行政院國家科學委員會專題研究計畫 成果報告

研究肺癌細胞對歐洲紫杉醇衍生物(剋癌易)產生抗藥性之機轉(第 3 年)
研究成果報告(完整版)

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計 畫 主 持 人：許國堂

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中 華 民 國 99 年 10 月 27 日

附件一

行政院國家科學委員會補助專題研究計畫 v 成果報告

研究肺癌細胞對歐洲紫杉醇衍生物(剋癌易)產生抗藥性之機轉（第 3 年）

計畫類別：v 個別型計畫　　□ 整合型計畫
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執行期間：96 年 08 月 01 日至 99 年 07 月 31 日

計畫主持人：許國堂

共同主持人：

計畫參與人員：碩士班研究生-兼任助理人員：廖福田、丘翎燕、黃馨儀、
黃依柔、曾媄慧

成果報告類型(依經費核定清單規定繳交)：□精簡報告　v 完整報告

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□赴大陸地區出差或研習心得報告一份
□出席國際學術會議心得報告及發表之論文各一份
□國際合作研究計畫國外研究報告書一份

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執行單位：中山醫學大學醫學分子毒理學研究所

中　華　民　國　99 年 10 月 30 日

中文摘要及關鍵詞 (keywords)：

Docetaxel（剋癌易 DOC）, Vincristine (VCR), MDR-1(P-glycoprotein, ABCB1), 抗藥性 (drug resistance), 肺癌, p53 promoter, reporter gene assay

本計畫之第一年目標為使用具有 Docetaxel (DOC),Vincristine (VCR)之抗藥性的肺癌細胞株做為材料，並且找出 p53、tubulin 和 MDR-1(P-glycoprotein, ABCB1)與 DOC/VCR 之抗藥性的關係。同時，利用 DNA 微探針陣列比對會被 DOC/VCR 轉錄活化的基因，並且逐步地在本計畫期間進一步比對和驗證其與抗藥性的關聯。我們已經建立了具有 DOC/VCR 之抗藥性的 A549 和 H1299 之肺癌細胞株，並且使用全人類基因 DNA 微探針陣列比對會被 DOC/VCR 轉錄活化的基因。我們發現 MDR-1(ABCB1)基因的過度表現是只與 A549 細胞對 DOC 的抗藥性能力相關，卻與 VCR 的抗藥性能力無關。應該還有其他的基因會影響細胞對 DOC 的抗藥性能力。我們也發現到抗 DOC/VCR 藥的肺癌細胞株其中的 p53、tubulin 的基因表現並未受到抗藥篩選的影響。接著使用鈣離子通道抑制劑(Verapamil, Diltiazem and Nifedipine)來降低其抗藥性。結果證明鈣離子通道抑制劑可以有效增加抗藥之肺癌細胞株對藥物感受性。此研究結果已經發表於 **Toxicology Letters** Volume 192, Issue 3, pages 408-418 **IF:3.249 (15/75)** 15 February, 2010。

本計畫之第二年目標為找出 DOC 如何控制 p53 的基因轉錄活性，因而調節藥物感受性。我們已經將 p53 的基因轉錄調控區分離出來，然後使用 reporter gene assay 尋找 DOC 的反應序列。初步結果證明 DOC 處理後會造成 p53 promoter 的基因轉錄活性。此部分之成果已經完成並且著手進行論文書寫預計 2011 年可以投稿。此稿之題目暫定為(Docetaxel induces human p53 tumor suppressor gene transcription via a short sequence at core promoter element) 。 我們也發現高度表現 clusterin 除了會降低肺癌細胞對化學治療藥物的感受性外，也會減少細胞移動的能力。此部分之成果已經完成論文書寫和投稿。此稿之題目為（Regulation of chemosensitivity and migration by clusterin in non-small cell lung cancer cells)。

本計畫之第三年目標為找尋逆轉抗藥性的方法和機制來讓具有對化學治療藥物產生多重抗藥性的癌細胞可以進行有效的治療。我們發現具有多重抗藥性的癌細胞可以被 endoplasmic reticulum stress (ER stress) inducers 誘導而死亡。此部分之成果已經完成並且著手進行論文書寫預計 2011 年可以投稿。此稿之題目暫定為(The effect of ER stress on docetaxel and vincristine-induced multidrug resistance in human lung cancer cell lines)。因此預估本項專題研究計畫應該會有四篇論文發表，其中已經發表壹篇，投稿審查中壹篇，草稿中兩篇。

英文摘要：

The purpose of this study is to analyze the expression of MDR-associated genes with docetaxel (DOC) or vincristine (VCR) selected A549 and H1299 non-small cell lung cancer (NSCLC) sublines that exhibit MDR phenotypes. Although all drug resistant sublines showed cross-resistance to DOC, VCR, and doxorubicin (DXR); the gene of ATP-binding cassette (ABC) transporter B1 (ABCB1, MDR-1) was only found strongly induced in DOC but not VCR resistant A549 sublines by DNA microarry analysis and quantitative reverse transcription real-time polymerase chain reaction (qRT-PCR). The results showed that MDR is regulated by ABC transporters and by other factors. Furthermore, verapamil (VER), the inhibitor of ABCB1 and an L-type calcium channel blocker, is capable to reverse the resistance in all drug-resistant sublines independent of ABCB1 expression levels. Other L-type calcium channel blockers, Diltiazem (DIL) and Nifedipine(NIF) also sensitizes MDR sublines without interfering ABCB1 activity but with less efficacy. Our data of the first year already reported in **Toxicology Letters** Volume 192, Issue 3, pages 408-418 **IF:3.249 (15/75)** 15 February, 2010.

We also characterized the promoter of p53 to define the cis-element responsible for DOC induction by luciferase reporter gene assay. The upstream region of p53 promoter was cloned and sequenced for characterization. We found that a 120-bp sequence upstream of transcription start site is important for p53 promoter basal activity. We also transfected p53 promoter deletion constructs into lung cancer lines with Doc treatment, the results showed that a 21- bp (PE 21) element can up regulate p53 promoter activity by treated with Doc. To identify the response element of Doc, we generated four mutation constructs (M1-M4) on PE 21 sequence, only M4 has no response to Doc stimulation. In comparison with previous studies, we found that 5-FU not only up regulates p53 RNA and protein, but also may act with similar sequences of Doc response element of p53 promoter. In further experiment we demonstrated that all the constructs are regulated by 5-FU except M4. Therefore, we hypothesized that Doc probably has same response element as 5-FU induction. The results will be submitted in year of 2011 as the manuscript titled (Docetaxel induces human p53 tumor suppressor gene transcription via a short sequence at core promoter element). Another manuscript was submitted with the title（Regulation of chemosensitivity and migration by clusterin in non-small cell lung cancer cells) that is reviewing process.

The third year project was to search the re-sensitization agents that are able to induce the death of the MER-associated NSCLC cells. Furthermore, we also proposed to define the mechanism that regulates the reversal of MDR resistance. We found that the endoplasmic reticulum stress inducers can reverse the MDR-associated resistance. The results is going to be submitted in year of 2011 with the title of (The effect of ER stress on docetaxel and vincristine-induced multidrug resistance in human lung cancer cell lines).

We estimate that from this three-year NSC funding of my project, we would be able to publish four related SCI papers. The first paper is already published and the second paper is submitted under reviewing process. There are two additional papers will be prepared and submitted in near future.

目錄:

報告內容：

壹、Ling-Yen Chiu, Jiunn-Liang Ko, Yi-Ju Lee, Tsung-Ying Yang, Yi-Torng Tee, Gwo-Tarng Sheu* **(2010)** L-type calcium channel blockers reverse docetaxel and vincristine-induced multidrug resistance independent of ABCB1 expression in human lung cancer cell lines **Toxicology Letters** Volume 192, Issue 3, 408-418 **IF:3.249 (15/75)** 15 February

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L-type calcium channel blockers reverse docetaxel and vincristine-induced multidrug resistance independent of *ABCB1* expression in human lung cancer cell lines

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ABSTRACT

Multidrug resistance (MDR) of cancer cells to cytotoxic drugs significantly impedes chemotherapeutic treatment. The purpose of this study is to characterize docetaxel (DOC) or vincristine (VCR) selected A549 and H1299 non-small cell lung cancer (NSCLC) sublines that exhibit MDR phenotypes and followed by re-sensitization study. Although all drug resistant sublines showed cross-resistance to DOC, VCR, and doxorubicin (DXR), the expression of ATP-binding cassette (ABC) transporter B1 (*ABCB1*) gene was found to be strongly induced in DOC but not in VCR resistant A549 sublines by quantitative reverse transcription real-time polymerase chain reaction (qRT-PCR). In DOC and VCR resistant H1299 sublines, moderate expression of *ABCB1* was detected. The levels of ABCB1 protein and efflux activities were further examined by immunoblotting and rhodamin-123 staining assay. The results showed that both ABC and non-ABC mediated MDR are existed. Furthermore, verapamil (VER), an inhibitor of ABCB1 and an L-type calcium channel blocker, is capable of reversing the resistance in all drug-resistant sublines independent of *ABCB1* expression. Importantly, VER only sensitizes resistant sublines but has no effect on parental cancer cells. Other L-type calcium channel blockers, such as diltiazem (DIL) and nifedipine (NIF), also sensitize MDR sublines without interfering with ABCB1 activity but with lower efficacy than VER. Our data showed that in addition to ABCB1, calcium channel activity may play a crucial role in DOC- and VCR-acquired MDR. Therefore, inhibition of calcium influx may provide a new target to modulate MDR in chemotherapy.

1. Introduction

Lung cancer is the second most frequently diagnosed cancer in men and women and accounts for one-third of all cancer-related deaths in the United States every year (Jemal et al., 2009). Eighty percent of lung cancer cases are non-small cell lung cancer (NSCLC) and the remaining 20% are small cell lung cancer (SCLC). The 5-year survival rate for lung cancer (15%) is the second to the lowest of all cancers, just above pancreatic cancer (Jemal et al., 2009).

Generally, chemotherapy is suggested for treatment of advanced-stage cancers. However, the ability of cancer cells to become simultaneously resistant to different drugs, a trait known as multidrug resistance (MDR), limits the efficacy of chemotherapy (Baird and Kaye, 2003). There are two types of MDR. In the first type, referred to as intrinsic resistance, the resistance is present in cancer cells at the time of first contact. The second is the acquired type when the cancer cells become insensitive to treatment after relapse (Fojo and Menefee, 2005). No matter the type, the mechanism of resistance involves many factors, including increased drug efflux, detoxification of the drug, target alteration, abrogation of apoptosis and other unknown factors (Stavrovskaya, 2000).

P-glycoprotein (P-gp), the product of the human *MDR-1* gene, is a member of the large ATP-binding cassette (ABC) family of membrane proteins (Johnstone et al., 2000; Stavrovskaya, 2000). ABC transporters include P-glycoprotein (*Pgp/ABCB1*), multidrug resistance proteins (*MRPs/ABCCs*), and breast cancer resistance protein (*BCRP/ABCG2*) which function as ATP-dependent drug efflux transporters, forming a unique defense network against multiple chemotherapeutic drugs and cellular metabolites (Tan et al.,

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2000). To date, 13 genes of ABC transporters (*ABCA2, ABCB1, ABCB4, ABCB11, ABCC1–6, ABCC11–12, and ABCG2*) have been found to be associated with drug resistance and drug transport (Gillet et al., 2007). It is reasonable that overexpression of one or more ABC proteins leads to excessive efflux and insufficient intracellular concentrations of chemotherapeutic agents, resulting in multidrug resistance (Gillet et al., 2007).

Docetaxel (Taxotere®, DOC) has anti-mitotic properties through the binding to microtubules (MTs) and prevention of depolymerization and stabilization of MTs (Fitzpatrick and Wheeler, 2003). These effects of DOC are correlated with mitotic arrest and cellular toxicity (Horwitz, 1992). Vincristine (VCR) is a classic anti-tubulin agent that induces disruption of MTs by binding to tubulin and inhibits tubulin polymerization/MT formation (Dumontet and Sikic, 1999). The action of VCR is to destabilize MTs that is in contrast to DOC. Both DOC (Davies et al., 2003; Green, 2002) and VCR (Kobayashi et al., 2000; Wood et al., 2001) have been applied clinically as part of various cancer chemotherapy regimens. However, both drugs are a substrate of ABCB1 transporter, therefore overexpression of ABCB1 in cancer cells is considered the major phenotype of MDR to DOC and VCR (Kartner et al., 1983; McGrogan et al., 2008).

To overcome the high prevalence of MDR, researchers have developed ABC transporter inhibitors to increase the intracellular concentration of chemotherapy drugs (Krishna and Mayer, 2000). However, much evidence suggests that MDR to DOC is mediated not only by ABC transporters (typical MDR) (Teodori et al., 2002) but also by non-ABC transporter associated (atypical MDR) factors (Krishna and Mayer, 2000). Atypical MDR has been reported as alterations of apoptotic pathway, with some metabolizing enzymes such as glutathione S-transferases, cytochrome P450-dependent oxidases, and topoisomerase activity (Krishna and Mayer, 2000). There is a debate about whether targeting ABC transporters is enough to benefit patients with MDR.

In the early 1980s, it was found that calcium channel blockers are inhibitors of MDR in leukemia cells (Tsuruo et al., 1981,1983). In fact, verapamil (VER) was the first compound to reach clinical trial for its ability to reverse MDR (Krishna and Mayer, 2000). Although, some of those clinical trials failed due to the high toxicity of VER or absence of improvement in the clinical outcome (Ozols et al., 1987), others showed that VER improves patient survival (Belpomme et al., 2000; Cairo et al., 1989; Salmon et al., 1991; Timcheva and Todorov, 1996), including a randomized study of NSCLC (Millward et al., 1993).

In order to establish the acquired MDR phenotype for genomic characterization of the ABC transporters with MDR, we used DOC and VCR as selection agents to treat two NSCLC cell lines (A549 and H1299). Under continuous exposure, several drug resistant sublines were obtained for investigation. The expression of ABC transporters and association with MDR, as well as the significance of calcium influx regulation in drug resistance were further characterized in this study.

2. Materials and methods

2.1. Drugs and chemicals

Docetaxel (DOC, 10 mg) was obtained from Aventis Pharmaceuticals Inc. (Bridgewater, NJ, USA). Doxorubicin (DXR), vincristine sulfate salt (VCR), verapamil hydrochloride (VER), nifedipine (NIF), cis-diltiazem hydrochloride (DIL) and rhodamine-123 were obtained from Sigma Chemical.

2.2. Establishment of the drug-resistant A549 and H1299 sublines

Human adenocarcinoma A549 cells and human large cell lung cancer H1299 cells were cultured at 37 °C in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1% NEAA, 1% sodium pyruvate, 1% L-glutamine, 100 IU penicillin mL^{-1} and 100 mg streptomycin mL^{-1}. The DOC and VCR resistant sublines were established from parental cells in a stepwise manner by exposure to increasing concentrations of DOC or VCR. For example, A549 cells in low cellular density were seeded onto 10-cm Petri dish and treated with 0.5 nM DOC until the surviving cells grew to an obvious colony. The selected colony was amplified in the presence of 0.5 nM DOC until confluence before the drug dose increased in multiples of two for next round of selection. The DOC-resistant sublines maintained at 16 and 32 nM DOC are denoted as A549/D16 and A549/D32, respectively. H1299/D8 subline was grown in the presence of 8 nM DOC but could not survive when selected with 16 nM DOC. Similar designations, A549/V16 and H1299/V16, were applied to VCR stably resistant sublines.

2.3. DNA microarray analysis and quantitative reverse transcription real-time polymerase chain reaction (qRT-PCR)

To analyze gene expression, A549/D16, A549/V16-1, H1299/D8 and H1299/V16 sublines were harvested, and total RNA was isolated using TRIZOL (Invitrogen, Carlsbad, CA). Microarray experiment and data analysis were performed by Welgene Biotech (Taipei, Taiwan) using the Agilent human whole genome oligo 4 × 4 K, which represents 41,000 unique human genes. To validate the results obtained from microarray analysis, we performed SYBR®-Green I chemistry verification for expression of 11 selected genes of ABC transporter (Szakacs et al., 2004) in all drug resistant cell lines, using an ABI 7500 thermal cycler (Applied Biosystems). For data analysis, SDS 2.2 software was used. Detection of PCR products was accomplished by measuring the emitting fluorescence (Rn) at the end of each reaction step (reaction cycles). Threshold cycle (Ct) corresponds to the cycle number required to detect a fluorescence signal above the baseline. We performed gene expression analysis using the comparative ($2^{-\Delta\Delta CT}$) Ct method. The extracted delta Ct values (which represent the expression normalized to the ribosomal 18S expression) were grouped according to the drug resistant cell lines.

2.4. Cytotoxicity assay (MTT assay)

Chemosensitivity to DOC or VCR was determined using MTT colorimetric assay. Approximately 2×10^4 cells per well were seeded onto 24-well plates. After 24-h incubation, the cells were exposed to various concentrations of DOC or VCR in fresh medium for 48 h. At the end of the exposure period, the supernatant was removed and cells were washed with PBS. Then, 300 μL MTT (1 mg/mL; Sigma) was added to each well and cells were incubated at 37 °C for 2.5 h. After the supernatant was removed and the cells were washed with PBS, 300 μL 2-propanol solution was added per well to dissolve the water-insoluble formazan salt. The plates were shaken at 70 rpm at room temperature for 10 min. Finally, the absorbance was measured at 570 nm using an ELISA plate reader (Molecular Devices SPECTRA max 340 PC). Mean values were calculated from three independent experiments. Chemosensitivity is expressed as the drug concentration for 50% cell survival (IC_{50}).

2.5. Re-sensitization by calcium channel inhibitors

Approximately 2×10^4 cells per well were seeded onto 24-well plates. After 24-h incubation, the cells were exposed to various concentrations of VER or NIF or DIL in fresh medium for 2 h. Following this, cells were exposed to various concentrations of DOC or VCR for 48 h. At the end of the exposure period, the supernatant was removed and cells were washed with PBS followed by MTT assay.

2.6. Rhodamin-123 flow cytometry assay

Suspensions of logarithmic phase cells were obtained from culture plates by trypsinization. During the accumulation period, cells were resuspended in rhodamine-containing medium (improved minimum essential medium with 10% FCS and 0.1 μg/mL rhodamine-123) and incubated in 5% CO_2 for 30 min. After the accumulation period, efflux was initiated by sedimentation at 800 rpm and resuspension in rhodamine-free medium (improved minimum essential medium with 10% FCS) at 37 °C in 5% CO_2 for 90 min. At the end of both the accumulation and efflux periods, cells were collected and washed in ice-cold Hanks' buffered salt solution. The washed cells were placed in Hanks' buffered salt solution with 10% FCS on ice, and kept in the dark until flow cytometric analysis. Samples were analyzed on a FACScan flow cytometer (Becton Dickinson) equipped with a 488-nm argon laser. The green fluorescence of rhodamine-123 was observed with a 530-nm band pass filter.

2.7. Protein extraction and Western blot analysis

Protein extracts were prepared from exponentially growing cells as described previously. Briefly, cells were collected and the cell pellets were resuspended in lysis buffer (50 mM Tris–HCl, pH 7.4, 2 mM EDTA, 1% NP-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate and 150 mM NaCl) plus protease inhibitors (Roche). Protein of 10–30 μg was used for SDS polyacrylamide gel electrophoresis. After transfer to PVDF membrane, the proteins were reacted with polyclonal anti-MDR1, anti-hnRNP H (Santa Cruz Biotechnology), monoclonal anti-tubulin-β (NeoMarker) and anti-p53 (DAKO) followed by anti-goat (Santa Cruz Biotechnology) or anti-mouse (Calbiochem) IgG conjugated to horseradish peroxidase. A chemiluminescence detection kit (ECL; Amersham Pharmacia Biotech) was used to determine the levels of protein expression.

Table 1
Drug sensitivity of parental cell lines and the drug resistant sublines.

Drug	$IC_{50} \pm SD^{*}$ (nmol/L)						
	A549	A549/D16	A549/D32	A549/V16	H1299	H1299/D8	H1299/V16
DOC	6.4 ± 0.1 (1.0)	730.0 ± 56.6 (114.1)	2035.0 ± 954.6 (318.0)	780.0 ± 51.3 (121.9)	12.9 ± 3.1 (1.0)	640.0 ± 135.3 (49.7)	620.0 ± 81.9 (48.1)
VCR	14.9 ± 0.1 (1.0)	770.0 ± 56.6 (51.9)	730.0 ± 28.3 (49.2)	760.0 ± 92.4 (51.2)	13.8 ± 0.9 (1.0)	1206.7 ± 317.2 (87.7)	470.0 ± 20.0 (34.2)
DXR	222.7 ± 23.2 (1.0)	3423.9 ± 152.3 (15.4)	7694.4 ± 148.0 (34.6)	1531.4 ± 80.8 (6.9)	139.5 ± 7.5 (1.0)	7904.2 ± 325.9 (56.7)	1289.8 ± 46.3 (9.2)

Note: Cell survival was determined by MTT assay as described in Section 2. (Fold resistance) was relative to the parental cell lines. DOC, Docetaxel; VCR, Vincristine: DXR, Doxorubicin.

* Data are the mean ± SD of at least three independent experiments done in triplicate.

3. Results

3.1. The toxicity profiles of DOC and VCR stably resistant NSCLC cells

The major limitation of using clinical samples to study gene expression profiles of drug resistance is the heterogeneity of the cells, which masks the significance of the obtained results. Therefore, we used a selection process that was not designed to cover the broad spectrum of clinical situations but rather to focus on the alteration of gene expression within individual cancer cell types. During the selection process, the A549 and H1299 cells were continuously exposed to an increasing concentration of DOC or VCR as described in Section 2. The concentration of drugs used in this selection is similar to clinically relevant concentrations of 5–200 nM of paclitaxel to produce mitotic arrest and cell death (Blagosklonny and Fojo, 1999). On MTT toxicity assay, the established A549 and H1299 sublines (Table 1) revealed their drug sensitivity in terms of IC_{50} (inhibition concentration). The DOC-selected A549/D16 subline showed 114.1-fold higher resistance to DOC and 51.9-fold higher resistance to VCR when compared with parental A549 cells. Not surprisingly, A549/D16 subline was also cross-resistant to DXR (15.4-fold). The VCR-selected A549/V16 subline showed 51.2-fold higher resistance to VCR, 121.9-fold higher resistance to DOC and 6.9-fold higher resistance to DXR. Concentration associated resistance of A549 subline to DOC was observed in A549/D32 subline (318-fold), which showed two-fold higher resistance to DOC when compared with A549/D16 subline. The H1299/D8 and V16 sublines also exhibited cross-resistance to all three drugs tested. These data provide evidence that DOC and VCR-selected sublines retain MDR phenotypes. Apparently, DOC-resistant sublines are cross-resistant to VCR and DXR, and VCR resistant sublines are cross-resistant to DOC and DXR.

3.2. Gene expression analysis of the ABC transporters using DOC and VCR resistant sublines

In order to obtain gene expression profiling associated with drug resistance, we used the human whole genome DNA microarray, which represents 41,000 unique human genes. The differentially expressed ABC transporter genes from parental and drug resistant cells were compared and summarized (Table 2). Thirteen genes of ABC subfamily A, 9 genes of subfamily B, 12 genes of subfamily C, 4 genes of subfamily D, 1 gene of subfamily E, 3 genes of subfamily F and 5 genes of subfamily G were analyzed on DNA microarray. Only genes of subfamilies A–C exhibited substantial up-regulation while genes of subfamilies D–G did not. The up-regulated ABC genes were further examined by qRT-PCR and the results are shown in Table 2. The expression efflux transporters of the ABC family such as *ABCC1*, *ABCC2* and *ABCG2* were not significantly induced by DOC or VCR. *ABCB1* of A549/D16 subline was significantly up-regulated on microarray analysis (41-fold) and qRT-PCR was performed on A549/D16 and D32 sublines to verify the significance of *ABCB1* expression. *ABCB1* is the most enhanced gene in A549/D16 and D32 followed by *ABCA8*. Interestingly, *ABCB1* up-regulation was not detected on microarray or on qRT-PCR analysis of A549 cells selected by VCR. Actually, the expressions of *ABCB1* in all three individual A549/VCR16 sublines (A549/V16-1, -2 and -3) were lower than in parental A549 cells on qRT-PCR. Individual sublines of A549/VCR16 were analyzed to avoid the possible artifact of the down-regulation of *ABCB1* expression from VCR-selection. Furthermore, all three A549/VCR16 sublines exhibited lower ABC transporter gene expression when compared with A549/D16 subline. Therefore, it is unclear if ABC transporters contribute to the MDR of A549/V16 sublines. Another NSCLC cell line, H1299 was also selected by DOC or VCR to obtain DOC-resistant (H1299/D8) and VCR resistant (H1299/V16) sublines. Both sublines were analyzed to reveal the expression profiles of ABC transporter genes. The expressions of *ABCB1* gene of H1299/D8 and V16 sublines exhibited 2–3-fold enhancements on microarray and qRT-PCR analysis.

3.3. Detection of ABCB1, p53, and tubulin protein levels by Western blot analysis

To confirm the alteration of *ABCB1* expression in drug resistant sublines, the ABCB1 protein was further analyzed by Western blotting (Fig. 1). A549/D16 and D32 expressed very high levels of ABCB1 protein. In contrast, A549/V16 expressed low level of ABCB1 protein. The ABCB1 proteins of H1299/D8 and V16-1 exhibited a level of

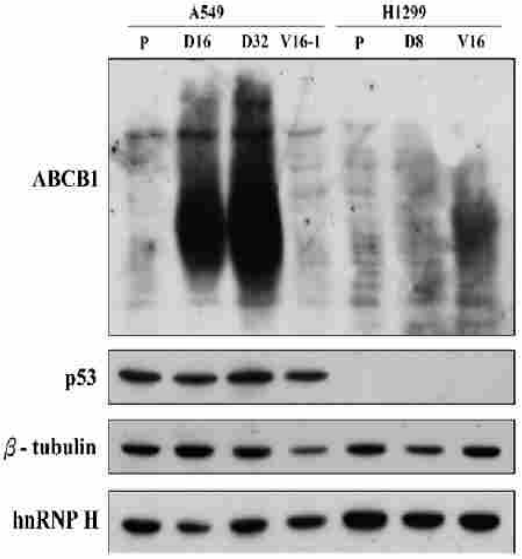

Fig. 1. Western blot analysis of ABCB1, p53, and tubulin expressions. Total lysates (100 μg) of parental cells (P), DOC-resistant sublines (D16, D32, D8) and VCR resistant sublines (V16, V16-1) were analyzed for ABCB1 detection. To detect p53, β-tubulin and hnRNP H, total lysate (10 μg), were loaded in each lane. Protein of hnRNP H is included as an endogenous control. Due to the hyperglycosylation of ABCB1 protein, the expression level only can be seen as its intensity of a smeared pattern.

Table 2
Relative expression (fold) of ABC transporter isotypes of parental cell lines and the drug resistant sublines.

Gene	Array (A549/D16)	Real-time (A549/D16)	Real-time (A549/D32)	Array (A549/V16-1)	Real-time (A549/V16-1)	Real-time (A549/V16-2)	Real-time (A549/V16-3)	Array (H1299/D8)	Real-time (H1299/D8)	Array (H1299/V16)	Real-time (H1299/V16)
ABCA1	5.2	30.9	138.4	0.4	0.9	0.9	7.7	1.5	1.3	0.9	0.2
ABCA2	1.0			1.3				1.0		0.8	
ABCA3	1.6			1.2				0.5		0.7	
ABCA4	7.4			4.7				1.0		1.0	
ABCA5	3.7	5.7	41.2	0.6	1.4	0.6	1.2	8.0	0.9	0.4	0.2
ABCA6	4.8			1.0				1.0		1.0	
ABCA7	1.7			1.5				0.5		0.8	
ABCA8	18.2	256.0	1028.4	0.3	0.3	<0.1	23.5	1.0	1.7	1.0	198.9
ABCA9	8.2			1.2				1.0		1.0	
ABCA10	0.6			1.1				2.1		1.2	
ABCA11	0.9			1.9				2.2		1.0	
ABCA12	1.3	3.6	28.9	3.2	12.1	2.7	15.7	1.0	0.1	1.0	0.9
ABCA13	1.0			1.0				1.0		1.0	
ABCB1	41.8	4455.8	33214.6	1.0	0.2	0.3	<0.1	2.5	2.2	2.6	2.9
ABCB4	1.6	43.5	300.6	1.0	3.7	0.1	0.1	1.0	1.6	1.0	1.1
ABCB5	1.0			1.0				1.0		1.0	
ABCB6	0.8	3.3	15.9	0.9	2.2	0.5	1.1	5.7	4.0	0.7	1.0
ABCB7	1.0			0.6				0.8		1.0	
ABCB8	0.3			1.1				1.2		0.8	
ABCB9	0.9			1.8				1.4		1.2	
ABCB10	0.7			1.3				0.9		1.1	
ABCB11	1.3			1.7				1.7		1.9	
ABCC1	1.0	3.5	39.1	1.5	3.5	1.1	4.5	0.6	1.9	0.9	0.5
ABCC2	1.1			1.2				0.4		2.6	
ABCC3	2.3	3.8	25.3	1.5	5.8	1.0	1.8	0.8	1.7	1.6	1.4
ABCC4	0.4			0.5				0.8		1.5	
ABCC5	2.2	3.3	60.4	1.7	5.3	1.2	2.2	0.7	0.7	0.7	0.2
ABCC6	4.2	5.9	30.2	0.5	0.2	0.1	0.1	0.9	0.1	1.2	0.3
ABCC8	1.5			0.6				1.1		1.2	
ABCC9	2.1			1.0				0.6		1.0	
ABCC10	1.3			1.4				0.5		0.6	
ABCC11	2.1			1.3				0.8		0.8	
ABCC12	1.0			1.0				1.0		1.0	
ABCC13	1.1			1.3				1.2		1.3	
ABCD1	1.6			1.1				1.0		1.0	
ABCD2	1.0			1.0				0.5		0.7	
ABCD3	0.7			0.5				1.2		1.2	
ABCD4	1.9			0.7				0.8		0.9	
ABCE1	0.5			1.4				1.2		1.3	
ABCF1	0.8			1.2				0.9		0.8	
ABCF2	0.6			1.0				1.9		0.8	
ABCF3	1.0			1.1				1.0		0.9	
ABCG1	1.3			0.2				1.3		1.3	
ABCG2	0.2			0.7				0.6		2.8	
ABCG4	0.8			0.3				1.0		1.5	
ABCG5	0.9			1.0				0.9		1.1	
ABCG8	1.0			1.0				1.0		1.0	

Note: Array was determined by Welgene Biotech as described in Section 2. Real-time results are the average of a least two separate experiments.

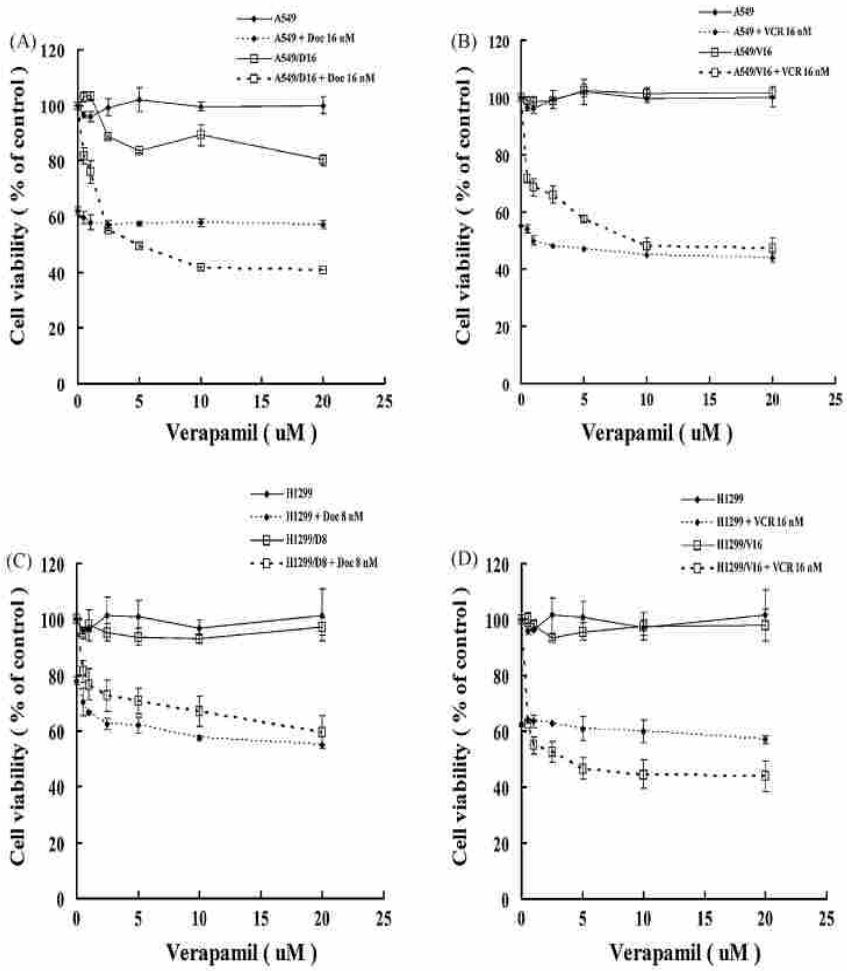

Fig. 2. Modulation of drug sensitivity by VER. To measure the cytotoxicity and sensitization of VER in parental and drug resistant cells, (A) parental A549 and A549/D16 cells were treated with various concentrations of VER without DOC treatment for cytotoxicity measurement. DOC (16 nM) was added to A549 and A549/D16 cells incubated with VER for drug sensitivity measurement. Similar conditions were also applied to (B) A549 and A549/V16 cells, (C) H1299 and H1299/D8 cells, and (D) H1299 and H1299/V16 cells.

expression higher than that in parental H1299 cells. Therefore, our results from mRNA analysis correlated well with protein expression data for ABCB1 in drug-resistant sublines. Previously, we have shown that with transient treatment with low concentrations of DOC and VCR, there is p53 induction in A549 cells and β-tubulin up-regulation in H1299 cells (Chang et al., 2006). In another study, β-tubulin protein increased in taxol-resistant cells (Kavallaris et al., 1997). Interestingly, from our microarray data, mRNA levels of p53 and β-tubulin did not increase (data not shown) in drug resistant sublines. Therefore, proteins of p53 and β-tubulin were further examined by Western blot analysis. Our data showed that β-tubulin expression was not altered significantly in DOC and VCR resistant sublines. Although the transcription of *ABCB1* mediated by p53 has been reported (Chin et al., 1992), p53 was not altered in either A549/DOC or VCR drug resistant sublines.

3.4. Reversal of drug resistance by ABCB1 inhibitor, verapamil (VER)

Verapamil (VER) is an ABCB1 inhibitor and calcium channel blocker that has been used to characterize ABCB1 activity (Tsuruo et al., 1983). Therefore, we pretreated cells with VER and then challenged them with DOC or VCR to measure cell survival and analyze whether drug resistance can be reversed. When parental A549 cells were treated with up to 20 μM VER, the viability of cells was not altered. Addition of DOC to VER-pretreated A549 cells did not enhance the sensitivity of the cells to DOC. Interestingly, A549/D16 cells treated with VER showed moderately reduced viability. Upon addition of DOC, the VER-pretreated A549/D16 cells re-sensitized to DOC toxicity with VER concentration dependence. VER-enhanced DOC efficacy resulted in lower viability of A549/D16 subline than parental cells treated under the same conditions (Fig. 2A). Similar conditions were applied to A549/V16 cells, and VER had no effect on A549/V16 cells. Surprisingly, A549/V16 cells with low level ABCB1 expression were also re-sensitized by VER pretreatment resulting in similar sensitivity to that of parental cells treated under the same conditions (Fig. 2B). Since A549/V16 has very low ABCB1 expression, the re-sensitizing effect of VER on these cells was mostly unrelated to inhibition of ABCB1 activity. Similar re-sensitization results by VER were also obtained in H1299/D8 (Fig. 2C) and H1299/V16 (Fig. 2D) sublines. Apparently, VER is capable of reversing DOC and VCR-induced resistance in all drug resistant sublines independent of the level of ABCB1 expression.

3.5. Other calcium channel blockers, DIL and NIF, reverse drug resistance with lower efficacy and higher toxicity than VER

There are three classes of L-type calcium channel blockers that have been used clinically (Pepine et al., 1983). Diltiazem

(DIL) is a benzothiazepine calcium channel blocker that is in an intermediate class between phenylalkylamine, such as VER, and dihydropyridines (nifedipine, NIF) in its selectivity for vascular calcium channels (Triggle, 2007). Thus, it is interesting to compare the reversal efficacies of DIL and NIF with that of VER using drug resistant sublines. A549/D16 subline treated with DIL showed moderately reduced viability and re-sensitized to DOC to a degree similar to that of parental cells with 20 μM DIL pretreatment (Fig. 3A). When A549/V16 subline was pretreated with DIL, the drug toxicity was enough to significantly reduce viability of A549/V16 cells, but the parental A549 cells were not affected. When parental H1299 cells were pretreated with DIL, high concentration of DIL resulted in cellular toxicity of parental H1299 cells. The efficacy of re-sensitization of H1299/D8 and V16 sublines by DIL was lower than that by VER, because higher DIL dosage was required. Viability of A549/D16 subline was not affected by NIF treatment. In addition, the re-sensitization of NIF-pretreated A549/D16 cells did not increase to the level of parental A549 cells (Fig. 3B). In drug resistant A549/V16 subline pretreated with NIF, similar responses were observed to those of DIL pretreatment. NIF had no effect on parental H1299 cells in contrast to significant toxicity by DIL. None of the three classes of calcium channel blockers showed significant toxicity to the parental A549 cells but the efficacies of re-sensitization of drug resistant A549/D16 subline were different (Figs. 2 and 3), with the highest efficacies for VER and the lowest for NIF. The efficacies were obtained from the results of 5 μM calcium channel blocker pretreatment. VER decreased cell survival to 50% of control (Fig. 2A). DIL decreased cell survival to 80% of control (Fig. 3A) and NIF decreased cell survival to 90% of control (Fig. 3B). These data showed that DIL and NIF alone are highly toxic to VCR resistant A549/V16 subline without DOC or VCR treatment. Although DIL and NIF exhibited lower efficacy in re-sensitizing drug resistant sublines and higher toxicity to parental cancer cells, they may have potential for treatment of DOC and VCR resistant NSCLC alone or in combination with chemotherapeutic drugs.

3.6. Characterization of ABCB1 activity by rhodamin-123 efflux assay

Since the expression levels of ABCB1 protein were significantly different between A549/D16 and A549/V16 sublines, the activity of ABCB1 was further examined by rhodamin-123 efflux assay (Fig. 4). Low retention of fluorescent dye inside of the cells indicates high activity of the energy-dependent ABCB1 pump. When A549 and A549/D16 were treated with rhodamin-123, the fluorescence level decreased in A549/D16 subline (Fig. 4A), which correlated with high ABCB1 expression status (Fig. 1). When A549/V16 subline was treated under the same conditions, the fluorescence level did not change, which reflected low level of ABCB1 protein expression. The rhodamin-123 fluorescence level decreased moderately in H1299/D8 subline when compared with parental H1299 cells. Although the ABCB1 protein levels were slightly higher in H1299/V16 than in H1299/D8 lines (Fig. 2), when H1299/V16 subline was treated under the same conditions, the fluorescence level significantly decreased to near the level of untreated H1299/V16 and parental cells. Therefore, it is logical to assume that pumping

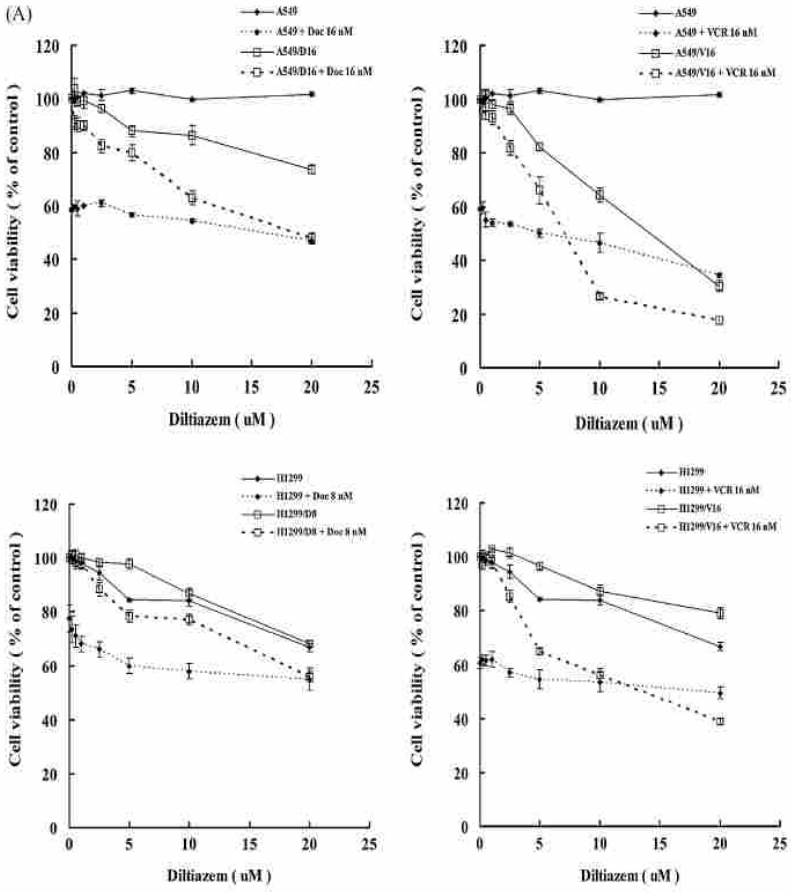

Fig. 3. Modulation of drug sensitivity by DIL and NIF. To measure the cytotoxicity and sensitization of DIL in parental and drug resistant cells, (A) parental and drug resistant cells were treated with various concentrations of DIL without DOC treatment for cytotoxicity measurement. The chemotherapeutic drugs were added to cells incubated with DIL for drug sensitivity measurement. Similar conditions were also applied to (B) cells treated with NIF.

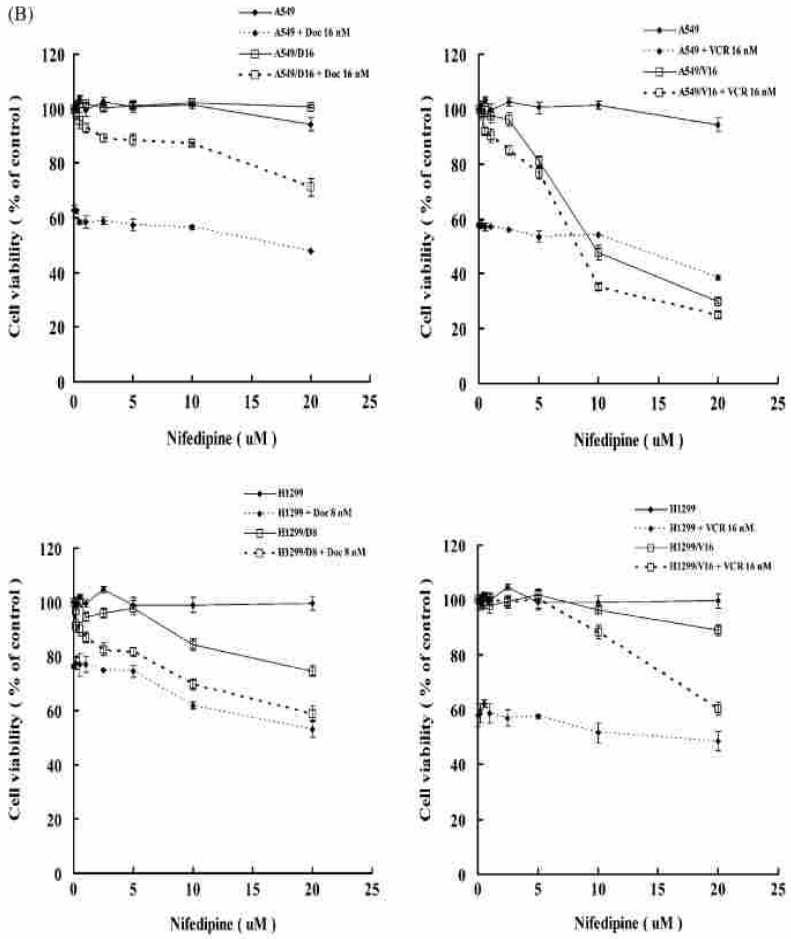

Fig. 3. (*Continued*).

activity of ABCB1 transporter differs between H1299/D8 and V16 sublines. Apparently, the rhodamin-123 efflux activities of drug resistant A549/D16 and A549/V16 sublines correlated with their ABCB1 expression levels. Therefore, rhodamin-123 assay can be used to monitor the specific activity of ABCB1 protein.

3.7. Calcium channel blockers sensitize drug resistant sublines via inhibition of ABCB1 and calcium channel activities

Our data not only demonstrated that VER reverses drug resistance of A549/D16 subline that exhibits high level of ABCB1 protein, but also reverses drug resistance of A549/V16 subline that expresses very low level of ABCB1 protein. Whether VER treatment affects ABCB1 activity in both sublines was further evaluated (Fig. 4B). The ABCB1 activity of parental A549 cells was not affected by either 2.5 μM or 10 μM VER treatment. When A549/D16 subline was treated with 2.5 μM or 10 μM VER, the fluorescence level increased correspondingly. This implies that VER inhibits ABCB1 pumping activity resulting in more fluorescent dye retention. Interestingly, when A549/D16 subline was treated with 10 μM VER, the fluorescence level was only partly enhanced when compared with A549 cells. Furthermore, when A549/D16 subline was treated with 10 μM VER, maximal level of re-sensitization of cells to DOC treatment was also observed (Fig. 2A). How can incomplete ABCB1 inhibition result in maximal re-sensitization? It is likely that VER modulates resistance through inhibition of ABCB1 activity and calcium channel in A549/D16 subline. To confirm that the inhibition of calcium influx modulates drug resistance, we have to demonstrate that reversal of drug resistance can be achieved without interfering with ABCB1 activity. Indeed, the peak level of fluorescence was not significantly altered when A549/V16 was treated with VER (2.5 and 10 μM) indicating that reversal of drug resistance by VER is not associated with ABCB1 activity in A549/V16 subline (Fig. 4B). Furthermore, neither DIL (Fig. 4C) nor NIF (Fig. 4D) affect ABCB1 activity in A549 and A549/D16 cells, which implies that the reversal of drug resistance by these two calcium channel blockers is not associated with ABCB1 activity.

4. Discussion

The mechanisms of MDR development in cancer cells are complex. They are not only associated with different types of cancers and cells but also with the cytotoxic action of the drugs. A genomic approach to identifying chemoresistance pathway has been shown to provide an understanding of chemotherapeutic resistance (Riedel et al., 2008). Thus, we sought to identify cellular resistance pathways by genomic analysis of acquired resistance to facilitate effective combination therapy.

DOC and VCR are two tubulin binding agents (TBA) that target MTs resulting in mitotic arrest of cancer cells (Chang et al., 2006). However, primary (intrinsic) and secondary (acquired) resistance is a problem in TBA therapy. We demonstrated that a human NSCLC of A549 cells selected with DOC and VCR resulted in similar MDR phenotypes but significantly different ABC transporter expression profiles. Although overexpression of ABCB1 is considered the major mediator of MDR, our data showed that VCR-selected A549/V16

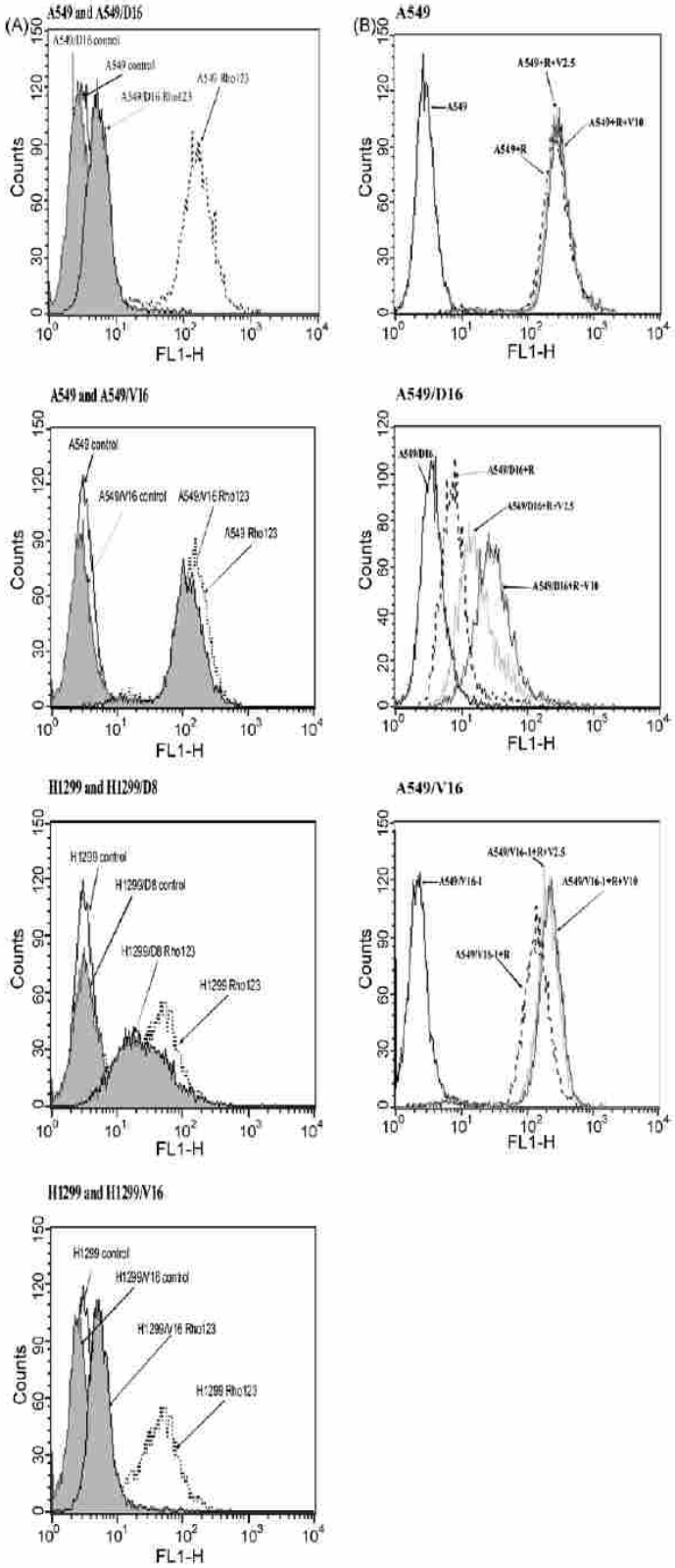

Fig. 4. Characterization of ABCB1 activity. (A)To correlate the ABCB1 activity to its protein expression, the fluorescent dye rhodamine-123 was incubated with parental and A549/D16 cells, parental and A549/V16 cells, parental and H1299/D8 cells, and parental and H1299/V16 cells. The accumulation of rhodamine 123, which is transported by ABCB1, was determined by a flow cytometer. Low level of the fluorescent dye inside of a cell indicates high activity of the energy-dependent ABCB1 pump. Solid line represents fluorescence background without rhodamine-123 incubation. Dashed line represents residue fluorescence measured by flow cytometry. (B) To measure VER modulation of ABCB1 activity, A549, A549/D16 and A549/V16 cells were treated with VER (2.5, 10 μM) for 90 min followed by rhodamin-123 (R) efflux assay. The ABCB1 overexpressing A549/D16 subline was further tested by DIL (C) and NIF (D) to determine their ability to modulate ABCB1 activity. Cells were treated with DIL (10 μM) or NIF (10 μM) followed by rhodamin-123 efflux assay.

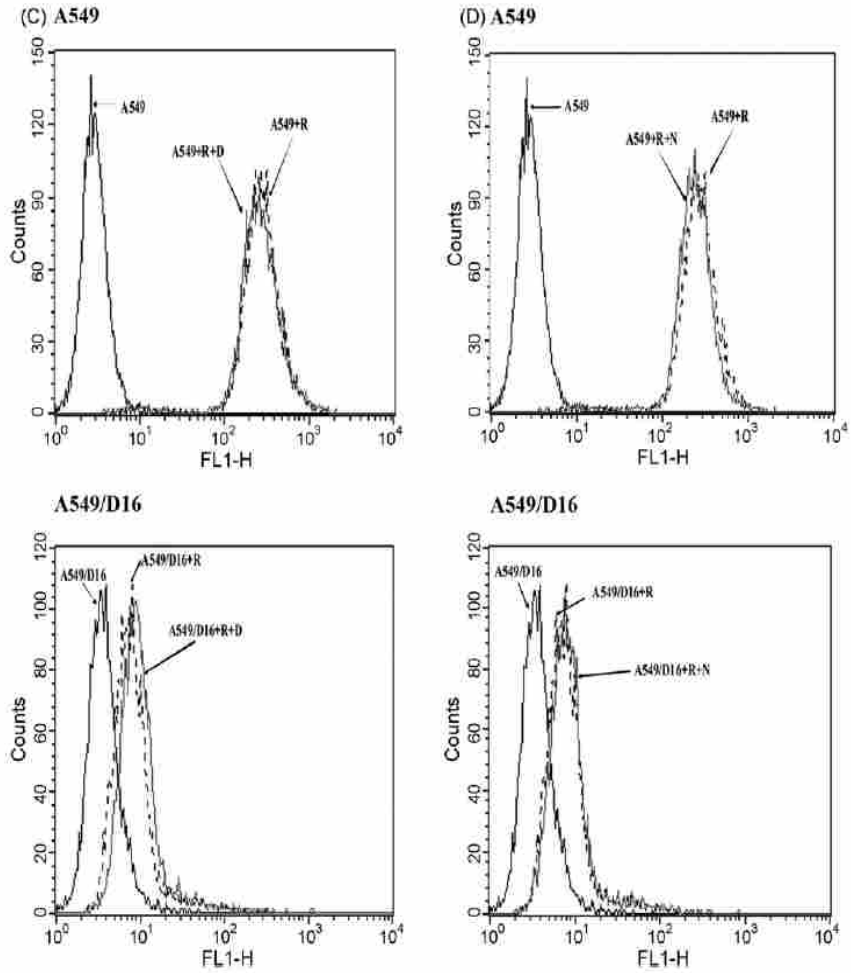

Fig. 4. (*Continued*).

cells are cross-resistant with DOC without ABCB1 overexpression. Another NSCLC of H1299/D8 cells selected with DOC also exhibited different expression profile of ABC transporters when compared with A549/D16 subline. In addition to our cell line data, it has been reported that there is no significant correlation between ABCB1 expression and response to DOC in patients with breast cancer (Noguchi, 2006). It has also been shown that human ovarian carcinoma cell line selected with taxol treatment exhibits ABCB1 and non-ABCB1-mediated mechanisms (Parekh et al., 1997). We could not detect ABCB1 overexpression in A549/V16 sublines. To avoid the possibility of *ABCB1* not responding to DOC and VCR, the parental A549 and H1299 cells were treated with 15 nM of each drug for 24 h and the transcription of *ABCB1* was analyzed by qRT-PCR. The transcription of *ABCB1* gene was significantly up-regulated in both cell lines (data not shown) suggesting that *ABCB1* can be induced by DOC and VCR treatment in parental cells. These results provide evidence that ABC transporters only partly contribute to MDR in these DOC and VCR resistant cells. Atypical and typical MDR can exist in the same cancer cell treated with DOC or VCR.

Regulation of *ABCB1* by p53 and the mechanism of p53 response element in *ABCB1* have been characterized in detail (Johnson et al., 2001). Mutant p53 protein stimulates the *ABCB1* promoter and wild-type p53 enables the repression of *ABCB1* (Bahr et al., 2001; Chin et al., 1992; Thottassery et al., 1997). It is of interest to determine if p53 plays a role in the regulation of ABCB1 overexpression in drug resistant subline. We hypothesized that up-regulated p53 is associated with low ABCB1 expression in A549/V16 subline. However, we found no alteration in p53 at the transcription (data not shown) and protein levels in A549/D16 and A549/V16 sublines. Our data showed that the expression of ABCB1 in A549 drug resistant sublines is not associated with p53 expression.

Previously, selection of A549 cells with 12 and 24 nM taxol displayed significant increases in class I, III, and IVa isotypes in taxol-resistant samples but no significant change in either α or β-tubulin concentrations when compared with the parental cells (Kavallaris et al., 1997). Lack of ABCB1 overexpression in A549 taxol resistant subline in that study was similar to the results of our A549/V16 subline but different from those of our A549/D16 subline. As the expression of total β-tubulin was not affected in our sublines, whether altered β-tubulin isotype expression is present and is involved in MDR of DOC and VCR-selected sublines needs further investigation.

It is important to study MDR in each cancer to develop tailor-made strategies to circumvent drug resistance and improve patient survival and life quality. VER has been applied to treat multiple myeloma (MM) since 1989, based on the overexpression of ABCB1 as the target for VER in patients with MM (Dalton et al., 1989; Gottesman and Pastan, 1989). Therefore, the application of VER to the reversal of MDR has to be limited by ABCB1 or other ABC transporter overexpression. Although all of our drug resistant sublines were sensitized by VER treatment regardless of ABCB1 expression, the hyperactive ABCB1 transporter in A549/D16 and H1299/V16

(Fig. 4) seemed more sensitive to VER treatment, resulting in lower cell viability (Fig. 2A and D, respectively). It should be noted that even under high concentration of VER, the viability of parental cells was not affected. Furthermore, VER could not sensitize the parental cancer cells to drug treatment. These results indicated that a prerequisite of refractory cancer for co-treatment of VER with chemotherapeutic drug is essential to prevent side effects of VER with non-responder.

VER is not only an ABCB1 inhibitor, but also an L-type calcium channel blocker. Since our data showed that VER modulates MDR without ABCB1 overexpression, it is logical to assume that calcium channel blocker activity is also involved in MDR reversal ability. Therefore, DIL and NIF were tested for their reversal ability with drug resistant sublines. We demonstrated that neither DIL nor NIF is an inhibitor of ABCB1 transporter but they still modulate MDR activity. Calcium homeostasis is tightly regulated in both normal and cancer cells for cell proliferation and viability (Monteith et al., 2007). However, the role of intracellular calcium in MDR of cancer cells has not been well investigated. Interestingly, two human breast carcinoma MCF-7 sublines selected for resistance against DXR with ABCB1 overexpression (MCF-7/DXR cells) and without ABCB1 expression (MCF-7/RT) have been shown to contain deficient intracellular calcium pool (Chen et al., 2002). It was hypothesized that MDR-positive cancer cells require small intracellular calcium pools to sustain cytotoxic effects of certain chemotherapeutic drugs (Chen et al., 2002). The treatment of A549 cells with paclitaxel generates reactive oxygen species (Alexandre et al., 2006) and thus may damage the calcium transport system localized in the endoplasmic reticulum, mitochondria, and plasma membranes resulting in cell death. Our data from previous studies allowed us to hypothesize that DOC and VCR treatment leads to a disruption of calcium homeostasis, thus low level intracellular calcium pool may permit MDR-positive cells to sustain free radical-induced damage with the help of other unidentified factors. The inhibition of calcium influx by calcium channel blockers disrupts calcium signaling thus sensitizing MDR-positive cells to death in combination with chemotherapeutic drugs.

In summary, our findings suggest that DOC and VCR-mediated NSCLC exhibit both ABC and non-ABC associated MDR phenotypes. Modulation of MDR activity by L-type calcium channel blockers reverses the resistance that does not require the overexpression of ABC transporters and marked the significant role of calcium in chemotherapy.

Conflict of interest

None.

Acknowledgements

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Regulation of chemosensitivity and migration by clusterin in non-small cell lung cancer cells

Running Title: Regulation of chemosensitivity and migration by clusterin

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Abstract

The most significant role of clusterin (CLU) plays in cells is to protect all cells from environmental stimuli and stress; unfortunately, cancer cells usually benefit from this protection upon medical treatment. Therefore, chemosensitivity of human non-small cell lung (NSCLC) cancer cell lines were examined for the association with the level of CLU. We found that H1355 cells with the highest level of CLU have the least sensitivity to adriamycin (ADR), docetaxel (DOC), and gemcitabine (GEM) treatment. Inhibition of CLU expression by short hairpin RNA interference (shRNAi) resulted in higher chemosensitivity. When CLU is stably expressed in A549 and H1299 cells, only the chemosensitivity of ADR is reduced. The DOC, GEM sensitivity were not markedly altered by CLU overexpression. These data indicate that inhibition of CLU expression enhances chemosensitivity, but effect of CLU overexpression is not always associated with chemosensitivity. Moreover, we found when A549 and H1299 cells overexpressing CLU, their migration were reduced significantly. The transcription of matrix metalloproteinase-2 (MMP2) was markedly reduced in CLU-overexpressing H1299 cells that indicate less metastasis potential of cancer cells when CLU level is high. A hypothesis could be proposed from our data that high level of CLU certainly reduces chemosensitivity and increases survival; but under unknown circumstance, high level of CLU may inhibit cancer cells migration. Therefore, cancer cells have to determine whether expressing more CLU is needed to protect them from toxicity of drugs or with less CLU that enables them to migrate to other tissue sites.

Key words: Lung cancer; Clusterin; Chemosensitivity; Migration

Introduction

Lung cancer is the second most frequently diagnosed cancer in men and women and accounts for one-third of all cancer-related deaths in the United States every year [1]. Eighty percent of lung cancer cases are non-small cell lung cancer (NSCLC) and the remaining 20% are small cell lung cancer (SCLC). The 5-year survival rate for lung cancer (15%) is the second to the lowest of all cancers, just above pancreatic cancer [1].

Clusterin (CLU) in humans a single copy gene located at chromosome 8 [2]. CLU is a secreted glycoprotein that is translated from a single mRNA as a preprotein [3]. This preprotein is an intracellular 449 amino acid polypeptide chain with an apparent MW of 60 kDa cytosolic clusterin (cCLU), where the first 22 amino acids represent the classical hydrophobic secretory signal sequence [4]. Proteolytically cleaved of mature glycosylated c-CLU into an α and β chain that are linked by five disulfide bridges [5] results in the mature form of the secreted clusterin protein (sCLU). The processed clusterin protein appears as a smear of about 40 kDa as detected by western blot [6]. Nuclear clusterin (nCLU) is translated from the second ATG from the mRNA without leader peptide. The n-CLU is unglycosylated and it is involved in apoptosis induction [6]. Overexpression of a truncated form of CLU lacking the hydrophobic secretion signal sequence that localizing to the nucleus of prostate epithelial cells, caused induction of apoptosis [7; 8].

The expression of CLU has been extensively investigated in prostate cancer [9; 10], cervical cancer [11], bladder cancer [12], renal cell carcinoma [13; 14] for its prognostic significance that including chemotherapeutic sensitivity and metastasis potential. Inhibition of CLU expression in cancer cells resulted in cells more sensitive to the stress and toxic conditions which enhance cell death [10].

When applied the association of CLU expression to lung cancer research, the investigators facing a dilemma with inconsistence results from *in vitro* and *in vivo* clinical data. Target the CLU gene expression in lung cancer cell lines by antisense oligonucleotides (ASO) and small-interfering RNAs (Si-RNA) resulted in sensitization of cancer cells to radiotherapy [15] and chemotherapy [16]; furthermore, reduced their migration and invasion ability [17]. Whereas, when tumor specimens of NSCLC were examined, patients with cytoplasmic CLU-positive staining had a better overall survival and less recurrence than those with CLU-negative patients [18]. Apparently, CLU expression seems to be a positive and favorable prognostic factor in patients of NSCLC. The contradictory between the promising in vitro cell lines data and clinical findings would jeopardize the development of a better therapy for lung cancer in future. Therefore, we are trying to further study the expression of CLU in human lung cancer cell lines and compare to their chemotherapeutic sensitivity with various therapeutic drugs, as well as the role of CLU in migration.

Materials and Methods:

Cell lines and culture

Human NSCLC cell lines (A549, H1299, H1355, H460 and Calu-1) and lung cells (BEAS-2B) from the American Type Culture Collection were cultured on Dulbecco's modified Eagle's medium (DMEM) (GIBCO, Rockville, MD). The human bronchial epithelial cell line BEAS-2B was cultured on LHC-9 medium (GIBCO, Rockville, MD). All lung cancer cell lines

were maintained at 37 ℃ in a 5% CO_2-humidified atmosphere on medium containing 10% fetal bovine serum (FBS) and 100ng/ml each of penicillin and streptomycin (Life Technologies, Rockville, MD).

Drugs and chemicals

DOC was obtained from Aventis Pharmaceuticals Inc. (Bridgewater, NJ, USA). ADR was purchased from Sigma Chemical. GEM was provided by Eli Lilly (Indianapolis, IN, USA).

Cell growth and viability for chemosensitivity (MTS assay)

Cell viability was assessed by CellTiter 96 AQeous One Solution Cell Proliferation Assay (Promega, Madison, WI), according to the manufacturer's instructions. In brief, 20 μl of MTS reagent was added into each well of the 96-well assay plate containing the samples in 100 μl of culture medium. Plates were incubated at 37 ℃ in a humidified, 5% CO_2 atmosphere for another 40 min. Absorbance at 490 nm was read by means of an ELISA plate reader. Each experiment was repeated at least three times. Final data were normalized and presented as percentage of controls.

Western blot analysis

The cells were washed with PBS and harvested for sonication in the presence of a protease inhibitor cocktail (Sigma-Aldrich). The concentration of protein was determined by Bradford assay and the amounts of cell lysates (15 μg) were applied to SDS-PAGE gels. After transferring proteins to a polyvinylidene fluoride (PVDF) membrane, proteins were reacted with anti-clusterin (Santa Cruz Biotechnology), or anti-β-actin (Sigma-Aldrich), followed by anti-mouse IgG conjugated with horseradish peroxidase (Calbiochem). A chemiluminescence detection kit (ECL, Amersham Pharmacia Biotech) was applied to determine the levels of protein expression.

Transfection of clusterin-shRNAi

The V2LHS-150636-clusterin lentiviral shRNAmir (CLU-shRNA) and Non-silencing shRNAmir negative control (RHS4346, shRNA control) were purchased from Open biosystems (Thermo Fisher Scientific). The TurboFectTM *in vitro* transfection reagent (Fermentas life sciences) was used to transfect shRNA control and CLU-shRNA vectors into H1355 cells ($4x10^5$) in 6-cm dishes (3 μg of DNA/dish) followed by 48 and 72 h incubation for western blot analysis. For MTS assay, H1355 cells ($7.5x10^4$) seeded in a 24-well plate were transfected (0.4 μg of DNA/well). After 60 h, the medium was collected for western blotting. The fresh medium with individual drug were added into the cells and incubated for 48 h (ADR, DOC) or 72 h (GEM) with indicated concentration of drug.

Clusterin overexpression

Full-length open reading frame of human clusterin cDNA was generated by RT–PCR from a human Huh7 cell line using the forward primers of 5- GTGACATA TGATGAAGACTCTG and reverse primer of 5- AACGCGTCGACATCTCACTC CTCCCT. To express the gene, the obtained DNA fragment was subcloned into p3XFLAG-CMV-10 (Sigma-Aldrich) followed by DNA sequencing. The CLU expression vector was transfected with TransFastTM Transfection Reagent (Promega) into A549 and H1299 followed by G418 selection (1.5 mg/ml, Sigma-Aldrich). We isolated the well separated clone of cells into a 96-well plate and reamplified until the cell grew in 10-cm dishes that could be examined by immunoblot analysis.

Cell migration assay with Boyden chamber

In each well of a 48-well bottom chamber, 32 μl DMEM with 10% FBS was added. We used forceps to handle the 8-μm porosity polycarbonate membrane and placed the silicone gasket over the membrane. Then, the top chamber was placed over the gasket, and the Boyden chamber was assembled. Total of 52 μl DMEM solution that containing 2% FBS and cancer cells were loaded into each well of the top chamber, and the whole chamber was incubated at 37°C and 5% CO_2 for time as indicated. Thereafter, the chamber was disassembled, and the membrane, through which cells had passed and adhered, was immersed in cooled 95% methanol for 10 min to fix migrated cells. This membrane was then stained in 20% Giemsa stain overnight. The stained cells on the underside of membrane were count under a microscope.

Gelatin zymography

Production of MMPs by cancer cells were analyzed by gelatin zymography. To avoid the MMPs contamination, cells were cultured in serum-free medium for 24 h, and conditioned media were collected. Equal amount of conditioned medium samples were mixed with SDS sample buffer containing 2% SDS without β-mercaptoethanol and applied to 10 % SDS polyacrylamide gels copolymerized with 0.1% gelatin (Sigma-Aldrich) without boiling. After electrophoresis, gels were washed for 1 h at room temperature with gentle agitation in renaturing buffer (2.7% Triton X-100 in H2O) to remove SDS. The gels were then equilibrated in developing buffer (50 mM Tris-HCl, pH 7.4, 0.2 M NaCl, 5 mM $CaCl_2$ 和 0.2% Brji 35) at 37°C overnight. The gels were then stained with 0.5% Coomassie Brilliant Blue and destained. The MMP activities were visualized as clear bands against the blue background of the stained gels.

Semi-quantitative RT-PCR of MMP2 and MMP9

To analyze the mRNA levels of MMP2 and MMP9, total RNA was extracted from H1299 cells that stably expressing clusterin using the TRIzol reagent (Gibco BRL). The purified RNA (2 μg) were reverse-transcribed to cDNA by oligo-dT primers. For MMP2, the forward primer (5'-TTTTCTCGAATCCATGATGG) and the reverse primer (5'-CTGGTGCAGCTCTCATATTT) were used. For MMP9, the forward primer (5'-AAGATGCTGCTGTTCAGCGGG) and the reverse primer (5'- GTCCTCAGGGCACTGCAGGAT) were used. For GAPDH, the forward primer (5'- GCCAAGGTCATCCATGACAAC) and the reverse primer (5'- CAGTAGAGGCAG GGATGATGTTC) were used as the calibration control. The amplified PCR products were analyzed with 1% agarose gel.

Results

Expression levels of CLU in human NSCLC cells and lung cells.

Adenocarcinoma cell lines of A549, H1355, large cell carcinoma cell lines of H1299, H460 and squamous cell carcinoma cell lines of Calu-1, were examined by immunoblot analysis for the levels of cytosolic and secreted CLU protein expression. The highest level of precursor form of the secreted CLU (pre-sCLU) is present in H1355 cells followed by A549, H460 and Calu-1, respectively (Fig. 1A). The mature forms of secreted CLU (sCLU) were present in cell lysate and culture medium (Fig. 1B). The human bronchial epithelial cell line BEAS-2B and H1299 cells contain only an insignificant level of cytosolic CLU. According to the CLU levels, we chose H1355, A549 and H1299 cells to investigate the correlation of CLU and chemosensitivity.

Chemosensitivity of NSCLC cells to ADR, GEM, and DOC reversely correlated with the CLU levels.

The sensitivity of H1355, A549 and H1299 cells to ADR (Fig. 2A), GEM (Fig. 2B), and DOC (Fig. 2C) were evaluated by MTS assay. The H1355 cells were the least sensitive cells to ADR, GEM and DOC. These data support the protection role of CLU in H1355 cells.

Inhibition of CLU expression results in sensitization of cells to drug treatment.

To determine the effect of CLU associated with drug sensitivity; H1355 cells were transiently transfected with small hairpin interference RNA construct (CLU- shRNA) that inhibit CLU expression. The expression of CLU was significantly reduced after 72 h of transfection that examined by immunoblotting (Fig. 3A). When CLU expression was reduced, the chemosensitivity of H1355 were increased to ADR (Fig. 3B), GEM (Fig. 3C) and DOC (Fig. 3D) by MTS assay. The levels of sCLU proteins in both medium were showed in the small window by western blot analysis.

Secretary CLU collected from H1355 medium reduces drug sensitivity of H1299 cells.

To examine whether the chemosensitivity is regulated by sCLU, the medium from H1355 cells after 24 h incubation was harvested as the source of sCLU. The conditioned medium used for cell treatment was a mixture of one part of fresh and one part of collected medium to avoid metabolic toxicity from the collected medium. Because the H1299 cells contain lower CLU level, therefore, chemosensitivity of H1299 cells were compared in the presence of conditioned medium and fresh medium. The chemosensitivity of H1299 cells were decreased to ADR (Fig. 4A), GEM (Fig. 4B) and DOC (Fig. 4C) treatment with addition of conditioned medium by MTS assay. Apparently, sCLU present in the conditioned medium has protective effect for H1299 cells to against these drugs.

Overexpression of CLU reduced ADR but not GEM and DOC chemosensitivity in A549 and H1299 cells

Since the sCLU is produced from pre-sCLU with modification, we have constructed a full-length gene of CLU that contains the leader signaling sequence for expression. Stably expression of pre-sCLU in A549 and H1299 individual clones were selected by G418 treatment and examined by western blotting (Fig. 5A). Two clones of A549 (A-1, A-2) and H1299 (H-1, H-2) cells that overexpressing CLU were applied to further chemosensitivity assay. The chemosensitivity to ADR was reduced in all A-1, A-2 (Fig. 5B), H-1 and H-2 (Fig. 5C) CLU-overexpressing cells. Interestingly, the chemosensitivity to GEM (Fig. 5D, E) and DOC were not significantly decreased in these cells (Fig. 5F, G).

Migration was reduced in CLU-overexpressing H1299 and A549 cells

The alteration of cell migration by CLU was further investigated in vitro with modified Boyden chamber inserted with polyethylene terephthalate filter. When compared with parental H1299 cells, both clones of H1299 cells that overexpressing CLU (H-1 and H-2) significantly reduced their migration ability (Fig. 6A). Only one clone of A549 cells that overexpressing CLU (A-2) showed reduced migration ability (Fig. 6B).

The transcription of MMP2 but not MMP9 was inhibited in H1299 cells that overexpssing clusterin

To further explore the factor that may be inhibited by clusterin, we performed gelatin zymography to detecte whether MMP2 and MMP9 activities was altered. Interestingly, the MMP2 activity was lost in H-1 and H-2 clustering overexpressing cells (Fig. 7A). The lost of MMP2 activity was further confirmed by RT-PCR analysis of MMP2 and MMP9 mRNA transcripts. Only the transcription of MMP2 was inhibited in CLU-overexpressing cells (Fig. 7B).

Discussion

Clusterin (CLU), a protein with many identities, present not only inside of cell, it also exist as part of circulating proteins. The extracellular form of CLU is a highly glycosylated α-β-heterodimer linked by five disulphide bonds [19]. Although clusterin expression level associated with the chemo- and radiosensitivity have been investigated extensively and reviewed [20; 21], those results were sometimes contradictory that needed further research. Especially, CLU-positive expression in human lung cancer patients showed better overall and disease-free survival than those CLU-negative patients [18]. Whereas, it was found that in a lung cancer cell line, CLU silenced by siRNA reduced migration and invasion [17]. From the results of these reports it missed a link between the effects of CLU inhibition and CLU overexpression. Therefore, clinical data did not correlate well to cell line data and whether CLU is a favorable prognostic marker can not be concluded.

We started with the examination of CLU level in several human lung cancer cell lines and identified H1355 as a CLU-rich cell line. Inhibition of CLU expression by transient shRNA transfection reduced the chemosensitivity of H1355 cells to ADR, GEM, and DOC. Addition of CLU-rich conditioned medium to H1299 cells resulted in reduced chemosensitivity. Our data is similar to the data reported from July et al that targeted A549 CLU by siRNA or antisense oligonucleotides sensitized paclitaxel or GEM sensitivity *in vitro* and *in vivo* [16]. To investigate whether high level of CLU could reduce chemosensitivity, we then expressed exogenous CLU in A549 and H1299 cells. Interestingly, the chemosensitivity of CLU overexpressing cells were not altered markedly. The possible explanation for the later is the nCLU in those CLU overexpressing cells may regulate pro-apoptotic activities simultaneously in contrast to sCLU. Cao et. Al. [15] also reported that when H460 cells treated with an antisense oligonucleotide against CLU (OGX-011) and followed by radiotherapy; induced tumor regression in xenograft model. Apparently, inhibition of CLU resulted in high chemosensitivity is true for H1355, A549 and H460 cells and that is not related to the level of CLU.

Interestingly, when the extracellular CLU concentration was high; chemosensitivity was also reduced in cancer cells with less endogenous CLU. This result suggested that as long as cancer cells exist in CLU-rich microenvironment, not each cancer cells necessary to express high level of CLU to protect them from medicine treatment. The sCLU from neighboring cells also provide shielding effect for those cells expressing low level CLU.

If high level of CLU benefits cancer cells, why we observed lung cancer cells expressing different levels of CLU? Is there possible disadvantage with high level of CLU to cancer cells? When the cells overexpressing CLU, we observed that they grew as bunch-like and clustered in petri-dish (data not shown). Therefore, we decided to examine the migration ability of the CLU-overexpressing cells. According to our data, the migration ability was reduced and MMP2

transcription was inhibited in the cells overexpressing CLU. We have not yet further identified whether the nCLU or sCLU is responsible for the migration inhibition. Obviously, CLU overexpression may reduce the ability of cancer cell to spread out and results in easier detection by immune system.

Human MMPs are a family of over 20 different endopeptidases that are able to degrade various components of the extracellular matrix (ECM). MMP2 is an enzyme that is supposed to have an important role in invasion to the basement membrane. The role of MMP2 on the survival of patients with NSCLC was studied by Qian et al. [22] This study supported the fact that MMP2 could be included in further prospective trials studying prognostic factors in NSCLC.

Our data point to a possible explanation of why better overall and disease-free survival found in human lung cancer patients with CLU -positive expression. In these patients, cancer cells may be less active in metastasis than patients with CLU -negative expression. Of course, the application of ASO against CLU may be required further discussion. Because when CLU is inhibited by ASO, those targeted cancer cells if not killed by medicine or radiation treatment, they might turn into migration active cells which could move to other tissues to rebuild tumor.

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Legends

Figure 1

Expression levels of clusterin were examined by western blot analysis. Cells of A549 and H1355 are lung adenocarcinomas; H1299 and H460 are large cell carcinoma and Calu-1 is a squamous carcinoma. BEAS-2B cells were isolated from normal human bronchial epithelium obtained from autopsy of non-cancerous individuals. (A) Total cellular lysate (15 μg) from the cultured cells were analyzed for cytosolic clusterin expression. (B) The medium collected from individual culture (15 μl) were also analyzed.

Figure 2

Chemosensitivity of A549, H1299 and H1355 cells were compared by MTS viability assay. Triplicate samples of each cancer cells ($2x10^3$ cells) were seeded in 96-well plate and (A) treated with 86, 172, 344, 688 and 1032 nM of ADR for 36 (B) 20, 40, 80, 160, and 200 nM of GEM for 48 h (C) 10, 20, 40, 80 and 100 nM of DOC for 48 h followed by MTS assay.

Figure 3

Inhibition of clusterin expression by short hairpin RNA interference (shRNAi) increases chemosensitivity. (A) To measure the effect of CLU inhibited by shRNAi, the H1355 cells were transfected with CLU-shRNA and Non-silencing shRNAmir negative control (control). After 48 and 72 h later, cells were harvested for western blot analysis. (B) For chemosensitivity, cells were treated with (B) 0.344 to 2.58 μM of ADR (C) 0.5 to 2 μM of GEM and (D) 10 to 80 nM of DOC as described in Materials and Methods.

Figure 4

Secretory CLU collected from H1355 medium reduces drug sensitivity of H1299 cells. Triplicate samples of H1299 cells ($2x10^3$ cells) were seeded in 96-well plate and conditioned medium was added together with (A) 86, 172, 344, 688 and 1032 nM of ADR for 36 h (B) 20, 40, 80, 160, and 200 nM of GEM for 48 h (C) 10, 20, 40, 80 and 100 nM of DOC for 48 h followed by MTS assay.

Figure 5

Overexpression of CLU reduced ADR but not GEM and DOC chemosensitivity in A549 and H1299 cells. (A) As described in material and methods, Two CLU-overexpressing H1299 sublines (H1, H2) and A549 sublines (A1, A2) were selected by G418 and verified by western blot analysis. Triplicate samples of cells ($2x10^3$ cells) were seeded in 96-well plate and treated with (B, C) 86 to 1032 nM of ADR for 36 h (D, E) 10 to 80 nM of GEM for 48 h (F, G) 5 to 60 nM of DOC for 48 h followed by MTS assay.

Figure 6

Migration ability was reduced in CLU-overexpressing H1299 and A549 cells. To measure the migration ability of CLU-overexpressing cells, modified Boyden chamber was used and the migrated (A) H1299, H-1 and H2 (B) A549, A-1 and A2 were count.

Figure 7

The transcription of MMP2 but not MMP9 was inhibited in H1299 cells that overexpssing clusterin. (A) The activities of MMP2 and 9 from H1299, H-1 and H-2 were characterized by gelatin zymography. Cells ($8x10^5$) were seeded in 35-mm dishes and incubated for 24 h with

serum-free medium. The conditioned medium collected from individual cells (45 μl) was analyzed. (B) Total RNA from H1299, H-1 and H-2 were purified for cDNA synthesis. Semi-quantitative RT-PCR of MMP2, MMP9 and GAPDH were analyzed.

Figure1AB
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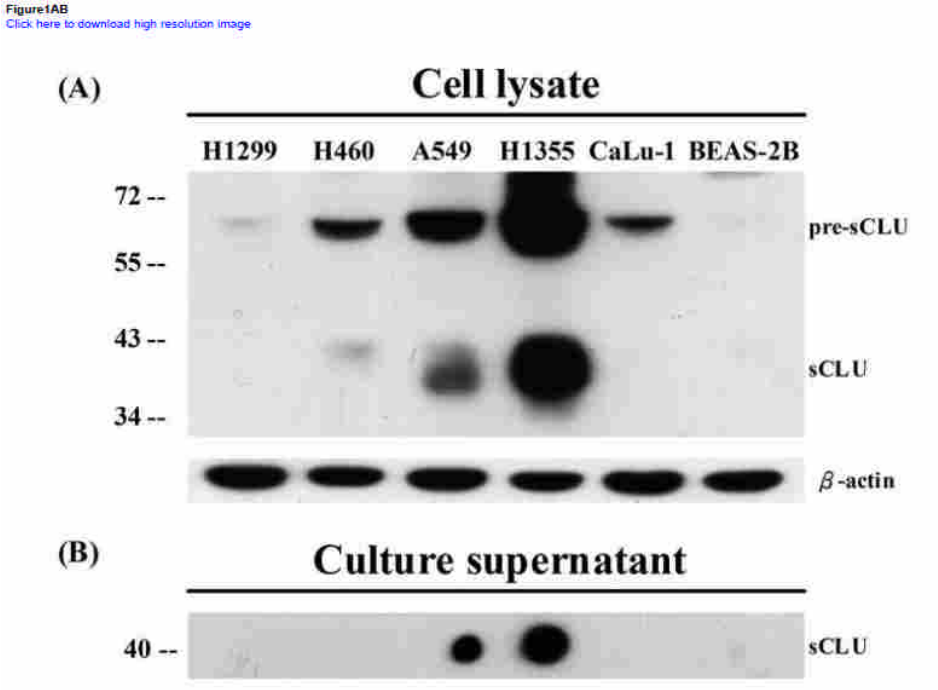

Figure2A
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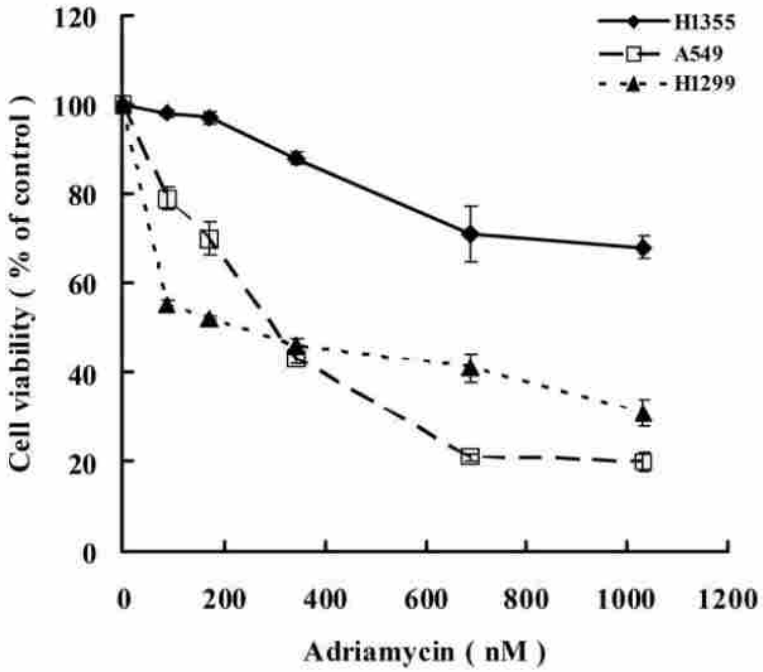

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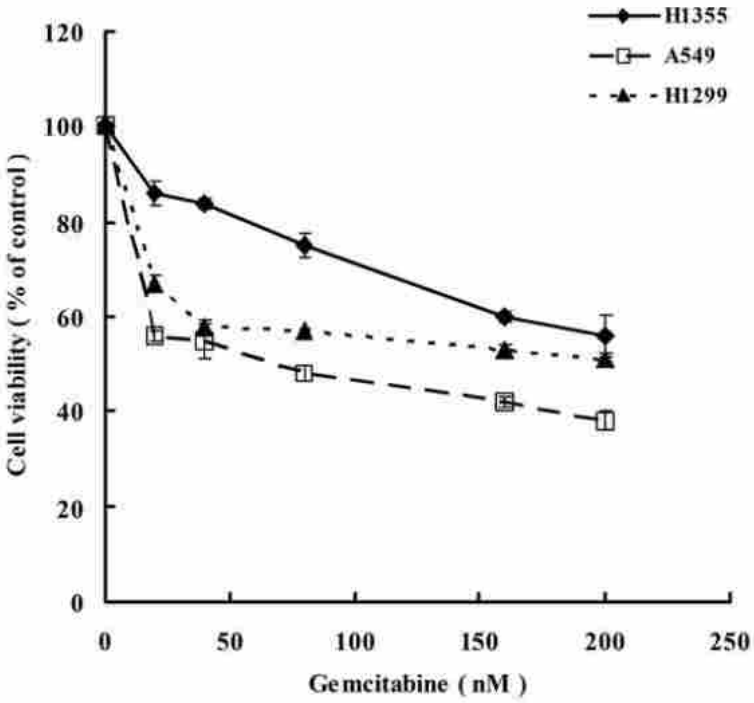

Figure2C
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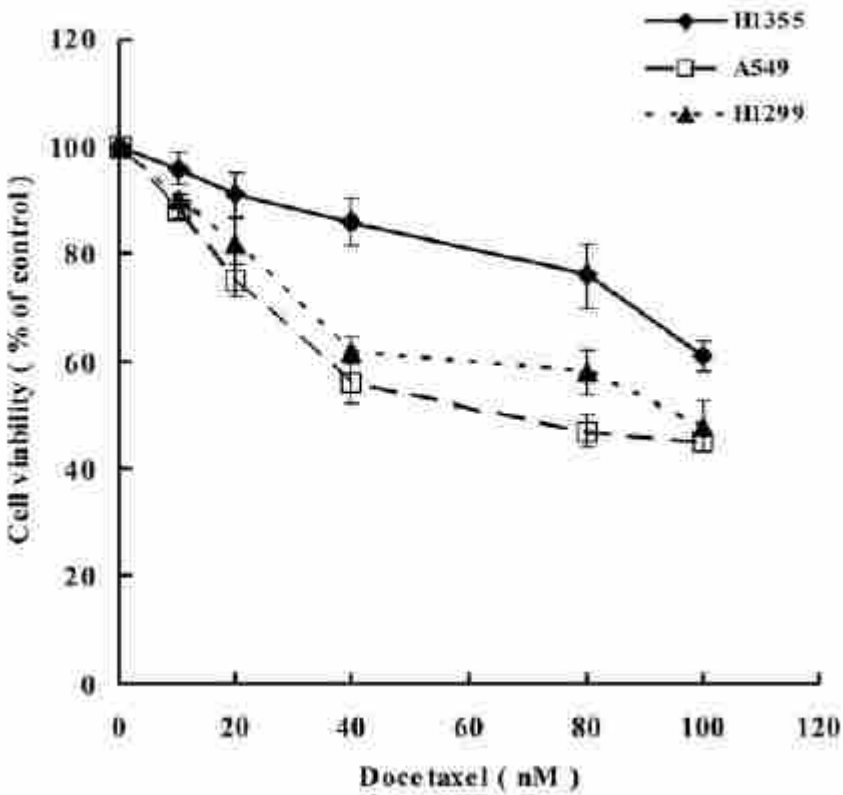

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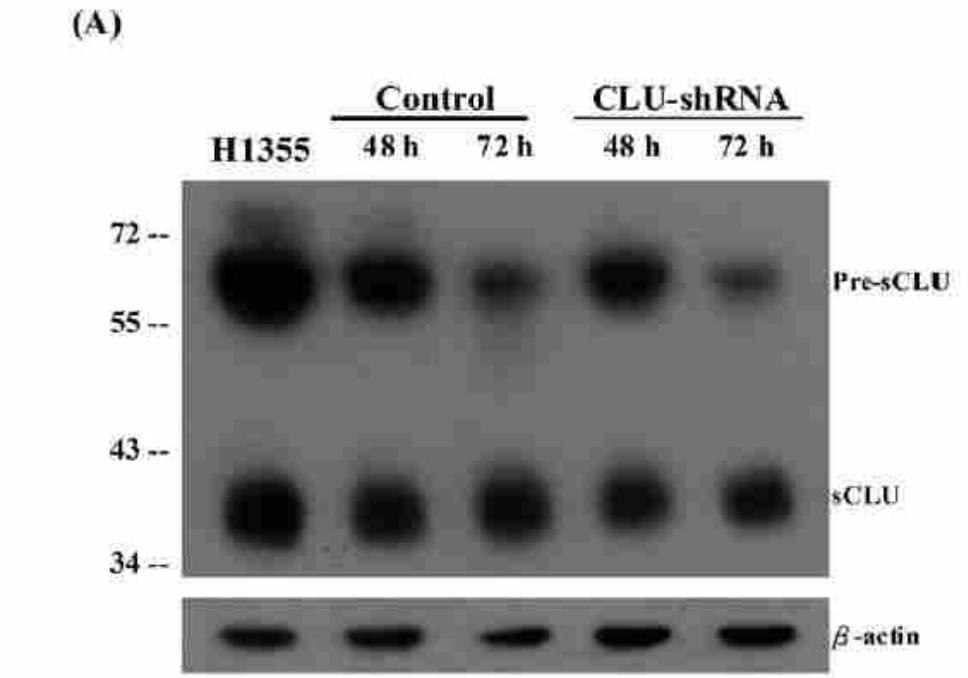

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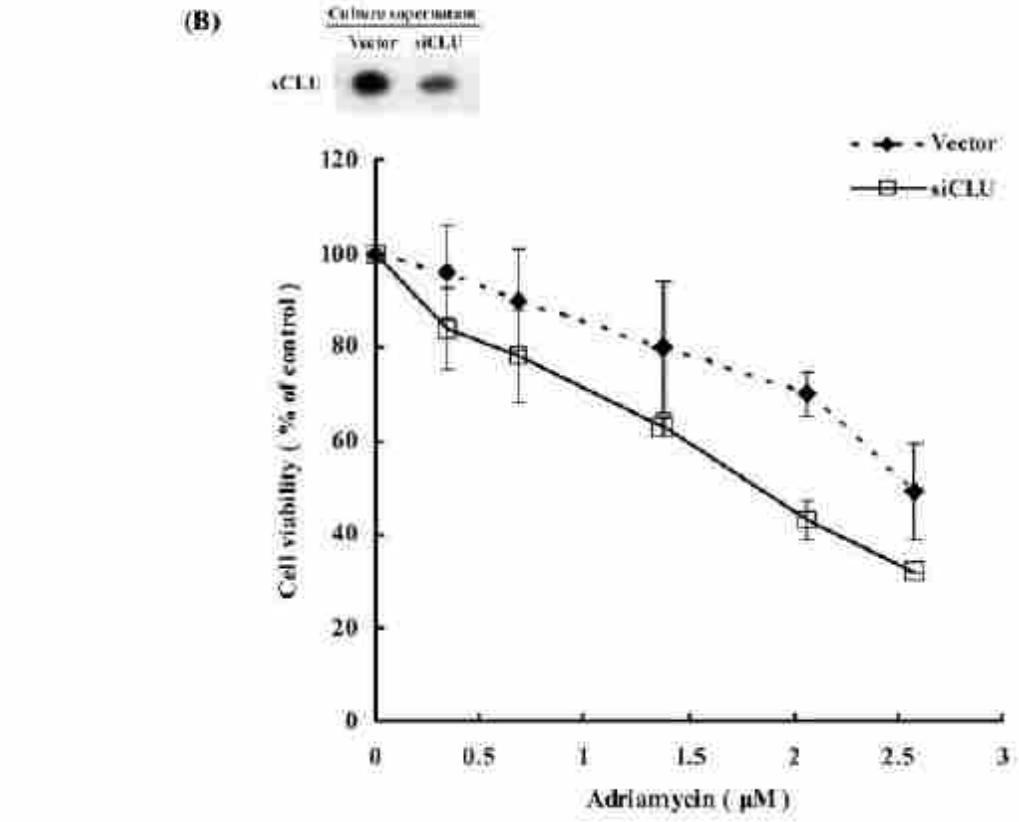

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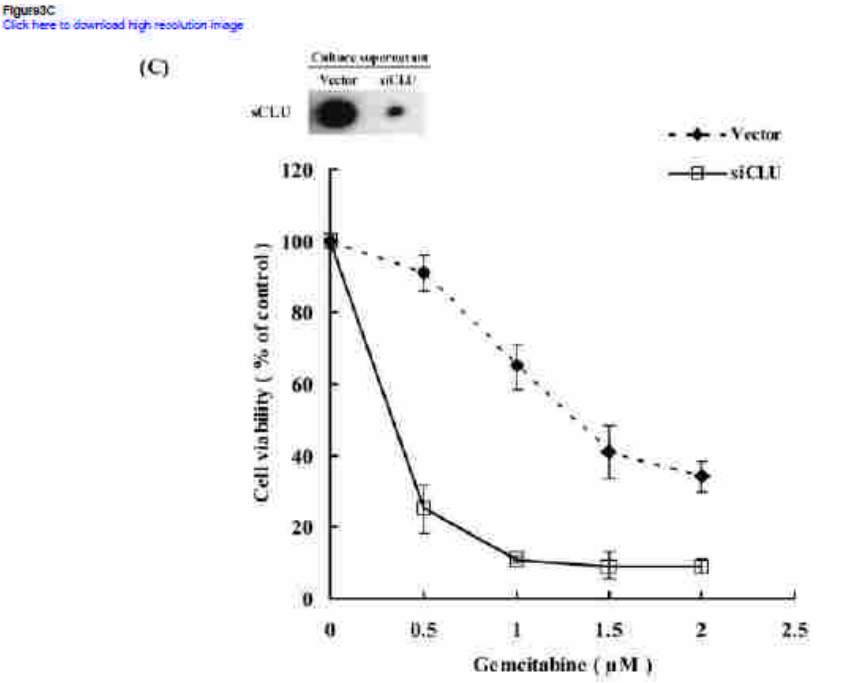

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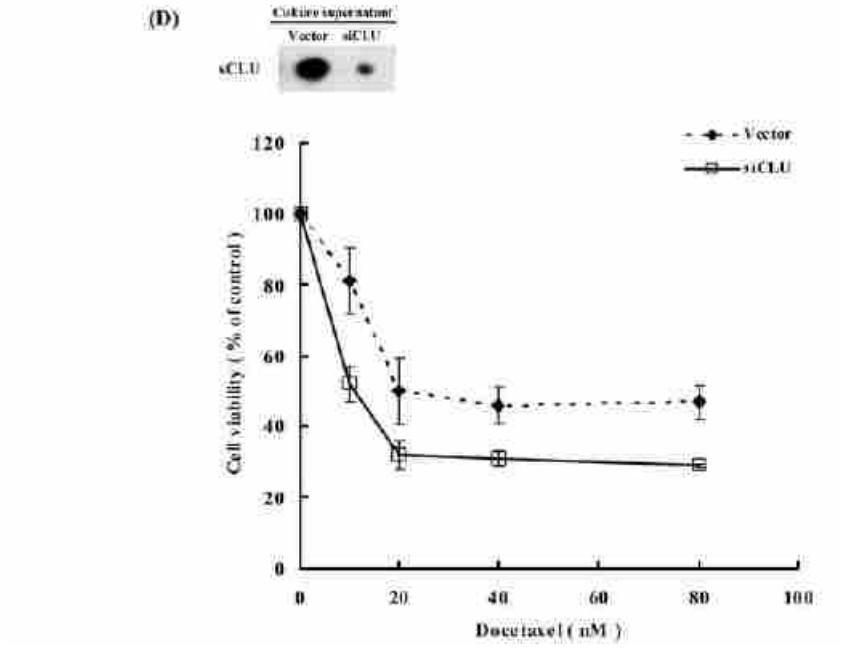

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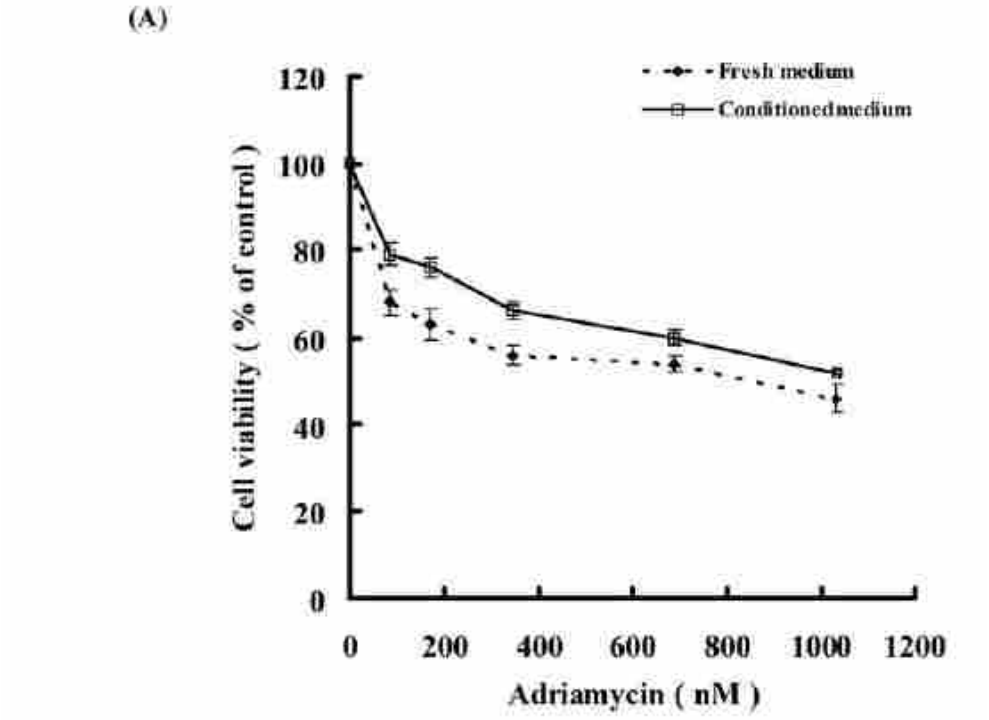

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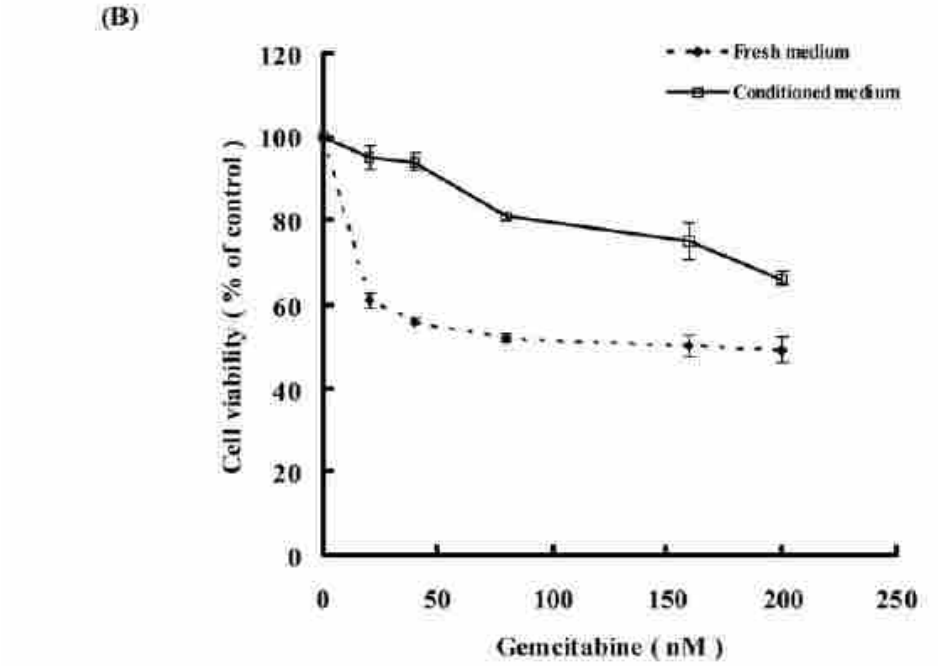

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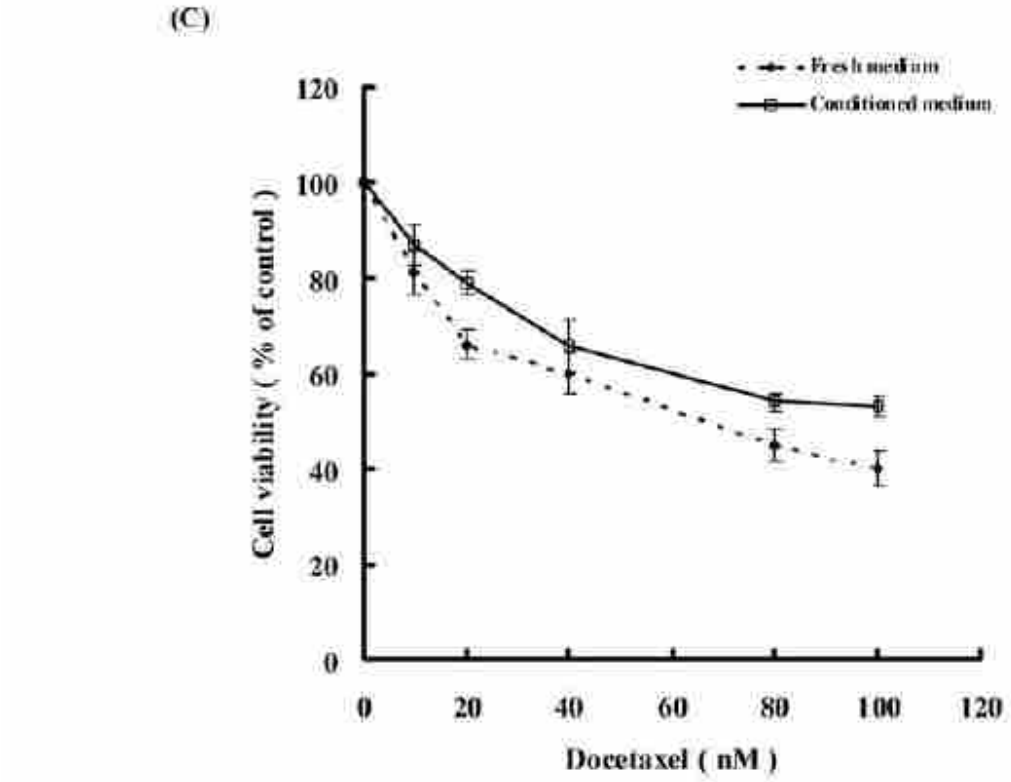

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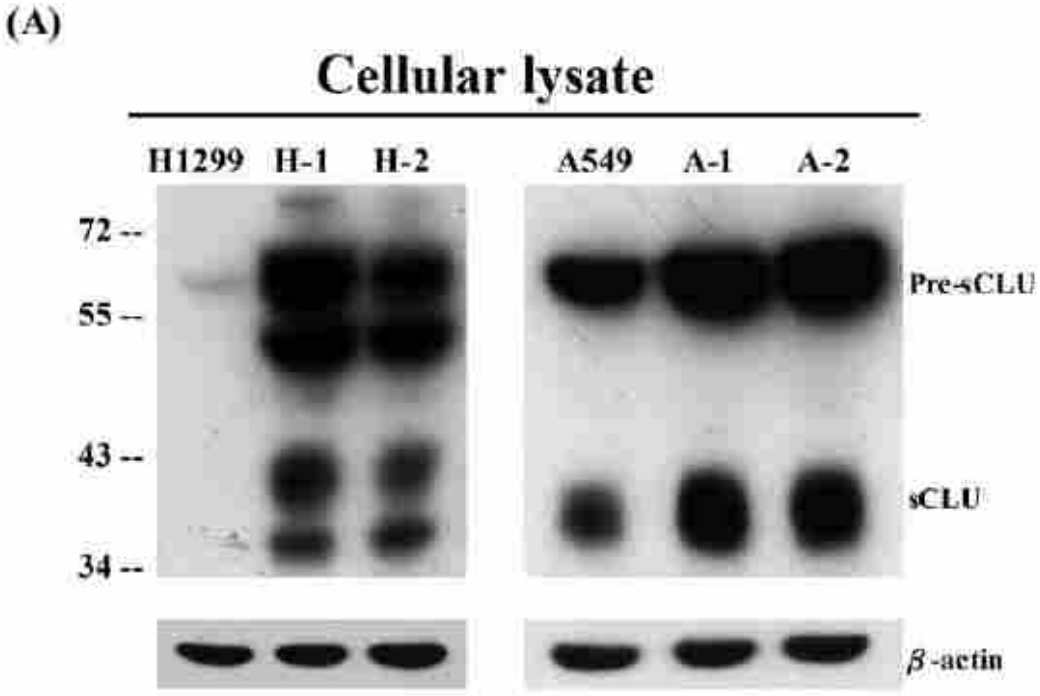
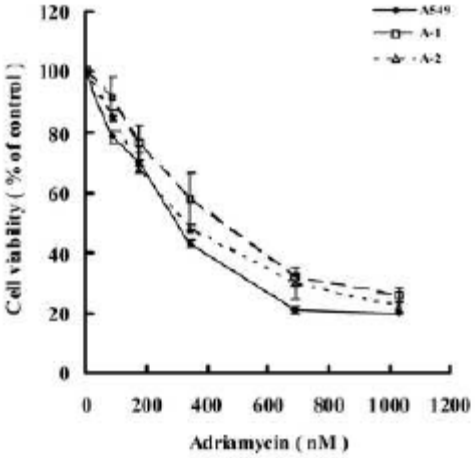


Fig 5B



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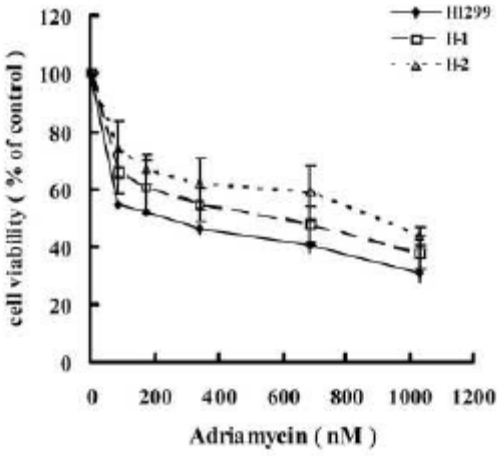

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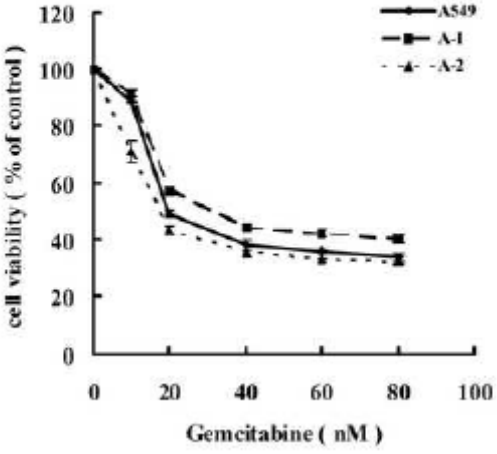

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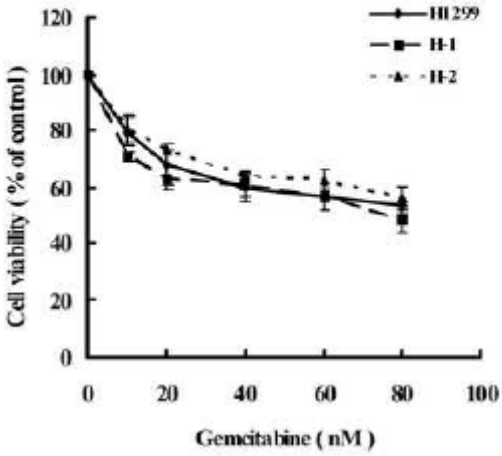

Fig 5F

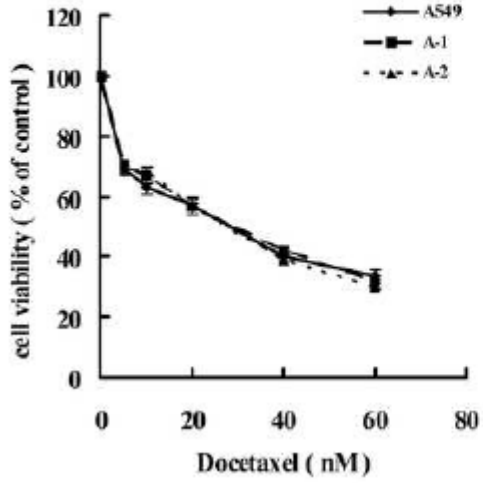

Fig 5G

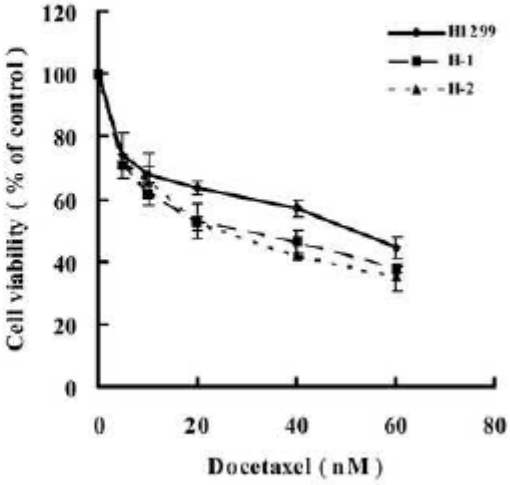

Fig 6A

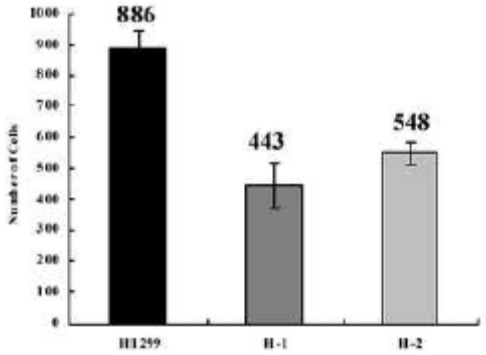

Fig 6B

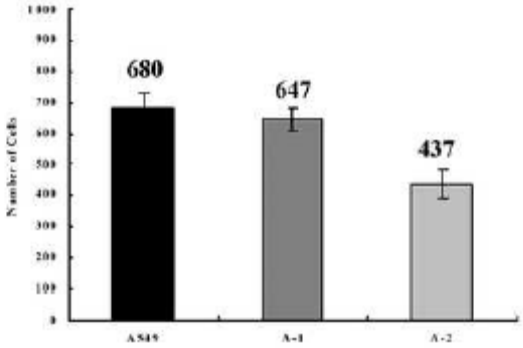

Figure7AB
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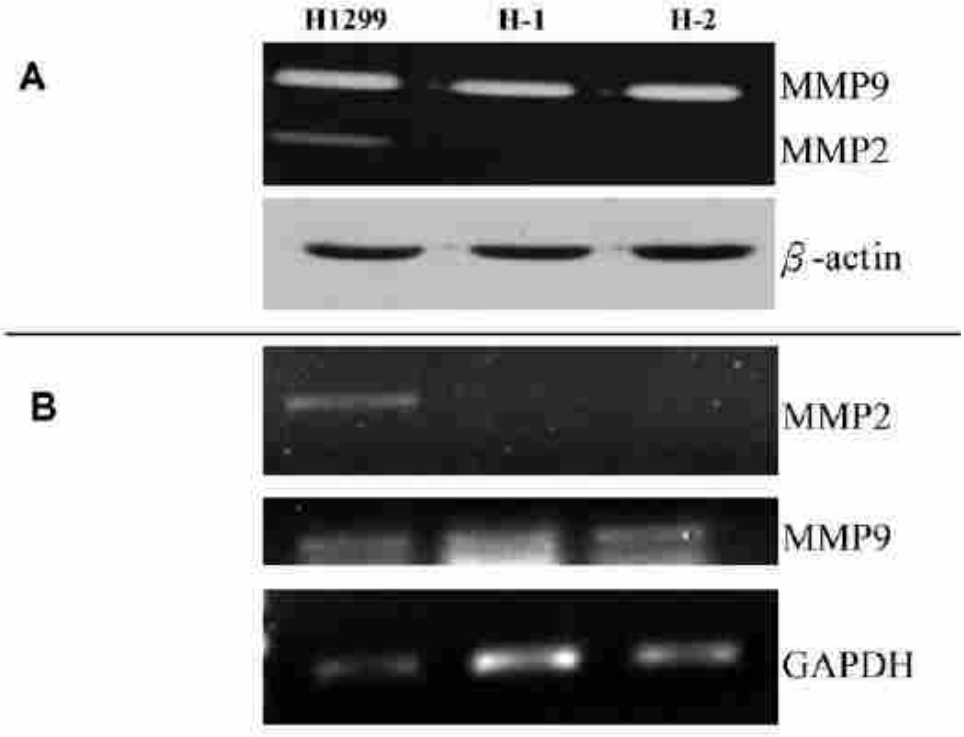

無衍生研發成果推廣資料

96 年度專題研究計畫研究成果彙整表

計畫主持人：許國堂	計畫編號：96-2314-B-040-017-MY3
計畫名稱：研究肺癌細胞對歐洲紫杉醇衍生物(剋癌易)產生抗藥性之機轉	

成果項目			量化			單位	備註（質化說明：如數個計畫共同成果、成果列為該期刊之封面故事...等）
			實際已達成數（被接受或已發表）	預期總達成數(含實際已達成數)	本計畫實際貢獻百分比		
國內	論文著作	期刊論文	0	0	0%	篇	
		研究報告/技術報告	0	0	0%		
		研討會論文	0	0	0%		
		專書	0	0	0%		
	專利	申請中件數	0	0	0%	件	
		已獲得件數	0	0	0%		
	技術移轉	件數	0	0	0%	件	
		權利金	0	0	0%	千元	
	參與計畫人力（本國籍）	碩士生	3	0	100%	人次	
		博士生	2	0	100%		
		博士後研究員	0	0	0%		
		專任助理	0	0	0%		
國外	論文著作	期刊論文	1	4	100%	篇	
		研究報告/技術報告	0	0	0%		
		研討會論文	0	0	0%		
		專書	0	0	0%	章/本	
	專利	申請中件數	0	0	0%	件	
		已獲得件數	0	0	0%		
	技術移轉	件數	0	0	0%	件	
		權利金	0	0	0%	千元	
	參與計畫人力（外國籍）	碩士生	0	0	0%	人次	
		博士生	0	0	0%		
		博士後研究員	0	0	0%		
		專任助理	0	0	0%		

其他成果 (無法以量化表達之成果如辦理學術活動、獲得獎項、重要國際合作、研究成果國際影響力及其他協助產業技術發展之具體效益事項等，請以文字敘述填列。)	無

	成果項目	量化	名稱或內容性質簡述
科教處計畫加填項目	測驗工具(含質性與量性)	0	
	課程/模組	0	
	電腦及網路系統或工具	0	
	教材	0	
	舉辦之活動/競賽	0	
	研討會/工作坊	0	
	電子報、網站	0	
	計畫成果推廣之參與（閱聽）人數	0	

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■達成目標

□未達成目標（請說明，以 100 字為限）

□實驗失敗

□因故實驗中斷

□其他原因

說明：

2. 研究成果在學術期刊發表或申請專利等情形：

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專利：□已獲得 □申請中 ■無

技轉：□已技轉 □洽談中 ■無

其他：（以 100 字為限）

估本項專題研究計畫應該會有四篇論文發表，其中已經發表壹篇，投稿審查中壹篇，草稿中兩篇

3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）（以 500 字為限）

我們希望提供未來化學治療的參考，希望經由這個長期的研究，可以延長病人的存活期，減少不良作用，達到控制病情的目的，自然而然地會改善肺癌患者的生活品質。