Expression of Dihydrodiol Dehydrogenase and Resistance to Chemotherapy and Radiotherapy in Adenocarcinoma Cells of Lung

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Abstract. *Background: The cytoplasmic enzyme dihydrodiol dehydrogenase (DDH) plays an important role in the detoxification process. Recently, the overexpression of DDH was detected in non-small cell lung cancer (NSCLC) cells and patients with DDH overexpression were shown to have a significantly higher incidence of early tumor recurrence and distant metastasis. In this study, the correlation between DDH expression and resistance to cisplatin, adriamycin and radiotherapy in NSCLC was examined. Materials and Methods: Seven lung adenocarcinoma cell lines (H23, H838, H1437, H1648, H2009, H2087 and H2126) were used in the study. The DDH level was determined by reverse transcription polymerase chain reaction and immunoblotting. Drug- and radiation-mediated cytotoxicity was measured by clonogenic assay. DDH isoforms (DDH1, DDH2 and DDH3) were transfected into H23 cells that did not express DDH to examine their effects on drug and radiation resistance. Results: DDH-overexpressed adenocarcinoma cells exhibited a much higher resistance to doxorubicin, cisplatin and irradiation than cells with lower DDH expression. The DDH2- and DDH1-transfectants showed higher drug and radiation resistance than the DDH3-trasnfectant. Conclusion: Resistance to both anticancer drugs and irradiation in lung adenocarcinoma cells was closely associated with DDH activity. DDH1 and DDH2 were the main isoforms responsible for these effects.*

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Lung cancer is one of the leading causes of cancer death worldwide. In Taiwan, the annual mortality rate represents about 20% of total cancer-related deaths (1). In the United States, the annual death rate from lung cancer is approximately 30%, the toll exceeding the total deaths, due to breast, prostate and colon cancer combined (2). Most patients are diagnosed at the late stage of the disease. However, some patients, who were diagnosed at the early stage and underwent curative resection, still died due to the early recurrence and metastasis of the cancer (3, 4). Even the combination modality with irradiation did not significantly improve patient survival. In addition to metastasis, resistance to chemotherapy and radiotherapy are considered major obstacles to achieving a complete cure for these patients.

Although advances in cancer epidemiology and preventive medicine have reduced the occurrence of most cancers, the incidence of lung cancer, in particular non-small cell lung cancer (NSCLC), remains high (3, 4). Several molecular methods, which can distinguish various gene expression patterns in two or more cell populations, have been used to identify and to characterize specific expression spectra that might reflect the clinical performance of these patients (5-9).

Using differential display, the overexpression of dihydrodiol dehydrogenase (DDH) was detected in both primary NSCLC and lung cancer cell lines and patients with DDH overexpression were demonstrated to have a significantly higher incidence of early tumor recurrence and distant metastasis. DDH overexpression was further shown to correlate with poor prognosis, in particular in patients at later stages (10, 11). Since DDH catabolizes xenobiotic compounds, the possibility of metabolizing anticancer drugs with similar structures was indicated (10, 12, 13). The identification of DDH overexpression in ethacrynic acid-induced drug-resistant colon cancer cells and in daunorubicin-resistant stomach

cancer cells further strengthened such speculation (14, 15). Interestingly, by using cDNA microarray, DDH overexpression was also detected in cisplatin-resistant ovarian cells, and the subsequent results of transfection confirmed the role of DDH in cisplatin resistance (16). However, the resistant mechanisms to daunorubicin and cisplatin are, respectively, associated with DNA topoisomerase IIα and the DNA repair system (17, 18), converging on DDH expression.

In this study, seven adenocarcinoma cell lines were examined with respect to their DDH activity and cytotoxicity following doxorubicin, cisplatin and radiation treatments. Our results indicated that resistance to anticancer drugs and radiation in these lung adenocarcinoma cells was closely associated with DDH activity. Although DDH expression is necessary for cytoprotection, cross-resistance to cisplatin and radiation indicates that other factors associated with DNA repair and caspase-independent apoptosis may also be important.

Materials and Methods

Materials, cell cultures and drug treatment. The culture media and fetal calf serum (FCS) were from Gibco Laboratories (Grand Island, NY, USA). All other materials were reagent grade from Sigma (St. Louis, MO, USA) and Merck (Darmstadt, Germany). The lung adenocarcinoma cells, H23, H838, H1437, H1648, H2009, H2087 and H2126, were purchased from the American Type Culture Collection (Manassas, VA, USA) and were grown as a monolayer in RPMI1640 plus 5% FCS. All the cultures were incubated at 37˚C and all the media were supplemented with 3 mM glutamine, penicillin (100 IU/ml) and streptomycin (100 μg/ml).

RNA extraction and signal amplification using reverse transcription polymerase chain reaction (RT-PCR). The expression of mRNA in the lung cancer cell lines was determined by RT-PCR. Briefly, total RNA was extracted from $1x10^7$ cancer cells using the SNAP RNA column (Invitrogen Corporation, San Diego, CA, USA). Following spectrophotometric determination of the RNA yield, cDNA was synthesized by random primer and AMV reverse transcriptase. An aliquot of cDNA was then subjected to 35 cycles of PCR using a standard procedure of denaturing at 94˚C for 1 min, hybridizing at 52˚C for 30 sec and elongating at 72˚C for 1.2 min with specific primer sets (the primer sequences and the length of the amplified gene fragments are listed in Table I). The amplified products were resolved in agarose gel and visualized by ethidium bromide staining. The specificity of the amplified fragment was determined by DNA sequencing (Perkin-Elmer, Foster City, CA, USA), and was matched with the database of GenBank (http://www.ncbi.nlm.nih.gov/blast).

Gene expression detected by immunocytochemical and immunoblotting methods. An immunoperoxidase procedure was used to detect protein expressions (19). The antibodies used were specific to the antigen [antibodies to β-actin and DDH were from Sigma Chemicals and Cashmere Scientific Company (Taipei, Taiwan), respectively]. The procedure for immunoblotting was described previously (20). Briefly, $5x10^6$ cells were washed with phosphate-buffered saline twice and lysed in loading buffer [50 mM Tris (pH 6.8), 150 mM NaCl, 1 mM disodium EDTA, 10% glycerol, 5% β-mercaptomethanol, 1 mM phenylmethylsulfonylfluoride, 1% SDS and 0.01% bromophenol blue]. Electrophoresis was carried out in 10% polyacrylamide gel with 4.5% stacking gel. After electrophoresis, the proteins were transferred to a nitrocellulose membrane. The membrane was then probed with the specific antibodies. The signal was amplified by biotin-labeled goat anti-mouse IgG and peroxidase-conjugated streptavidin. The protein was visualized by exposing the membrane to an X-Omat film (Eastman Kodak, Rochester, NY, USA) with enhanced chemiluminescent reagent (Pierce, Rockford, IL, USA).

Table I. *Sequences of the primer sets and length of the amplified gene fragment.*

mRNA	Primer sequences	Fragment length
DDH	5'-GTGTGAAGCTGAATGATGGTCA-3' 5'-TCTGATGCGCTGCTCATTGTAGCTC-3'	815 bp
β-actin	5'-TGAAGTACCCCATCGAGCACG-3' 5'-AGTGATCTCCTTCTGCATCCTGT-3'	755 bp

Drug-sensitivity and radiation-sensitivity assay. The drug and radiation-sensitivity were measured by the number of cells killed (21). The cells were seeded at 100, 1,000 and 10,000 cells/6-cm plate 6 h prior to the drug challenge. The cells were treated with various doses of radiation or concentrations of anticancer drugs, such as adriamycin and cisplatin, for 2 h before removing drugs. The negative control groups included cells without radiation or cells treated with the same dilution of DMSO that was used as the drug solvent. The total cell survival was determined 7 to 10 days following drug challenge by crystal violet staining. The percent survival of cells was quantified by comparing with the control group.

Gene cloning into mammalian expression system. To enhance the DDH expression in low-expressing H23 cells, the ecdyson-induced expression system and PerFect Lipid Transfection kit (Invitrogen) were used to promote gene expression. Transfectants containing the *DDH* gene were selected by bleomycin. Expression of the *DDH* gene was monitored by gel electrophoresis to determine the extent and the correct molecular weight of the protein. This expression was verified by immunocytochemistry. The exact composition of recombinant protein, isolated by immunoprecipitation, was determined by partial trypsin digestion and amino acid sequencing.

Results

Correlation of DDH expression with drug and radiation resistance as determined by immunoblotting and RT-PCR in lung adenocarcinoma cells. Among the seven lung adenocarcinoma cell lines screened by immunoblotting, six (H1437, H1648, H2087, H2126, H23 and H838) expressed DDH. The expression of DDH was high in H838, H1437

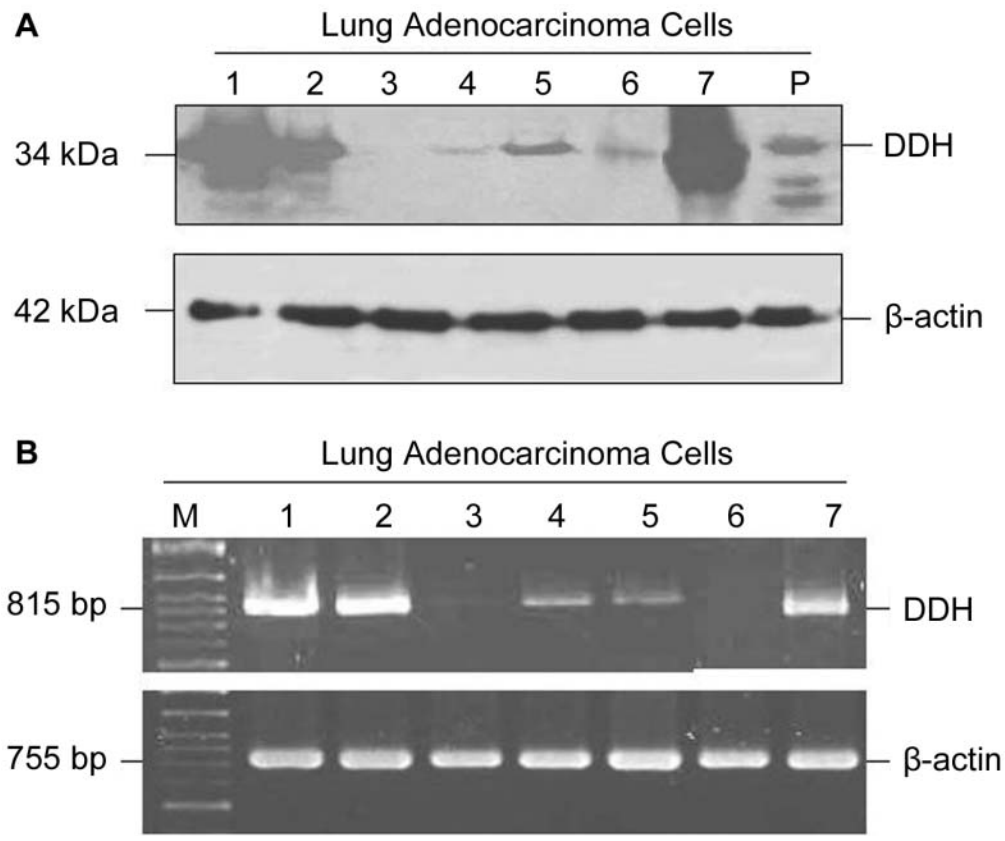

Figure 1. *Dihydrodiol dehydrogenase (DDH) protein and gene expression in lung adenocarcinoma cell lines. (A) The expression of DDH protein was determined by immunoblotting with specific monoclonal antibodies. (B) DDH gene expression was determined by reverse transcription polymerase chain reaction. Lane 1: H1437; lane 2: H1648; lane 3: H2009; lane 4: H2087; lane 5: H2126; lane 6: H23; and lane 7: H838. P: positive control (column-purified DDH fraction from pig liver), M: marker.*

and H1648 and weak in H2126, H2087 and H23. DDH was not detected in the H2009 cells (Figure 1A). The results were further confirmed by RT-PCR (Figure 1 B). The specificity of the amplified fragment was determined by DNA sequencing and the DNA sequence was matched to the particular DDH listed in the database of GenBank (http://www.ncbi.nlm.nih.gov/). Interestingly, resistance to adriamycin (H838>H1437=H1648>H2126>H2087=H23), cisplatin (H838=H1437=H1648>H2126>H23>H2087) and irradiation (H838>H1437>H1648=H2126>H2087=H23) corresponded well to the extent of DDH expression in these cells (Figure 2). Because the H2009 cells did not form a discernible colony, no clonogenic result could be obtained.

Effect of different DDH isoforms on drug and radiation resistance. To determine whether a specific DDH isoform mediated the development of drug and radiation resistance in lung adenocarcinoma cells, full-length cDNAs of DDH1, DDH2 and DDH3 were inserted into an ecdyson-induced expression system to promote DDH expression in H23 cells. Since DDH4 was not detected in the lung cancer cells, it was not cloned into the expression system. The transfectant selected by bleomycin was further monitored by gel electrophoresis for the extent and molecular weight of the expressed protein. Among various mixtures of cationic lipid and L-dioleoyl phosphatidylethanolamine (DOPE), pFx-3 rendered the best transfection results (Figure 3 A), so pFx-3 treated cells were used in the following experiments. DDH expression in the transfectant was further verified by immunocytochemistry (Figure 3 B). The exact composition of recombinant protein was isolated by immunoprecipitation and was determined by partial trypsin digestion and amino acid sequencing (data not shown). Although both DDH1- and DDH2-transfectants were more resistant than the parental H23 cells to adriamycin and radiation, no significant differences in drug resistance was found between the parental H23 and DDH3-transfected cells. The order of resistance was determined as DDH2->DDH1->DDH3-transfectant (Figure 3 C).

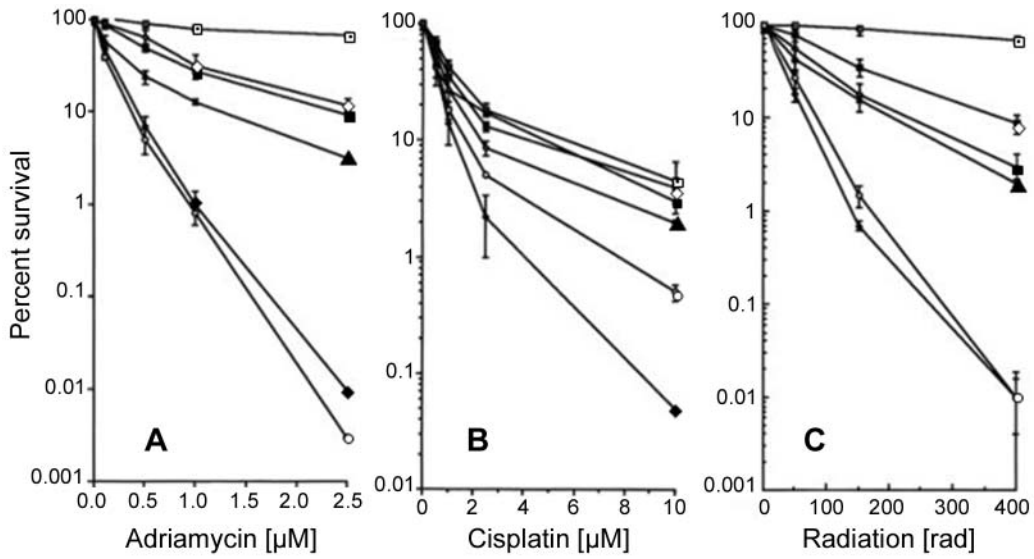

Figure 2. *Sensitivity of lung adenocarcinoma cells to adriamycin, cisplatin and ionizing radiation. The percent survival is plotted against dose of drug (in μM) or γ-ray (in rad). The survival curves are the mean of triplicate experiments. The cell lines that expressed more DDH were more resistant to the drugs and irradiation. H838 showed the strongest expression of DDH and was most resistant to cisplatin, adriamycin and irradiation. H23 showed almost no DDH expression and was least resistant to adriamycin and irradiation. ⊡: H838, ◇: H1437, ■: H1648, ◆: H2087, ▲: H2126, ○: H23.*

Discussion

In our previous work, we showed that NSCLC patients with DDH overexpression had a significantly higher incidence of early tumor recurrence and distant metastasis. In patients at later stages of the disease, DDH overexpression was even correlated with poor prognosis (10, 11). In the current study, DDH expression was demonstrated in lung adenocarcinoma cells. The cells with higher expression of DDH were more resistant to cisplatin, adriamycin and radiation. After transfection of the *DDH* gene into cells without DDH expression, increased resistance to cisplatin, adriamycin and radiation was demonstrated. The expression of DDH after transfection was confirmed by immunocytochemistry. Resistance to drug treatment and radiation was mainly related to DDH2 and DDH1.

DDH is an aldo-keto reductase that generally catabolizes xenobiotic compounds, such as polycyclic aromatic hydrocarbons (PAH). DDH activity could be detected in human liver, but was barely detectable in human lung (22, 23). DDH converts PAH into catechol in the human liver (12, 24). Further oxygenation of catechol into PAH o-quinone can be detoxified by conjugation with glutathione, sulfate or glucuronide (13, 25, 26). The ability of DDH to generate reactive oxygen species (ROS) during the oxidation of PAH *trans*-dihydrodiols may have important implications for tumor initiation and promotion (27, 28). In humans, at least four isoforms, DDH1 to DDH4, have been identified (22). DDH2 overexpression was more frequently detected in esophageal squamous cell carcinoma (29), while DDH1 overexpression was more frequent in lung cancer (10, 30). DDH1 and DDH2 both exhibit PGF synthase activity, which converts PGD2 into 9α, 11β-PGF_2 (31). This suggests a possible role of PG in the regulation of cell proliferation and tumorigenesis.

Our gene transfer results clearly indicated that DDH expression is required for increased cytoresistance to adriamycin, cisplatin and radiation. However, the mechanisms of this effect are not well known. The forced overexpression of DDH induced high levels of cisplatin resistance in DDH-transfected human ovarian carcinoma cells (16). A review of the literature on DDH and cisplatin resistance suggests the involvement of free radical detoxification in this process (32). Cisplatin is an alkylating agent, the cytotoxic effect of which *via* binding to DNA and induction of DNA strand breaks. Cisplatin has also been shown to increase the generation of ROS in tumor cells leading to an up-regulation of the apoptotic process (33). An increase in the activity of DDH in NSCLC cells lines might be sufficient to repair the biochemical changes induced by cisplatin (due to the generation of free radicals), thus leading to the development of drug resistance.

A literature review of DDH and adriamycin resistance suggests that conversion of daunorubicin to daunorubicinol is a significant step in detoxification, since the products of anthracycline carbonyl reduction, the 13-hydroxy derivatives, are significantly less cytotoxic. Up-regulation of

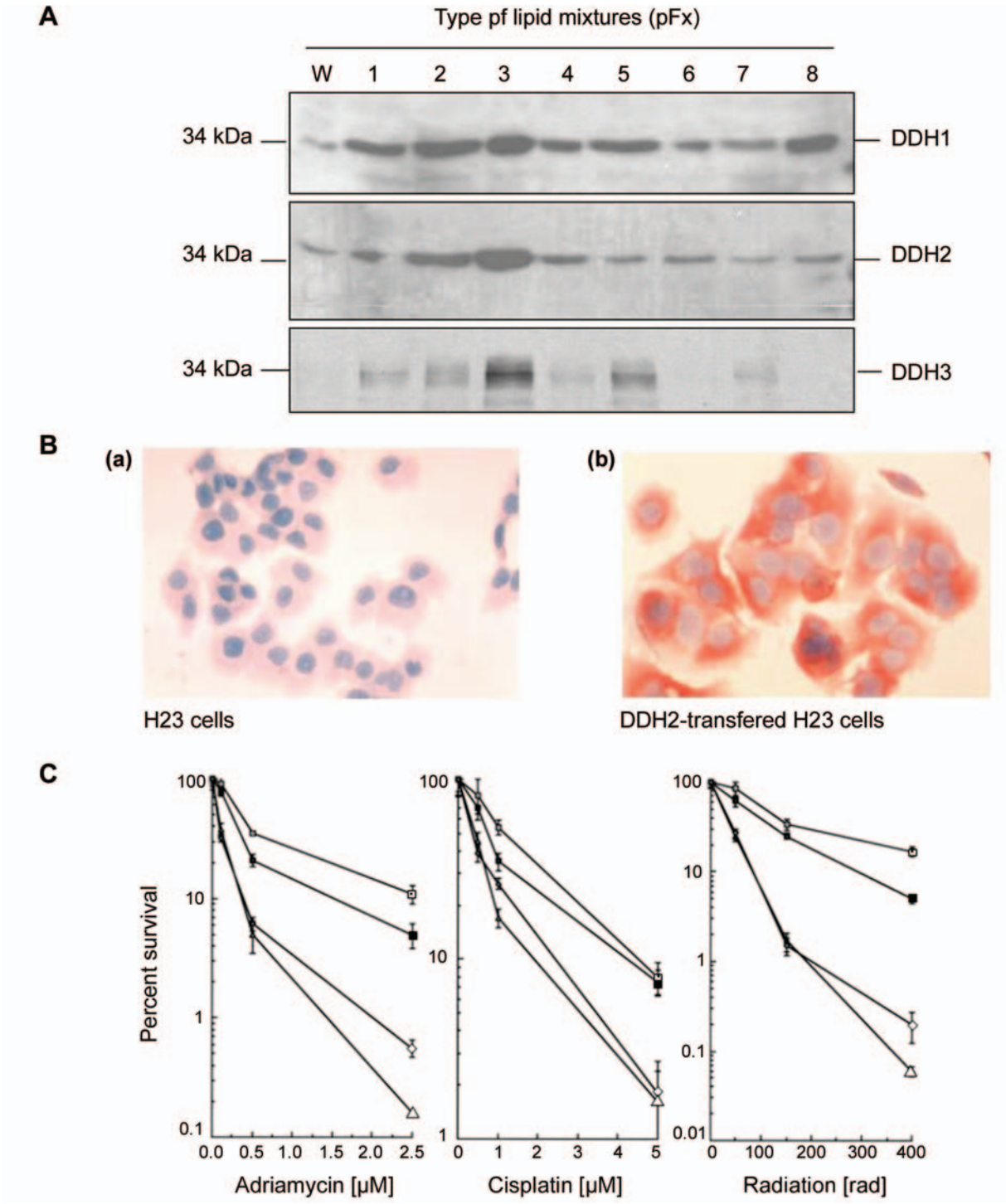

Figure 3. *Effect of different isoforms of DDH on drug- and radiation-resistance. (A) Full-length cDNA of DDH1, DDH2 or DDH3 was cloned into an ecdyson-induced expression system. The plasmid containing full-length DDH cDNA was transfected into H23 cells using various mixtures of cationic lipid and L-dioleoyl phosphatidylethanolamine (DOPE) as the delivering vehicle to promote DDH expression. The transfectant, which was selected by bleomycin, was monitored by immunoblotting to determine the extent and the correct molecular weight of the expressed protein. The lipid mixture pFx-3 rendered the best transfection results for DDH1, DDH2 and DDH3. (B) DDH expression in transfectant was verified by immunocytochemistry. Panel a: H23, panel b: DDH2-transfected H23 cells. (C) Sensitivity of H23 and DDH-transfectants to adriamycin, cisplatin and ionizing radiation. Percent survival is plotted against dose of drug (in μM) or γ-ray (in rad). Survival curves are the mean of triplicate experiments. Both DDH1- and DDH2-transfectants were more resistant than the parental H23 cells. No significant difference of cytotoxicity was found between parental H23 and DDH3-transfected cells. ■: DDH1-transfected H23 cells, ⊡: DDH2-transfected H23 cells, ◇: DDH3-transfected H23 cells, △: untransfected H23 cells.*

a carbonyl reductase has been reported to induce the development of doxorubicin resistance in tumor cells (15). The examination of specific carbonyl reducing enzymes revealed that the increased mRNA expression of carbonyl reductase, aldose reductase and DDH2 were involved in daunorubicin detoxification (15).

In conclusion, our results demonstrated that, in lung adenocarcinoma cells, both drug and radiation resistance was closely associated with DDH expression, in particular with DDH1 and DDH2. DDH expression can be directly or indirectly associated with drug inactivation as well as mechanisms of DNA repair and, possibly, apoptosis. These associations provide an interesting target for elucidating the mechanism of cytoprotection.

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References

1 Annual Reports of the Department of Health, the Executive Yuan, Republic of China, 2000.

2 Schiller JH, Harrington D, Belani CP *et al*: The Eastern Cooperative Oncology Group. Comparison of four chemotherapy regimens for advanced non-small-cell lung cancer. N Engl J Med *346*: 92-98, 2002.

3 Pairolero PC, Williams DE, Bergstralh EJ *et al*: Postsurgical stage I bronchogenic carcinoma: morbid implications of recurrent disease. Ann Thorac Surg *38*: 331-336, 1984.

4 Harpole DH Jr, Herndon JE II, Young WG Jr *et al*: Stage I non small cell lung cancer. A multivariate analysis of treatment methods and patterns of recurrence. Cancer *76*: 787-796, 1995.

5 Wu CG, Hakvoort TB, Lamers WH *et al*: Isolation of up- and down-regulated cDNAs associated with hepatocellular carcinoma by a subtraction-enhanced display technique. Biochim Biophys Acta *1315*: 169-175, 1996.

6 Velculescu VE, Zhang L, Vogelstein B *et al*: Serial analysis of gene expression. Science *270*: 484-487, 1995.

7 Maeda S, Otsuka M, Hirata Y *et al*: cDNA microarray analysis of *Helicobacter pylori*-mediated alteration of gene expression in gastric cancer cells. Biochem Biophys Res Commun *284*: 443-449, 2001.

8 Paweletz CP, Charboneau L, Bichsel VE *et al*: Reverse phase protein microarrays which capture disease progression show activation of pro-survival pathways at the cancer invasion front. Oncogene *20*: 1981-1989, 2001.

9 Liang P, Averboukh L and Pardee AB: Distribution and cloning of eukaryotic mRNAs by means of differential display: refinements and optimization. Nucleic Acids Res *21*: 3269-3275, 1993.

10 Hsu NY, Ho HC, Chow KC *et al*: Overexpression of dihydrodiol dehydrogenase as a prognostic marker of non-small cell lung cancer. Cancer Res *61*: 2727-2731, 2001.

11 Chen CY, Hsu CP, Hsu NY *et al*: Expression of dihydrodiol dehydrogenase in the resected stage I non-small cell lung cancer. Oncol Rep *9*: 515-519, 2002.

12 Shou M, Harvey RG and Penning TM: Contribution of dihydrodiol dehydrogenase to the metabolism of (±)-*trans*-7,8-dihydroxy-7,8-dihydrobenzo[a]pyrene in fortified rat liver subcellular fractions. Carcinogenesis *13*: 1575-1582, 1992.

13 Flowers-Geary L, Harvey RG and Penning TM: Cytotoxicity of polycyclic aromatic hydrocarbon o-quinones in rat and human hepatoma cells. Chem Res Toxicol *6*: 252-260, 1993.

14 Shen H, Kauvar L and Tew KD: Importance of glutathione and associated enzymes in drug response. Oncol Res *9*: 295-302, 1997.

15 Ax W, Soldan M, Koch L *et al*: Development of daunorubicin resistance in tumour cells by induction of carbonyl reduction. Biochem Pharmacol *59*: 293-300, 2000.

16 Deng HB, Parekh HK, Chow KC *et al*: Increased expression of dihydrodiol dehydrogenase induces resistance to cisplatin in human ovarian carcinoma cells. J Biol Chem *277*: 15035-15043, 2002.

17 Chow KC, MacDonald TL and Ross WE: DNA binding by epipodophyllotoxins and N-acyl anthracyclines: implications for mechanism of topoisomerae II inhibition. Mol Pharmacol *34*: 467-473, 1988.

18 Hill BT, Shellard SA, Hosking LK *et al*: Enhanced DNA repair and tolerance of DNA damage associated with resistance to *cis*-diammine-dichloroplatinum (II) after *in vitro* exposure of a human teratoma cell line to fractionated X-irradiation. Int J Radiat Oncol Biol Phys *19*: 75-83, 1990.

19 Hsieh CC, Chow KC, Fahn HJ *et al*: Prognostic significance of HER-2/neu overexpression in stage I adenocarcinoma of lung. Ann Thorac Surg *66*: 1159-1163, 1998.

20 Chow KC and Ross WE: Topoisomerase specific drug sensitivity in relation to cell cycle progression. Mol Cell Biol *7*: 3119-3123, 1987.

21 Chow KC, King CK and Ross WE: Abrogation of etoposide-mediated cytotoxicity by cycloheximide. Biochem Pharmacol *37*: 1117-1122, 1988.

22 Hara A, Taniguchi H, Nakayama T *et al*: Purification and properties of multiple forms of dihydrodiol dehydrogenase from human liver. J Biochem (Tokyo) *108*: 250-254, 1990.

23 Penning TM and Sharp RB: Characterization of dihydrodiol dehydrogenase in human liver and lung. Carcinogenesis *11*: 1203-1208, 1990.

24 Vogel K, Bentley P, Platt KL *et al*: Rat liver cytoplasmic dihydrodiol dehydrogenase. J Biol Chem *255*: 9621-9625, 1980.

25 Glatt HR, Vogel K, Bentley P *et al*: Reduction of benzo(*a*)-pyrene mutagenicity by dihydrodiol dehydrogenase. Nature *277*: 319-320, 1979.

26 Penning TM: Dihydrodiol dehydrogenase and its role in polycyclic aromatic hydrocarbon metabolism. Chem-Biol Interact *89*: 1-34, 1993.

27 Penning TM, Ohnishi T, Ohnishi T *et al*: Generation of reactive oxygen species during the enzymatic oxidation of polycyclic aromatic hydrocarbon *trans*-dihydrodiols catalyzed by dihydrodiol dehydrogenase. Chem Res Toxicol *9(1)*: 84-92, 1996.

28 Seike K, Murata M, Hirakawa K *et al*: Oxidative DNA damage induced by benz[α]anthracene dihydrodiols in the presence of dihydrodiol dehydrogenase. Chem Res Toxicol *17*: 1445-1451, 2004.
29 Wang LS, Chow KC, Wu YC *et al*: Inverse expression of dihydrodiol dehydrogenase and glutathione-s-transferase in patients with esophageal squamous cell carcinoma. Int J Cancer *111*: 246-251, 2004.
30 Deng HB, Adikari M, Parekh HK *et al*: Ubiquitous induction of resistance to platinum drugs in human ovarian, cervical, germ-cell and lung carcinoma tumor cells overexpressing isoforms 1 and 2 of dihydrodiol dehydrogenase. Cancer Chemother Pharmacol *54*: 301-307, 2004.
31 Suzuki T, Fujii Y, Miyano M *et al*: cDNA cloning, expression, and mutagenesis study of liver-type prostaglandin F synthase. J Biol Chem *274*: 241-248, 1999.
32 Gebauer G, Mirakhur B, Nguyen Q *et al*: Cisplatin-resistance involves the defective processing of MEKK1 in human ovarian adenocarcinoma 2008/C13 cells. Int J Oncol *16*: 321-325, 2000.
33 Cuello M, Ettenberg SA, Nau MM *et al*: Synergistic induction of apoptosis by the combination of TRAIL and chemotherapy in chemoresistant ovarian cancer cells. Gynecol Oncol *81*: 380-390, 2001.

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