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 dimmly 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 lowering 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.

Key words: gemcitabine, non-small cell lung cancer, A549 cells

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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% CO₂ 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 (5x10³) were inoculated into each well. After overnight incubation (37°C in 5% CO₂), 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′-gtcttcaccaccatggagaagg-3′, reverse 5′-gg caggtcaggtccaccactga-3′; CYP3A5, forward 5′-ctggccactcaccctgatgtc-3′, reverse 5′-atct atgctgctctctctctttctt-3′; p21, forward 5′-ctcttccaggtccacctgg-3′, reverse 5′-agagttctccaggtccacctgg-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 guide-line 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.

Results

Establishment of gemcitabine-resistant non-small cell lung cancer A549 cells. To isolate gemcitabine-resistant A549/GR cells, 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.
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).

**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-[methyl-
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).

**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 hydrolase 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/GR cells by RT-PCR. Induction of 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...
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 acquired 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 correlation 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 the 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
damage (27-29) and overexpression leads to G1 and G2 or (26). p21 plays an essential role in growth arrest after DNA Cip/Kip family of cyclin-dependent kinase inhibitors (CKIs) inhibitors of CYP3A5.

clarify whether gemusitabine affected the substrate or substrates and modulators (25). Additional study is needed to the same cells (or tissues) and share a large number of CYP3A and drug transporters are frequently co-expressed in their respective contribution to overall CYP3A-mediated drug and similar substrate specificity makes it difficult to dissect ximately 90% sequence identity of its DNA with CYP3A4, and alkaloids (23). Most importantly, CYP3A5 shares appro-

of CYP3A5 in the cells induced resistance to anthracyclines cloned and established stable cell lines (23). Overexpression xet al (Figs. 6B and 7A). As shown in Fig. 7B, the expression of A549/GR cells was more increased than that 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 G1 and G2 or S-phase arrest (30,31). It has been reported that p21 interaction 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 NH2 terminus of p21 protein (34). We demonstrated that the expression of p21 in A549/G 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 benz(o)pyrene-derived benzo(a)pyrene 7,8-exoxide to the less toxic trans-dihydridiol derivative, benzo(a)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/G cells will help in selection of an effective chemotherapy and to design treatment to reverse clinical gemcitabine resistance in NSCLC.
References


