行政院國家科學委員會專題研究計畫　成果報告

Caveolin-1 造成癌細胞多重抗藥性機制的探討

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Introduction:

Although chemotherapy has improved the prognosis of lung cancer patients, there are still many patients who have initial resistance to chemotherapy or develop the drug resistance after several courses of chemotherapy (1). Understanding and control of drug resistance has become an important improvement in cancer therapy.

Caveolin-1, a 21- to 24-kD protein, is the principal component of caveolae, which are special invaginated microdomains of the plasma membrane present in most mammalian cells.(2) The role of caveolin-1 in cancer cells is quite diverse. Caveolin-1 functions as a tumor suppressor by contact inhibition of signaling molecules by its scaffolding domain.(3) Up-regulation of caveolin-1 prevents anchorage-independent growth (4) and sensitizes apoptotic machinery through phosphatidylinositol 3 kinase (PI-3K) in different cells.(5) However, there is also substantial evidence that caveolin-1 expression increases metastatic ability and increases the survival rate in prostate cancer and other malignancies. (6, 7)

Recent studies have shown that acquisition of the MDR phenotype is associated with the up-regulation of lipids including glucocylceramide (GlcCer) and cholesterol, which constitute lipid rafts and caveolae. (8) Some multidrug resistant cancer cell lines express very high caveolin-1 levels.(8-10) The up-regulation of caveolin-1 is accompanied by a several fold increase in the density of caveolae in these MDR

cancer cells.(9, 10) In addition, P-glycoprotein, a plasma membrane drug efflux ATPase confering multidrug resistance, is enriched in caveolae within some MDR cancer cells. However, caveolin-1 expression is not necessarily correlated with P-glycoproein, because caveolin-1 is up-regulated in MDR cells with little P-glycoprotein expression.

Cisplatin based combination chemotherapy displays significant anti-tumor activity against lung cancer and remains one of the regimens in lung cancer patients. Although caveolin-1 is over-expressed in cisplatin treated cancer cells compared with parent cells, there is no direct correlation between caveolin-1 expression and cisplatin resistance. In this study, we observe the cavoelin-1 and P-glycoprotein expression in the paraffin-embedded specimens from pathologically proven lung cancer patients who received cisplatin plus gencitabine after biopsy. Correlation between caveolin-1 expression and the chemotherapy resistance is established.

Materials and Methods:

Cell Culture

Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum and 2 mM L-glutamine at 37^{o}C, 5% CO_2 in a humidified incubator. Different concentrations of cytotoxic drugs such as cisplatin (Sigma, Oakville, Ontaria, Canada) and gemcitabine (Lilly, Fegersheim, France) were

added in the culture medium.

An SV-40-transformed, nontumorigenic bronchial epithelial cell line, BEAS-2B (CRL9609; American Type Culture Collection, Rockville, MD), was grown in modified F12 medium admixed with Hepes stock solution (1.5 mol/L, pH 7.2 to 7.4) and supplemented with growth factors as previously described.

SDS-PAGE and Western Blotting

The cells ($1x10^6$) were harvested and prepared by application of 500 μl of boiling 2X concentrated electrophoresis sample buffer (125 mM Tris-HCl, pH 6.8, 2% SDS, 5% glycerol, 0.003% bromophenol blue, and 1% β-mercaptoethanol) to each 10-cm diameter dish. Protein samples were separated by SDS-PAGE and transferred to a PVDF membrane, which was subjected to immunoblotting by anti-human caveolin-1 antibody (1:1000 dilution, BD Transduction Laboratories, Lexington, KY), followed by blotting with HRP-conjugated rabbit anti-human antibody (1: 3500 dilution, Amersham, Buckinghamshire, United Kingdom). An enhanced chemiluminescence (ECL) reaction (Amersham) was applied for signal detection. Immunoblotting with anti-human α-tubulin antibody (1:1000 dilution, Oncogene, Darmstadt, Germany) was used as an internal marker both for quantity and quality control.

Immunohistochemistry

Formalin-fixed, paraffin-embedded, surgical biopsy specimens from 73 patients

diagnosed as non-small cell lung cancer were obtained from the Department of Pathology, National Taiwan University Hospital, during the period of 1998 to 2000. Tumor staging was performed using the TNM classification system of international union against cancer. The follow-up data was obtained from medical records. All patients had advanced diseases, at least stage IIIb, in the initial presentation and did not receive total excision for the tumor. They all had good performance and received chemotherapy with the regimen of gencitabine plus cisplatin after diagnosis.

Immunohistochemistry

Paraffin-embedded 5 μm-thick sections were deparaffinized, heated in citrate buffer (0.01M), treated with 0.3% H_2O_2, and re-hydrated. After blocking, serial sections from the same patients were incubated respectively with anti-caveolin-1 IgG (clone 2297, 1:500 dilution, BD Transduction Laboratories, Lexington, KY) and anti-P-glycoprotein IgG (clone C494, 1:250 dilution, Signet Laboratories, Dedham, MA) for 1 hour at room temperature, washed, and then blotted with biotin-labeled bridge antiserum (1:200, Vector Laboratories, Burlingame, CA). After several washes with PBS, sections were incubated with a solution of avidin and biotin-conjugated peroxidase complex (Vector Laboratories) for 30 minutes at room temperature. DAB colorization was applied, and slides were further counter-stained with hematoxylin. After serial dehydration, slides were mounted for microscopic examination (Olympus).

For each specimen, the entire population of cancer cells in the lung tissue was scanned. All specimens were evaluated without knowledge of the patients' clinical data. Caveolin-1 immunoreactivity was normally localized to fibroblasts, type I pneumocytes, and endothelial cells of blood vessels in all tissue specimens examined, which served as an internal quality control in immunohistochemistry. We artificially chose 30% caveolin-1 immunoreactivity as a cutoff value for the assignment of positivity (or negativity) of caveolin-1 staining. Although antibody against P-glycoprotein had shown to cross-react with Pyruvate Carboxylase, unequivocal plasma membrane patterns of immunostaining represent true P-glycoprotein expression. Positive staining of P-glycoprotein was defined if 30% or more of the neoplastic cells showed membrane staining.

Statistical Analysis

The correlation of caveolin-1 immunoreactivity with patients' clinical variables was analyzed by χ^2-test or Fisher's exact test (2-sided). Survival curve was plotted by the Kaplan-Meier product-limit method and the statistical significance was assessed by the log-rank test. Univariate and multivariate analyses were performed using the Cox proportional-hazards regression model. $P < 0.05$ was considered to indicate statistical significance in all of the analyses. All analyses were done with statistical software (Statview version 5.0; SAS Institute, Cary, NC)

Result:

Up-regulation of caveolin-1 expression by cytotoxic drugs in drug-sensitive cancer cells

We treated the lung cancer cell lines A549 with cisplatin and gemcitabine cytotoxic drugs. As shown in Figure 1, drug-sensitive A549 cells had less or absent caveolin-1 expression. After exposure of the cancer cells to these cytotoxic drugs, up-regulation of caveolin-1 expression was found. Higher concentration of cytotoxic drug and longer exposure time also increased the expression of caveolin1.

Clinical Finding

We examined the status of caveolin-1 expression in specimens obtained from patients who received chemotherapy. In this retrospective study, 73 specimens diagnosed as lung cancer were obtained from the Department of Pathology, National Taiwan University Hospital, during the period of 1998 to 2000. All patients had advanced diseases, good performance and received chemotherapy with the regimen of gencitabine plus cisplatin. The patients consisted of 41 man and 32 women. The mean age was 57 years old (range 25-83). Forty-four patients had an age lower than 65 years old with 29 patients older than 65 years old. The pathological findings disclosed adenocarcinoma in 49 patients, squamous cell carcinoma in 18 patients, and large cell carcinoma in 3 patients. Pathological staging was performed according the TNM

classification of the International Union against cancer. Eleven patients were in stage IIIb, and 59 patients were in stage IV. Twenty-three patients had response to chemotherapy, 32 patients remained stable during chemotherapy and 19 patients had disease progression during chemotherapy.

Expression of cavoelin-1 in advanced lung cancer

Caveolin-1 immunoreactivity was normally localized to fibroblasts, type I pneumocytes, and endothelial cells of blood vessels in all tissue specimens examined, which could serve as an internal quality control in immunohistochemistry. We artificially chose 30% caveolin-1 immunoreactivity as a cutoff value for the assignment of positivity (or negativity) of caveolin-1 staining. Twelve of 73 patients had caveolin-1 expression (Fig. 2). To determine the significance of caveolin-1 expression, all patients were divided into two groups according their cavoelin-1 expression: caveolin-1 negative group (n=61 patients) and cavoelin-1 positive group (n=12 patients). The clinicopathologic variables of the caveolin-1 negative and caveolin-1 positive group are listed in table 1.

Correlation of caveolin-1 expression and poor response to chemotherapy

Among the 12 patients with caveolin-1 expression, only four patients remained stable disease during chemotherapy. The other eight patients had progressive disease during chemotherapy. In 23 patients who responded to chemotherapy, there was no

caveolin-1 expression found in their specimens. Correlation between caveolin-1 expression and progress disease during chemotherapy was established (P=0.01, table 1).

We also examined the status of P-glycoprotein expression in these specimens and 4 patients had unequivocal plasma membrane patterns of immunostaining represent true P-glycoprotein expression. In these 4 patients, one patient had progress disease during chemotherapy, two patients had partial response during chemotherapy and one patient remained stable during chemotherapy. Only one patient had caveolin-1 and P-glycoprotein co-expression and this patient remained stable during chemotherapy.

Prognostic significance of caveolin-1 expression

Analysis of the association of caveolin-1 expression with patients' survival rate was performed on the 73 patients of non-small cell lung cancer. The survival time after chemotherapy for these 12 patients with caveolin-1 expression and 61 patients without caveolin-1 expression was calculated by the Kaplan-Meier method (Fig. 3). Multivariate analysis was also performed for caveolin-1 immunoreactivity and other factors as stage, age, sex, and chemotherapy response. Performance status was excluded for analysis because all of the patients were good enough to received chemotherapy at the beginning. The univariate log-rank test for each variable was tested, and the result is listed in Table 2. When all variables were evaluated, the

multiple cog regression model suggested that the expression of caveolin-1 was an independent factor for prediction of poor survival in patients with non-small cell lung cancer (hazard ratio 3.516; *P*=0.0008) (Table 2)

Discussion:

In this study, immunohistochemical staining was performed in 73 specimens from lung cancer patients with advanced disease receiving chemotherapy. Only 12 patients had caveolin-1 immunostaining. Among them, four patients had stable disease during chemotherapy and eight had disease progression during chemotherapy. Caveolin-1 expression is correlated with a poor response to chemotherapy. Although up-regulation of caveolin-1 was found in many MDR cancer cell lines (8-11), this is the first study with clinical data to demonstrate the relationship between caveolin-1 expression and possible drug resistance.

Caveolin-1 is thought to be a tumor suppressor gene, and its expression is down-regulated in oncogenically transformed fibroblast, as well as breast and lung cancer (12-14). However, after cytotoxic drug stress, up-regulation of caveolin-1 could be seen both in cancer lines and in vivo (15, 16). It has been reported that little or no caveolin-1 immunostaining is in non-small cell lung cancer patients before chemotherapy. But re-expression of caveolin-1 has been observed in half of the patients who receive chemotherapy and/or radiotherapy before operation (16).

Up-regulation of caveolin-1 after cytotoxic drugs is thought to be an early cellular response before drug resistance is manifested. However, in this study, 12 of 73 patients who had advanced non-small cell lung cancer (at least stage IIIb) without chemotherapy before biopsy showed positive caveolin-1 immunostaining. Many of them had disease progression during chemotherapy. Expression of caveolin-1 before administration of cytotoxic drugs is a good predictor for drug resistance.

Several mechanisms have been proposed for caveolin-1 up-regulation in MDR cells. P-glycoprotein (P-gp), an ATPase that pumps out drugs used in chemotherapy, has been found to be enriched in caveolin-1 rich membrane domain in some MDR cells (9). But up-regulation of caveolin-1 is correlated with little P-gp expression in other MDR cells and over-expression of P-gp is not localized in caveolae (17). Reintroduction of caveolin-1 expression into drug sensitive cancer cells does not increase the expression of P-gp (9, 18). In the specimens of ovarian cancer patients, caveolin-1 expression is not correlated with P-gp expression (19). In our study, P-glycoprotein was present in 4 patients without correlation of caveolin-1 expression.

It is interesting that caveolin-1 and cholesterol could regulate each other reciprocally (20-23). Induction of caveolin-1 expression enhances the cholesterol efflux and reduction of their expression with caveolin-1 antisense DNA, oxysterols or vanadate is coupled to a comparable decrease in free cholesterol efflux (24).

Caveolin-1 is also transcriptionally regulated by sterol-regulatory-element-binding-protein (SREBP) (20). A high cholesterol efflux pathway may serve as a platform for lipophilic drugs transport from intracellular compartment to plasma membrane against a steep concentration gradient, although there is still no direct evidence to elucidate this. However, some cytotoxic drugs used to establish MDR cancer cell lines with caveolin-1 up-regulation have low lipophilicity including doxorubicin and cisplatin.

Another possibility is that caveolin-1 expression exerts negative regulation in apoptotic machanisms, although such results are in recent conflict. Down-regulation of caveolin-1 by anti-sense vector prevents staurosporine-induced apoptosis in NIH-3T3 and T24 cells (25). Caveolin-1 sensitizes Rat-1 fibroblast to ceramide-induced apoptosis and also increases 293 cells and HeLa cells to arsenite cytotoxicity through PI-3K pathway (5, 26). However, Caveolin-1 could inhibit anoikis in MCF-7 breast cancer cells (27), and suppresses *c-myc*-induced apoptosis (28). In acid sphingomyelinase-deficient ($asmase^{-/-}$) mice, loss of caveolae and glycolipid-enriched membrane microdomains (GEM) increases susceptibility to apoptosis after T cell receptor stimulation probably through decrease of MAPK activation (29). A recent paper reported caveolin-1 has anti-apoptotic effect in prostate cancer cell through stimulate Ark activity leading to phosphorylation of GSK3, FKHR

and MDM2 (30). These conflicting reports may indicate caveolin-1 effect are cell type- and expression level-dependent (5, 28, 31).

In summary, our study reveals that up-regulation of caveolin-1 in cancer cells has more cytotoxic drug resistance no matter hydrophilic or lipophilic drugs. Caveolin-1 is down-regulated in newly-transformed cells and almost absent in the early stages of lung cancer (14). However, its re-expression in the late stage disease before chemotherapy is correlated with the drug resistance. Caveolin-1 may be one component of mechanisms in multidrug resistance and may serve as a therapeutic target for preventing multidrug resistance.

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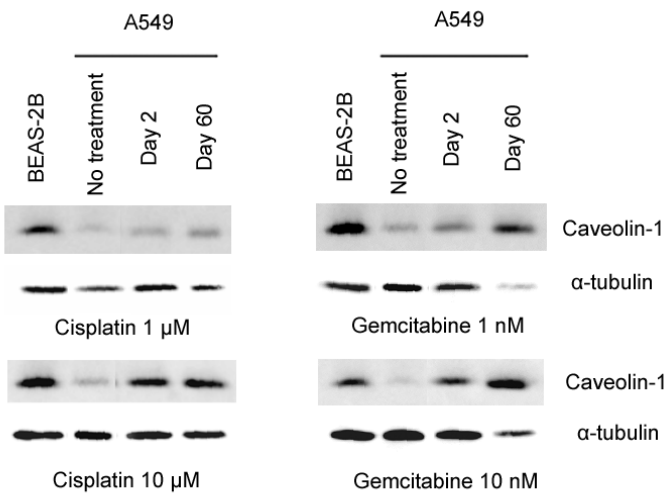
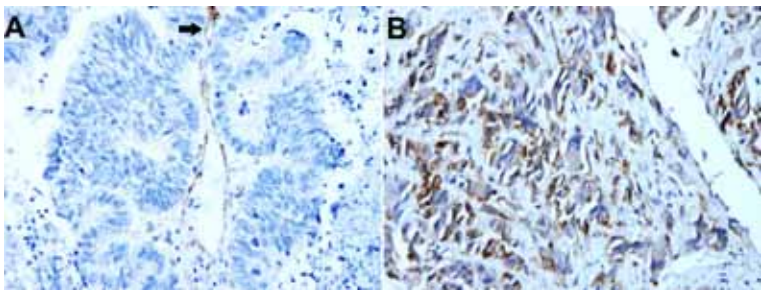
Figure 1.



Western blotting revealed caveolin-1 expression in BEAS-2B cells, but little expression in A549. After exposure to different cytotoxic drugs including cisplatin and gemcitabine, up-expression of caveolin-1 in A549 was found. Expression of caveolin-1 was higher in high dose cytotoxic drug and longer exposure time. Staining of α-tubulin was used as control.

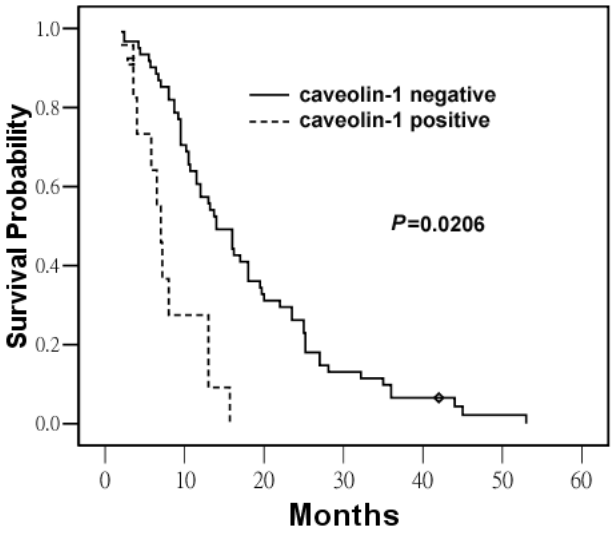
Figure 2.



Immunohistochemical staining for caveolin-1. **A.** Negative or faint staining of caveolin-1 in tumor cells from the patient who was response to chemotherapy. Positive staining of endothelial cells as internal control (arrow). **B.** Cancer cell cytoplasm stained with caveolin-1 from the patient who was resistant to

chemotherapy.

Figure 3.



Comparison of overall survival curves for patients with caveolin-1-positive and negative tumors in 73 patients who received cisplatin plus gemcitabine chemotherapy.

Table 1. Relationship between Clinicopathologic Features and Caveolin-1 Expression in Biopsy Specimens of Non-small Cell Lung Cancer

Characteristic	Caveolin-1 (-) No. of Patients (%)	Cavelin-1 (+) No. of Patients (%)	*P* value
Age	57.9 ± 13	62.7 ± 14	0.14
Gender			
Male	34 (82.9)	7 (13.1)	1
Female	27 (84.4)	5(15.6)	
Stage			
IIIb	11 (91.7)	1 (8.3)	0.68
IV	50 (82)	11 (18)	
Cell Type			
Adenocarcinoma	39 (84.8)	7 (15.2)	0.75
Others	22 (81.5)	5(18.5)	
Chemotherapy response			
Progress disease	12 (63.2)	7(36.8)	0.01[§]
Others	49 (90.7)	5(9.3)	

[§] Significance

Table 2. Analysis of parameters including caveolin-1 immunoreactivity and other clinical prognostic factors in 73 patients with advance lung cancer

Factors	Cog Regression	
	Hazard Ratio (95%CI[a])	*P*
Caveolin-1[b]	3.516 (1.686-7.331)	0.0008*
Age	0.994 (0.975-1.012)	0.4952
Gender	1.264 (0.759-2.103)	0.3681
Pathology[c]	1.038 (0.614-1.756)	0.8882
Stage	1.499 (0.776-2.897)	0.2286

[a] CI, confidence interval

[b] Cut-off value: 30%

[c] Adenocarcinoma vs. non adenocarcinoma

*Significant