Secreted Clusterin (sCLU) Gene Silencing Enhances Chemosensitivity of A549 Cells to Cisplatin through AKT and ERK1/2 Pathways In Vitro

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Abstract

Several studies have shown Secreted Clusterin (sCLU) silencing directed against sCLU mRNA in sCLU-rich lung cancer cell lines sensitized cells to chemotherapy. However, the molecular mechanisms underlying the effect of sCLU silencing on lung cancer cell chemosensitivity is not known. In the present study, we aimed to determine that sCLU overexpression by pCDNA3.1-sCLU transfection on chemosensitivity to cisplatin (DDP) in A549 cells in vitro. We down-regulated sCLU expression by short hairpin RNA against sCLU (sCLU-shRNA) and investigated the effects on chemosensitivity to DDP in A549 cells and A549ICP in vitro. In order to confirm the correlation between sCLU and AKT and ERK1/2 signals, cells were treated with wortmannin and U0126. We found the chemotherapeutic agent DDP activated sCLU. Overexpression of sCLU increased cellular DDP chemoresistance in the A549ICP and pCDNA3.1-sCLU transfected A549 cells via inhibition DDP-induced apoptosis. Whereas sCLU knockdown induced chemosensitization in the S549 and A549ICP cells via increase of DDP-induced apoptosis. sCLU overexpression activated pAkt Ser473 and pERK1/2 Thr202/Tyr204, and vice versa. Inhibition of pAkt Ser473 and pERK1/2 Thr202/Tyr204 was sufficient to induce significant recovery in chemosensitivity to DDP in A549ICP in the presence of sCLU overexpression. The chemotherapeutic agent DDP activated sCLU, which directly regulated pAkt and pERK1/2. This novel finding suggests that therapies directed against sCLU and its downstream signaling targets pAkt and pERK1/2 may have the potential to enhance the efficacy of DDP-based chemotherapy.

Keywords: Lung cancer; Chemotherapy; Clusterin; ERK; AKT

Introduction

Lung cancer, the leading cause of cancer death worldwide, can be divided into two types: Non-Small Cell Lung Cancer (NSCLC) and Small Cell Lung Cancer (SCLC). Non–Small-Cell Lung Cancer (NSCLC) comprises >75% of lung cancer cases making it the leading cause of cancer-related death. The prognosis for NSCLC patients is highly dependent on the stage at diagnosis, and despite efforts to develop early screening tools; a majority of tumors are detected at an advanced stage [1]. The therapies available to date for NSCLC treatment are surgery, radiotherapy, and chemotherapy. Because of the size and distribution of lung cancer, the cytoreductive surgery is not very effective for this disease and therefore chemotherapy and/or radiation are the only treatments of choice. Despite major advances in patient management, chemotherapy and radiotherapy, nearly 80% of the patients still die within 1 year of diagnosis and long-term survival is obtained only in 5-10% of the cases [2]. Chemoresistance occurs not only to clinically established therapeutic agents but also to novel targeted therapeutics. However, extensive stage disease has initial response rates to chemotherapy exceeding 70%; the disease almost invariably progresses and becomes fatal [3]. Both intrinsic and acquired mechanisms have been implicated in drug resistance but it remains controversial which mechanisms are responsible that lead to failure of therapy in cancer patients [4-6].

Clusterin is an enigmatic glycoprotein with a nearly ubiquitous tissue distribution. It plays important roles in various pathophysiological processes, including tissue remodeling, reproduction, lipid transport, complement regulation, and apoptosis. Clusterin appears to have two main isoforms that result from alternative splicing: The secreted and nuclear forms of clusterin have been reported to play different roles in various pathophysiological processes, including tissue remodeling, reproduction, lipid transport, and cell distribution. It plays important roles in various pathophysiological processes, including tissue remodeling, reproduction, lipid transport, complement regulation, and apoptosis. Clusterin appears to have two main isoforms that result from alternative splicing: the secreted and nuclear forms of clusterin have been reported to play different roles in various pathophysiological processes, including tissue remodeling, reproduction, lipid transport, and apoptosis. Clusterin appears to have two main isoforms that result from alternative splicing: the secreted and nuclear forms of clusterin have been reported to play different roles in various pathophysiological processes, including tissue remodeling, reproduction, lipid transport, and apoptosis.
and its downregulation, by means of PI3K inhibitors, lowers resistance to various types of therapy in tumour cell lines [22–24]. Mitogen-activated protein kinase/Extracellular signal Regulated Kinase (ERK) pathway plays an essential role in the development and progression of various tumors. ERK1/2 is a key component of this pathway. The hyperactivation of ERK1/2 also has been shown to promote resistance to chemotherapy drugs in many cancer cells [25–27]. Inhibiting the action of ERK1/2 prevents tumor cell proliferation, promote apoptosis and reverse resistance to therapy [28,29].

In lung cancer, phosphorylated ERK1/2 (p-ERK1/2) and Akt (p-Akt) can be further stimulated by chemotherapeutics, and targeting the ERK1/2 or Akt pathway has been reported to sensitize cancer cells to therapy [30–32]. We have also recently found ERK1/2 and Akt pathways contribute to cisplatin resistance in human small cell lung cancer A549 cells, and blocking these pathways may be an effective strategy for improving the efficacy of cisplatin as anticancer treatment [33]. Tang, et al. [28] has found knockdown of clusterin sensitizes pancreatic cancer cells to gemcitabine by inhibition of gemcitabine-induced clusterin-pERK1/2 activation. In breast cancer, clusterin may regulate the aggressive behaviour of human breast cancer cells through modulation of ERK1/2 signalling and MMP9 expression [34]. In lung cancer cells, clusterin regulate EMT and aggressive behaviour through modulating ERK1/2 signalling and Slug expression [16]. We therefore suggested ERK1/2 signalling is regulated by clusterin. Many studies in literature have reported that Akt pathway may also be regulated by clusterin in cancers including lung cancer [17,35,36].

Given the importance of clusterin in regulation of AKT and ERK1/2 signaling, we hypothesized that targeting clusterin might represent a novel approach to modulate the chemoresistance of lung cancer cells to cisplatin. We present the first evidence indicating a role for clusterin in determining lung cancer cellular chemoresistance to cisplatin, the mechanism by which is via regulating AKT and ERK1/2 signaling.

Materials and Methods

Cell lines

Human lung adenocarcinoma bronchiolosalveolar carcinoma A549 cells and cisplatin (DDP) resistant A549 cells (A549DDP) were obtained from the American Type Culture Collection (Manassas, VA) and cultured at 37°C in a humidified atmosphere containing 5% CO2 in RPMI 1640 complete medium supplemented with sodium bicarbonate (2.2%, w/v), L-glutamine (0.03%, w/v), penicillin (100 units/ml), streptomycin (100 μg/ml), and fetal calf serum (10%).

Reagents

Akt (Ab-27) antibody (E021054-2), ERK1/2 (Ab-202/204) Antibody (E022017-2), ERK1/2 (Phospho-Thr202/Tyr204) Antibody (E012017-2), Akt (Phospho-Ser473) Antibody (E011054-2) and β-actin Antibody(S57) (E12-041-3) were purchased from EonoGene, Shanghai, China. Clusterin (A-9)(sc-166907, 1:200) was purchased from Santa Cruz (Shanghai, China). A549 DDP cells were transfected with sCLU-shRNA or control scrambled using the Lipofectamine 2000 (Invitrogen), according to the manufacturer’s protocol. A549 cells were transfected with endotoxin-free preparations of pcDNA3.1-sCLU or pcDNA3.1 (control). Transiently transfected cells were harvested 48 h after transfection. Transfection efficiency was assessed by detection of Green Fluorescence Protein (GFP) expression by fluorescence microscopy. Positively transfected cells were routinely more than 50% of the cell population. For selection of stably transfected cell populations, G418 was added to the culture medium 48 h after transfection at a concentration of 400 μg/ml for A549 cells. The cells were selected with 400 μg/ml G418 for 14 d. Selected colonies were screened by immunoblotting to identify stable clones expressing sCLU.

Drug treatments

Cisplatin was purchased from Sigma-Aldrich Co. Dimethyl sulfoxide was used to dilute cisplatin to a 20 mM stock. A549, A549DDP, A549/pCDNA3.1-sCLU, A549DDP/sCLU-shRNA, A549/pCDNA3.1 and A549DDP/pCDNA3.1 cells were treated with cisplatin (1-50 μM) for 72 h or with the same concentration of cisplatin for the same duration followed by wortmannin (400 nM) for 4 h or U0126 (25 μM) for 8 h in the continuous presence of cisplatin. The concentration and duration of cisplatin treatment was chosen based on preliminary studies examining its effects on cell growth inhibition and induction of apoptosis.

Western blot analysis

For Western blot analysis, cells or xenograft tissues were rinsed in ice-cold PBS twice and lysed in cell lysis buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% sodium deoxycholate, 1% Nonidet P-40, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 50 mM NaF, 2 mM Na3 VO4, 2 mM EGTA, 2 mM EDTA, and 0.25 mM PMSF) for 30 min. Samples were sonicated for 30 s and centrifuged for 20 min at 12,000 × g at 4°C. Samples were electrophoresed on a 12% SDS-polyacrylamide gel and electrophoretically blotted on nitrocellulose membrane. Membranes were blocked in TBS/Tween-20 with 5% milk and incubated with primary Abs diluted in TBS/Tween-20/BSA overnight at 4°C. The following primary antibodies were used: (β-actin: 1 : 10000; sCLU, Akt
cisplatin resistant A549 cells (A549 DDP) (1.26 ± 0.21) compared to the
washed twice in PBS and resuspended in PBS containing RNAse A (20
ml-1) for 10 min at room temperature. The samples underwent FACS
analysis (FL-3 channel) using a Beckman Coulter Counter Epics XL flow
cytometer. For each sample, 10,000 events were collected and stored for
subsequent analysis using EXPO software. Data were elaborated using
Autfit feature of the Multicycle for Windows software and expressed as
fraction of cells in the different cycle phases. The percentage of cells
in the sub-G0 phase was quantitated as an estimate of cells undergoing
apoptosis.

Terminal dUTP nick-end labeling (TUNEL) assay

Fixed cells were incubated with an equilibrium buffer for 5
min using the in situ apoptosis detection kit. Apoptosis of the cells
was evaluated on the basis of the TUNEL assay according to the
manufacturer's instructions. For quantifying apoptotic cells, apoptotic
and total cells were counted in 5 random fields scoring between 300
and 500 cells, and the numbers of apoptotic cells were expressed as
percentages of the total cell population. TUNEL staining slides were
observed with microscope. All assays were performed in quadruplicate.

Statistical analysis

Student’s t-test was used to compare mean values where appropriate
using SPSS11.0 software. P values <0.05 were considered significant.
All data are expressed as means ± SD for a series of experiments.

Results

sCLU was overexpressed in DDP resistant A549 cells
(A549DDP)

As shown in Figure 1A, sCLU protein was overexpressed in
cisplatin resistant A549 cells (A549DDP) (1.26 ± 0.21) compared to the
A549 cells (0.12 ± 0.03) (P<0.05). (Figure 1A).

Successful overexpression of sCLU through pCDNA3.1-sCLU
transfection in A549 cells

Fluorescence microscopy was used to detect the pCDNA3.1-
sCLU transfection efficiency in A549 cells. After 48 h transfection, the
green staining of more than 75% of cells was confirmed in pCDNA3.1
transfected A549 cells (Figure 1B).

In the pCDNA3.1-sCLU clones, expression of exogenous clusterin
was significantly higher compared to the endogenous clusterin levels in
the controls (pCDNA3.1). As shown in Figure 1C, sCLU protein
was overexpressed in pCDNA3.1-sCLU transfected A549 cells (1.06 ±
0.24) compared to the A549 cells (0.12 ± 0.03) and control pCDNA3.1
transfected A549 cells (0.14 ± 0.03) (P<0.05).

Efficient silencing of the sCLU expression in A549DDP cells by
using shRNA

A549DDP cells were stably transfected with scrambled control
or shRNA of sCLU. Protein expression was evaluated in whole-cell
extracts from A549DDP cells. Comparing to the scrambled control, sCLU
knockdown was shown to be successful across the A549DDP cells (Figure
2). Furthermore, sCLU mRNA gene expression was also inhibited after
stably shRNA of sCLU transfection(data not shown).

sCLU silencing increases DDP-induced apoptosis in A549 cells

To characterize the role of sCLU in DDP resistance, A549 cells were
stably transfected with control vector or sCLU-shRNA vector against
sCLU to generate A549/shRNA or A549/sCLU-shRNA stable clone,
respectively (Figure 2). The A549/sRNA or A549/sCLU-shRNA stable
clones were treated with 1-50 µM cisplatin for 72 h. Cytotoxicity of
DDP in control and sCLU knockdown cells were measured by MTT
assays. As shown in Figure 3A, A549 cells depleted of sCLU displayed
decreased cell survival after DDP treatment for 72 hs, compared with
those of control cells and control-shRNA transfected A549 cells. sCLU-
shRNA or shRNA transfection alone did not affect the survival after
DDP treatment (data not shown).

To examine whether depletion of sCLU can re-sensitize DDP
resistant A549DDP cells, A549DDP was stably transfected with control
or sCLU shRNA to establish control and sCLU knockdown cell lines
(Figure 2). As shown in Figure 3B, depletion of sCLU in A549DDP cells
dramatically decreased the survival of DDP in sCLU knockdown cells
measured by MTT assays.

sCLU overexpression confers resistance to DDP in vitro in
A549 cells

To examine whether sCLU overexpression can be resistant to DDP
treatment, A549 cells was stably transfected with pCDNA3.1 control or
pCDNA3.1-sCLU to establish control and sCLU overexpressed A549
cells (Figure 1C). As shown in Figure 3C, overexpression of sCLU
in A549 cells dramatically decreased cell survival of DDP in sCLU
overexpressed cells measured by MTT assays. pCDNA3.1 control or
pCDNA3.1-sCLU transfection alone did not affect the survival after
DDP treatment (data not shown). The data presented above suggest
that stable overexpression of clusterin or stable silencing of clusterin
alone did not affect cell survival of the A549 cells, but sCLU confers
DDP resistance.

sCLU silencing increases DDP-induced apoptosis in A549 cells

In addition to testing apoptosis levels of sCLU silencing on DDP
induced apoptosis, the A549DDP cells, sCLU-shRNA stably transfected
A549 cells and their controls were chosen for study.

We first analyzed the sCLU alone on apoptosis in A549 cells. A549
cells were transiently transfected with sCLU-shRNA, and its controls

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for 72 hours, no significant apoptotic cells was increased by TUNEL staining and FACS assay (data not shown).

To analyze the extent of the DDP-mediated cell death, we scored apoptosis by TUNEL and FACS. A549DDP cells were treated with 1-50 µM cisplatin for 72 h, no significant TUNEL positive cells were shown. To observe whether depletion of sCLU can re-sensitize DDP resistant
Figure 2: Effective silencing of the sCLU expression in A549DDP cells after shRNA transfection. sCLU expression was knockdown by western blot assay after stably shRNA of sCLU transfection (*P<0.05).

Figure 3: sCLU level is associated with DDP sensitivity in A549 cells. (A) A549/shRNA or A549/sCLU-shRNA stable clone were seeded into 96-well plates. The following day, 1-50µM cisplatin were applied. After incubation for 72 hours, cell survival was analyzed by MTT. Cell growth in the absence of DDP corresponds to 100. (B) A549DDP was stably transfected with control or sCLU shRNA, then treated with 1-50 µM cisplatin for 72 h. MTT assays were performed the cell survival. (C) A549 cells was stably transfected with pCDNA3.1 control or pCDNA3.1-Sclu, then treated with 1-50 µM cisplatin for 72 h. MTT assays were performed the cell survival. Boxes, mean; bars, ± SD. *P<0.05, **P<0.01 (compared with control or cells transfected with mock expression plasmid).
A549<sup>DDP</sup> cells, A549<sup>DDP</sup> was stably transfected with control or sCLU shRNA to establish control and sCLU knockdown cell lines (Figure 2). The results showed depletion of sCLU in A549<sup>DDP</sup> cells dramatically increased TUNEL positive cells in the A549<sup>DDP</sup>/sCLU shRNA cells (Figure 4A). Similar results were also shown by FACS assay (data not shown).

A549 cells treated with 1-50 µM cisplatin for 72 h induced a few apoptotic cells, however, in the sCLU-shRNA stably transfected A549 cells, treated with 1-50 µM cisplatin for 72 h induced much more TUNEL positive cells (Figure 4B). Similar results were also shown by FACS assay (data not shown). These results above are consistent with cell growth inhibition studies by MTT, suggesting that the loss of viable cells by sCLU-shRNA transfection is partly due to the induction of an apoptotic cell death mechanism.

**Cisplatin activates sCLU and sCLU-dependent ERK1/2 and AKT phosphorylation in A549 cells**

A549 cells were treated with cisplatin (DDP) (1-50 µM) for 72 times. Protein expression was evaluated in whole-cell extracts from A549 cells by Western blotting. As shown in Figure 5A, sCLU, pERK1/2 and pAKT protein expression was elevated in A549 cells after 1,5,10 and 50 µM cisplatin treatment for 72 h.

To determine if pERK1/2 and pAKT protein expression was sCLU dependent, A549 cells were stably transfected with control vector or sCLU-shRNA vector against sCLU, then treated with 1-50 µM cisplatin for 72 h. Western blot result shown no statistical sCLU, pERK1/2 and pAKT protein expression upregulation was found in sCLU-shRNA vector transfected cells compared to the cells treated with DDP alone (Figure 5B) or control vector transfected cells treated with DDP (data not shown).

When the A549 cells were treated with wortmannin (400 nM) for 4 h or U 0126 (25 µM) for 8 h, then treated with 1,5,10 and 50 µM cisplatin for 72 h, pERK1/2 and pAKT protein expression was inhibited, however, sCLU was activated in the continuous presence of cisplatin all the same (data not shown). These results demonstrate that cisplatin activates sCLU-dependent pERK1/2 and pAKT expression in A549 cells.

**sCLU silencing sensitizes A549 cells to DDP via inactivtion of ERK1/2 and AKT phosphorylation**

To determine the biological significance of the functional link between clusterin and AKT and ERK, we have evaluated the effects of sCLU knockdown DDP induced A549 cell death. As shown in Figure 6A, A549<sup>DDP</sup> has higher sCLU levels, the phosphorylated ERK1/2 and AKT was also activated in the A549<sup>DDP</sup> cells. In sCLU-shRNA stably transfected A549<sup>DDP</sup> cells, the sCLU, pERK1/2 and pAKT was specifically silenced. In contrast, inhibition of phosphorylated ERK1/2 and AKT by wortmannin or/and U 0126 did not affect sCLU levels in the A549<sup>DDP</sup> cells, suggesting that ERK1/2 and AKT is a critical downstream mediator of sCLU.

It has shown above that A549<sup>DDP</sup> cells depleted of sCLU displayed decreased cell survival and increased apoptotic cells after DDP treatment for 72 h, compared with those of control cells and control-shRNA transfected A549 cells.

We then investigated if concurrent blockage of pERK1/2 and pAkt cooperatively potentiates DDP (5 µM)-induced cancer A549<sup>DDP</sup>
cells death and survival inhibition. A549DDP cells were treated with wortmannin or U0126 alone or combined wortmannin and U0126 to simultaneously block pAkt and pERK1/2 and examined the effect of which on DDP-induced cancer cell apoptosis and survival inhibition.

Treatment with DDP caused no detectable apoptotic cells in the A549DDP cells. When treatment with DDP and wortmannin, 8.9% of the apoptosis cells was detected (P<0.05), when combined with U0126, 9.4% of the apoptosis cells was detected (P<0.05), however, when cells were treated with DDP and in combination with wortmannin and U0126, a synergistic cytotoxicity that 19.6% of the apoptosis cells was detected (P<0.01) (Figures 6B and 6C). This potentiated cytotoxicity by double pathway blockage is much higher than that of individual blockage of either pERK1/2 or pAKT. Similar results were also shown in the cellular viability inhibition (data not shown).

Discussion
This development of cisplatin resistance is a complex phenomenon involving multiple alterations. It can be intrinsic owing to germ-line genetic variation or acquired through altered mRNA or protein expression in key pharmacokinetic or pharmacodynamic pathways. It can also be the reduction of intracellular drug accumulation, increased DNA damage repair, and up-regulation of antiapoptotic genes [6,38]. Moreover, activation of survival-associated signal transduction pathways influences the cellular response to cisplatin treatment [39]. The crucial point in these events may be determined by signaling events downstream of the drug-DNA interactions. Acquisition of resistance by tumor cells to anticancer drugs may involve modifications in the cells ability to trigger downstream signaling events. It is thus the balance between the various proteins present in the cell that finally decides whether the cell should live or die.

Recent focus has turned to Clusterin (CLU) as a key contributor to chemoresistance to anticancer agents. Its role has been documented in prostate cancer for paclitaxel/docetaxel resistance [40] as well as in renal [41], and melanoma [42], breast tumor cells [9,43]. Most significantly, sCLU expression is documented to lead to broad-based resistance to other unrelated chemotherapeutic agents such as doxorubicin [9,44], cisplatin [9,44,45], and etoposide [46].

In the present study, we found cisplatin treatment increased the levels of sCLU in the A549 cells which made the A549 cells acquire the resistance to cisplatin. Overexpression of sCLU by sCLU transfection...
protected human lung cancer cells from cisplatin-induced cytotoxicity. In addition, inhibition of sCLU by shRNA to sCLU transfection could restore cisplatin sensitivity in cisplatin cisplatin-sensitive and resistant A549cisplatin cells. Although the involvement of sCLU acquires resistance to cisplatin in A549 cells, however, the molecular mechanisms underlying the effect of sCLU on lung cancer cell chemosensitivity is not known.

ERK1/2 activation results in the phosphorylation of many intracellular proteins that regulate various cellular functions ranging from proliferation and differentiation to apoptosis [47,48]. Cisplatin can activate ERK1/2 in various cell types. Some studies have shown that activation of ERK1/2 is associated with an increase in cell survival in cisplatin-treated cells [49,50]. Accumulating evidence has indicated that the activation of resistance to chemotherapeutic drugs also involves the activation of the PI3K/Akt pathway [51]. Combining PI3K/Akt inhibitors with standard chemotherapy has been successful in increasing the efficacy of chemotherapeutic agents both in vivo and in vitro [52,53].

Our previous studies have reported [33] that cisplatin activate both p-ERK1/2 and p-Akt in A549 cells. Blockade of either of these pathways with chemical inhibitors moderately sensitized A549 cells to cisplatin induced apoptosis and reduced cell viability. Strikingly, much more effective potentiation of cytotoxicity to cisplatin was achieved when p-ERK1/2 and p-Akt were concurrently blocked in vitro and vivo. Our study demonstrated that cisplatin-induced ERK1/2 and Akt activation participates in protection from cisplatin-mediated cytotoxic effect in A549 cells.

PI3K/Akt and ERK1/2 is believed to act downstream of sCLU [16,17,34-36]. In this study, cisplatin could up-regulate the expression of sCLU, which is correlated with the activation of pAkt and pERK1/2. When p-ERK1/2 and p-Akt protein was prevented by U0126, a MKK1/2 inhibitor, and wortmannin an inhibitor of phosphatidylinositol 3-kinase (PI3K), cisplatin could not induce p-ERK1/2 and p-Akt upregulation, though sCLU was induced by cisplatin. In the cisplatin resistant A549cisplatin cells and sCLU stably transfected A549 cells, p-ERK1/2 and p-Akt was also activated. However, when sCLU was inhibited by shRNA transfection, p-ERK1/2 and p-Akt was inhibited. Therefore, we suggest that p-ERK1/2 and p-Akt was regulated by sCLU.

In the DDP resistant A549cisplatin cells and sCLU stably transfected A549 cells, high sCLU expression level was found. Silencing of sCLU expression can re-sensitize the two cells to cisplatin. In addition, inhibition of p-ERK1/2 or p-Akt alone could in part sensitize the two cells to cisplatin, however, when p-ERK1/2 and p-Akt were concurrently blocked, significant recovery in chemosensitivity to DDP in A549cisplatin and sCLU stably transfected A549 cells. Therefore, we suggest that sCLU protects lung cancer cells from cisplatin-induced cytotoxicity, which might be by activation of ERK1/2 and Akt.

Taken together, activation of sCLU may contribute to cisplatin resistance. Our results identify a new mechanism showing that the sCLU-AKT and sCLU-ERK1/2 signaling pathway is responsible for cisplatin resistance and suggest that targeting the sCLU-AKT and sCLU-ERK1/2 signaling pathway may overcome cisplatin resistance in human lung cancer.

References


