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Biochemical Characterization of Adriamycin-Resistance in PC-14 Human Lung Adenocarcinoma Cell Line

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To investigate the mechanism of adriamycin (ADM) resistance in the ADM resistant subline PC-14/ADM, we examined the expressions of p-glycoprotein (P-gp), topoisomerase I (Topo I) and II (Topo II), glutathione-S-transferases (GSTs), tissue transglutaminase (t-TG), epidermal growth factor receptor (EGFR), and E-cadherin and the activity of superoxide dismutase (SOD) in PC-14 and PC-14/ADM cells. There was no change in the cellular levels of P-gp, Topo I, Topo II, and the two isoforms of GSTs. However, SOD activity in PC-14/ADM cells was 2.38 fold higher than that in PC-14 cells. A marked induction of the t-TG expression was also observed in PC-14/ADM cells. In addition to those changes, expressions of EGFR and E-cadherin were down regulated in PC-14/ADM cells. Therefore, molecular modifications such as an increase in SOD activity, induction of the t-TG expression, and down regulation of EGFR and E-cadherin expressions may play important roles in PC-14/ADM cells during the development of ADM resistance.

Keywords: Adriamycin, Drug resistance, Epidermal growth factor receptor, PC-14 cell line, Tissue transglutaminase.

Introduction

Development of drug resistance is one of the major barriers in cancer chemotherapy (Moscow and Cowan, 1988). Clinically, its development has usually been associated with a short duration of response and little responsiveness to the subsequent chemotherapy. Mechanisms by which cells become resistant to drugs are also complex and a number of specific mechanisms have been elucidated (Ling, 1982; Beck, 1987; Myers *et al.*, 1987; Mehta, 1994; St. Croix and Kerbel, 1997; Kang *et al.*, 1997). For effective chemotherapy treatment, it is important to investigate the mechanism of drug resistance and to design methods to overcome it based on resistance mechanisms.

ADM, an anthracycline antitumor agent, is clinically active against a variety of human malignancies. Several mechanisms have been proposed to explain the antitumor activity of ADM, including DNA damage mediated by Topo II (Tewey *et al.*, 1984), interaction with membranes (Tritton, 1991), and generation of oxygen free radicals (Sinha *et al.*, 1989). The emergence of resistance to ADM severely limits the use of this agent for the successful treatment of cancer and is mostly associated with multidrug resistance (MDR). Although mechanisms of ADM resistance are not completely understood, they are known to be multifactorial: (1) They reduce the accumulation of ADM in cytoplasm and/or nucleus by the overexpression of P-gp (Chan *et al.*, 2000; Guo-chang and Chu-tse, 2000). (2) They reduce the Topo II activity or expression (De Jong *et al.*, 1990; McPherson *et al.*, 1993; Son *et al.*, 1998). (3) They enhance the activation of the intracellular detoxification system by induction of antioxidant enzymes such as catalase, GST, and SOD (Anuszewska *et al.*, 1997; Kobayashi *et al.*, 1997; Guo *et al.*, 1998). In addition, it has been reported that ADM resistant cells showed cell cycle arrest (OLounghlin *et al.*, 2000) and expressed a high level of t-TG (Mehta, 1994).

In an attempt to understand the development of acquired ADM resistance in human lung adenocarcinoma, we examined the involvement of P-gp, Topo I, Topo II, GSTs, SOD, t-TG, EGFR, and E-cadherin in PC-14 cell line and its ADM resistant subline, PC-14/ADM.

Materials and Methods

Cell culture PC-14 human lung adenocarcinoma cell line and MKN-45 human stomach adenocarcinoma cell line were kindly provided by Dr. N. Saijo (National Cancer Center Research Institute, Tokyo, Japan). SW620 human colon adenocarcinoma cell line and its ADM resistant subline AD300 were kindly

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provided by Dr. Y. G. Kang (Asan Medical Center, Seoul, Korea). Cells were propagated in a RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C in a balanced air humidified incubator with an atmosphere of 5% CO_2.

Establishment of an ADM resistant subline from PC-14 cell line An ADM resistant subline was developed by continuous exposure of PC-14 cell line to ADM (Sigma Chemical Co, St Louis, USA) starting at 0.01 μg/ml and increasing in a stepwise manner to 0.6 μg/ml. The resistant subline, which was maintained in the medium containing ADM for more than 3 months, was considered established. The ADM resistant subline is referred to as PC-14/ADM hereafter. PC-14/ADM cells were challenged monthly with ADM at 0.6 μg/ml. Experiments with this subline were performed after maintenance in an ADM-free medium for 2-3 wks.

Antibodies The monoclonal antibody against P-gp was purchased from Centocor (Malvem, USA). The polyclonal antibodies against Topo I and Topo II were purchased from TopoGEN Inc. (Columbus, USA). The polyclonal antibodies against GSTπ and catalase were purchased from UBI (Lake Placid, USA) and Biodesign (Kennebunk, USA), respectively. The antisera against GST-L and t-TG were kindly donated by Dr. S. C. Park (Seoul National University, Seoul, Korea). The anti-human EGFR and E-cadherin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, USA) and TaKaRa Shuzo Co. (Shiga, Japan), respectively.

Microculture tetrazolium (MTT) assay The assay method was essentially the same as previously reported (Mosmann, 1983). Cells were seeded at a density of 5×10^3 cells in 96 well plates, which give an optical density value in the range of 0.8 to 1.2 at 540 nm. Cells were treated with ADM (0.01, 0.03, 0.1, 0.3, 1 μg/ml), cisplatin (CDDP; 0.1, 0.3, 1, 3, 10, 30 μg/ml), carboplatin (CBDCA; 1, 3, 10, 30, 100, 300 μg/ml), pirarubicin (THP; 0.01, 0.03, 0.1, 0.3, 1, 3 μg/ml), mitomycin-C (MMC; 0.01, 0.03, 0.1, 0.3, 1, 3 μg/ml), 5-fluorouracil (5-FU; 0.03, 0.1, 0.3, 1, 3 μg/ml), and etoposide (VP-16; 1, 3, 10, 30, 100, 300 μg/ml) and incubated for 4 d in a humidified CO_2 incubator. Each experiment was performed in triplicate. IC_{50}s were defined as 50% reduction in optical density in each test.

Fractionation of cellular proteins For the preparation of cytosolic proteins, 2×10^7 cells were washed 3 times with a phosphate buffered saline (PBS), disrupted by repeated freeze/thaw in a hypotonic solution (10 mM KCl, 10 mM Tris-HCl pH 7.4, and 1.5 mM $MgCl_2$) supplemented with 2 mM phenylmethyl-sulfonylfluoride (PMSF; Boehringer Mannheim, Germany), and centrifuged at 15,000 rpm for 1 h at 4°C. The supernatant was saved for the immunoblot analysis of GSTπ, GST-L, t-TG, and catalase, and the measurement of SOD activity. For the preparation of membrane associated proteins, the pellet was extracted with the immunoprecipitation buffer (1% Triton X-100, 0.5% sodium deoxycholate, 0.2% sodium dodecylsulfate (SDS), 0.15 M NaCl, and 50 mM Tris-HCl pH 7.4) containing 2 mM PMSF and centrifuged at 15,000 rpm for 1 h at 4°C. The supernatant was saved for the immunoblot analysis of P-gp, E-cadherin, and EGFR. The nuclear preparation was made by lysis in a NP-40 lysis buffer (10 mM Tris-HCl pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, and 0.5% NP-40) on ice for 10 min. Nuclei were extracted by sonication in the NP-40 lysis buffer supplemented with 1% SDS and incubated at 65°C for 5 min. Nuclear proteins were utilized for the immunoblot analysis of Topo I and Topo II. The protein concentration was measured by the bicinchoninic acid (BCA) method.

Cell labeling and immunoprecipitation of P-gp For the analysis of P-gp, SW620, AD300, PC-14, and PC-14/ADM cells were labeled with 50 μCi/ml of [^{35}S] methionine for 16 h. The membrane proteins (1 mg) were extracted with the immunoprecipitation buffer and incubated with the antibody to P-gp at 4°C for 2 h. Immunocomplexes were collected with protein A sepharose-4B (Sigma Chemical Company, St. Louis, USA) at 4°C for 1 h. After resolution of the proteins by 8% SDS-polyacrylamide gel electrophoresis (PAGE), the biosynthetically labeled P-gp was examined by fluorography. SW620 and its ADM resistant subline, AD300, were used as a positive control for P-gp immunoprecipitation.

Immunoblot analysis 30~50 μg of cell lysates were used for the immunoblot analysis. Samples were boiled in a Laemmli sample buffer containing 10% β-mercaptoethanol, separated on SDS-PAGE gels, and transferred to nitrocellulose membranes. Membranes were blocked with 5% FBS in PBST (PBS containing 0.1% Tween 20) at room temperature for 1 h. The blots were incubated for 2 h with a primary antibody, washed for 30 min with three exchanges of PBST, incubated for 1 h with a secondary anti-mouse or anti-rabbit antibody conjugated with alkaline phosphatase (AP), and then washed for an additional 30 min with three exchanges of PBST. The P-nitroblue tetrazolium chloride (NBT; Biorad Laboratory, Richmond, USA) and 5-bromo-4-chloro-3-indoylphosphate p-toluidine salt (BCIP; Biorad Laboratory, Richmond, USA) were used as color developing reagents. The procedure followed the manufacturer's specifications.

Measurement of SOD activity The SOD activity in the cytosolic fraction was measured by the previously reported method (Paoletti and Mocali, 1990). The absorbance at 340 nm was read by a Hewlett Packard 8452 diode array spectrophotometer. One unit of SOD activity is defined as the amount of enzyme required to inhibit the rate of NAD(P)H oxidation of the control by 50% based on the calculation; sample rate/control rate × 100 = % inhibition. The measurement was performed in duplicate.

Results

Sensitivity to various chemotherapeutic agents To investigate the resistance to various chemotherapeutic agents in PC-14 and PC-14/ADM cells, cells were treated with ADM, CDDP, CBDCA, THP, MMC, 5-FU, and VP-16 and IC_{50}s were evaluated (Table 1). PC-14/ADM subline was approximately 8.40-fold more resistant to ADM than its

Table 1. IC_{50}s and relative resistance to various chemotherapeutic agents in PC-14 cell line and PC-14/ADM subline

Drugs	IC_{50} (μg/ml)[a]		RR[b]
	PC-14	PC-14/ADM	
ADM	0.081±0.008	0.680±0.016	8.40
CDDP	0.403±0.068	1.533±0.047	3.80
CBDCA	4.900±1.098	16.900±1.143	3.45
THP	0.014±0.003	0.135±0.022	9.64
MMC	0.038±0.012	0.197±0.030	5.13
5-FU	0.593±0.151	1.416±0.023	2.38
VP-16	9.866±1.239	29.500±4.262	2.99

[a]IC_{50} values were evaluated by MTT assay and results were presented as means±S.D. of three independent experiments.
[b]Relative resistance: IC_{50} of PC-14/ADM/ IC_{50} of PC-14

parent cell line, 3.80-fold more resistant to CDDP, 3.45-fold more resistant to CBDCA, 9.64-fold more resistant to THP, 5.13-fold more resistant to MMC, 2.38-fold more resistant to 5-FU, and 2.99-fold more resistant to VP-16 based on the MTT assay. Therefore, PC-14/ADM subline was not only resistant to ADM, but also other chemotherapeutic agents when it was compared to PC-14 cell line.

No induction of P-gp, Topo I, and Topo II in PC-14/ADM subline Since P-gp is known to be implicated in the multidrug resistance of various types of cancer cells, it was determined by the immunoprecipitation method whether or not the overexpression of P-gp is accompanied with the development of ADM resistance in PC-14/ADM cells. The specificity of the P-gp antibody was confirmed by the immunoprecipitation of P-gp in [^{35}S] methionine labeled detergent extracts of SW620 cell line and its ADM resistant subline, AD300, whose P-gp overexpression was previously

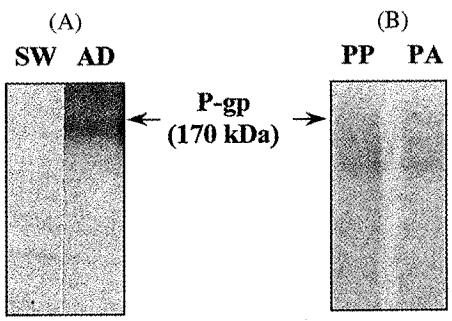


Fig. 1. Expression of P-gp in PC-14 and PC-14/ADM cells. (A) Immunoprecipitation of P-gp in the detergent soluble fractions of [^{35}S] methionine labeled SW620 human colon adenocarcinoma cells (SW) and AD300, its ADM resistant subline (AD). (B) Immunoprecipitation of P-gp in the detergent soluble fractions of [^{35}S] methionine labeled PC-14 (PP) and PC-14/ADM (PA) cells. Estimated molecular mass of P-gp was denoted in kDa.

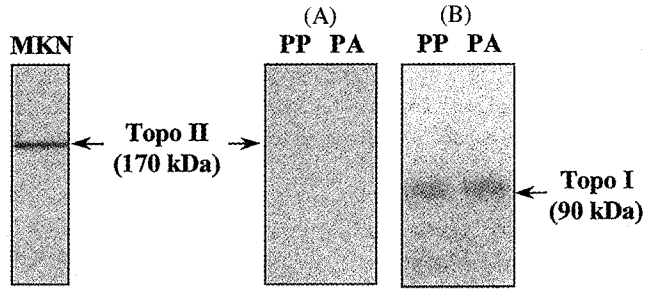


Fig. 2. Immunoblot analysis of Topo I and Topo II in PC-14 and PC-14/ADM cells. The nuclear fractions of PC-14 (PP) and PC-14/ADM (PA) cells were immunoblotted with antibodies to Topo I (A) and Topo II (B). Nuclear fractions of MKN-45 cells were used as a positive control in detection of Topo II. Estimated molecular masses of Topo I and Topo II were denoted in kDa.

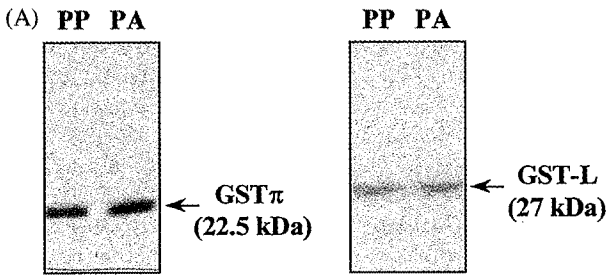
reported (Bates *et al.*, 1993; Barnes *et al.*, 1996; Komatsubara *et al.*, 1999) (Fig. 1A). Under the same condition, PC-14 and PC-14/ADM cells showed a very faint 170 kDa band (Fig. 1B). Thus, P-gp is not considered to be involved in the development of ADM resistance in PC-14/ADM subline.

Topo II, which is an intracellular target of ADM, and whose expression is also related to the cell cycle, was examined in PC-14 and PC-14/ADM cells (Fig. 2A). The specificity of the Topo II antibody was confirmed by the immunoblot analysis of Topo II in nuclear extracts of MKN-45, whose Topo II expression was previously reported (Son *et al.*, 1998). Topo II expressions were not detected in both PC-14 and PC-14/ADM cells. Additionally, Topo I expressions were similar in both cells (Fig. 2B). Therefore, the ADM resistance in PC-14/ADM cells is not caused by the induction of Topo I and Topo II.

Enhanced SOD activity in PC-14/ADM subline To investigate if expressions of GSTs are increased during the development of ADM resistance in PC-14/ADM subline, expressions of GSTπ and GST-L were examined by an immunoblot analysis (Fig. 3A). PC-14 and PC-14/ADM cells expressed similar levels of GSTπ and GST-L. We also observed the expression of catalase, but no difference in this protein was detected between PC-14 and PC-14/ADM cells (data not shown).

The activity of SOD in the cytosolic fraction was measured by the method based on NAD(P)H oxidation (Fig. 3B). PC-14 cells showed 1.19 unit/100 μg cytosolic proteins, but PC-14/ADM cells showed 2.83 unit/100 μg cytosolic proteins. This corresponds to a 2.38 fold more induction than its parent line. These results suggest that in PC-14 lung adenocarcinoma cell line, SOD activity is increased in the acquisition of ADM resistance.

Expressions of drug resistance related proteins In order to investigate whether or not expressions of other drug resistance related proteins may be induced during the development of ADM resistance in PC-14/ADM subline,



(B)

Cell strain	SOD activity (unit/100 μg cytosolic proteins)	Relative ratio[a]
PC-14	1.19	1.00
PC-14/ADM	2.83	2.38

[a]Relative ratio: SOD activites of PC-14/ADM/PC-14

Fig. 3. Expressions of GSTπ and GST-L and SOD activity in PC-14 and PC-14/ADM cells. (A) Immunoblot analysis of GSTπ and GST-L in the cytosolic fractions of PC-14 (PP) and PC-14/ADM (PA) cells. Estimated molecular masses of GSTπ and GST-L were denoted in kDa. (B) SOD activity in the cytosolic fractions of PC-14 and PC-14/ADM cells. One unit of SOD defines the amount of enzyme required to inhibit the rate of NAD(P)H oxidation of the control by 50%. The SOD activity was presented as means of two independent experiments.

expressions of t-TG, EGFR, and E-cadherin were examined by immunoblot analysis (Fig. 4). Expression of t-TG was markedly induced in PC-14/ADM cells compared to its parental line (Fig. 4A). However, expressions of EGFR and E-cadherin were much lower in PC-14/ADM cells than in its parental cells (Fig. 4B and 4C). These results suggest that induction of the t-TG expression and down regulation of EGFR and E-cadherin expressions contribute to the development of ADM resistance in PC-14/ADM subline.

Discussion

Tumor cells may acquire drug resistance by a variety of biochemical mechanisms that act on the intracellular drug concentration, bioactivation/inactivation steps, and cell growth/cell death program. Our biochemical characterization of the ADM resistant subline showed an increase in SOD activity, overexpression of t-TG, and down regulation of EGFR and E-cadherin.

In this work, we used an ADM resistant subline, PC-14/ADM, from a human lung adenocarcinoma cell line. PC-14/ADM cells were also cross-resistant to other chemotherapeutic agents although the relative levels of resistance were different (Table 1). This MDR of PC-14/ADM cells was unexplained by the overexpression of P-gp, because the expression of P-gp was similar in both PC-14 and PC-14/ADM cells (Fig. 1).

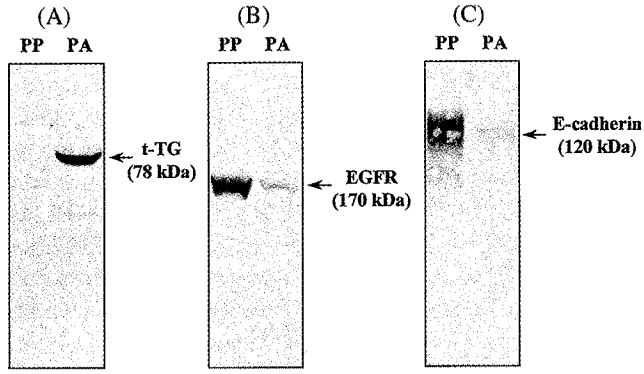


Fig. 4. Immunoblot analysis of t-TG, EGFR, and E-cadherin in PC-14 and PC-14/ADM cells. The cytosolic fractions for detection of t-TG (A) and the membrane fractions for EGFR (B) and E-cadherin (C) of PC-14 (PP) and PC-14/ADM (PA) cells were immunoblotted with appropriate antibodies. Estimated molecular masses of t-TG, EGFR, and E-cadherin were denoted in kDa.

The decrease in protein levels or enzyme activity of Topo II has been primarily implicated in the acquired resistance to ADM (Deffie *et al.*, 1989a; Deffie *et al.*, 1989b; Liu, 1989; De Jong *et al.*, 1990; McPherson *et al.*, 1993; Nitiss *et al.*, 1993; Son *et al.*, 1998). However, in our system, the Topo II expression was undetected in PC-14/ADM subline as well as its parent cell line (Fig. 2).

Since ADM can also influence free radical formation (Meijer *et al.*, 1987; Sinha *et al.*, 1989), overexpressions of radical scavenging enzymes such as GST, glutathione peroxidase (GPO), SOD, and catalase have been implicated in the acquired resistance to ADM (Ramu *et al.*, 1984; Doroshow, 1986; Deffie *et al.*, 1988; Mimnaugh *et al.*, 1989; Sinha *et al.*, 1989; Hao *et al.*, 1994; Zyad *et al.*, 1994; Ban *et al.*, 1996; Anuszewska *et al.*, 1997; Kobayashi *et al.*, 1997). We investigated two isoforms of GSTs from placental tissues (GSTπ) and rat liver tissues (GST-L). Earlier studies have shown that GSTa overexpression is associated with resistance to nitrogen mustards (Buller *et al.*, 1987) and CDDP (Kodera *et al.*, 1994). Also, GSTπ overexpression is associated with resistance to ADM (Cowan *et al.*, 1986), nitrosoureas (Smith et al., 1989), and CDDP (Kodera et al., 1994; Yi *et al.*, 1996). In our observations, however, no difference in expression of GSTπ, as well as GST-L, was observed between PC-14 and PC-14/ADM cells (Fig. 3A). The SOD activity was also measured. As we expected, a 2.38 fold more induction of SOD activity was observed in PC-14/ADM cells than in PC-14/ADM cells (Fig. 3B). Therefore, evidence is presented in this study that the ADM resistance in PC-14 cells is correlated with SOD activity, but not with GSTs.

t-TG is a Ca^{2+}-dependent enzyme that catalyzes irreversible cross-linking of proteins by promoting the formation of isopeptide bonds between protein-bound glutamine and lysine residues (Lornad and Conard, 1984; Greenberg *et al.*, 1991).

Roles of this enzyme have been proposed in diverse cellular functions as a regulation of cell growth and differentiation (Birckbichler and Patterson, 1978; Moore *et al.*, 1984), cellular adhesion and morphology (Gentile *et al.*, 1992), wound healing (Bowness *et al.*, 1988), and apoptosis (Autuori *et al.*, 1998). t-TG has also been shown to be overexpressed in ADM resistant cells (Mehta, 1994; Han and Park, 1999a; Han and Park, 1999b). In accordance with the study of Han and Park (1999b), we detected high levels of the t-TG expression in PC-14/ADM cells (Fig. 4A). Since it has been known that several effective cancer chemotherapeutic agents (including ADM, actinomycin D, mithramycin, and bleomycin) act as substrates in t-TG-catalyzed reactions (Russell and Womble, 1982), it is possible that t-TG present in drug resistant cells may utilize ADM as an amine substrate and thus render it inactive or prevent its sequestration to intracellular target sites.

Another strategy of cell survival against anticancer drugs is the regulation of cell cycle. Recent reports confirmed that cell cycle delay by the down regulation of EGFR was correlated with a reduction of sensitivity to CDDP in breast and cervical carcinoma (Dixit *et al.*, 1997; Donato *et al.*, 2000). Also, cell cycle arrest was observed in ADM resistant human lung carcinoma (OLoughlin *et al.*, 2000). In agreement with these previous studies, we also observed reduction of EGFR expression in PC-14/ADM cells (Fig. 4B). Actually, doubling time of PC-14/ADM subline was markedly increased when compared to its parent line (data not shown). These results suggest that down regulation of EGFR and delayed cell cycle may make cells avoid the attack of anticancer drugs that target cells with a high proliferative potential or aberrant cell cycle control. Therefore, it is necessary that other cell cycle regulators (including Erk, Cyclins and Cdks) are investigated in future study.

Finally, we observed E-cadherin expression in PC-14 and PC-14/ADM cells. Contrary to our expectation and previous reports (Dimanche-Boitrel *et al.*, 1994; St. Croix and Kerbel, 1997), E-cadherin expression was decreased in PC-14/ADM cells (Fig. 4C). Since down regulation of E-cadherin expression is correlated with tumor invasion (Takeichi, 1993; Birchmeier and Behrens, 1994; Perl *et al.*, 1998), the development of ADM resistance in PC-14/ADM cells may play a role in the invasive transition of it. In future studies, therefore, the relationship of the acquired drug resistance and the invasive transition in tumor cells need to be investigated.

In summary, the present study suggests that increased SOD activity, induction of t-TG expression, and down regulation of EGFR and E-cadherin may be involved in ADM resistance in PC-14/ADM cells.

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