Knockdown of CABYR-a/b Increases Chemosensitivity of Human Non–Small Cell Lung Cancer Cells through Inactivation of Akt

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Abstract

CABYR is a calcium-binding tyrosine phosphorylation–regulated protein that was identified as a novel cancer testis antigen in lung cancer in our previous study. However, the role of CABYR as a driver of disease progression or as a chemosensitizer is poorly understood. This study sought to investigate the relationship between the expression levels of CABYR-a/b, which are the two predominant isoforms of the five isoform proteins encoded by CABYR, and chemosensitivity in non–small cell lung cancer cells. We found that the short hairpin RNA–mediated knockdown of CABYR-a/b significantly inhibited the proliferation of NCI-H460 and A549 cells and resulted in the attenuation of Akt phosphorylation, which is constitutively active in lung cancer cells. The silencing of CABYR-a/b expression notably impacted the downstream components of the Akt pathways: decreasing the phospho-GSK-3β (Ser9) levels and increasing the expression of the p53 and p27 proteins. Furthermore, CABYR-a/b knockdown led to a significant increase in chemosensitivity in response to chemotherapeutic drugs and drug-induced apoptosis, both in vitro and in vivo. Conversely, the transient transfection of CABYR-a/b–depleted cells with constitutively active Akt partially restored the resistance to cisplatin and paclitaxel and significantly decreased the activation of GSK-3β and cleaved PARP. Taken together, our results suggest that the inhibition of CABYR-a/b is a novel method to improve the apoptotic response and chemosensitivity in lung cancer and that this cancer testis antigen is an attractive target for lung cancer drug development.

Implications: Suppression of CABYR-a/b expression increases chemosensitivity of lung cancer cells by inhibiting Akt activity. Mol Cancer Res; 12(3); 335–47. ©2013 AACR.

Introduction

Lung cancer is the leading cause of cancer-related death worldwide (1). Chemotherapy is an established treatment for advanced non–small cell lung cancer (NSCLC), and both cisplatin (DDP) and paclitaxel (Taxol) are widely used as anticancer agents for the treatment of NSCLC in clinical settings. However, the development of resistance to chemotherapy is a major hurdle limiting treatment success (2, 3). Therefore, the identification of molecules related to chemoresistance and a further understanding of the underlying mechanisms will facilitate the development of strategies to overcome this problem. Cancer testis antigens are a class of tumor antigens with restricted expression in the testis and a variety of cancers (4), and these antigens have given rise to new treatment options, such as cancer immunotherapy, targeted agents, and antibody-based and antibody–drug conjugates therapies. However, scarce data related to the roles of cancer testis antigens in drug resistance have been obtained. It has been reported that the paclitaxel-sensitive human ovarian cancer cell line OVCAR8 overexpresses MAGE2 and MAGE6, which leads to enhanced resistance of these cells to paclitaxel and doxorubicin but not other drugs, such as topotecan and cisplatin (5). Furthermore, the inhibition of MAGE-A and GAGE expression was shown to increase apoptosis in medulloblastoma cells and to sensitize these cells to cisplatin and etoposide (6). Similarly, the downregulation of CAGE in the drug-resistant human melanoma cell line Malme3M and the human hepatic cancer cell line SWU387 was found to increase drug sensitivity resulting from apoptosis induction (7). Therefore, the currently available data suggest that cancer testis antigens play an important role in the chemoresistance of cancer cells.

CABYR is a calcium-binding tyrosine phosphorylation-regulated protein that was first isolated from human spermatozoa. There are 6 transcript variants of CABYR that encode 5 protein isoforms, including CABYR-a, -b, -c, -d, and -e (2 of the transcript variants encode the same isoform, CABYR-c; ref. 8). CABYR was initially reported to be...
specific and localized to the principal piece of the sperm flagellum that associates with the fibrous sheath during capacitation (8). In our previous study, we confirmed that CABYR is a novel cancer testis antigen in lung cancer (9). In addition to lung cancer and brain tumors (9, 10), CABYR was also shown to be aberrantly expressed in liver cancer and esophageal cancer (11). The knockdown of CABYR-c in HepG2 cells resulted in the inhibition of cell growth (12). However, there are no reports about the biologic functions of CABYR in lung cancer, particularly in drug resistance. Therefore, we hypothesized that CABYR may act in a similar manner as other cancer testis antigens, such as MAGE and CAGE, about their role in the chemoresistance of cancer cells. To evaluate this hypothesis, we examined the role of CABYR-a/b in lung cancer drug resistance and investigated the mechanism through which CABYR-a/b may promote such resistance.

Materials and Methods

Chemicals, antibodies, and plasmids

Cisplatin (DDP), paclitaxel (Taxol), vincristine (VCR), and vindesine (VDS) were obtained from Sigma (St. Louis). The Annexin V-FUROUS Staining Kit was purchased from Roche (Indianapolis); G418 was obtained from Merck; poli vinylidine difluoride membranes were obtained from Millipore; the Lipofectamine 2000 Transfection Reagent was purchased from Invitrogen; and all other chemicals were obtained from Millipore; the Lipofectamine 2000 Transfection Reagent was purchased from Invitrogen; and all other chemicals were obtained from Sigma. Mouse anti-CABYR-a/b antiserum was kindly provided by Dr. Y.K. Pak (Kyung Hee University, Seoul, South Korea).

Cell culture and transfection

NCI-H460 and A549 cells were purchased from the Chinese Academy of Sciences Committee Type Culture Collection Cell Bank and the cells were authenticated by short tandem repeat analysis in HK Gene Science Technology Co. The cells were cultured in Dulbecco’s Modified Eagle Medium containing 10% fetal calf serum (FCS), penicillin (100 units/mL), streptomycin (100 units/mL), and 2 mmol/L glutamine and grown at 37°C in a humidified atmosphere with 5% CO₂. To silence the expression of CABYR-a/b, 3 CABYR-a/b-specific short hairpin RNA vectors [SureSilencing short hairpin RNA (shRNA) plasmids] and a scrambled shRNA vector were purchased from Gene Pharma. The scrambled and targeted sequences were as follows: scrambled: GCTGTCCTGCTGGCTGGCT; shRNA, GCAACTCGCCAGAACAATAGT; shRNA1, GCTGTCCTGCTGGCTGGCT; shRNA2, GCAACTCGCCAGAACAATAGT. CABYR-a/b-shRNA was designed according to the sequence provided under GenBank accession number NM_012189.2 CABYR. For the generation of stable transfectants, NCI-H460 and A549 cells were transfected with CABYR-a/b-shRNA and scrambled shRNA vector using the Lipofectamine 2000 Transfection Reagent (Invitrogen) according to the manufacturer’s protocol. After 24 hours, the cells from each transfection were split into 3 separate culture dishes to ensure that independent lines were established. The stable cell lines were selected based on their growth in the presence of 0.4 mg/mL G418 (Merck), and individual cell lines were isolated using cloning discs (PGC Scientific).

Reverse transcription (RT)-PCR

The total RNA was extracted from the cells for the generation of single-stranded cDNA, and PCR was performed using primers specific for CABYR-a/b (forward: 5’-CGGAATTCTACATTCTTCAAGCGGACACTT-3’, reverse: 5’-TCATGGGCCCCTATTACGCTGGTTGATTC-3’) and glyceraldehyde-3-phosphate dehydrogenase (forward: 5’-AGGTCGGAATCAAGGGATTTG-3’, reverse: 5’-GTGATGCGATGGACACTGTGTG-3’). One fifth of the PCR product was subjected to electrophoresis on a 1% agarose gel.

Cell-proliferation assay

Cells (5 × 10³/well) stably expressing the scrambled shRNA vector (sh-vec) or shRNA against CABYR-a/b (shRNA, shRNA 1, and shRNA 2) were seeded into 24-well plates for growth analyses. The cell proliferation was evaluated by counting the number of cells every day using a Z2 Coulter Particle Count and Size Analyzer (Beckman Coulter). The cells were trypsinized and counted at the indicated time points, and all of the experiments were performed in triplicate.

Assessment of cell viability using the MTT assay

The cytotoxicity of chemotherapeutic agents was determined through the MTT assay. The cells were seeded at 5 × 10⁴ to 10 × 10⁴ cells in 100 μL of complete medium per well into 96-well plates. After overnight incubation, various concentrations of drugs, including cisplatin (0–50 μmol/L), paclitaxel (0–60 nmol/L), vincristine (0–100 ng/L), and vindesine (0–100 ng/L), were added to each well, and dimethyl sulfoxide (DMSO) was added as a control. Each condition was tested in 4 replicates. After incubation for 24 hours, the culture media was supplemented with 1 mg/mL MTT for 3 hours at 37°C. The media was then removed, and the cells were solubilized with DMSO. The absorbance was then measured at 450 and 630 nm, and the results are expressed as a percentage of the absorbance reading of the control cells (% survival).

Flow cytometric analysis of cell cycle and apoptosis

After treatment with 15 μmol/L cisplatin or 5 nmol/L paclitaxel for 24 or 48 hours and culturing in fresh culture medium containing 0.5% FCS for an additional 18 hours, both attached and detached cells were harvested and washed.
with PBS buffer containing 5 mmol/L EDTA. The cells were then fixed with 70% cold ethanol at 4°C for 12 hours. After washing twice with cold PBS, the cells were collected, resuspended in PBS containing 250 μg/ml RNase A, and incubated at 37°C for 30 minutes. The cells were then stained with propidium iodide for 30 minutes, and a cell-cycle analysis was performed using flow cytometry with a FACSScan instrument (BD Biosciences). The data were analyzed, and the proportion of apoptotic cells was calculated according to the apoptotic peak (sub-G1 peak) in the cell distribution compared with the propidium iodide fluorescence per cell.

**Annexin V/propidium iodide binding assay**

The cells were treated with 15 μmol/L cisplatin or 5 nmol/L paclitaxel for 24 hours, and both detached and attached cells were collected and washed with PBS. The cells were then stained with Annexin V-Alexa Fluor 488 conjugate and propidium iodide according to the manufacturer’s protocol and analyzed by flow cytometry using a FACSScan instrument (BD Biosciences).

**Colony-formation assay**

To measure the colony formation, stable CABYR-a/b-silenced and control cells were exposed to cisplatin (7.5 μmol/L) or paclitaxel (5 nmol/L) for 24 hours. Subsequently, the cells were trypsinized, counted, and seeded for a colony formation assay in a 6-well plate (1,000/well or 500/well), and cultured for 2 to 3 weeks. The colonies were fixed, stained with crystal violet, and counted. Only those colonies containing 50 cells were counted. The number of colony-forming units was expressed as the percentage of the controls. Each result was the average of at least 3 independent experiments.

**Western immunoblot analysis**

The cells were lysed, and equal amounts of the sample proteins were separated by SDS-PAGE and then transferred to a polyvinylidene difluoride membrane. The following reagents (1:200 to 1:2,000 dilution) were separately incubated with the specimens, and biotinylated secondary antibodies were applied at a dilution of 1:200 before HRP application (Vectastain ABComplex; Vector Laboratories, Inc.), resulting in a positive brown stain. The following primary antibodies were used: anti-CABYR-a/b antisera and anti-Akt, anti-p53, anti-p27, anti-catenin, anti-β-catenin, anti-α-catenin, anti–actin β, and anti–actin α (1:200 to 1:2,000 dilution). The following secondary antibodies were used: secondary antibodies conjugated to HRP were added and incubated for anti-catenin, anti-Akt, anti-p53, and anti–actin α (1:200 dilution) and then incubated with a HRP-conjugated streptavidin (1:50 dilution) (1:200 before HRP application). Secondary antibodies conjugated to HRP were added and incubated for 10 minutes. Color development of developed membranes was quantified by densitometry. The following antibodies were used: anti-CABYR-a/b antisera, anti-Akt, anti-p53, anti-p27, anti–actin β, and anti–actin α (1:200 to 1:2,000 dilution) were incubated with the control cells (50,001). Cells with stably silenced CABYR-a/b and mock control cells (15,013) were separately incubated with the specimens, and biotinylated secondary antibodies were applied at a dilution of 1:200 before HRP application (Vectastain ABComplex; Vector Laboratories, Inc.).

**Immunohistochemistry and TUNEL assay**

Immunohistochemistry was performed as described in our previous study (9). Mouse anti-CABYR-a/b antisera (1:100 dilution), anti-Akt (1:500 dilution), and anti-pAkt (1:50 dilution) were separately incubated with the specimens, and biotinylated secondary antibodies were applied at a dilution of 1:200 before HRP application (Vectastain ABCComplex; Vector Laboratories, Inc.), resulting in a positive brown stain. The specimens were stained with hematoxylin and eosin for morphology. Apoptosis was detected in tumor tissue sections of cisplatin- or paclitaxel-treated mice using the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) enzyme reagent (Fisher Scientific) according to the manufacturer’s instructions. The microscopy digital images were captured using an upright Zeiss Axio imager.

**Statistical analysis**

All of the experiments were repeated at least twice. The statistical significance was evaluated using Student t test (2-tailed) for comparisons between 2 groups of data. The asterisks indicate significant differences between the experimental groups and the corresponding control condition. The statistical analysis was performed using the GraphPad Prism software (GraphPad). Differences were considered statistically significant at a P value of less than 0.05.

**Results**

**Silencing of CABYR-a/b expression inhibits cell proliferation and impairs the Akt signaling pathway**

To explore the biologic functions of CABYR in lung cancer, 2 predominant isoforms, CABYR-a/b, were selected for investigation because of their high degree of nucleotide sequence similarity. Stable CABYR-a/b-knocked down cell lines were established in H460 and A549 cells (Fig. 1C and D), which express higher levels of endogenous CABYR-a/b than the other lung cancer cell lines tested. The cell growth curve analysis revealed significantly decreased growth in the CABYR-a/b-knockdown group compared with the control group in both H460 and A549 cells (Fig. 1A and B). However, the inhibition of CABYR-a/b had no effect on the rate of apoptosis in either cell line (data not shown). The Akt pathway is essential for cell proliferation and survival, and its activity is constitutive in NSCLC cells (14–16). To determine whether the Akt pathway is
attenuated by CABYR-a/b downregulation, we examined the activation of Akt and its downstream targets. The Akt activity was assessed by immunoblotting with a phospho-specific antibody against phosphorylated Ser473, which has been reported to be constitutively active in H460 and A549 cells under normal growth conditions (17, 18). Interestingly, the suppression of CABYR-a/b led to a marked decrease in the level of phosphorylated Akt and GSK-3β and, conversely, to an increase in the expression of p53 and p27 in both H460 and A549 cells (Fig. 1E and F). These results indicate that the expression level of CABYR-a/b impacts the proliferation of H460 and A549 cells and that this effect is at least partially mediated by the Akt pathway.

**Downregulation of CABYR-a/b enhances sensitivity to drugs and drug-induced apoptosis in lung cancer cells**

To determine whether the knockdown of CABYR-a/b sensitizes H460 and A549 cells to chemotherapy, the cells were treated with a DNA damaging agent (DDP), a microtubule-polymerizing agent (Taxol), or 2 microtubule-disassembling agents (VDS and VCR). As shown in Fig. 2A–H, the survival rate of the cells was inversely
correlated with the dose of all 4 drugs treatments evaluated in H460 and A549 cells. The increase in chemosensitivity resulting from CABYR-a/b silencing did not seem to depend on distinct chemotherapeutic mechanisms. To quantitatively measure the level of apoptosis, H460 cells were incubated with 15 μmol/L DDP or 5 nmol/L Taxol for 24 or 48 hours, stained with propidium iodide, and analyzed by flow cytometry. These doses of DDP and Taxol were selected because they caused minimal amounts of apoptosis in the control groups for both cell lines, as assessed by the dose–response curves.

As shown in Fig. 3A and B, the sub-G1 population was increased in a time-dependent manner after treatment with DDP or Taxol. Furthermore, CABYR-a/b knockdown combined with DDP treatment resulted in a greater fraction of apoptotic cells relative to Taxol treatment in both H460 and A549 cells (Fig. 3C and D). These results were further confirmed by staining for Annexin V, which showed that the percentage of H460 cells in early apoptosis was significantly increased when CABYR-a/b was silenced in combination with DDP or Taxol treatment for 24 hours; in comparison, less than 1% of the cells in the H460-vector-shRNA group were observed to be in early apoptosis after treatment with both drugs (Fig. 4A). A similar result was obtained for A549 cells (Fig. 4B).

As a marker of caspase activation, PARP cleavage was evaluated by monitoring the presence of the Mr 89,000 cleavage product by Western blotting. These results demonstrated that PARP cleavage only occurred in H460 and A549 cells after the silencing of CABYR-a/b plus DDP or Taxol treatment, whereas cleaved PARP was virtually undetectable in either of the corresponding single-treatment groups (Fig. 4C and D).

To further determine whether there is a direct correlation between the expression of CABYR-a/b and chemosensitivity, a colony-formation assay in which H460 and A549 cells were treated with or without DDP or Taxol was conducted. Although the CABYR-a/b-silenced cells were less efficient at forming colonies compared with the control cells (Fig. 5A and B), the numbers of colonies formed by these silenced cells were significantly decreased after DDP or Taxol treatment (Fig. 5C–F). These results further strengthen the evidence that the silencing of CABYR-a/b increases the chemosensitivity of lung cancer cells.

Taken together, these results strongly suggest that the downregulation of CABYR-a/b not only increases the sensitivity of lung cancer cells to chemotherapy but enhances drug-induced apoptosis in lung cancer cells.

**CABYR-a/b knockdown increases tumor sensitivity to DDP and Taxol in vivo**

The chemosensitivity of cells with silenced CABYR-a/b after treatment with DDP or Taxol was further evaluated in H460 cell xenografts in nude mice. Similar to previous reports (19), relatively lower doses of drugs, that is, 2.5 mg/kg DDP or 5 mg/kg Taxol, were given via the tail vein after the tumor diameters reached 0.5 cm, and the drugs were administered once every 4 days. Similar to the results obtained in vitro, the silencing of CABYR-a/b alone significantly delayed tumor growth and decreased the total weight of the tumors in H460 xenografts compared with control animals, and these inhibitory effects were increased when the mice were treated with DDP or Taxol. The mean tumor volume in the animals receiving CABYR-a/b-silenced cells in combination with drug treatment was significantly lower than that found in the corresponding single-treatment groups (\(^{***}, P < 0.001\); \(^{**,} P < 0.01\)) after day 7 (Fig. 6A and B). Moreover, CABYR-a/b silencing combined with DDP treatment showed greater suppression of tumor growth relative to Taxol treatment, and these animals demonstrated the lowest tumor weight among all of the groups (Fig. 6C and D).

These data strongly suggest that the silencing of CABYR-a/b and treatment with a chemotherapeutic drug exhibit a synergistic effect in H460 cells. To confirm the relationship between the expression level of CABYR-a/b and tumor growth in vivo, the expression of CABYR-a/b was examined in tumor sections by immunohistochemical staining. In agreement with the in vitro results, the expression of CABYR-a/b was significantly decreased in xenografts derived from CABYR-a/b-silenced cells compared with control cells. A panel of representative tumor tissue sections from different groups is shown in Fig. 6E. The phospho-Akt (Ser473) expression was downregulated after CABYR-a/b silencing, and Akt expression was almost invariable by immunohistochemical staining (Fig. 6F). These findings are consistent with the results in vitro. Next, we sought to confirm whether the observed reduction in tumor size was the result of increased apoptosis using a TUNEL assay. As shown in Fig. 6G, the proportion of apoptotic cells was significantly higher in the groups that received CABYR-a/b silencing plus DDP or Taxol treatment compared with a single treatment (positive nuclear staining represents apoptosis). Therefore, these results demonstrated that the downregulation of CABYR-a/b also sensitized lung cancer cells to chemotherapeutic drugs in vivo through the induction of apoptosis.

**Transfection of constitutively active Akt restores the resistance to DDP and Taxol in CABYR-a/b-silenced H460 cells**

Based on the result that the silencing of CABYR-a/b results in the inactivation of the Akt pathway in both H460 and A549 cells and because Akt is known to perform an important role in the chemoresistance of cancer cells (20), we performed restoration experiments to examine whether the Akt pathway contributes to the effect of CABYR-a/b knockdown on chemoresistance. A plasmid expressing constitutively active Akt (Myr-Akt) was transiently transfected into cells, and as expected, the chemoresistance of CABYR-a/b-silenced H460 cells was significantly restored (to the level of the control group) when lower doses were used (less than 20 μmol/L DDP and 5 nmol/L Taxol). With a higher dose (50 μmol/L DDP and 10 nmol/L Taxol), the resistance was also...
Figure 2. Suppression of CABYR-a/b expression increases the sensitivity of lung cancer cells to chemotherapeutic agents in vitro. The survival rates of NCI-H460 and A549 cells following treatment with graded concentrations of DDP (A and E), Taxol (B and F), VCR (C and G), or VDS (D and H) were measured using the MTT assay after treatment for 24 hours. The cell survival percentage was obtained in comparison to that of the respective untreated sample. The data are presented as the means ± SD of 3 independent experiments. *, P < 0.05 and **, P < 0.01 versus the CABYR-a/b-shRNA and sh-vec groups.
partially restored, although a notable difference was observed between the 2 groups (Fig. 7A and B). Following the restoration of phosphorylated Akt, PARP cleavage was significantly decreased after the cells were treated with DDP or Taxol (Fig. 7C and D), and phospho-GSK-3β (Ser9) was significantly increased (Fig. 7E). Taken together, these results strongly suggest that the CABYR-a/b-mediated chemoresistance in lung cancer cells is mediated by the Akt pathway.

Discussion

To address the role of CABYR in drug resistance and explore the underlying mechanisms, CABYR-a/b was knocked down in 2 commonly studied lung cancer cell lines, H460 and A549, and 2 different types of chemotherapeutic drugs (DNA damage-inducing and mitotic arrest-inducing drugs) were used to evaluate the chemoresistance. Three new findings were presented in this study. First, the expression level of CABYR-a/b was positively correlated with
the growth ability of lung cancer cells both in vitro and in vivo. Second, the downregulation of CABYR-a/b enhanced the sensitivity of NSCLC cells to chemotherapy both in vitro and in vivo. Third, the CABYR-a/b–mediated resistance to chemotherapy-inducing apoptosis was partially mediated through the Akt pathway.

CABYR was identified as a novel cancer testis antigen in lung cancer in our previous study, and this protein was later
found to be overexpressed in various types of cancers, including brain, liver, and esophageal cancer. However, no previous studies investigated the role of CABYR in lung cancer. To explore the biologic functions of CABYR in lung cancer, the 2 predominant isoforms of the 5 CABYR isoforms, namely CABYR-a and -b, were selected for evaluation because of their nucleotide sequence similarity. We first established H460 and A549 cells with stable silencing of CABYR-a/b and observed that CABYR-a/b silencing decreased the growth of H460 and A549 cells both in vitro and in vivo compared with the mock control. Because the Akt pathway is known to be essential for cell proliferation and survival and its activity is constitutive in NSCLC cells (14–16, 20), we focused on this pathway in this study. Akt requires phosphorylation at 2 specific amino acid residues, namely threonine 308 and serine 473;
phosphatidylinositol-dependent kinase 1 (PDK1) phosphorylates threonine 308, and the mTOR complex-2 (mTORC2) phosphorylates serine 473 (21). Because reactivity with phospho-specific Akt antibodies correlates strongly with in vitro kinase activity and A549 does not exhibit Thr308 phosphorylation under normal culture conditions (20), only the phosphorylation of Akt at Ser473 was examined in this study. Our findings demonstrated that phosphorylated Akt and GSK-3β were significantly decreased in CABYR-a/b-silenced H460 and A549 cells, whereas the expression of p27 and p53 was increased. These results suggest that the inhibition of cell growth via the down-regulation of CABYR-a/b was mediated by inactivation of the Akt pathway.

NSCLC cells are known to be resistant to chemotherapy-induced apoptosis, and Akt is a central regulator in cell survival when cells are exposed to various apoptotic stimuli, such as chemotherapeutic agents. A review of the literature identified previous reports that showed that a direct inhibition of Akt in NSCLC cells can enhance apoptosis in response to chemotherapeutic agents (22–24). Therefore, we also investigated whether CABYR-a/b downregulation can increase the chemotherapeutic sensitivity of lung cancer cells. Two types of anticancer drugs commonly used in the

Figure 6. Suppression of CABYR-a/b in NCI-H460 cells markedly reduces tumor growth and increases the chemosensitivity of cells in vivo. The volumes of tumor xenografts were measured on the indicated day, and the mice were treated with 0.9% NaCl, DDP (A), or Taxol (B) for 23 days after inoculation; the arrow (“#”) indicates the day of drug administration. The control groups in (A) and (B) are the same. The tumors were weighed after the mice were euthanized (C), and the mean ± SD of the tumor weights are presented. **P < 0.05 and ***P < 0.01 versus the CABYR-a/b-shRNA combination and the CABYR-a/b-shRNA alone groups (D). The expression of CABYR-a/b (E) and Akt and p-Akt (F) in the tumor sections was examined by immunohistochemistry, and apoptosis was evaluated with the TUNEL assay (G). Scale bar = 20 μm.
The xenografts from CABYR-a/b decreased. In agreement with the results obtained (Ser473), GSK-3 sh-vec groups. The total Akt, p-Akt versus the CABYR-a/b-shRNA and (C) analyzed by Western blotting (Ser9), and cleaved PARP were present in the principal piece of the mouse epididymal spermatozoa, the effects of murine CABYR on the growth of those of the control groups. Because murine CABYR is only present in the principal piece of the mouse epididymal spermatozoa, the effects of murine CABYR on the growth of human lung cancer cells were neglected in the xenograft experiments. Taken together, our results suggest that the expression levels of CABYR-a/b may have predictive value for evaluating clinical responses to chemotherapy among patients with lung cancer.

To further evaluate whether CABYR-a/b regulates the chemosensitivity of NSCLC cells through the Akt pathway, constitutively active Akt (Myr-Akt) was transiently transfected into CABYR-a/b–silenced H460 cells. As expected, the resistance of these cells to DDP or Taxol treatment was partially restored at various concentrations of DDP (approximately 50 μmol/L) and Taxol (approximately 20 nmol/L). This study is therefore the first to report that the modulation of CABYR-a/b can alter Akt activity and sensitivity to chemotherapy in NSCLC cells. To date, few reports, with the exception of studies performed on human spermatozoa, have evaluated the biologic functions of CABYR. For example, it was reported that CABYR-a interacts with α-enolase in 293T cells (11) and that CABYR-c binds GSK-3β only in yeast 2-hybrid and GST pull-down assays but not in cells
(10). Based on our results, we hypothesize that the regulation of Akt activation by CABYR-a/b is likely posttranslational, and this hypothesis is also supported by observations that gene amplification (data not shown) and total Akt protein levels do not change in CABYR-a/b–silenced cells compared with mock controls. One possible mechanism may be that CABYR-a/b indirectly regulates Akt dephosphorylation by modulating proteins that terminate Akt signaling through the dephosphorylation and inactivation of Akt, such as PHLPP (25), CTMP (26), PHLDA3 (27), and FKBP51 (28). Conversely, CABYR-a/b may also influence the activation of Akt directly or indirectly. Although the mechanism through which CABYR-a/b regulates Akt activation remains to be elucidated, our results, at least in part, suggest that CABYR-a/b is an important upstream regulator of the Akt pathway that is involved in both cell proliferation and the chemosensitivity of lung cancer cells. In our study, we successfully used the RNA interference technique to induce the specific suppression of CABYR-a/b expression and increase the chemosensitivity of lung cancer cells. We could also use specific CABYR-a/b–targeting siRNA as a small molecule drug combined with chemotherapeutics to increase the cytotoxicity of lung cancer cells.

Taken together, the present findings demonstrate that the expression levels of CABYR-a/b not only influence the proliferation of lung cancer cells but also alter their response to chemosensitivity through the Akt pathway, and these data strongly support the potential value of CABYR-a/b as a target for lung cancer chemotherapy. In addition, it would be valuable to determine whether the knockdown of CABYR-a/b would enhance other apoptotic stimuli, such as TRAIL and TNF-α, and whether other isoforms of CABYR, such as CABYR-c, exhibit similar effects; these studies are currently underway in our laboratory.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: Z. Qian, D. He, X. Xiao
Development of methodology: Z. Qian, M. Li
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Z. Qian, M. Li, R. Wang, Q. Xiao, J. Wang, M. Li
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Z. Qian, M. Li
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