Zymed, Invitrogen). Five random non-overlapping views were chosen per slide; at least 100 cells were observed and examined per view at x400 magnification. Labelling scores were determined by the percentage of positive cells per slide. Results were compared using the Shimizu *et al* standard (17).

RT-PCR. Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. RT-PCR was performed using the RNA PCR kit (AMV) version 3.0 (Takara) following the manufacturer's instructions. RT-PCR products were analyzed on 1% agarose gels. The sequences of the PCR primers are: RhoGDI2-upstream (5'-ATCGAATTC atgactgaaaaagccccagag-3'), RhoGDI2-downstream (5'-ATT CTCGAGtcattctgtccactccttc-3'), GAPDH-upstream (5'-GAA GGTGAAGGTCGGAGTC-3'), and GAPDH-downstream (5'-GAAGATGGTGATGGGATTTC-3').

Cell culture. A549, SPC-A-1, 95D and NCI-H446 cells were cultured in RPMI-1640 medium containing 10% fetal calf serum, 100 IU/ml penicillin and 100 µg/ml streptomycin. The cells were grown in a humidified incubator at 37°C and 5% CO₂ air atmosphere. Cells were grown on sterilized tissue culture petri dishes and passaged once every 2-3 days. Various treatment of cells included the addition of rhHGF (10 and 30 ng/ml), LY294002 (5, 20, 50 µmol/l), or rapamycin (10, 20, 40 nM), or the combination of LY294002 and rapamycin (LY 5 µmol/l +R 10 nM, LY 10 µmol/l +R 10 nM).

Immunofluorescence. Coverslips with grown cells were washed with PBS and fixed with 4% paraformaldehyde at room temperature for 20 min before being soaked in anti-RhoGDI2 antibody (1:100) in the wet box at 4°C overnight. After washing away unbound primary antibodies, FITC-labeled goat anti-rabbit secondary antibody was added in the dark room and incubated at 37°C for 30 min. After washing off the unbound secondary antibodies, the results were observed via fluorescence microscopy.

Western blotting. Western blotting was performed using standard techniques as previously described. Briefly, cells were washed twice with PBS buffer and lysed in RIPA lysis buffer (50 mM Tris-Cl pH 7.4, 150 mM NaCl, 0.5% sodium deoxycholate, 1% NP-40, 0.1% SDS, 1 mM EDTA, 100 mM NaF, 1 mM Na₃VO₄, 1 mM PMSF, and 2 µg/ml aprotinin) on ice. Total proteins (50 µg) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes. Membranes were blocked with 5% nonfat milk in TBST (10 mM Tris, pH 7.4, 150 mM NaCl and 0.1% Tween-20) at room temperature for 2 h and incubated with the indicated primary antibodies at 4°C overnight with gentle rocking. After washing with TBST, the membrane was reacted with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h at room temperature. After extensive washing with TBST, proteins were visualized by the enhanced chemiluminescence (ECL) detection kit in accordance with the manufacturer's recommendations.

Monolayer wound healing assay. 95D cells were incubated in 6-well plates to reach full confluence in monolayer. The monolayers were then disrupted by scraping with a pipette tip, incubated with or without drugs, and observed every 2 h until the wound was completely closed.

Matrigel invasion assay. Diluted matrigel was added to the upper chambers of a 24-well transwell. In the upper chambers, 200 µl cells were grown in serum-free medium on 8 µm porous polycarbonate membranes, which were coated with matrigel basement membrane matrix (BD Biosciences, Bedford, MA). The lower chambers were filled with RPMI-1640 medium containing 20% fetal calf serum. After incubation for 24 h at 37°C in a humid atmosphere of 5% CO₂, the cells that had migrated through the pores were fixed with ethanol for 30 min and stained with crystal violet. The number of cells was then counted visually under microscope using an averaged reading obtained in five different viewing fields.



	Case no.			χ^2	P
	Total	-	+		
Gender					
Male	76	46	30	0.393	0.531
Female	36	24	12		
Age					
≤60	59	39	20	0.690	0.406
>60	53	31	22		
Cancer type					
Squamous cell lung cancer	59	34	25	1.263	0.261
Adenocarcinoma	53	36	17		
Cell differentiation level					
High	20	8	12	16.123	<0.01
Medium	73	43	30		
Low	19	19	0		
TNM grade					
I-II	89	48	41	13.573	<0.01
III-IV	23	22	1		
Spread to lymph nodes					
Yes	55	51	4	42.130	<0.01
No	57	19	38		

siRNA knock-down of RhoGDI2 expression in lung cancer cells. Synthetic siRNA (sequences GCAGCUUUGACCA CUCUAA and CCACCAUCACAGAGUGUCU) targeting RhoGDI2 coding region were transfected into A549 cells using the Lipofectamine 2000 kit (Invitrogen) according to manufacturer's instructions. Cells were allowed to recover for 24 or 48 h prior to being subjected to fluorescence-activated cell sorting (FACS) analysis. Each experiment setting was repeated in triplicates and the percentage of cells in different phases of the cell cycle was averaged and recorded.

Statistical analysis. All statistical calculations were performed using SPSS13.0 software. $P>0.05$ were considered statistically significant.

Results

Expression level of RhoGDI2 is inversely correlated with the malignancy grade of the lung cancer tissues studied. We first sought to analyze the expression level of RhoGDI2 in various clinical lung cancer specimens via immunohisto-staining. A strong correlation is observed between the down-regulation of RhoGDI2 expression and the higher malignancy grade (III-IV) of lung cancer ($p<0.01$, see Table I). Accordingly, lower cell differentiation level and greater lymph node metastasis propensity are also associated with lower level of RhoGDI2 expression ($p<0.01$, Table I). Other

factors, such as age and gender, do not seem to be linked with RhoGDI2 expression ($p>0.05$, Table I). Interestingly, RhoGDI2 expression seems to be stronger in squamous carcinoma than in adenocarcinoma specimens. However, the result of statistical analysis prevents us from assigning any meaningful significance to this finding ($p=0.261$, Table I). A representative comparison in RhoGDI2 expression between cancerous and paracancerous lung tissues is shown in Fig. 1.

We were interested in finding out whether the down-regulation of RhoGDI2 expression occurs at mRNA level as well. For this purpose, total RNAs were extracted from lung cancer specimen and normal lung tissue of the same patient, respectively. RT-PCR results indicate that RhoGDI2 mRNA level is significantly lower in lung cancer tissue than that in normal tissue. A representative gel of the RT-PCR products obtained from clinical specimens is shown in Fig. 2A. Whether the low level of RhoGDI2 mRNA present in lung cancer cells is a result of down-regulated transcription activity or of reduced mRNA processing/stability remains unclear.

Expression and intracellular localization of RhoGDI2 in lung cancer-derived cell lines. We next examined the expression level of RhoGDI2 in lung cancer-derived cell lines using RT-PCR and Western blots. While RhoGDI2 is weakly expressed in A549 and NCI-H446, the expression levels in 95D and SPC-A-1 seem to be 2- to 3-fold higher than those observed

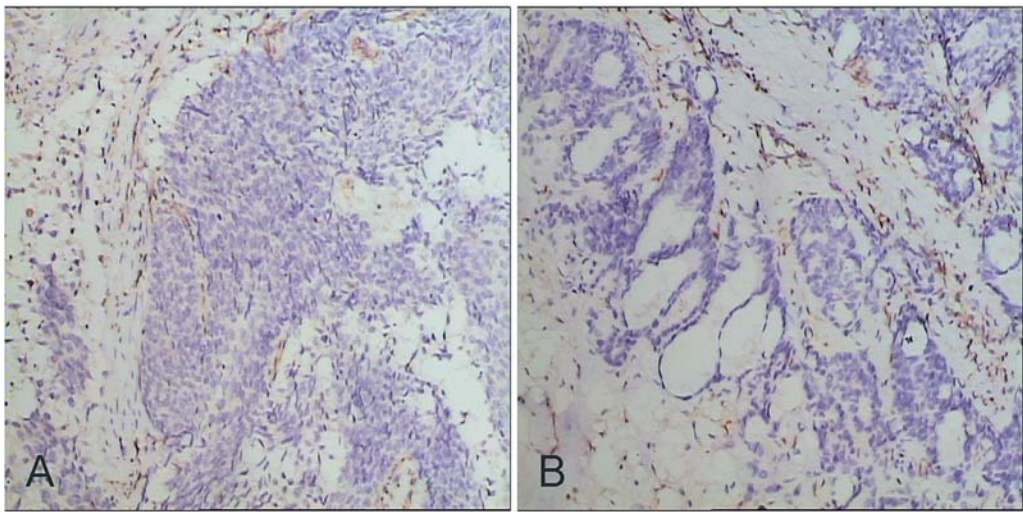


Figure 1. Expression of RhoGDI2 is down-regulated in both squamous lung cell carcinoma and adenocarcinoma. (A) Squamous lung cell carcinoma; (B) adenocarcinoma.

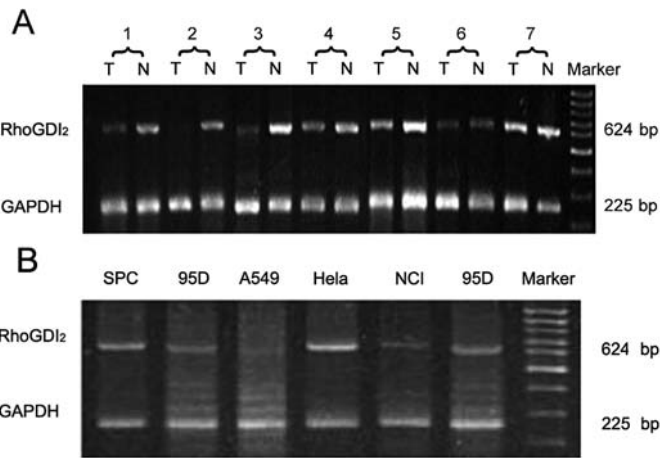


Figure 2. mRNA levels of RhoGDI2 are down-regulated in lung cancer cells compared to normal tissue as detected by RT-PCR. RT-PCR was performed on total RNA extracted from (A) clinical specimens and (B) cultured cell lines. T, tumor site extraction; N, normal tissue near the tumor site.

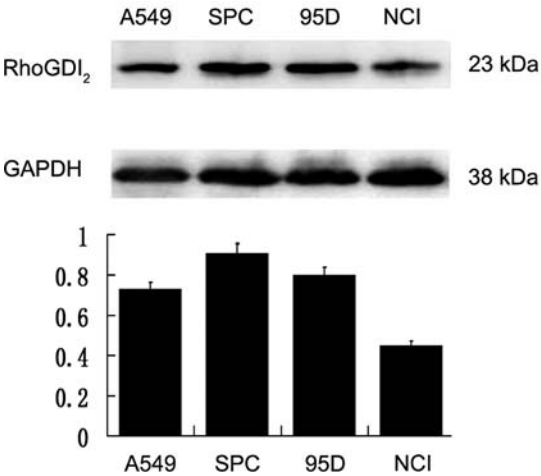


Figure 3. Expression of RhoGDI2 in four lung cancer-derived cell lines. The expression of RhoGDI2 in A549, SPC-A-1, 95D, and NCI-H446 was examined via Western blotting and quantified using endogenous GAPDH as reference.

in the former cell lines (Figs. 2B and 3). To investigate whether lung cancer-derived cell lines express RhoGDI2 in its cognate intracellular compartment, we performed immunofluorescence microscopy on cultured A549 cells. We found that RhoGDI2 is localized primarily in the cytoplasm of A549 cells (data not shown), which is in accordance to its known regulatory functions.

Potential relationship between RhoGDI2 and PI3K/Akt/mTOR signalling pathway. Given its prominent role in the oncogenesis and development of many lung cancers, the PI3K/Akt/mTOR pathway was chosen as a potential signalling event that cross-talks with RhoGDI2 in lung cancer cells. Recombinant human hepatocyte growth factor (rhHGF) is known to promote the growth, motility and invasive ability of 95D cells through the activation of Akt pathway, whereas

LY294002 and rapamycin negatively affect these attributes by inhibiting PI3K activity and mTOR pathway, respectively. In serum-free culture of 95D cells, the expression of rhoGDI2 is higher than that in 95D cells cultured in the presence of 10% fetal bovine serum. rhHGF treatment (10 ng/ml) did not affect the expression of RhoGDI2 compared to that in 95D cell culture in 10% FBS, whereas 30 ng/ml rhHGF significantly reduced it (Fig. 4). This suggests that there may exist an antagonistic relationship between the PI3K/Akt/mTOR pathway and RhoGDI2 expression in lung cancer cells. This position is further supported by the observation that treatment of 95D cells with LY294002 increased RhoGDI2 expression in a dose-dependent manner (Fig. 5). Moreover, low dosages of rapamycin treatment (10 and 20 nM) enhanced RhoGDI2 expression, whereas higher dosage of rapamycin at 40 nM actually reduced the level of RhoGDI2, probably because of significant cell deaths (data not shown) or other

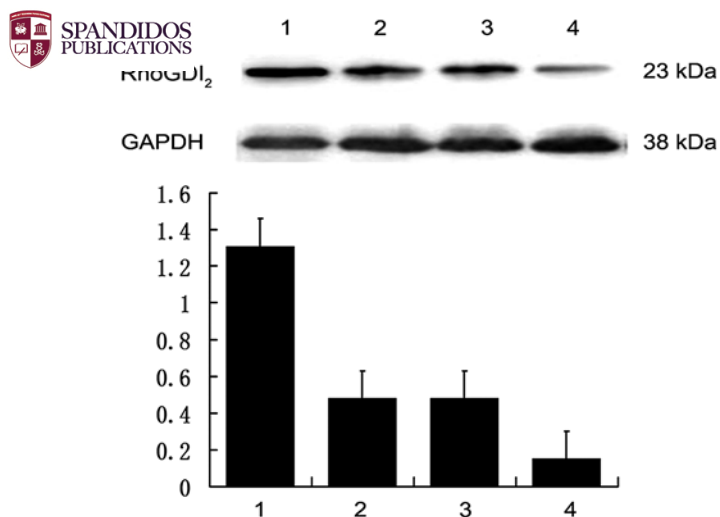


Figure 4. Effect on RhoGDI2 expression in 95D treated with PI3K/Akt/mTOR agonist. The expression levels of RhoGDI2 as examined via Western blotting and charted results below. 1, Serum-free culture; 2, 10% FBS supplemented; 3, 10 ng/ml rhHGF supplemented; 4, 30 ng/ml rhHGF supplemented.

signalling pathways that had become activated at this concentration of rapamycin (see Discussion). Finally, combined treatment of LY294002 and rapamycin additively increased RhoGDI2 expression, implying that the target molecules of these inhibitors each contribute via a different path to the down-regulation of RhoGDI2 in lung cancer cells.

Expression of RhoGDI2 does not positively contribute to cancer cell growth in lung cancer-derived cell lines. Since elevated RhoGDI2 expression was found in breast cancer cell lines and positively linked to breast cancer cell invasion (10,18), we were interested in checking whether RhoGDI2 expression is required for the growth of lung cancer-derived cells. When endogenous RhoGDI2 expression was knocked down by transfecting synthetic RhoGDI2-specific siRNAs into A549 cells, the distribution of S-phase vs. G2-phase cells did not change significantly from those of mock-transfected cells as analyzed by FACS (data not shown). Furthermore, RhoGDI2 knock-down cells did not exhibit higher degree of cell death either in the presence or absence of rapamycin or LY294002. These results indicate that RhoGDI2 does not positively contribute to the growth and survival in lung cancer cells *in vitro*.

RhoGDI2 is involved in cell mobility and invasion of lung cancer-derived cell lines. We were also interested in finding whether RhoGDI2 knock-down cells exhibited elevated activities in cell invasion (30% more than mock-transfected A549 cells). In addition, the siRNA-treated cells showed a marked shortening of time required for wound healing compared to native or mock-transfected A549 cells. These results suggest that RhoGDI2 is a candidate metastasis suppressor in lung cancer. In agreement with this, the treatment of 95D cells with rhHGF enhanced cell invasion and wound healing, whereas the addition of LY294002 and/or rapamycin inhibited these activities (Fig. 6).

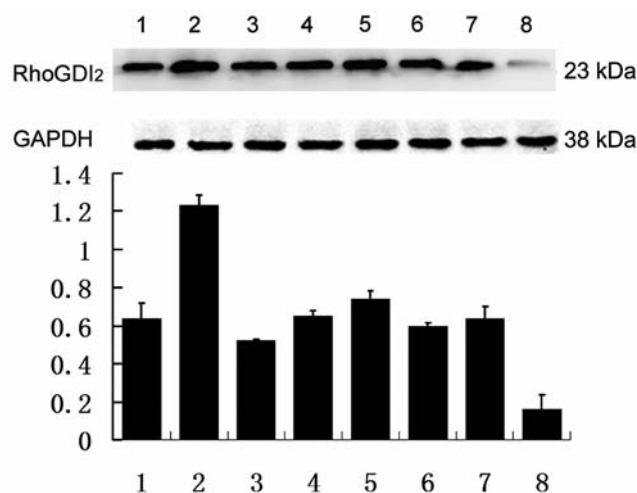


Figure 5. Effects on RhoGDI2 expression in 95D treated with PI3K/Akt/mTOR antagonist. The expression levels of RhoGDI2 as examined via Western blotting and charted results below. 1, LY294002 (LY) 5 μmol/l + rapamycin (R) 10 nM; 2, LY 10 μmol/l + R 10 nM; 3, LY 5 μmol/l; 4, LY 20 μmol/l; 5, LY 50 μmol/l; 6, R 10 nM; 7, R 20 nM; 8, R 40 nM.

Discussion

RhoGDI2 is known to be a metastasis suppressor in bladder cancer. In sharp contrast, RhoGDI2 promotes cell invasion in breast, ovarian and gastric cancers. However, its role in the oncogenesis, development and metastasis in lung cancer has not been systematically examined so far. In the present study, we aimed to address the following questions: 1) Is RhoGDI2 involved in the oncogenesis, development and metastasis of lung cancer? 2) What are the signalling events involving RhoGDI2 in lung cancer? The expression profile of RhoGDI2 in clinical specimens showed a strong correlation of RhoGDI2 down-regulation and higher malignancy grade of the tumor. This suggests that RhoGDI2 may be involved the development of lung cancer. Expression of RhoGDI2 is also inversely correlated with degree of cell differentiation and propensity of lymph node metastasis in tumor specimens, further suggesting that RhoGDI2 may be involved in lung cancer metastasis. When we examined expression of RhoGDI2 in cultured lung cancer-derived cells, we found that RhoGDI2 expresses at varying levels from cell line to cell line. On one hand, this phenomenon is similar to the varying levels of RhoGDI2 expression detected in clinical specimens. On the other hand, since all four cell lines examined so far showed at least some degree of RhoGDI2 expression, we posit that the anti-metastatic signalling events downstream of RhoGDI2 could have been countered in these cells via some unknown yet reversible mechanisms.

We next focused our study on examining possible signalling events that may involve RhoGDI2 in the progression of lung cancers. Akt signalling pathway is often found activated in most lung cancers. Up-regulation of Akt signalling is positively associated with the malignancy of lung cancers: high level of phosphorylated Akt was detected in excised non-small cell lung cancer (NSCLC) tumor tissue via immunohistochemical method (19). Furthermore, activation of Akt signalling is reported in bronchioloalveolar carcinoma but

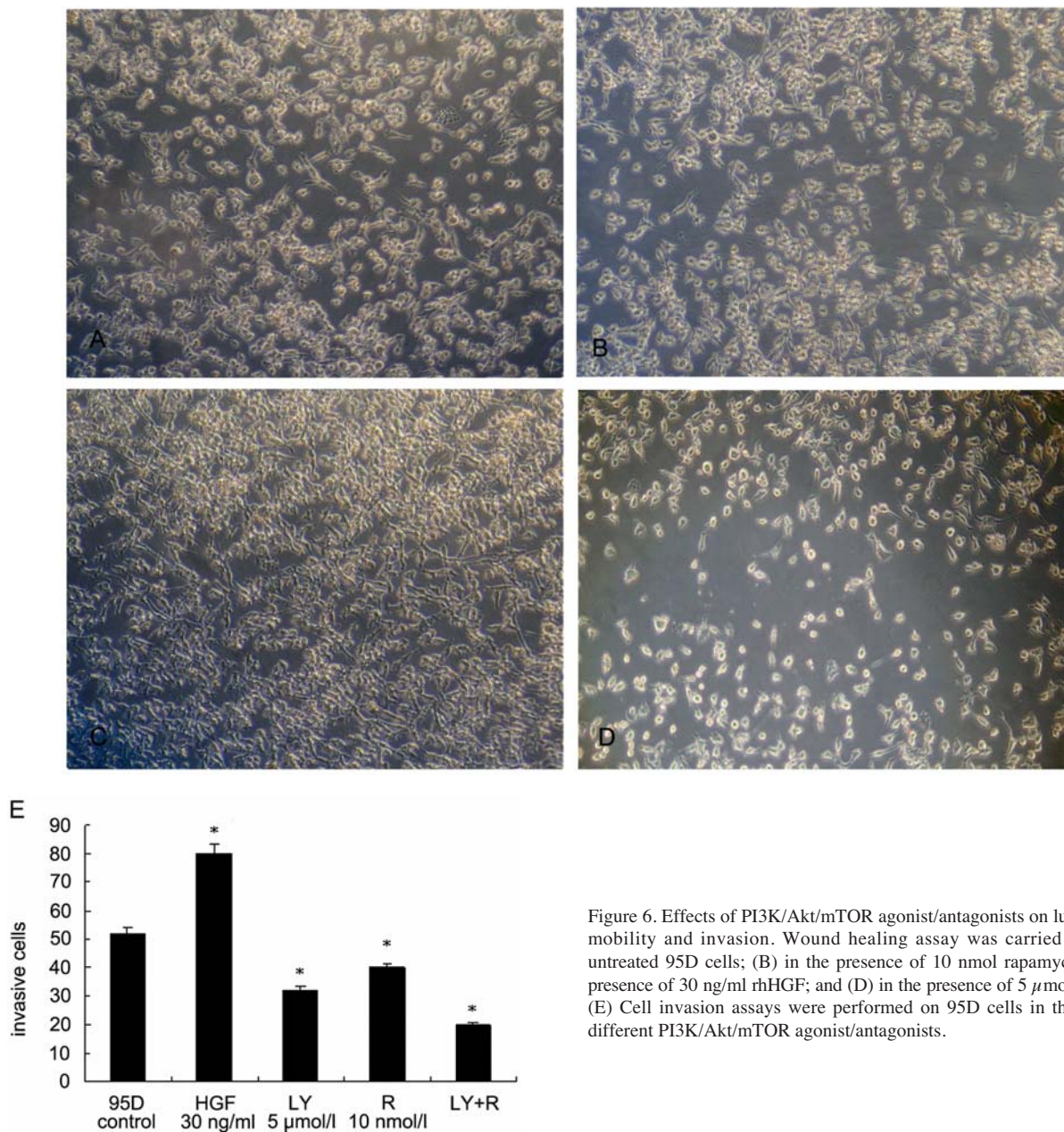


Figure 6. Effects of PI3K/Akt/mTOR agonist/antagonists on lung cancer cell mobility and invasion. Wound healing assay was carried out with (A) untreated 95D cells; (B) in the presence of 10 nmol rapamycin; (C) in the presence of 30 ng/ml rhHGF; and (D) in the presence of 5 μ mol/l LY294002. (E) Cell invasion assays were performed on 95D cells in the presence of different PI3K/Akt/mTOR agonist/antagonists.

not the corresponding normal cells, suggesting that Akt may play a role in cancer progression to malignancy (20). We were therefore very interested in finding out whether RhoGDI2 signalling events cross-talk with the PI3K/Akt/mTOR pathways. Using specific agonist/antagonists of the PI3K/Akt/mTOR pathways, we were able to show that the expression of RhoGDI2 is inversely correlated to the level of activity of the Akt/mTOR pathway. Inhibition of PI3K or mTOR alone leads to elevated RhoGDI2 expression and negative impact on cell mobility and invasion *in vitro*, with the combined inactivation of PI3K and mTOR achieving the best anti-metastasis effects. This suggests that for lung cancer patients with functional RhoGDI2 gene, it may be possible to reactivate RhoGDI2 expression thus inhibit metastasis and cancer progression.

Interestingly, our results with the double inhibition of mTOR and PI3K echoes the recent finding that dual inhibition of parallel signalling pathways in lung cancer such as those via the activation of PI3K and MAPK can prevent undesired

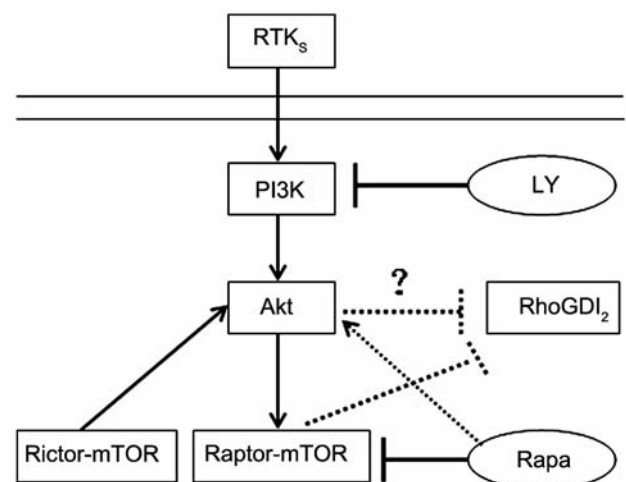


Figure 7. The possible interactions between RhoGDI2 and PI3K/Akt/mTOR pathway. Arrows indicate stimulation, whereas T-lines indicate inhibition. Dotted lines are hypothetical interactions proposed in text.



SPANDIDOS PUBLICATIONS pathway activation induced by inhibitor-mediated negative feedback loops (21). These authors further proposed that the combined inhibition of PI3K and MAPK pathways may provide much better therapy against tumors as parallel signalling pathways are blocked simultaneously. In a similar thread of thought, we think that there might be a clinical benefit in applying combinatorial treatments targeting different components of the PI3K/Akt/mTOR signalling pathways in the treatment of lung cancer.

Based on our data, we propose a potential interaction map between the known components of PI3K/Akt/mTOR pathway and RhoGDI2 in lung cancer metastasis (Fig. 7). In this hypothetical model, both mTOR and Akt can independently antagonize the metastasis suppressor activity of RhoGDI2 by down-regulating its expression. This explains the observation that the dual inhibition by LY294002 and rapamycin led to an additive enhancement on RhoGDI2 expression. Since rapamycin is active on Raptor-mTOR but not Rictor-mTOR complex, higher concentration of rapamycin may shift the intracellular balance from Raptor-mTOR to Rictor-mTOR, which activates Akt by phosphorylating Ser473 in Akt (22). Furthermore, the release of feedback loop inhibition by mTOR inhibitors in cancer cells can lead to upstream receptor tyrosine kinase activation, which again leads to activation of Akt (23). Either or both of these mechanisms could be the reason underlying our observation that RhoGDI2 expression was lower when higher dosage of rapamycin (40 nM) was added to 95D cells.

Despite recent advances in research, lung cancer remains a top killer in cancer-related deaths. In China, the 5-year survival rate of lung cancer patients is <10%. WHO warns that lung cancer and AIDS will be the two top causes of death in the 21st century. Metastasis is found in 90% of lung cancer-caused deaths. Since metastasis involves different cellular processes than those that participate in the onset and early stages of tumorigenesis, metastatic cancers can be more effectively treated if the metastasis process can be specifically targeted by therapeutics. Moreover, because the survival rate for patients diagnosed with metastatic lung cancer is dramatically lower than that of patients diagnosed with pre- or non-metastatic forms, identifying key molecules involved in metastasis shall greatly help early diagnosis of metastatic lung cancers, thus reducing patient fatality. Our results point to the direction that RhoGDI2 may function as a potential molecular marker in metastatic lung cancer, which warrants further investigation of its interaction with other signalling pathways involved in lung cancer.

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