Isolation and characterization of gemcitabine-resistant human non-small cell lung cancer A549 cells

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Abstract. Gemcitabine is an effective chemotherapy against non-small cell lung cancer (NSCLC). However, resistance to gemcitabine reduces its efficacy. We have isolated gemcitabine-resistant human non-small cell lung cancer A549 cells, termed A549/GR cells. A549/GR cells were resistant to gemcitabine as well as paclitaxel and docetaxel but not carboplatin and irinotecan. The expression level of multidrug resistance protein 7 (MRP7) in A549/GR cells was higher than that in A549 cells, and the inhibitor of MRP7 by cepharanthine increased the sensitivity to gemcitabine in A549/GR cells. These findings indicate that cepharanthine reversed gemcitabine resistance. To determine predictive molecular markers of gemcitabine resistance for more effective treatment of these tumors, we performed PCR array. We identified that *CDKN1A/p21*, *CYP3A5*, *microsomal epoxide hyrolase 1 (EPHX1)* and *ABCC6 (MRP6)* were up-regulated >5-fold in A549/GR cells. Gemcitabine also induced the expression of *p21* and *CYP3A5* in A549 cells. A better understanding of the characterization and mechanism of the resistance to gemcitabine in A549/GR cells may help identify agents that reverse clinical gemcitabine resistance in NSCLC.

Introduction

Lung cancer is the most common cause of cancer death in developed nations. In the United States, an estimated 219,440 new cases, and 159,390 deaths are projected for 2009 (1,2). Non-small cell lung cancer (NSCLC) presents commonly as incurable locally advanced or metastatic disease. Despite major research efforts, survival prospects remain dismally small, and 14% of all patients with lung cancer are expected to live five years after diagnosis (3). In patients with advanced, incurable NSCLC, cisplatin-based combinations have resulted in improved survival rates, and platinum combined with any anti-cancer agents such as paclitaxel, docetaxel, gemcitabine, irinotecan, vinorelbine or pemetrexed (3), in combination with bevacizumab, bevacizumab-eligible patients is recommend as first line therapy for most patients (3). Although various chemotherapeutic agents and treatment regimens improved outcomes for patients with advanced NSCLC, the treatments ultimately fail in most patients because of resistance or intolerable toxicity. Chemoresistance, whether inherent or acquired, is known to be a major reason for the failure of anti-cancer therapies. Gemcitabine, a deoxycytidine nucleotide analog of cytosine arabinoside (4), is a chemotherapeutic agent used in the treatment of advanced NSCLC. The active metabolites of gemcitabine, gemcitabine diphosphate and gemcitabine triphosphates, block ribonucleotide reductase lowing levels of native deoxycytidine (5).

To understand the molecular basis of gemcitabine resistance, we have isolated gemcitabine-resistant cells from human non-small cell lung cancer A549 cells (A549/GR). The development of gemcitabine resistance was accompanied by cross-resistance to paclitaxel and docetaxel but not carboplatin and irinotecan. Since MRP7 is implicated in both gemcitabine and taxane resistance, we hypothesized that increased MRP7 expression explained gemcitabine resistance in the A549/GR cell line.

To determine predictive molecular markers of gemcitabine resistance for more effective treatment of these tumors, we performed PCR array. We identified that *p21/CDKN1A*, *CYP3A5*, *microsomal epoxide hyrolase 1(EPHX1)* and *ABCC6 (MRP6)* were up-regulated >5-fold in A549/GR cells. Gemcitabine also induced the expression of *p21* and *CYP3A5* in A549 cells. A better understanding of the characterization and mechanism of the resistance to gemcitabine in A549/GR cells may help identify agents that reverse clinical gemcitabine resistance in NSCLC.

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Materials and methods

Reagents. Paclitaxel, irinotecan hydrochloride, docetaxel and ethyl methanesulfonate and 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT) were obtained from Sigma Chemical (St. Louis, MO). Gemcitabine hydrochloride was from LKT Laboratories, Inc. (Minnesota, USA). Carboplatin was from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), penicillin-streptomycine solution (10,000 U/ml penicillin and 10,000 μg/ml streptomycin) were from Hyclone (Utah, USA).

Cell culture. The A549 cell line, derived from non-small cell lung cancer, was maintained in DMEM containing 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin at 37˚C in a 5% CO_2 humidified atmosphere.

Cell proliferation by 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT) assay. Cell proliferation *in vitro* was measured by MTT colorimetric assay in 96-well plates. The cells ($5x10^3$) were inoculated into each well. After overnight incubation (37˚C in 5% CO_2), anti-cancer agents were added to the culture and were then incubated for 3 days. Thereafter, 50 μl of MTT (1 mg/ml) was added to each well and the plates were incubated for additional 4 h. After aspiration of culture medium, the resulting formazan was dissolved with 100 μl of dimethylsulfoxide. The plates were read at 570 nm using a micro-plate reader.

Chronic gemcitabine exposure. Gemcitabine-resistant A549/GR cells were isolated by the A549 cells with increasing concentrations of gemcitabine following ethyl methanesulfonate-induced mutagenesis, and then incubated in a selection medium with gemcitabine (0.1-100 μM).

RT-PCR method. Total cellular RNA was extracted by RNeasy Mini kit (Qiagen Sciences, Maryland, USA). RNA quality and concentration were confirmed in NanoDrop ND-100 Spectrophotometer (Thermo Scientific, Wilmington, DE). For RT-PCR, 1 μg of total RNA was used for cDNA synthesis using iScript cDNA synthesis kit (Bio-Rad, CA, USA), according to the manufacture's protocol. The conditions for the RT-PCR were as follows: 5 min at 95˚C, and then 28 cycles of amplification in PCR master mix (Promega, WI, USA) at 95˚C for 30 sec, annealing at 52˚C for 30 sec and extension at 72˚C for 1 min. The primers used for this analysis are as follows: MRP7, forward 5'-gtgcgaatgctcattcttcctc-3', reverse 5'-tgtacagctgtg catcaaatgt-3'; GAPDH, forward 5'-gtct tcaccaccatggagaagg-3', reverse 5'-gg caggtcaggtccaccactga-3'; CYP3A5, forward 5'-ctggccactcaccctgatgtc-3', reverse 5'-atct atgctgtccttcttctt-3'; p21, forward 5'-ctcttcggcc cagtggacagc-3', reverse 5'- agagtctccaggtccacctgg-3'.

PCR array. Total cellular RNA was extracted by RNeasy Mini kit (Qiagen Sciences). RNA quality and concentration were confirmed in NanoDrop ND-100 Spectrophotometer (Thermo Scientific). For RT-PCR, 1 μg of total RNA was used for cDNA synthesis using iScript cDNA synthesis kit (Bio-Rad), according to the manufacture's protocol. After cDNAs were mixed with SYBR-Green Supermix, mixtures were added to the plates of RT-2 Profiler PCR array human cancer drug resistance and metabolism. The conditions for real-time PCR were as follows: 10 min at 95˚C and then 40 cycles at 95˚C for 15 sec, 60˚C at 1 min. The data from PCR array were normalized according to the manufacture's guideline using software from SABioscience.

Statistical analysis. Data are presented as the mean ± SD. Statistical analysis was performed using StatView 5.0. (SAS Institute Inc., Cary, NC, USA). Differences were considered significant at $P<0.05$.

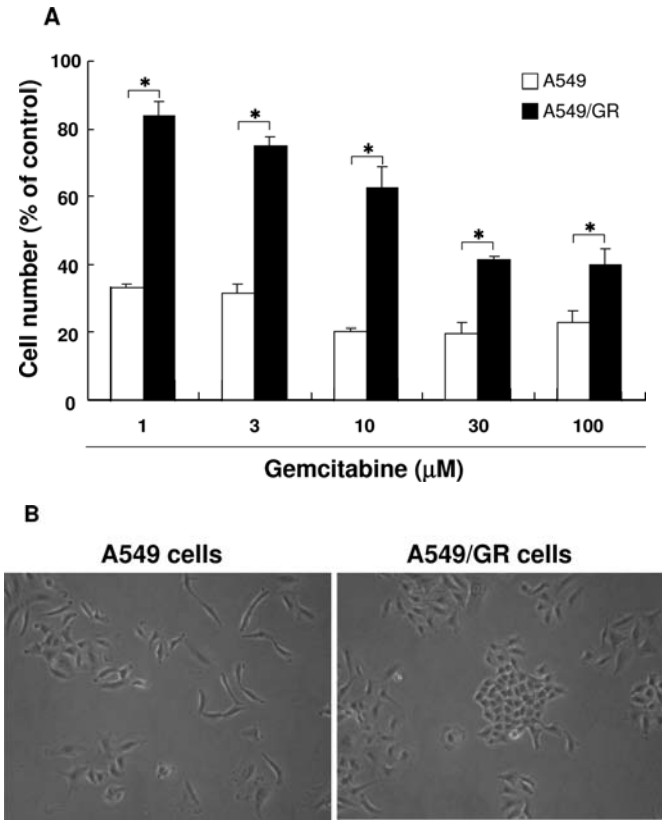

Figure 1. Sensitivity of gemcitabine and morphology in A549 and A549/GR cells. (A) A549 and A549/GR cells were treated with various concentrations of gemcitabine for 72 h, and cells viability was determined using MTT assay in each cell line. Each column and bar represents the mean ± SD. *P<0.05. (B) Morphology of A549 and A549/GR cells.

Results

Establishment of gemcitabine-resistant non-small cell lung cancer A549 cells. To isolate gemcitabine-resistant A549/GR cells, A549 cells were cultured in selection medium containing stepwise increases in gemcitabine concentration from 0.1 to 100 μM. We examined the sensitivity to gemcitabine of each cell line. A549/GR cells were more resistant than parental A549 cells, despite retaining a similar microscopic appearance to the parent A549 cells (Fig. 1A and B).

Cross resistance to gemcitabine. We tested the drug sensitivity of each cell line by MTT assay. Fig. 2 showed sensitivity

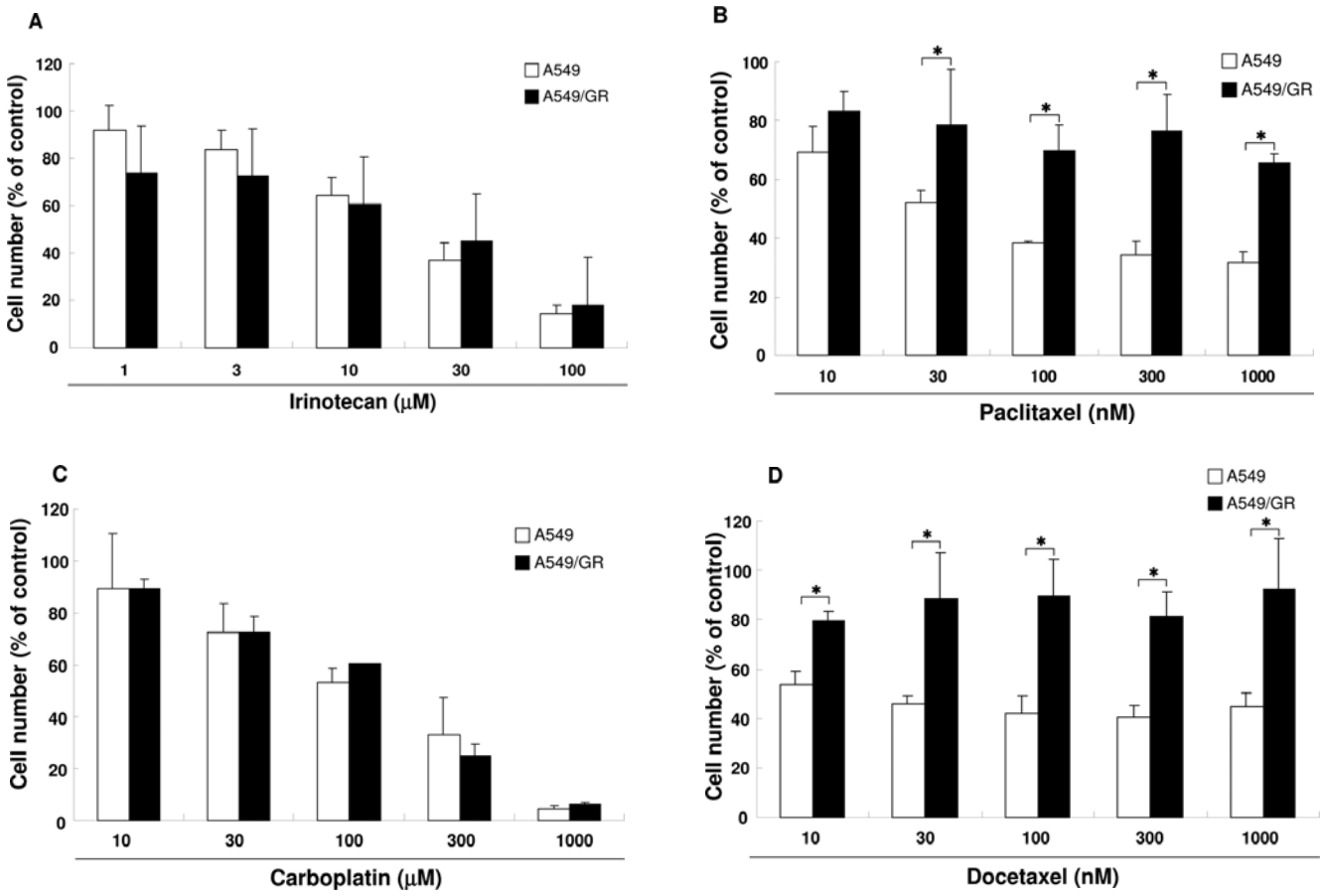

Figure 2. Sensitivity of anti-cancer agents on A549 and A549/GR cells. A549 and A549/GR cells were treated with various concentrations of irinotecan (A), paclitaxel (B), carboplatin (C) and docetaxel (D) for 72 h and cells viability was determined using MTT assay in each cell line. Data are mean of triplicates, and bar represents the mean ± SD. *P<0.05.

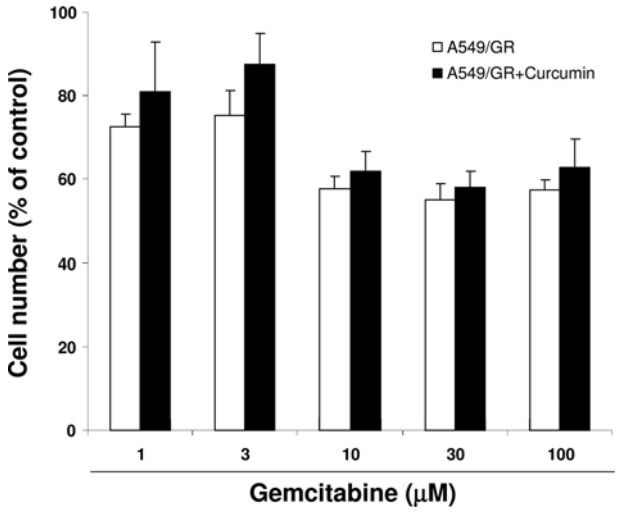

Figure 3. Effect of curcumin on the sensitivity in A549/GR cells. A549/GR cells were treated with gemcitabine in the presence or absence of curcumin for 72 h. Cell viability was determined using MTT assay in each cell line. Data are mean of triplicates and bar represents the mean ± SD.

of various anti-cancer drugs for the parental and resistant cell lines. Interestingly, A549/GR cells were resistant to paclitaxel and docetaxel but not irinotecan and carboplatin as compared to A549 cells (Fig. 2A-D).

The effect of curcumin on the sensitivity in A549/GR cells. The transcription factor nuclear factor-κB (NF-κB) has been linked with cell proliferation and chemoresistance such as gemcitabine, paclitaxel and docetaxel (12-14). Since curcumin has been shown to suppress NF-κB activation, we investigated the effect of curcumin on the sensitivity to gemcitabine in A549/GR cells. Curcumin did not affect the sensitivity to gemcitabine-resistance cells (Fig. 3). These results suggest that activation of NF-κB might not relate the resistant to gemcitabine in A549/GR cells.

Expression levels of MRP7 in the A549 cells and A549/GR cells. ABC transporters promote the active efflux of a wide variety of solutes across cellular membranes. To date, MRP7 have been implicated of gemcitabine resistance (12). Therefore, we examined the expression levels of *MRP7* in A540 and A549/GR cells by RT-PCR. Compared to A549 cells, *MRP7* showed increased expression in A549/GR cells (Fig. 4A).

Effect of cepharanthine on the sensitivity in A549/GR cells. Cepharanthine (6',12'-dimethoxy-2,2'-dimethyl-6,7-[methyle-

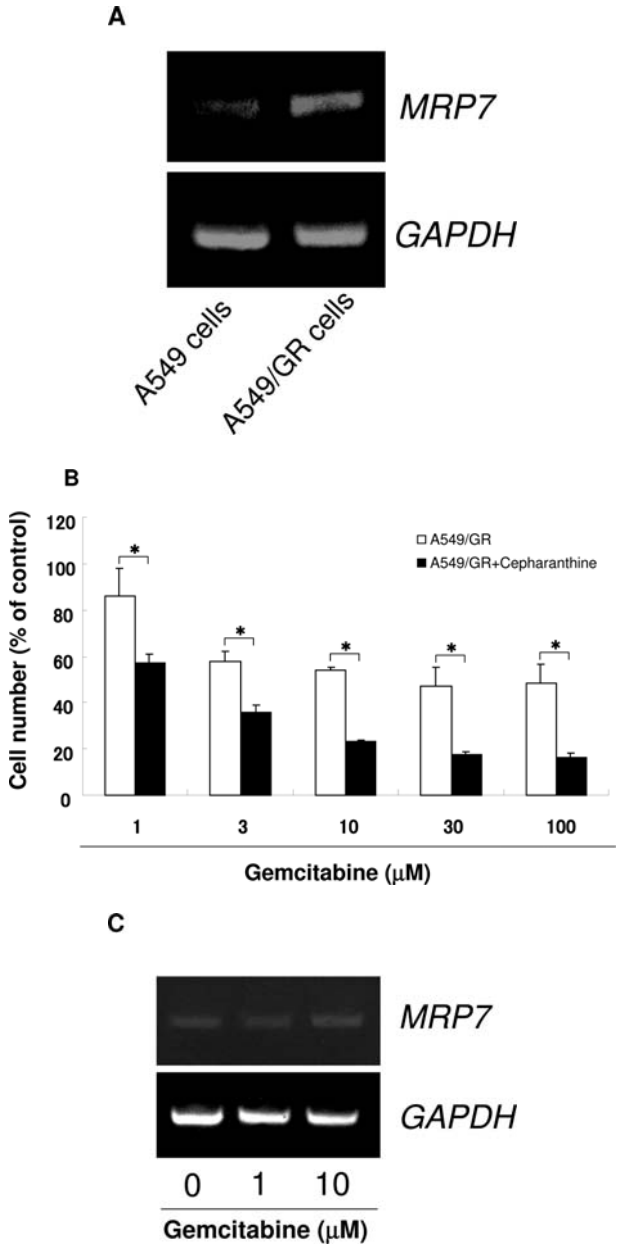

Figure 4. Expression of *MRP7* in A549 and A549/GR cells, and the effect of cepharanthine on the sensitivity to gemcitabine in A549/GR cells. (A) Total RNAs were isolated and expression of *MRP7* in A549 and A549/GR cells was detected by RT-PCR. The expression of *GAPDH* was also examined as a loading control. (B) A549/GR cells were treated with gemcitabine in the presence or absence of cepharanthine for 72 h. Cell viability was determined using MTT assay in each cell line. Data are mean of triplicates and bar represents the mean ± SD. *P<0.05. (C) A549 cells were treated with gemcitabine at 1 or 10 μM for 24 h. After extracted with total RNA, expression of *MRP7* in A549 cells was detected by RT-PCR. The expression of *GAPDH* was also examined as a loading control.

nebis(oxy)]oxyacanthan) is a biscoclaurine alkaloid extracted from the roots of *Stephania Cepharantha Hayata* (6). Since cepharanthine has been shown to be an inhibitor of MRP7 (7), we examined the effect of cepharanthine on the sensitivity to gemcitabine using MTT assay. Cepharanthine treatment at 3 μg/ml resensitized resistant cells to gemcitabine (Fig. 4B).

Effect of gemcitabine on the expression of MRP7 in A549 cells. Since the expression of MRP7 increased in A549/GR cells, we investigate the effect of gemcitabine on the expression of MRP7 in A549 cell. Gemcitabine does not alter expression of MRP7 in A549 cells (Fig. 4C).

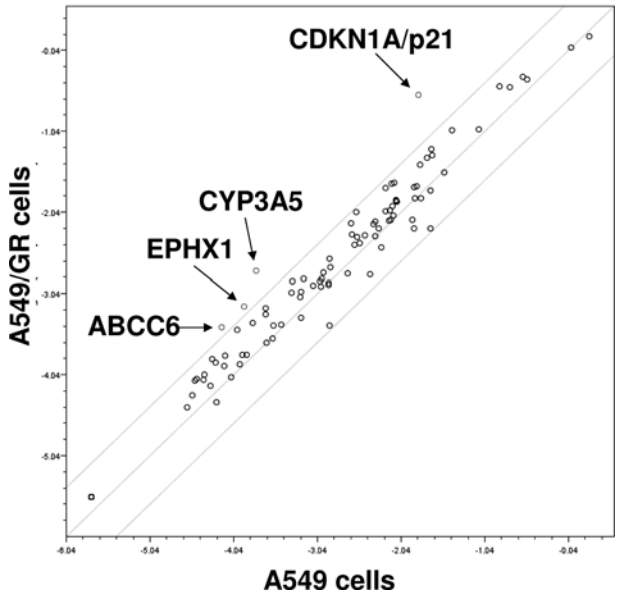

Figure 5. Gene expression pattern in A549 and A549/GR cells. The scatter plot shows mean signal intensities of each gene primers using data obtained from the PCR array.

PCR array and the effect of gemcitabine on the expression of p21 and CYP3A5. To determine predictive molecular markers of gemcitabine resistance for more effective treatment of these tumors, we have isolated gemcitabine-resistant cells from human non-small cell lung cancer A549 cells (A549/GR) and performed PCR array that covers 84 genes encoding enzymes for drug resistance, drug metabolism, DNA repair, cell cycle, growth factor receptor, hormone receptor and transcription factors. We identified 4 genes, *CDKN1A/p21*, *CYP3A5*, *microsomal epoxide hyrolase 1 (EPHX1)* and *ABCC6 (MRP6)* that were up-regulated >5-fold in A549/GR cells (Fig. 5). As shown in Fig. 6A, the expression of *ABCC1 (MRP1)*, *ABCC2 (MRP2)*, *ABCC3 (MRP3)*, *ABCC5 (MRP5)* and *MVP (LRP)* were up-regulated >2-fold in A549/GR cells. In the expression of phase I and II metabolism genes, *CYP3A5* and *EPHX1* were more expressed in A549/GR cells than that in A549 cells. Cell cycle regulators, including those for CCND1 (cyclin D1), CCNE1 (cyclin E1), CDK2, CDK4, CDKN1A (p21Waf1, p21Cip1), CDKN1B (p27Kip1), CDKN2A (p16Ink4) and CDKN2D (p19Ink4d), involved in aspects of drug resistance. As shown in Fig. 6C, the expression of *CDKN1A/p21* was more increased in A549/GR cells than that in A549 cells. We also examined the expressions of *CDKN1A/p21* and *CYP3A5* in A549 cells and A549/GR cells by RT-PCR. The expressions of CDKN1A and CYP3A5 in A549/GR cells was also more increased than that in A549 cells (Fig. 7A). To determine if the expression of *CDKN1A/ p21* and *CYP3A5* was altered by gemcitabine, we examined the effect of gemcitabine on the expression levels of *CDKN1A/p21* and *CYP3A5* in A549 cells by RT-PCR. Induction of the

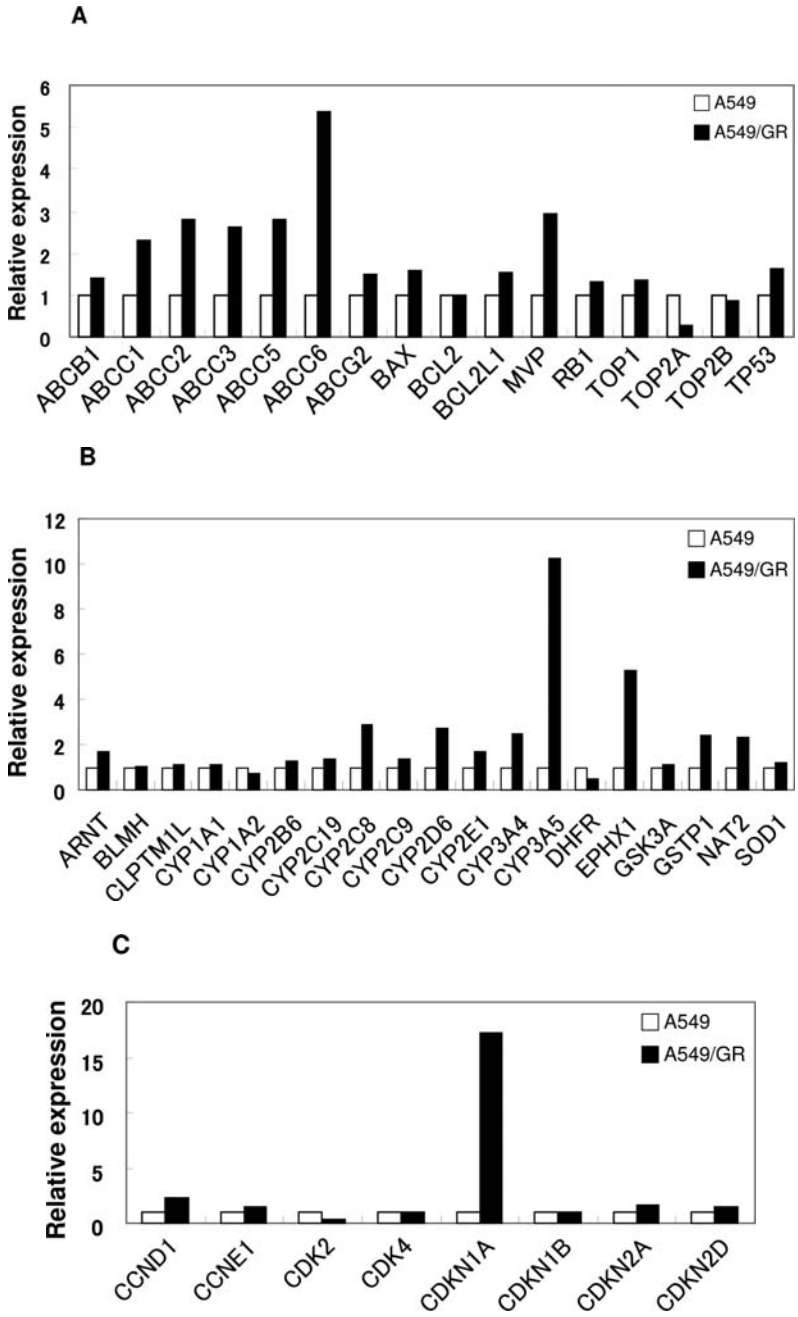

Figure 6. Expression pattern of drug resistance, drug metabolism and cell cycle-related genes in A549 and A549/GR cells. After total RNA was prepared, 1 μg of total RNA was used for cDNA synthesis. The expression of drug resistance (A), drug metabolism (B) and cell cycle (C)-related genes were determined by PCR array as described in Materials and methods. Data are normalized to the expression of each gene in parental A549 cells.

A549 cells by gemcitabine induced the expression of *CDKN1A/p21* and *CYP3A5* in A549 cells (Fig. 7B).

Discussion

Although gemcitabine is a promising treatment for NSCLC, the mechanism of action for gemcitabine, especially the mechanism of acquired drug resistance, is not well-known. In the present study, we sought to clarify the mechanism of gemcitabine-resistance by using A549/GR cells in which gemcitabine resistance was generated by long-term exposure to gemcitabine. To evaluate whether gemcitabine-resistant cells aquired multi-drug resistance, we performed sensitivity testing to paclitaxel, docetaxel, irinotecan and carboplatin in A549 cells and A549/GR cells. A549/GR cells were also resistant to paclitaxel and docetaxel but not irinotecan and carboplatin. A recent report showed that there is a strong correction between NF-κB activation and gemcitabine-, paclitaxel- and docetaxel-resistance in several cancer cell lines (8-10). Curcumin, a yellow coloring agent in turmeric, has been shown to inhibit activation of NF-κB (11). Although we investigated the effect of curcumin on the sensitivity to gemcitabine in A549/GR cells, curcumin did not affect of sensitivity to gemcitabine in A549/GR cells.

It was reported that multidrug resistance protein 7 (MRP7: ABCC10) is an ABC transporter that confers resistance to

gemcitabine (12). To examine the correlation between gemcitabine-resistance and the expression of MRP7, we compared the expression of MRP7 in A549 and A549/GR cells. The expression of *MRP7* was higher than that of in A549 cells. Interestingly, the expression of MRP7 also caused resistance to paclitacel and docetaxel (13,14). Cepharanthine is known as a membrane-interacting agent with membrane-stabilizing activity, and is widely used in Japan for the treatment of snake venom-induced hemolysis, nasal allergy, leukopenia induced by anticancer drugs and radiation therapy (6). We previously reported that cotreatment of cepharanthine with doxorubicin and vincristine resulted in the enhancement of cytotoxicity the anti-cancer drugs and the apoptosis induced in K562 cells (15). Cepharanthine also reversed paclitaxel resistance in MRP7-transfected cells (7). Therefore, we examined the effect of the sensitivity to gemcitabine in A549/GR cells. Cepharanthine reversed gemcitabine resistance in A549/GR cells. Although cepharanthine has been also reported to be an inhibitor of the P-gp efflux pump (16), NSCLC cell lines transfected with *MDR1* gene had augmented sensitivity to gemcitabine (17). These findings suggest that cepharanthine might be effective in restoring the sensitivity of tumors to gemcitabine by inhibiting the drug efflux activity of MRP7 but not P-gp.

To determine the novel molecular maker to gemcitabine resistant non-small cell lung cancer cells, we performed PCR array. As shown in Fig. 6A, the expression of *ABCC6 (MRP6)* in A549/GR cells was more increased than that in A549 cells. Mutations in human ABCC6 (MRP6), a member of the MRP family to drug efflux pumps, are the genetic basis of *Pseudoxanthoma Elasticum*, a disease that affects elastin fibers in the skin, retina and blood vessels (18-21). MRP6-transfected cells also revealed low levels of resistance to anti-cancer agents, including etoposide, teniposide, doxorubicin and daunorubicin (22). These results suggest that MRP6 confer resistance to multi-drug resistance for anti-cancer agents. MRP6 possibly is a molecular marker associated with gemcitabine-resistant in human non-small cell lung cancer.

In genes of drug metabolism, expression of *CYP3A5* in A549/GR cells was more increased than that in A459 cells (Figs. 6B and 7A). As shown in Fig. 7B, the expression of CYP3A5 was induced by gemcitabine in A549 cells. Wang *et al* have reported that full-length cDNA CYP3A5 gene was cloned and established stable cell lines (23). Overexpression of CYP3A5 in the cells induced resistance to anthracyclines and alkaloids (23). Most importantly, CYP3A5 shares approximately 90% sequence identity of its DNA with CYP3A4, and similar substrate specificity makes it difficult to dissect their respective contribution to overall CYP3A-mediated drug metabolism (24). Of importance for drug disposition is that CYP3A and drug transporters are frequently co-expressed in the same cells (or tissues) and share a large number of substrates and modulators (25). Additional study is needed to clarify whether gemusitabine affected the substrate or inhibitors of CYP3A5.

p21 (also called WAF1) is the founding member of the Cip/Kip family of cyclin-dependent kinase inhibitors (CKIs) (26). p21 plays an essential role in growth arrest after DNA damage (27-29) and overexpression leads to G_1 and G_2 or S-phase arrest (30,31). It has been reported that p21 interaction

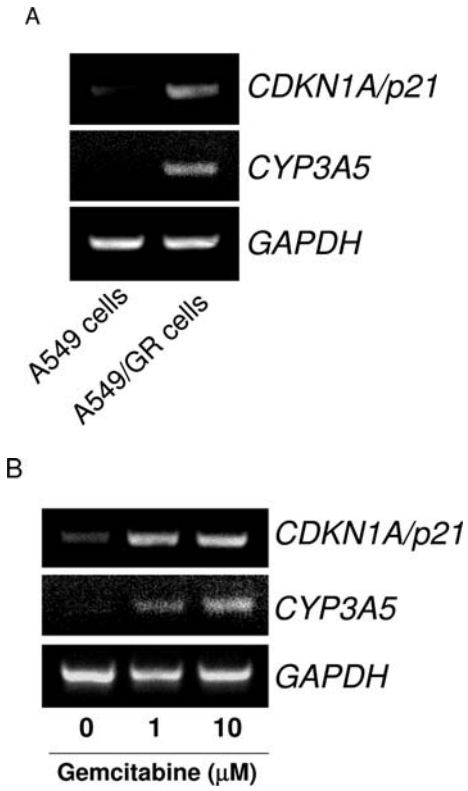

Figure 7. Expression of *CDKN1A/p21* and *CYP3A5* in A549 and A549/GR cells and the effect of gemcitabine on the expression of *CDKN1A/p21* and *CYP3A5* in A549 cells. (A) Total RNAs were isolated and expression of *CDKN1A/p21* and *CYP3A5* in A549 and A549/GR cells was detected by RT-PCR. (B) A549 cells treated with gemcitabine at 1 or 10 μM for 24 h. After extracted with total RNA, expression of *CDKN1A/p21* and *CTP3A5* in A549 cells was detected by RT-PCR. The expression of *GAPDH* was also examined as a loading control.

with procaspase 3 leads to resistance to Fas-mediated cell death, and stabilization of the apoptotic inhibitor protein c-IAP1 (32,33). Overexpression of p21 completely blocked DR4 TRIL receptor cytoplasmic domain (CD)-induced cleavage of caspase-8 and DR4-CD-induced apoptosis, and this activity resides within 91 amino acids of the NH_2 terminus of p21 protein (34). We demonstrated that the expression of *p21* in A549/GR cells was more increased than that in A549 cells, and gemcitabine induced the expression of *p21* in A549 cells (Figs. 6C, 7A and B). These results indicate that the overexpression of p21 might be molecular makers associated with gemcitabine-resistant human non-small cell lung cancers.

The expression of EPHX1 was also more increased than that in A549 cells. EPHX1 metabolizes a broad array epoxide substrates, including polycyclic aromatic hydrocarbons (PAH), carcinogens found in cigarette smoke (35). EPHX1 converts the tobacco combustion product benzo(α)pyrene-derived benzo(α)pyrene 7,8-exoxide to the less toxic trans-dihydrodiol derivative, benzo(α)pyrene 7, 8 diol (36). Further study is needed to elucidate the roles of the overexpression of EPHX1 in A549-gemcitabine resistance cells. A better understanding of the characterization and mechanism of the resistance to gemcitabine in A549/GR cells will help in selection of an the effective chemotherapy and to design treatment to reverse clinical gemcitabine resistance in NSCLC.

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