Expression analysis of Cdc42 in lung cancer and modulation of its expression by curcumin in lung cancer cell lines

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Abstract. Cdc42, a Rho GTPase family member, is involved in cell transformation, proliferation, survival, invasion and metastasis of human cancer cells. Overexpression of Cdc42 has been reported in several types of human cancer. However, the underlying mechanisms are not well understood. The present study showed that Cdc42 was overexpressed in 80 of 110 primary lung cancer patients, and overexpression of Cdc42 was significantly associated with high TNM stage and lymph node metastasis. Moreover, RNAi-mediated suppression of Cdc42 expression reduced actin filopodia formation, migration and invasion potential of a highly metastatic lung cancer cell line, 801D. In parallel, 801D cells were treated with curcumin and the effect on the expression of the Cdc42 gene at the transcriptional and translational levels was analyzed by RT-PCR and Western blotting. Curcumin inhibited cell migration, invasion and downregulated Cdc42 gene and Cdc42-related target gene expression in 801D cells. It also induced rearrangements of the actin cytoskeleton. These effects mimicked those of Cdc42 knockdown. Furthermore, xenograft experiments confirmed the suppression of tumor growth and invasion in vivo, which was due to the effect of curcumin and the inhibition of Cdc42 by curcumin. Our results showing the downregulation of Cdc42 expression by curcumin in lung cancer cells taken together with the clinical data suggest a potential therapeutic role for curcumin in inducing Cdc42-mediated inhibition of invasion of lung cancer cells.

Introduction

Cdc42 is a member of Rho family of GTPases, which belongs to the Ras superfamily, Cdc42 acts as an important molecular switch (1). Some studies in primary gene targeted mammalian cells have shown that Cdc42 is critically involved in actin filopodia formation, cell motility, directional migration and cell growth (2). Other researchers also found that Cdc42 was up-regulated in several types of human cancers, the overexpression of Cdc42 is associated with carcinogenesis and progression of many human tumor types (3-5). Furthermore, the up-regulated Cdc42 activity may impair c-Cbl-mediated EGFR degradation, contribute to EGFR hyperactivity and induce proteasomal degradation of p21(CIP1) leading to an increase in cell proliferation and migration (6,7). In contrast, downregulation of Cdc42 signals can inhibit anchorage-independent growth and induce apoptosis via the PI(3)K-Akt and Erk signaling cascades and the p53 tumor suppressor (8). Consistent with these reports, Cdc42 silencing by small hairpin RNA was able to reverse the metastatic and growth behavior of human colorectal cancer cells and bladder cancer cells (9,10). Taken together, the observed effects of Cdc42 over-expression and silencing on the cell malignant transformation indicate a role for Cdc42 in regulating tumor metastasis and progression.

Curcumin (diferuloylmethane), an active component of the spice turmeric (Curcuma longa), possesses biological activities include chemopreventive and therapeutic properties against many tumors in both in vitro and in vivo models and in clinical trials (11,12). Curcumin has been reported to inhibit cell proliferation and induce apoptosis in different cancer cells (11-13). Furthermore, curcumin has been shown to inhibit invasion, angiogenesis, and metastasis of cancer cells (14). Many mechanisms are involved in the biological activities of curcumin including the nuclear factor-κB, IκB kinase, Akt, activator protein-1, mitogen-activated protein kinases (MAPK), cyclooxygenase-2 lipoxygenase, inducible nitric-oxide synthase, urinary plasminogen activator, tumor necrosis factor, chemokines, and cell cycle machinery have been suggested as the targets of curcumin (15-17). Our previous work showed that curcumin mediated inhibition of cell proliferation and induction of apoptosis via modulation of reactive oxygen species-dependent mitochondrial signaling pathway.

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in lung cancer (13). Some studies on the anti-cancer effect of curcumin have focused on its inhibition of invasion and metastasis in several cancer cell lines (18-20). However, the precise impact and related molecular mechanism of curcumin on tumor metastasis remain to be elucidated.

In the present study, our findings showed that overexpression of Cdc42 was widespread in primary lung cancer patients. Knocking down of Cdc42 expression by shRNA against Cdc42 suppressed lung cancer cell migration, invasion and induced rearrangements of the actin cytoskeleton. Furthermore, we found that curcumin could efficiently suppress the migration, invasion and the formation of actin filopodia in lung cancer cells. In parallel, we observed that curcumin down-regulated the Cdc42 gene and Cdc42-related target genes expression in 801D cells. These effects mimicked those of Cdc42 knockdown, suggesting that the inhibition of endogenous Cdc42 expression in vitro and in vivo by curcumin may contribute to the inhibition of cell migration and invasion in these cells.

Materials and methods

Reagents and cell lines. Cdc42, E-cadherin, uPA, uPAR, cathepsin B and cathepsin D antibody, TRITC-phalloidin, 4% paraformaldehyde, crystal violet, hematoxylin and eosin (H&E) were purchased from Sigma. Human lung cancer cell lines (95D, 801D, A549, and 95C) and human bronchial epithelial cells (BEAS-2B) (a gift from Dr Guohua Lu, Zhejiang University) were cultured in RPMI-1640 medium at 37°C in 5% CO2, Cdc42-shRNA (Cdc42 target sequence 5'-CTTGCCAAGAACAAACAGA-3') plasmid (pGPU6/Neo-Cdc42) and empty plasmid vector (pGPU6/GFP/Neo-control) were obtained from Zhe Jiang Chinese Medical University.

Patients and immunohistochemistry. This study was approved by the Institutional Review Board of the 117th Hospital of PLA (Hangzhou, China). Surgically resected specimens from 110 patients with lung adenocarcinoma (between June 2002 and December 2005) were examined. All cases were staged according to the International Staging System (21). None of these patients received adjuvant or neoadjuvant chemotherapy or radiation treatment prior to surgery. In our series, all of the patients had complete medical records and follow-up. Immunohistochemical staining and evaluation were described in previous studies (22).

Cell transfections. The shRNA expression plasmids were transfected into 801-D cells using Lipofectamine 2000 (Invitrogen, USA) as described previously (23). Stable GFP-positive clones were obtained by flow sort. The expanded cells were then used for subsequent studies, Cdc42 expression was determined by Western blotting.

Migration and invasion assay. Wound healing experiment and transwell insert (24-well insert; pore size 8 μm, Corning, USA) assay were used to explore the effect of Cdc42 silence and curcumin on migration and invasion of 801D cells, respectively as described previously (23). Briefly, for wound healing experiment, cells were grown to confluence and then wounded using a pipette tip and all were photographed at 0 h and subsequent time-points, cell migration was evaluated by measuring the width of the wound at the identical position, and for invasion assay, chambers of matrigel coated invasion plates were used, 50,000 cells were added to upper chamber in serum-free media and invasion at 37°C towards 10% FBS containing growth media was determined after 24 h. Cells invaded through the membrane were fixed, stained with crystal violet and counted under a light microscope. All experiments were carried out in triplicate.

Western blot analysis. Cells were lysed in a lysis buffer (50 mM Tris-HCl, pH 7.8, 150 mM NaCl, 1% NP40, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride), as previously described (16). Briefly, the lysates were resolved on 10% SDS-PAGE gels, then transferred to PVDF membranes, blocked with 5% non-fat milk/BSA, probed with primary and corresponding secondary antibodies and detected by ECL reagent.

Semi-quantitative RT-PCR analysis. Total RNA was isolated from each group of cells using TRIzol reagent, according to the manufacturer's instructions. Obtained cDNAs were amplified using specific primers, primers for human Cdc42: 5'-GCCCGTGACCTGAAGGCTGTCA-3' (sense) and 5'-TGCTTTTAGTATGATGCCGACACCA-3' (antisense). Following three washes with PBS, cells were permeabilized for 1 min at 55˚C and finally extension for 1 min at 72°C.

In vivo xenograft experiments. Approximately 1x107 logarithmically growing 801D cells were inoculated in BALB/c nude/nude mice. Each experimental group consisted of three mice. After 4 weeks of observation, the mice were sacrificed and tumors were stripped (24). Tumor volume was measured and tumor volume was calculated according to the formula: Tumor volume (mm³) = L x W²/2 (where L is the length and W is the width).

Statistical analysis. SPSS version 11.5 for Windows was used for all analyses. The χ² test was used to examine possible correlations between the expression profiles of Cdc42, and any abnormal expression, as well as in comparison with clinicopathologic factors. The data were expressed as mean ± SD and One way ANOVA test was used to determine the significance of the multiple comparisons. The Kaplan-Meier method was used to estimate the probability of patient survival, and the log-rank test was used to evaluate differences in survival between patient subgroups. Differences were considered significant at P<0.05.

Results

Immunohistochemical analysis of Cdc42 expression in lung cancer samples. To study the role of Cdc42 in lung cancer, we
analyzed its expression pattern in 110 lung adenocarcinoma cases and 30 normal paraneoplastic lung tissues (>1.5 cm away from the tumor). Among these 30 cases normal lung tissues, weak to no expression was observed (Fig. 1A). However, 110 primary lung adenocarcinoma samples were found to be Cdc42-positive (72.72%, 80/110) (Fig. 1B-D), suggesting that Cdc42 was overexpressed in lung adenocarcinoma patients. Cdc42 high expression was significantly associated with lymph nodal metastasis and TMN staging, but not correlated with other factors (such as age, sex, tumor size, and differentiation) (Table I). Additionally, Kaplan-Meier analysis revealed that the 5-year survival rate of patients with negative shRNA expression was 36.67%, which was significantly higher than that of patients with Cdc42-positive expression (13.75%, $\chi^2=7.16$, $P=0.007$) (Fig. 1E). These results showed that Cdc42 expression levels could be a prognostic marker for metastasis in lung cancer patients.

Expression of Cdc42 in human lung cancer cell lines with different metastasis potentials. To clarify the functional relationship between Cdc42 expression and the ability of tumor metastasis in human lung cancer cell lines 801D, 95D, A549 and 95C, Cdc42 expression in protein levels were detected by Western blotting (Fig. 2A). Of four human lung cancer cell lines, Cdc42 expression in highly metastatic cells 801D and 95D was the strongest, whereas the weakest in the low-metastatic 95C cells, suggesting that Cdc42 expression positively associated with metastatic potentials of human lung cancer cells. Interestingly, Cdc42 expression in human bronchial epithelial cells (BEAS-2B) was much weaker. In addition, expression of Cdc42 protein decreased significantly in 801D cells with transfected Cdc42-shRNA plasmid compared to the cells with shRNA-control, shown by Western blot analysis (Fig. 2B). Together, these results suggested that Cdc42 was overexpressed in highly metastatic lung cancer cells. Thus, 801D cells were selected to further investigate the effect of specific silencing of Cdc42 on metastasis behavior such as tumor cell invasion and cell motility.

Silencing of Cdc42 suppresses the ability of cell migration and invasion of 801D cells. Increased cell motility and invasion of
carcinoma cells are key steps in the metastatic cascade. We next examined the role of Cdc42 in the ability of cell invasion of 801D cells. The invasive cell number of 801D transfected with Cdc42-shRNA was significantly less than that of 801D cells transfected with negative-shRNA plasmid (Fig. 3A). Consistent with the invasion results, wound healing experiment results showed that Cdc42-shRNA expressing 801D cells closed the scratch-wounds more slowly than cells untreated or transfected with negative-shRNA plasmid (Fig. 3B). These data demonstrated the importance of Cdc42 in lung cancer cell migration and invasion.

Silencing of Cdc42 suppresses rearrangements of the actin cytoskeleton in lung cancer cells. Because actin cytoskeletal reorganization is considered to be one of the most important functions of RhoGTPases (25), we stained F-actin in the untreated and shRNA-transfected cells with TRITC-conjugated phalloidin visualizing under a confocal microscope. As shown in Fig. 4A and B, 801D-negative-shRNA control cells and 801D cells showed a cross-linked actin network. Deletion of Cdc42 reduced the appearance of a cross-linked actin network and induced the reorganization of actin in 801D-shRNA cells (Fig. 4C). In addition, the formation of actin filopodia was also blocked. Moreover, we also determined the roles of Cdc42 in rearrangements of the actin cytoskeleton by stimulating cells with epidermal growth factor (EGF). Deletion of Cdc42 by shRNA inhibited the EGF-induced filopodia and pseudopodia formation (Fig. 4D), but this phenomenon was also shown in control 801D cells (Fig. 4E). These results suggested that Cdc42 played a crucial role in the cytoskeleton reorganization.

Curcumin inhibits invasion, and migration of 801D cells. To measure the effect of curcumin on the invasive ability of 801D cells, a Boyden chamber coated with Matrigel was used in a dosage experiment. 801D cells were plated on the upper chamber with 10 µM curcumin, 24 h later, the number of cells which moved to the underside of the coated membrane was counted under inverted microscopy. The results showed that the number of cells invaded to the lower chamber was significantly reduced by a 24 h treatment of curcumin (Fig. 5A). The effect of curcumin on 801D cell migration was further determined by wound-healing assay (Fig. 5B). These data demonstrated that curcumin significantly inhibited the invasion and migration of highly metastatic 801D cells.

Curcumin inhibits actin cytoskeleton reorganization. We next assessed the effect of curcumin on the cell morphology and actin cytoskeleton arrangement by immunofluorescence staining assay. 801D cells were treated with different concentra-
tions of curcumin (5 and 10 µM) for 24 h. Actin fibers were stained with TRITC-conjugated phalloidin. As shown in Fig. 4F-H, drastic reorganization of the F-actin network was observed: the cytoplasm stress fibers disappeared and F-actin patches appeared underneath the plasma membrane and the membrane filopodia formation was also blocked. Moreover, we also stimulated 801D cells with EGF. The results showing numerous filopodia were produced in control, while not in curcumin treated 801D cells.

Figure 4. Suppression of Cdc42 expression by shRNA or curcumin induces rearrangements of the actin cytoskeleton in lung cancer cells and curcumin inhibited the Cdc42 expression and invasion of lung cancer in vivo. 801D cells and negative control cells showed a cross-linked actin network (A and B). Deletion of Cdc42 by shRNA reduced the appearance of a cross-linked actin network and the formation of filopodia in 801D-shRNA cells (C). Deletion of Cdc42 by shRNA inhibited the EGF-induced filopodia and pseudopodia formation (D), but this phenomenon was also shown in control 801D cells (E). In addition, 801D cells were treated with 5 µM of curcumin (F), 10 µM of curcumin (G), or curcumin (10 µM) + EGF (100 ng/ml) (H), respectively. Curcumin inhibited the formation of filopodia. In addition, hematoxylin and eosin staining (HE) revealed the cellular heterogeneity in pathological sections of the excised tumor tissue. Untreated groups displayed characteristics of invasion (I). In contrast, curcumin treated groups showed clear boundaries with less invasiveness (J). Immunohistochemistry showed that the tumors formed by untreated groups were positive or strongly positive with Cdc42 (K); whereas the tumors formed by curcumin treated groups were weakly positive with Cdc42 stain (L) (original magnification x100).

Figure 5. Curcumin inhibited cell migration and invasion. 801D cells were scratched with a pipette tip and then treated with various concentrations of curcumin (5 and 10 µM). Cells were photographed under phase-contrast microscopy. The invasion ability of 801D cells was determined by invasion assay. Cells in low surface of the Borden chamber were stained and photographed under a light microscope. Data are presented as means ± SD of three independent experiments and expressed as percentage (A). For migration assay, confluent cell monolayers were wounded with a pipette tip. Representative images showing the inhibitory effect of curcumin on migration of 801D (B). Magnification x200. *P<0.05, compared with the untreated control.

Curcumin inhibits endogenous Cdc42 expression in 801D cells. Cell migration is regulated by Cdc42. We therefore investigated whether the effects reported above in the case of 801D cell migration and actin cytoskeletal disruption was paralleled by variations in the expression of Cdc42 using RT-PCR at the mRNA level and Western blot analysis at the protein level. In untreated cells, the expressing of Cdc42 protein and mRNA levels were high. As shown in Fig. 6, the
results of an mRNA and protein analysis of Cdc42 are illustrated under the treatment with 5-20 µM of curcumin. These data reveal a decrease in the expression of Cdc42 at protein level and mRNA level in a dose-dependent manner.

Curcumin suppresses Cdc42-regulated invasive and metastasis gene expression in lung cancer cell. As Cdc42 had been demonstrated to play a key role in curcumin contribution, here we also explored whether curcumin affects the Cdc42 down-stream functional proteins involved in invasion and metastasis. 801D cells were transfected with Cdc42-shRNA or treated with curcumin, and the phosphorylation and/or the expression levels of certain proteins were examined by Western blotting. As shown in Fig. 7A, Western blot analysis revealed that curcumin significantly inhibits the phosphorylation and/or enhances the expression levels of expression of Cdc42 target genes, such as invasion related genes Pak1, cofilin, and E-cadherin (26,27) in a dose-dependent manner. In contrast, the expression of uPA, uPAR, cathepsin B and cathepsin D (Fig. 7B), which are not regulated by Cdc42, were not affected by curcumin. These signaling effects again mimicked those of Cdc42 knockdown, suggesting that curcumin negatively regulates invasion and metastasis of the lung cancer cells and this may, at least in part, be due to its targeting effect on Cdc42.

Curcumin inhibits lung cancer growth and metastasis in vivo. Overgrowth and metastasis are two major characteristics of malignant tumors. We therefore investigated whether curcumin could suppress lung cancer tumor growth and invasion in vivo in an orthotopic transplanted nude mouse tumor model. The results showed that tumor volumes in control group increased from 166.64±3.54 to 882.28±5.60 mm³, whereas tumors in curcumin-treated group increased only from 158.32±6.25 to 420.54±7.89 mm³ (P<0.05, **P<0.01, compared with the untreated control).
to 224.17±2.55 mm³. The average weight of tumors from the control group was 0.685±0.125 g, whereas the average weight in curcumin-treated group was only 0.4105±0.113g, suggesting that curcumin strongly inhibited tumor growth in xenograft mouse lung tumor model. In addition, H&E staining showed that the tumors developed in curcumin treatment cells had clear boundaries with less invasiveness (Fig. 4I). In contrast, tumors arising from control groups displayed characteristics of invasion, indicating that curcumin inhibited invasion of lung cancer in vivo (Fig. 4I).

To determine the relationship among Cdc42 expression, lung tumor growth, and curcumin treatment in vivo, we examined the expression of Cdc42 in the orthotopic lung tumors in the nude mouse models. We found that the tumors formed showed strongly positive staining in the controls (Fig. 4K), but the tumors exhibited only weak immunoreactions in curcumin-treated cells (Fig. 4L). These data suggest that Cdc42 is involved in lung invasion and Cdc42 expression can be suppressed by curcumin in vivo.

Discussion

Overexpression of Cdc42 has been detected in many human tumors, suggesting that it may be key element in the process of tumorigenesis (28). Therefore, Cdc42 may become an important target molecule in cancer therapy. In the current study, we found that Cdc42 expression was correlated with lymphatic metastasis and TNM stage, while no association was observed between Cdc42 expression and any other clinicopathologic parameters. These observations are well-correlated with the recent studies demonstrating that overexpression of Cdc42 played a crucial role in progression and metastasis of lung cancer cells (5). Furthermore, in our experiments, we found that Cdc42 expression positively associated with metastatic potential of human lung cancer cells in vitro. Knocking down of Cdc42 expression by shRNA against Cdc42 suppressed lung cancer cell migration and invasion. These results indicated that Cdc42 played a key role in the vigorous migration and invasion of lung cancer cells.

The Rho GTPases are reported to be key regulators of actin dynamics that lead to organized actin-based structures associated with cell migration. Activated Cdc42 stimulate filopodia formation (29). In the present study, we reported the roles of Cdc42 in the organization of the actin cytoskeleton and cell migration in 801D cells. Our results showed that shRNA-mediated deletion of Cdc42 induced less cross-linked actin network and strongly inhibited actin-positive membrane filopodia formation. Conversely, expression of the Cdc42 protein in control 801D cells led to the formation of filopodia. We also determined the roles of Cdc42 in rearrangements of the actin cytoskeleton by stimulating cells with EGF. EGF increased lamellipodia and filopodia formation in vector control while not in Cdc42-shRNA 801D cells. Consistent with our findings, the activation of Cdc42 is required for the EGF-induced morphological changes by regulating the organization of the actin cytoskeleton (30).

Curcumin has been reported to possess anti-tumor effects in various cancer cells in vitro and in vivo (11,12). However, the precise impact and related molecular mechanism of curcumin on tumor metastasis remain to be elucidated. In the current study, we found that curcumin could efficiently suppress the migration, invasion and the formation of actin filopodia in lung cancer cells. In parallel, we observed that curcumin down-regulated the expression of Cdc42 gene at both mRNA and protein levels in 801D and A549 cells in a dose-dependent manner. These effects mimicked those of Cdc42 knockdown, suggesting that the inhibition of endogenous Cdc42 by curcumin may contribute to the inhibition of cell migration and invasion in these cells.

As Cdc42 had been demonstrated to play a key role in curcumin contribution, here we also explored whether curcumin affect the Cdc42 downstream functional proteins which involved in invasion and metastasis. Western blotting on several Cdc42-related or non-related genes further confirmed that Cdc42 was inhibited by curcumin. The expression of Cdc42 targeting genes PAK1, cofilin, and E-cadherin changed markedly in curcumin-treated cells, while that of non-Cdc42 targeting genes like uPA, uPAR, cathepsin B and cathepsin D had little change. These results suggested that curcumin could exert its antimetastatic activity by regulating gene expression through suppressing the transcriptional level of Cdc42.

We further observed the effect of curcumin on growth and invasion of lung cancer in vivo. The tumor inhibition rate and the frequency of xenograft invasion in mice after curcumin treatment was significantly decreased compared to the control group. Because Cdc42 is believed to be involved in cancer cell invasion, and metastasis, (2), our results also showed that curcumin inhibited tumor growth and invasion in vivo via their inhibition of the expression of Cdc42. These data suggested that Cdc42 is involved in lung cancer growth, migration, invasion and Cdc42 expression can be suppressed by curcumin in vitro and in vivo.

In conclusion, our data demonstrated that Cdc42 was overexpressed in tumor tissues, and its expression was directly correlated with tumor stage, lymph node metastasis, and patient survival. The suppression of Cdc42 was able to decrease the invasive potential and induce the rearrangements of actin cytoskeleton in lung cancer cells. Curcumin could markedly suppress Cdc42 genes and Cdc42-related target gene expression and effectively inhibit the migration/invasion of lung cancer cells in vitro, these effects mimicked those of Cdc42 knockdown. Furthermore, our results showed that curcumin inhibited tumor growth and invasion in vivo via their inhibition of the expression of Cdc42. These results suggested that curcumin might serve as a useful tool in exploring the potential therapeutic benefits of Cdc42 targeted therapy in lung cancer.

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