Abstract
Cisplatin [cis-diaminodichloroplatinum (II) (CDDP)] is one of the most widely used and effective therapeutic agents for many kinds of cancers. However, its efficiency is limited due to development of drug resistance. In this study, we showed that CDDP resistance was associated with AKT1 overexpression and gene amplification in human lung cancer cells that acquired the drug resistance. We showed that AKT1 forced expression in the cells was sufficient to render the cells CDDP resistant, and that AKT1 inhibition by its dominant negative mutant reversed the CDDP-resistant cells to be CDDP sensitive. These results show that AKT1 activity is essential for regulating CDDP resistance in cultured lung cancer cells. To study whether these results were correlated with human lung cancer tumors, we randomly selected tumor samples from human lung cancer patients to study the correlation of AKT activation and CDDP resistance in clinical tumor samples. We showed that AKT activation was highly related to CDDP chemosensitivity in human tumor tissues. Our results further showed that AKT1 induced lung cancer cells to become resistant to CDDP through the mammalian target of the rapamycin (mTOR) signaling pathway. These studies conclude that AKT amplification and the mTOR pathway play an important role in survival when cancer cells are exposed to different kinds of apoptotic stimuli, such as the withdrawal of extracellular signaling factors, oxidative and osmotic stress, irradiation, matrix adhesion, ischemic shock, and chemotherapeutic drugs (16–21). Recent studies have shown that AKT activation seems to be related to CDDP resistance of ovarian cancer cells (22, 23). To study the mechanism of CDDP in this study, we have established a human lung cancer line, A549/CDDP, resistant to CDDP treatment. We found that the CDDP-resistant cells show greatly increased AKT1 expression and gene amplification when compared with its parental cell line A549. We further showed that AKT1 activation is essential to the CDDP resistance in cancer cells. Similar results were found in tumor samples from human lung cancer patients. Our findings identify a new mechanism of CDDP resistance in human lung cancer cells through AKT1 amplification and the mammalian target of rapamycin (mTOR) pathway. This study may provide useful information to develop a potential new therapeutic approach for lung cancer patients by targeting the AKT/mTOR pathway to overcome CDDP resistance.

Introduction
Lung cancer is the most prevalent form of cancer worldwide and is the leading cause of cancer death in men and the second leading cause in women (1, 2). Non–small-cell lung cancer is the most common type of lung cancer, accounting for 75% to 80% of all lung cancer occurrences (3). cis-Diaminodichloroplatinum (II) (CDDP; cisplatin) is one of the most effective and widely used DNA-damaging anticancer drugs used for the treatment of various human cancers including lung cancer (4). However, the ability of cancer cells to become resistant to CDDP remains a significant impediment to successful chemotherapy. Intensive studies have been done to clarify the mechanisms of CDDP resistance. Up to now, multiple mechanisms have been proposed in CDDP resistance, including reduction of platinum accumulation related to the alteration of transmembrane pumps, increase of thiol-containing biomolecules such as glutathione and metallothionemins, enhancement of the DNA damage repair, and blockade of apoptosis induction (5–7). A better understanding of the processes and mechanisms leading to CDDP resistance of non–small-cell lung cancer is necessary to develop effective therapies that can improve the prognosis of patients with this deadly disease.

Serine-threonine kinase AKT transmits survival signals from growth factors and regulates metabolism, cell cycle progression, and cellular survival (8–11). AKT is the cellular homologue of the product of the v-akt oncogene and has three isoforms: AKT1, AKT2, and AKT3 (12–14). Previous studies indicate that dysregulation of AKT is a prominent feature of many human cancers including non–small-cell lung cancer (15). The AKT pathway plays an important role in survival when cancer cells are exposed to different kinds of apoptotic stimuli, such as the withdrawal of extracellular signaling factors, oxidative and osmotic stress, irradiation, matrix adhesion, ischemic shock, and chemotherapeutic drugs (16–21). Recent studies have shown that AKT activation seems to be related to CDDP resistance of ovarian cancer cells (22, 23). To study the mechanism of CDDP in this study, we have established a human lung cancer line, A549/CDDP, resistant to CDDP treatment. We found that the CDDP-resistant cells show greatly increased AKT1 expression and gene amplification when compared with its parental cell line A549. We further showed that AKT1 activation is essential to the CDDP resistance in cancer cells. Similar results were found in tumor samples from human lung cancer patients. Our findings identify a new mechanism of CDDP resistance in human lung cancer cells through AKT1 amplification and the mammalian target of rapamycin (mTOR) pathway. This study may provide useful information to develop a potential new therapeutic approach for lung cancer patients by targeting the AKT/mTOR pathway to overcome CDDP resistance.

Materials and Methods
Cell culture. Human non–small-cell lung cancer A549 cells (American Type Culture Collection) were cultured in RPMI 1640 containing 10% heat-inactivated fetal bovine serum, 100 units/mL penicillin G, and 100 mg/mL streptomycin in a 37°C CO2 incubator. Cells were selected by stepwise exposure to increasing CDDP concentrations up to 5 μg/mL. Then, the cells were maintained at least for 6 months in the medium containing 5 μg/mL CDDP. The CDDP-resistant cells were cultured without CDDP for 1 month before the analysis was done.

Reagents. Antibodies against j-actin, CDDP, LY294002, and rapamycin were purchased from Sigma. CDDP, LY294002, and rapamycin were dissolved in DMSO and stored at –20°C. Antibodies against phosphorylated AKT (Ser473), total AKT, and phosphorylated p70S6K1 (Thr421/Ser424) were dissolved in DMSO and stored at –20°C.  

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obtained from Cell Signaling Technology. Antibodies against total p70S6K1 were from Santa Cruz Biotechnology. Lipofectamine and TRIzol reagent were from Invitrogen. Wizard Genomic DNA Purification Kit and moloney murine leukemia virus (MMLV) reverse transcriptase were from Promega. DNA Marker, Taq polymerase, and oligo(dT)18 were from Takara. Adeno-X Rapid Titer Kit was from BD Biosciences Clontech.

**Patients and surgically resected tissues and primary culture.** The study set consisted of 45 primary human lung cancer samples taken from patients undergoing surgery in Xinqiao Hospital and Southwest Hospital of the Third Military Medical University, China, from January 2001 to May 2002. Written informed consent was received from each patient before the experiment. The clinical data included age, sex, date of diagnosis, histology, and smoking history. Tissue blocks from formalin-fixed, paraffin-embedded lung cancer specimens were cut into 5-μm sections, fixed on glass slides, and shipped to our laboratory for further processing. Part of the tissues was used to obtain primary cell culture for testing the sensitivity to CDDP treatment by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described (24). In brief, part of the lung cancer tissues from operation was immersed in D-Hanks solution with antibiotics; after being washed several times, tissues were cut into <1 mm3 sections. After the removal of red cells by hypotonic method, tumor tissues were treated by collagenase digestion and density gradient centrifugation to obtain the primary cancer cell suspension. The cells were seeded into 96-well plates. After culture for 24 h, various concentrations of CDDP were added and incubated for 48 h. Cell proliferation was determined by MTT assay. Only 27 of diagnostic lung cancer samples which had results of phospho-AKT staining and chemosensitivity were included in our data analysis.

**Adenovirus preparation and transduction efficiency.** The construction of constitutively active AKT (Myr-AKT) and dominant-negative AKT (AKT-K179M) has previously been described (25). Recombinant adenoviruses were made using the AdEasy system (26). Briefly, the constructs were subcloned into the shuttle vector pADTrack-CMV, then transferred to AdEasy-1 plasmid through homologous recombination as previously described (26). The viral vectors were then transfected into 293 cells to generate viruses. A control virus carrying the green fluorescent protein (Ad-GFP) was derived from the same vector system. Viral titres were determined using the BD Adeno-X Rapid Titer Kit per manufacturer’s instructions. Adenoviral transduction efficiency for the cell lines was determined using the Ad-GFP vector. A549 and A549/CDDP cells were infected with Ad-Myr-AKT1 and Ad-AKT-DN at 10 and 30 multiplicities of infection (MOI), respectively.

**MTT assay.** Cells were seeded into 96-well plates at 3 × 104 per well overnight and incubated with different concentrations of CDDP from 0 to 32 μmol/L with six replicate wells per treatment. For the cultured primary lung cancer cells, cells were seeded into 96-well plates at 7.5 × 104 per well and treated with six different concentrations of CDDP from 0 to 660 μmol/L (0, 6.6, 13.2, 66, and 660 μmol/L). All measurements were done in triplicate wells. After culture for 48 h, MTT dye solution was added to each well and samples were incubated at 37°C for 4 h. The formazan product was dissolved by adding 100 μL of DMSO to each well and the plates were read at 570 nm. Each treatment included six wells and the results were calculated from three independent experiments. The IC50 value was calculated from the 50% formazan formation compared with a control treatment included six wells and the results were read at 570 nm. Each treatment included six wells and the results were calculated from three independent experiments. The IC50 value was calculated from the 50% formazan formation compared with a control.

**Results and Discussion**

CDDP-resistant lung cancer cells show AKT1 overexpression and gene amplification. To study the molecular mechanism of CDDP resistance, we established CDDP-resistant cells, A549/CDDP cells from A549 human lung cancer cells, by stepwise exposure to increasing concentrations of CDDP. As shown in Fig. 1A, the IC50 concentration of A549/CDDP cells was ~4-fold that of A549 (30.74 versus 8.11 μmol/L) when the cells were treated with CDDP. To test what signaling molecules are involved in CDDP resistance, we found that levels of phospho-AKT and total AKT expression were 2-fold that of the control, which suggested that AKT activation and overexpression are associated with CDDP resistance in the cells (Fig. 1B). To study how AKT protein expression was induced in CDDP-resistant cells, we found that AKT1 steady-state mRNA level was increased ~2-fold in the cells, which correlated with the elevation of AKT protein level in A549/CDDP cells (Fig. 1C). It is known that AKT includes AKT1, AKT2, and AKT3 isoforms that are expressed in various tissues and cancer cells (30). We found that AKT2 and AKT3 mRNA levels were not induced in A549/CDDP cells (data not shown). To determine whether
increased AKT1 mRNA level was due to AKT1 gene amplification, genomic DNA was isolated from A549 and A549/CDDP cells and analyzed by PCR. There have been a few reports of AKT gene amplification being related to carcinogenesis. For example, AKT1 gene amplification has been detected in a single gastric carcinoma and glioblastoma (31, 32) and AKT2 gene amplification has been reported in ovarian, pancreatic, and breast cancers (33, 34). Overexpression of AKT3 mRNA was identified in breast and prostate cancers (35). Recent study has indicated that AKT2 is involved in CDDP resistance in ovarian cancer cells (36). We found that AKT1 gene was amplified 2-fold in A549/CDDP cells when compared with A549 cells (Fig. 1D). This result shows that AKT1 gene amplification is a new mechanism of CDDP resistance in human lung cancer cells that are developed by CDDP selection and colonial expansion.

AKT1 expression is sufficient and necessary for regulating CDDP resistance in cancer cells. To determine whether direct expression of AKT1 in the cells is sufficient to induce CDDP resistance, A549 cells were infected with adenovirus carrying GFP or a constitutive active form of AKT1 (Myr-AKT1). The effect of CDDP on cell proliferation was analyzed. AKT1 forced expression decreased the sensitivity of A549 cells to CDDP treatment with a 2.5-fold increase of IC50 when compared with the control (Fig. 2A). To further confirm the effect of AKT1 overexpression in other cancer cells, human prostate cancer DU145 cells were used. Overexpression of AKT1 in DU145 cells also induced CDDP resistance in the cells with a 2-fold induction of IC50 (Fig. 2B). These results indicated that AKT1 expression was sufficient to render DU145 cells CDDP resistant. To study whether AKT is required for A549/CDDP cells to become CDDP resistant, A549/CDDP cells were infected with adenovirus carrying AKT1 dominant negative mutant. The expression of AKT1 dominant negative mutant reversed the CDDP resistance to sensitivity with a 2-fold decrease of IC50 value with CDDP treatment (Fig. 2C), showing that AKT1 is required for the CDDP-resistant A549/CDDP cells to become CDDP resistant. Human ovarian cancer A2780 cells show amplification of PI3KCA, which codes for phosphatidylinositol 3-kinase (PI3K), an upstream molecule of AKT. To test whether AKT1 is required for the CDDP resistance in other cancer cells, A2780 cells were infected with adenovirus carrying GFP or AKT1 dominant negative mutant. Similarly, the expression of AKT1 dominant negative mutant in A2780 cells made the cells susceptible to CDDP treatment with a decrease of IC50 from 15.63 to 8.65 μmol/L (Fig. 2D). Taken together, these results suggest
that AKT1 activity is sufficient and essential for regulating CDDP resistance in human cancer cells.

AKT activation in human lung tumor tissues correlated with the CDDP resistance of tumor cells. To study whether AKT activation is correlated with CDDP resistance in human lung cancer tissues, we collected 27 primary human lung tumors from the surgery. Parts of the tumors were embedded in paraffin, and sections were stained for immunohistochemical studies using phospho-AKT antibodies; the other parts were used for the preparation of primary human lung cancer cells. The sensitivity of primary lung cancer cells to CDDP treatment was analyzed with the MTT assay. The relative intensity of phospho-AKT signals in the tumor tissues was scored from − (negative, no staining) to +++ (strong granular cytoplasmic staining in >20% of tumor cells; Fig. 3). The levels of phospho-AKT signals in human lung tumor tissues were highly related to CDDP resistance in primary lung cancer cells (Table 1), suggesting that AKT activation regulated CDDP resistance in human lung tumor tissues and cells in vivo. This result is consistent with the data obtained from A549 and A549/CDDP cells as described above. Elevated AKT activity was also detected in different kinds of human cancers that were associated with a poor prognosis (37–39). This indicates that AKT activation may be a common mechanism that could regulate CDDP resistance in other human cancer cells.

AKT-mediated CDDP resistance depends on PI3K activity. To test whether AKT-induced CDDP resistance depends on PI3K activity, parental A549 cells and A549/CDDP cells were cultured in the absence or presence of PI3K inhibitor LY294002 and treated with different concentrations of CDDP. The addition of low concentration of LY294002 greatly increases the sensitivity of A549/CDDP cells inhibited by CDDP treatment (Fig. 4A). In contrast, LY294002 treatment only slightly affected A549 cell proliferation (Fig. 4B). This result showed that A549/CDDP cells were more sensitive than A549 cells in response to LY294002 treatment and that AKT-induced CDDP resistance still required PI3K activity in the cells. This is consistent with AKT activation through phosphorylation that depends on PI3K activity in the cells. To analyze the AKT phosphorylation that correlates with AKT activity, parental A549 and A549/CDDP cells were infected with adenovirus carrying Myr-AKT (Ad-Myr-AKT) at 10 MOI. Cells infected with Ad-GFP were used as a control. The cells were trypsinized and seeded into 96-well plates 24 h after infection and analyzed for the sensitivity to CDDP treatment as described in Fig. 1. IC50 against cisplatin (CDDP) was calculated using the data from three replicate experiments. The IC50 in A549 infected with Ad-GFP and Ad-Myr-AKT was 7.65 and 18.77 μmol/L, respectively (A). IC50 against CDDP in DU145 infected with Ad-GFP and Ad-Myr-AKT was 6.97 and 12.76 μmol/L, respectively (B). A549/CDDP (C) and A2780 cells were infected with adenovirus carrying AKT dominant negative mutant (Ad-AKT-DN) at 30 MOI. Cells infected with Ad-GFP were used as a control. The cells were seeded into 96-well plates 24 h after infection and analyzed for the sensitivity to CDDP treatment. Triplicate experiments were done. AKT1 inhibition by its dominant negative mutant (AKT-DN) enhanced the chemotherapeutic effect of CDDP in both A549/CDDP and A2780 cells. IC50 value decreased from 32.05 to 16.74 μmol/L when AKT-DN was expressed in A549/CDDP cells. IC50 value decreased from 15.63 to 8.65 μmol/L when AKT-DN was expressed in A2780 cells.
activation, A549 and A549/CDDP cells were cultured in serum-free medium overnight, followed by the addition of CDDP and serum in the absence or presence of LY294002. Similarly, the higher basal levels of phospho-AKT and total AKT were observed in A549/CDDP cells than in A549 cells as we previously described (Fig. 1B). LY294002 and CDDP combination treatment decreased phospho-AKT to very low levels in both A549 and A549/CDDP cells (Fig. 4C). The reduction rate of phospho-AKT levels in A549/CDDP cells was much greater that that in A549 cells (Fig. 4C). These results were consistent with the MTT assay, which showed that LY294002 and CDDP combination treatment had much greater inhibition effect on A549/CDDP cells than on A549 cells.

To study the effect of LY294002 on tumor growth in vivo, A549 and A549/CDDP cells were injected into nude mice to initiate the tumor. When the diameter of tumors reached ~5 mm, the mice were treated with the combination of LY294002 and CDDP by i.p. injection and tumor volumes were analyzed. CDDP treatment alone did not inhibit tumor growth induced by A549/CDDP cells, but the combination of CDDP and LY294002 induced the regression of tumors to smaller size from their initial size when the treatment was started (Fig. 4D), and the effect of LY294002 treatment in inhibiting A549/CDDP cell–induced tumor growth was much greater than with A549 cell–induced tumor growth (Fig. 4D and E). These results show that the lung cancer cells with AKT amplification is hypersensitive to LY294002 and CDDP combination treatment, suggesting that AKT-induced CDDP resistance also requires PI3K activity in tumor xenografts.

Table 1. Results of the nonparametric approach (Kruskal-Wallis test)

<table>
<thead>
<tr>
<th>Rank of immunohistochemistry</th>
<th>n</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; against cisplatin (mean ± SE), mmol/L</th>
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<tr>
<td>–</td>
<td>7 (3 AC, 3 SC, 1 BC)</td>
<td>4.52 ± 1.34 (1.00; 10.02; 6.68; 7.34; 1.94; 3.67; 1.00)</td>
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<tr>
<td>+</td>
<td>7 (3 AC, 3 SC, 1 BC)</td>
<td>11.70 ± 2.31 (5.91; 8.25; 10.35; 23.70; 7.04; 14.92; 11.75)</td>
</tr>
<tr>
<td>++</td>
<td>8 (3 AC, 2 SC, 3 BC)</td>
<td>34.42 ± 7.73 (18.60; 36.39; 40.06; 40.67; 40.73; 20.43; 19.03; 17.69)</td>
</tr>
<tr>
<td>+++</td>
<td>5 (1 AC, 1 SC, 2 BC, 1 LC)</td>
<td>522.66 ± 298.61 (837.94; 1,559.03; 75.78; 90.14; 50.41)</td>
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Abbreviations: AC, adenocarcinoma; SC, squamous cell carcinoma; BC, bronchioloalveolar carcinoma; LC, large-cell carcinoma.

**mTOR and p70S6K1 are downstream targets of AKT1 in mediating CDDP resistance.** Serine/threonine kinase mTOR is activated by AKT in response to growth factors (40). mTOR, in turn, activated p70S6K1 through its phosphorylation. To test whether mTOR is the downstream target of AKT in mediating AKT-induced CDDP resistance, A549 and A549/CDDP cells were treated without and with rapamycin. A549/CDDP cells have higher level of p70S6K1 phosphorylation than A549 cells, and rapamycin treatment decreased p70S6K1 activation (Fig. 5A and B). This result indicated that CDDP resistance is associated with mTOR and p70S6K1 activation in the cells. To determine whether activation of the mTOR/p70S6K1 pathway is required for AKT-induced CDDP resistance, the cells were treated with CDDP alone or the combination of CDDP and rapamycin, and the IC<sub>50</sub> for inhibiting cell proliferation was measured. Rapamycin treatment slightly decreased the IC<sub>50</sub> than CDDP treatment alone in A549 cells (Fig. 5D), whereas rapamycin addition decreased the IC<sub>50</sub> to ~3-fold in A549/CDDP cells than CDDP treatment alone (Fig. 5C). This result showed that mTOR and p70S6K1 activation is required for AKT-induced CDDP resistance, suggesting that the mTOR/p70S6K1 pathway is downstream of AKT in mediating CDDP resistance in lung cancer cells.

It is still a major hurdle that cancer cells become resistant to chemotherapeutic agents during treatment. CDDP resistance is currently known to be associated with CDDP detoxification, cellular drug transport, DNA replication and repair, and anti-apoptosis pathways (6, 7). However, the molecular mechanisms of
AKT-mediated CDDP resistance depends on PI3K activity. A, LY294002 greatly increases the sensitivity of A549/CDDP cells to CDDP treatment. A549/CDDP cells were seeded into 96-well plates and treated with CDDP as described in Fig. 1 in the absence or presence of LY294002 (0, 5, and 10 μmol/L). Cell proliferation was measured by MTT assay in three replicate experiments. B, LY294002 treatment only slightly increases the sensitivity of A549 cells to CDDP treatment. A549 cells were treated with CDDP in the absence or presence of LY294002 as above. C, A549 and A549/CDDP cells were cultured in serum-free medium for 20 h, switched to medium without serum (-) or LY294002 (-) or with 5% fetal bovine serum (+) and 10 μmol/L LY294002 (+) for 2 h. Total cellular lysates were prepared and subjected to immunoblotting analysis with antibodies against p-AKT and total AKT. D, LY294002 treatment enhances the effect of CDDP in inhibiting tumor growth induced by A549/CDDP cells. Nude mice were injected s.c. into both flanks with 2 × 10^6 A549/CDDP cells. When the diameter of any tumor reached 5 mm, the mice were treated by i.p. injection of 4 mg/kg CDDP once a week for 3 successive weeks. At the same time, mice were injected i.p. twice a week for 3 wks with equal volume of solvent or 25 mg/kg LY294002 as indicated. Each treatment group contained five mice. The mice were euthanized on day 20 after drug treatment. Tumor volumes were measured by the width and length of tumors after the treatment. Points, mean from 8 to 10 tumors, normalized to the tumor volume on the first day of the treatment; bars, SE. *, P < 0.01, tumor volumes significantly different when compared with those of CDDP treatment alone. E, LY294002 only slightly increases the effect of CDDP in inhibiting tumor growth induced by A549 cells. Tumor growth was induced by the injection of A549 cells. The mice were treated as described above. *, P < 0.01, tumor volumes significantly different when compared with those of CDDP treatment alone.
Figure 5. The AKT/mTOR pathway was involved in the chemosensitivity of A549 and A549/CDDP to CDDP treatment. A, rapamycin inhibited phospho-p70S6K1 expression in a dose-dependent manner. A549 and A549/CDDP cells were treated with rapamycin (0, 5, 10, and 20 nmol/L) for 2 h. Total cellular proteins were extracted and analyzed by immunoblotting. B, the intensity of phospho-p70S6K1 protein signals obtained was quantified using Chem Doc densitometry software (Quantity One, Bio-Rad) from three replicate experiments. The phospho-p70S6K1 densitometry signals were normalized to those of total 70S6K1, then normalized to those of untreated A549 cells (100%). Columns, mean from three independent experiments; bars, SE. C, rapamycin enhanced the effect of CDDP in inhibiting A549/CDDP cell proliferation. A549/CDDP cells were seeded into 96-well plates and treated as described in Fig. 1, except the use of rapamycin (0 and 10 nmol/L). Cell proliferation was measured by MTT assay, and IC50 was calculated using the data from three replicate experiments. D, rapamycin slightly increased the effect of CDDP in inhibiting A549 cell proliferation. A549 cells were treated to study cell proliferation in the absence or presence of CDDP and rapamycin as described above.

its resistance remain to be identified. In this study, we showed that AKT1 amplification was responsible for CDDP resistance in human lung cancer cells, which represents a novel mechanism of CDDP resistance in cancer cells. AKT is known to be a major antiapoptotic signal in response to growth factors. AKT regulates cell survival, proliferation, and protein synthesis (41–43). Our results showed direct evidence that AKT1 was sufficient and necessary in regulating CDDP resistance. Recent studies showed that the PI3K/AKT pathway is related to chemoresistance mainly through inhibition of cellular apoptosis (44–46). AKT activation has been indicated to be correlated with chemoresistance (47, 48). These results are consistent with our data showing that AKT1 amplification and activation are involved in CDDP resistance. We also showed that AKT activation is associated with CDDP resistance in primary cultured lung cancer cells and human tumor tissue samples, confirming the importance of our finding in the clinical setting.

Acknowledgments

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AKT1 Amplification Regulates Cisplatin Resistance in Human Lung Cancer Cells through the Mammalian Target of Rapamycin/p70S6K1 Pathway

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