Increased expression of T-plastin gene in cisplatin-resistant human cancer cells: identification by mRNA differential display

Terumasa Hisano[a], Mayumi Ono[a], Masaharu Nakayama[a], Seiji Naito[b], Michihiko Kuwano[a], Morimasa Wada[a,*]

[a] *Department of Biochemistry, Kyushu University School of Medicine, Fukuoka 812-82, Japan*
[b] *Department of Urology, Kyushu University School of Medicine, Fukuoka 812-82, Japan*

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Abstract **The cellular resistance to the potent anticancer agent *cis*-diamminedichloroplatinum(II) (cisplatin) is thought to be mediated by multiple mechanisms. The technique of differential display of mRNAs was applied to various cisplatin-resistant cell lines and the corresponding parental sensitive human bladder, prostatic, and head and neck cancer cells in order to identify genes that underlie cisplatin resistance. Twenty-four clones were confirmed by Northern blot analysis to be expressed differentially between resistant and the corresponding sensitive cells. Partial DNA sequences of the eight clones that showed a threefold or greater increase in expression in either the resistant cells (seven clones) or sensitive cells (one clone) revealed that two were derived from the T-plastin gene and one from the tissue factor gene. The abundance of T-plastin mRNA in cisplatin-resistant T24/DDP10 cell was ~12 times that in the parental T24 cells. Transfection of T24/DDP10 cells with a vector encoding full-length T-plastin antisense RNA demonstrated that reduced T-plastin expression was associated with increased sensitivity to cisplatin. These results are consistent with the hypothesis that several mechanisms participate cooperatively in the acquisition of cisplatin resistance in human cancer.**

Key words: Cisplatin; Differential display; Plastin; Drug resistance

1. Introduction

The potent anticancer agent *cis*-diamminedichloroplatinum(II) (cisplatin) is widely used for treating a variety of malignant tumors, including those of the testes, head and neck, esophagus, small cell lung, ovary, and bladder [1]. However, clinical use of this drug for long periods is often limited because of the appearance of cisplatin-resistant tumor cells [2]. Cisplatin resistance in such cells appears to be mediated through various mechanisms, including inactivation of cisplatin by thiol-containing molecules such as glutathione [3–6] and metallothionein [7,8], increased DNA repair [9–11], decreased drug accumulation [12–15], increased expression of DNA topoisomerase I [16], and increased abundance of thioredoxin [17]. Moreover, it has been proposed that cisplatin could be transported out of the cell after conjugation with glutathione [18] and a putative corresponding pump has been isolated and identified as human canalicular multispecific organic anion transporter (cMOAT) [19]. Several of these mechanisms appear to operate in individual resistant cell lines, with each mechanism alone accounting for only a small portion of total resistance. Thus, in contrast to the multidrug-resistance phenotype conferred by expression of the human multidrug resistance 1 gene (*MDR1*) [20–23] or multidrug resistance-associated protein gene (*MRP*) [24,25], cisplatin resistance appears to require the simultaneous operation of multiple mechanisms.

The techniques of subtractive hybridization [26] and differential hybridization [27] have been used to isolate genes that are expressed differentially between two cell populations. However, these methods are associated with incomplete recovery of the candidate clone and require large quantities of mRNA, and are time consuming and labor intensive. The technique of differential display was recently developed to overcome these problems [28,29]. We have now used differential display to isolate and characterize genes that are expressed differentially in cisplatin-resistant cell lines and their parental counterparts.

2. Materials and methods

2.1. Cell culture and cell lines

Cisplatin-resistant cell lines were derived from various drug-sensitive parental counterparts as described previously ([5,15–17], respectively): T24/DDP5, T24/DDP7, and T24/DDP10 from human bladder cancer T24 cells [16]; P/CDP5 from human prostatic cancer PC-3 cells [5,15]; KCP-4 from human head and neck cancer KB cells [13]; and KK47/CB60 from human bladder cancer KK47 cells [17] (Table 1). These cell lines were cultured at 37°C under a humidified atmosphere of 5% CO_2 in Eagle's minimum essential medium (Nissui Seiyaku, Tokyo, Japan) containing 10% fetal bovine serum (Sera-lab, Sussex, UK), Bactopeptone (1 mg/ml) (Difco, Detroit, MI, USA), glutamine (0.292 mg/ml), kanamycin (100 mg/ml), and penicillin (100 U/ml).

2.2. Differential display

Differential display was performed as described [29] with the use of an RNAmap kit (GenHunter, Brookline, MA, USA). Briefly, purified polyadenylated RNA (0.02 μg) was reverse transcribed with 1 μM of either $T_{12}MG$, $T_{12}MA$, $T_{12}MT$ or $T_{12}MC$ oligonucleotide (where M is 3-fold degenerate for G, A and C) as primer. The solution was heated to 65°C for 5 min and cooled to 37°C for 10 min, and then 200 units of reverse transcriptase was added. After incubation at 37°C for 1 h, the mixture was heated to 95°C for 5 min and stand on ice immediately. PCR was then performed as follows. Reaction mixtures contained 0.1 volume of reverse transcription reaction, 1 × PCR buffer, 20 μM each dGTP, dATP, dTTP and dCTP, 4 μCi of [^{32}P]dCTP, 1 μM of the respective $T_{12}MN$ oligonucleotide (M = G, C or A; N = G, A, T or C), 0.2 μM specific arbitrary 10-mer, and 10 units of AmpliTaq DNA polymerase. Conditions of amplification were 40 cycles of 94°C for 30 s, 40°C for 2 min and 72°C for 30 s; and 1 cycle of 72°C for 5 min. 3.5 μl of sample was mixed with 2 μl of loading dye, heated at 80°C for 2 min immediately before loading onto a 6% DNA sequenc-

*Corresponding author. Fax: (81) (92) 632-4198.
E-mail: wada@biochem1.med.kyushu-u.ac.jp

Abbreviations: Cisplatin, *cis*-diamminedichloroplatinum(II); PCR, polymerase chain reaction; CFTR, cystic fibrosis transmembrane conductance regulator

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ing gel. Gels were run for about 3 h at 60 W constant power, dried and exposed directly to X-ray film overnight at room temperature. Recovery and reamplification of cDNA fragments from dried DNA sequencing gels were also performed as described previously [29].

2.3. Northern blot analysis

Total RNA was isolated by single-step acid guanidinium thiocyanate-phenol-chloroform extraction, as previously described [30], fractionated on a 1% agarose gel containing 2.2 M formaldehyde, and transferred to a Hybond N^+ membrane (Amersham, Buckinghamshire, UK). RNA was cross-linked to the membrane by ultraviolet irradiation. ^{32}P-Labeled DNA probes were prepared by random priming [31] with a DNA labeling kit (Amersham) and [α-^{32}P]dCTP (Dupont Biotechnology Systems, Boston, MA, USA), and were derived from the inserts of differential display (dd) clones propagated in the pGem-T vector (Promega, Madison, WI, USA) or from PCR products. After hybridization, the amount of mRNAs corresponding to the dd clones was quantitated with a BAS2000 Bioimaging Analyzer (Fuji, Tokyo, Japan).

2.4. DNA sequencing

dd clones were purified with QIAGEN-tip 20 (QIAGEN, Chatsworth, CA). Chain elongation and termination were performed with a DyeDeoxy Terminator Cycle Sequencing kit, and nucleotide sequencing was performed with a DNA sequencer (model 373A; Applied Biosystems, Foster City, CA).

2.5. Isolation of full-length T-plastin cDNA

Human T-plastin cDNA was generated by reverse transcription and PCR with mRNA extracted from the T24/DDP10 cell line. Reverse transcription was performed with a mixture containing 5 μg of mRNA, 20 μmol of random hexanucleotides, 200 μmol of each deoxynucleoside triphosphate, 50 mM Tris-HCl (pH 8.3), 10 mM $MgCl_2$, 50 mM KCl, and 40 U of reverse transcriptase. The mixture was incubated at 42°C for 1 h, heated at 95°C for 10 min, and treated with 10 mg of DNase-free RNase. The resulting cDNA was precipitated and resuspended in 50 μl of water, and 10 μl of the resulting suspension were subjected to amplification with two different sets of primers, yielding two overlapping cDNA fragments encoding full-length human T-plastin. In the first amplification, the sense primer (5′-ATTCCGAGGTGCAGAAGTTG-3′) corresponded to nucleotides 5–24 [32] and the reverse primer (5′-CTATCCGTGGTTCACCTTCC-3′) was complementary to nucleotides 1032–1051 of human T-plastin cDNA [37]. In the second amplification, the sense primer (5′-AGCAGGAATGAAGCCTTGG-3′) corresponded to nucleotides 822–840 and the reverse primer (5′-TAGCTGACCTGCGAATCATG-3′) was complementary to nucleotides 2019–2038 of human T-plastin cDNA. Amplification was performed with a Perkin-Elmer (Norwalk, CT) thermal cycler for 25 cycles of 94°C for 1 min, 54°C for 1 min, and 72°C for 1 min. The products of the first and second amplifications were digested with *Pst*I alone or *Pst*I and *Eco*RI, respectively, and the resulting fragments were ligated via the *Pst*I site and incorporated into the *Pst*I and *Eco*RI sites of the pMOSBlue plasmid (Amersham). The full-length human T-plastin cDNA sequence was checked by double-strand sequencing with the DyeDeoxy Terminator Cycle Sequencing kit and a DNA sequencer (model 373A, Applied Biosystems); no discrepancies were detected.

2.6. Construction of and transfection with antisense T-plastin plasmid

The T-plastin cDNA was isolated and purified from the pMOSBlue plasmid after digestion with *Bam*HI and *Nde*I, after nucleotide addition with the Klenow enzyme and ligation to *Xba*I linkers, the cDNA was cloned into *Xba*I sites of the expression vector pRC/CMV (Invitrogen, San Diego, CA) in the antisense orientation. The plasmid was then transfected into T24/DDP10 cells and stable transfectants were isolated as described previously [6,17].

2.7. Immunoblot analysis of T-plastin

Whole-cell extracts from each cell line were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 10% gels, and separated proteins were transferred to a nylon membrane in 25 mM Tris-HCl (pH 8.3), 192 mM glycine, and 20% methanol for 1 h at 300 mA [6,17]. The filters were incubated with 5% nonfat milk in Tris-buffered saline for 30 min at room temperature, and then with antibodies to plastin for 40 min. After washing twice with Tris-buffered saline, the filters were incubated with horseradish peroxidase-conjugated secondary antibodies and immune complexes were detected by enhanced chemiluminescence (ECL detection kit; Amersham). We used rabbit polyclonal antibodies that recognize T-plastin or L-plastin specifically [33], as well as antibodies to T-plastin [34] (kindly provided by M. Arpin) that recognize both T- and L-plastin (T. Hisano and M. Wada, unpublished data).

2.8. Cell survival assay by colony formation

Cells (400–1000) were seeded in 35-mm dishes, and incubated at 37°C in the absence of drug for 18 h and then in the presence of cisplatin for an additional 6 days; the number of colonies was then counted after Giemsa staining, as described previously [17]. The plating efficiency for T24 and T24/DDP10 cells transfected with the expression vector pRC/CMV alone [T24(vector), T24/DDP10(vector)-1 and -2, respectively] or for T24/DDP10 cells transfected with the vector containing T-plastin cDNA in the antisense orientation [T24/DDP10(AS)-1, -2 and -3] were 67%, 50%, 52%, 48%, 54% and 51%, respectively.

3. Results

Various cisplatin-resistant cell lines and their sensitive parental counterparts (Table 1) were subjected to differential mRNA display analysis. A total of 15 arbitrary 10-nucleotide PCR primers (15 for PC3, P/CDP5, T24, T24/DDP10, KK47 and KK47/CB60, and 4 for KB and KCP-4, respectively) were used in combination with two T_{12}MN reverse transcription primers, T_{12}MG and T_{12}MC, chosen because they yielded the largest numbers of display bands. Each differential display lane yielded ~150–200 discrete bands, allowing evaluation of >6000 RNA species, or 30–60% of the estimated 10000–20000 cellular mRNAs [35], for each comparison. From 150 differentially displayed bands on sequencing gels, we recovered 107 clones, 64 of which hybridized to cellular RNAs on Northern blot analysis. Twenty-four of these clones were confirmed to be expressed differentially between drug-resistant cell lines and their sensitive counterparts, eight of which (des-

Table 1
Human cancer cell lines resistant to cisplatin

Cell line	Relative resistance[a]	Derivation
T24	1.0	Bladder cancer cell[b]
T24/DDP5	2.2	Increased expression of topoisomerase I, decreased cisplatin accumulation
T24/DDP7	5.2	Increased expression of topoisomerase I, decreased cisplatin accumulation
T24/DDP10	8.4	Increased expression of topoisomerase I, decreased cisplatin accumulation
PC-3	1.0	Prostatic cancer cell[b]
P/CDP5	23.0	Decreased cisplatin accumulation, increased thioredoxin level
KB	1.0	Head and neck cancer cell[b]
KCP-4	62.5	Decreased cisplatin accumulation, increased thioredoxin level
KK47		Bladder cancer cell[c]
KK47/CB60	19.5	Increased metallothionein expression, no reduced cisplatin accumulation

[a]Relative resistance to cisplatin was calculated as IC_{50} of drug-resistant cell line divided by IC_{50} of each parental cell line, IC_{50} being the dose required to reduced the initial survival fraction to 50%.
[b]The isolation and properties of cisplatin-resistant cell lines derived from their drug-sensitive counterparts have been described previously ([16,15,13,6], respectively).
[c]S. Kotoh, S. Naito and J. Kumazawa, unpublished data.

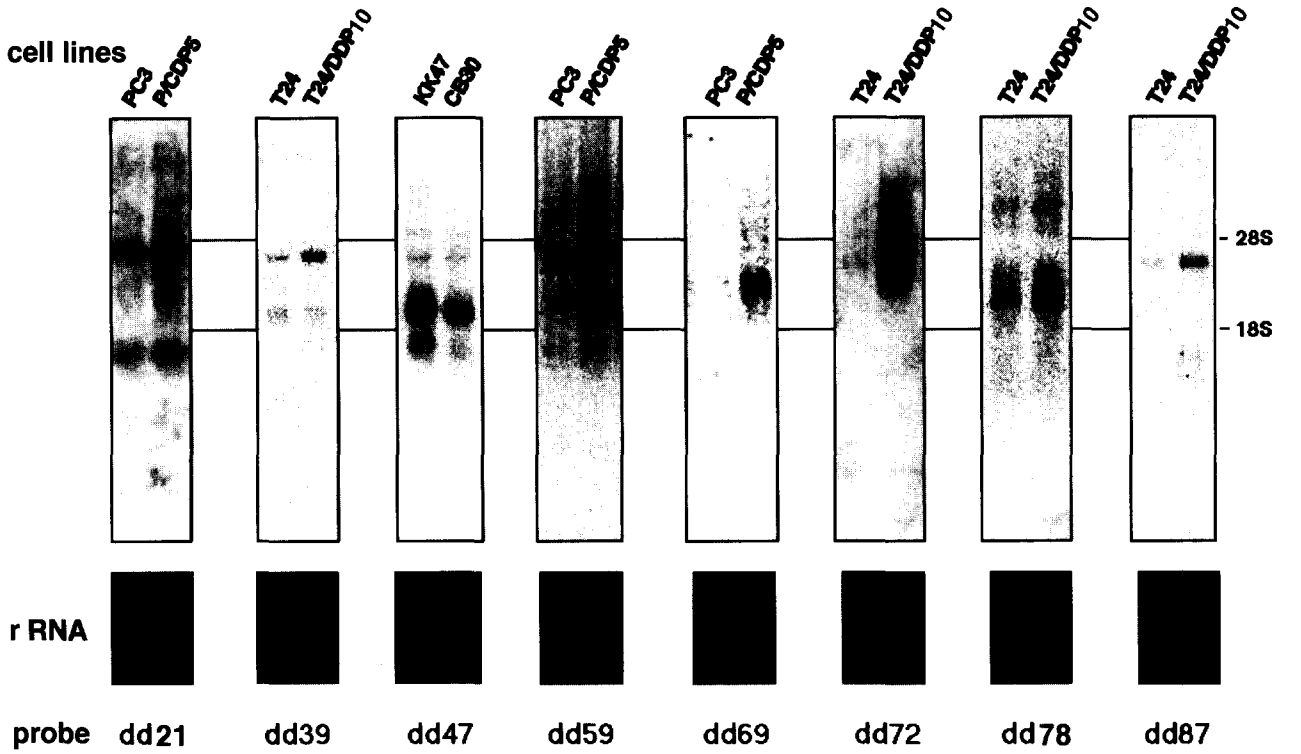

Fig. 1. Northern blot analysis of total RNA from the indicated cell lines with eight differentially expressed cDNA fragments as probes. The positions of 28S and 18S rRNA are indicated. Lower panels show ethidium bromide staining of rRNA in the gel prior to blotting.

ignated dd21, dd39, dd47, dd59, dd69, dd72, dd78, and dd87) showed a threefold or greater increase in expression in either the resistant cell lines (seven clones) or their sensitive counterparts (dd47) by Northern blot analysis (Fig. 1, Table 2).

Database searches revealed that clones dd72 and dd87 were derived from the human T-plastin gene, dd78 from the human tissue factor gene, and dd21 from the human mitochondrial DNA D-loop region, clone dd59 showed 60% identify to the *Escherichia coli N*-acetylglucosamine deaminase gene [36], clones dd47 and dd69 were identical to human cDNAs generated by random sequencing projects, and clone dd39 showed no homology to previously characterized sequences.

The sequences of dd72 and dd78 are aligned with those of human T-plastin and human tissue factor genes in Fig. 2. The only sequence differences corresponded to the primer regions of the dd clones, as might be expected from the low-stringency conditions of PCR at the early steps of differential display. The DNA sequence of clone dd72 corresponded not to the 3′ end of the cDNA but to an internal coding region, with the poly(T) primer corresponding to an internal A-rich region and not to the 3′ poly(A) tail (Fig. 2). The size of the mRNA (3.4 kb) corresponding to clone dd72 as determined by Northern blot analysis (Fig. 1) is also consistent with that previously determined for T-plastin mRNA [37].

The amount of T-plastin mRNA in cisplatin-resistant T24/DDP10 cells is ~12 times that in the parental T24 cells (Fig. 1, Table 2). The abundance of T-plastin mRNA was also increased in the cisplatin-resistant T24/DDP5, T24/DDP7, KCP-4, and P/CDP5 cell lines relative to that in the corresponding parental cells (Fig. 3); such increase in T-plastin mRNA was not apparent in cisplatin-resistant KK47/CB60 cells compared to the sensitive parental KK47 cells. Northern blot analysis with a dd87 probe, the nucleotide sequence of which was also identical to that of the T-plastin gene, gave results (data not shown) identical to those obtained with the dd72 probe (Fig. 3).

We investigated the expression of T-plastin protein by immunoblot analysis. Rabbit polyclonal antibodies generated against T-plastin but which recognize both T- and L-plastin [34] reacted with a 68-kDa protein in T24/DDP10 and T24 cells solubilized with SDS; the intensity of the immunoreactive band in the cisplatin-resistant cells was ~5 times that in the parental cells (data not shown) (T. Hisano and M. Wada, unpublished data). The amounts of T- and L-plastin were then determined separately with antibodies specific for each of these proteins [33]. The intensities of both the T- and L-plastin bands in T24/DDP10 cells were approximately three times those of the corresponding bands in T24 cells (Fig. 4A).

To evaluate whether overexpression of T-plastin in T24/DDP10 cells is functionally associated with cisplatin resistance, we generated T24/DDP10 cell lines that stably expressed full-length T-plastin antisense RNA. In two G418-resistant cell lines, T24/DDP10(AS)-1 and -2, the amounts of T-plastin was reduced to 30%, respectively; in another G418-resistant cell line, T24/DDP10/AS-3, the amounts of T- and L-plastin were similar to those in mock transfectant of T24/DDP10 cells, T24/DDP10(vector)-1 and -2 (Fig. 4A). The dose-response relations for the cytotoxic effect of cisplatin on T24/DDP10(AS)-1, -2 and -3 cells were determined by colony formation assays (Fig. 4C). T24/DDP10(AS)-1 and -2 cells were approximately twice as sensitive to the cytotoxic effect of cisplatin as were T24/DDP10(vector)-1, -2 or T24/DDP10(AS)-3 cells. In contrast, T24/DDP10(AS)-1 and -2 cells showed similar sensitivities to anticancer agents that tar-

A

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dd72      :     1 AGCCAGCGAAGGAACACAGGATTCTTACTCAGAGGAAGAAAAATATGCTT 50
T-plastin:   158 GT-------------------------------------------------- 207
dd72      :    51 TTGTTAACTGGATAAACAAAGCTTTGGAAAATGATCCTGATTGTAGACAT 100
T-plastin:   208 -------------------------------------------------- 257
dd72      :   101 GTTATACCAATGAACCCTAACACCGATGACCTGTTCAAAGCTGTTGGTGA 150
T-plastin:   258 -------------------------------------------------- 307
dd72      :   151 TGGAATTGTGCTTTGTAAAATGATTAACCTTTCAGTTCCTGATACCATTG 200
T-plastin:   308 -------------------------------------------------- 357
dd72      :   201 ATGAAAGAGCAATCAACAAGAAGAAACTTACACCCTTCATCATTCAGGAA 250
T-plastin:   358 -------------------------------------------------- 407
dd72      :   251 AACTTGAACTTGGCACTGAACTCTGCTTCTGCCATTGGGTGTCATGTTGT 300
T-plastin:   408 -------------------------------------------------- 457
dd72      :   301 GAACATTGGTGCAGAAGATTTGAGGGCTGGGAAACCTCATCTGGTTTTGG 350
T-plastin:   458 -------------------------------------------------- 507
dd72      :   351 GACTGTTTTGGGACTAGCTTTGGCAGATCATTAAGATCGGTTTGTTCGCT 400
T-plastin:   508 -------------------------------------------------- 557
dd72      :   401 GACATTGAATTAAGCAGGAATGCAGGAATGAAGCCTTGGCTGCTTTACTC 450
T-plastin:   558 -------------------------------------------------- 607
dd72      :   451 CGAGATGGTGAGACTTTGGAGGAACTTATGAAATTGTCTCCAGAAGAGCT 500
T-plastin:   608 -------------------------------------------------- 657
dd72      :   501 TCTGCTTAGATGGGCAAACTTTCATTTGGAAAACTCGGGCTGGCAAAAAA 550
T-plastin:   658 -------------------------------------------------- 707
dd72      :   551 AAAAAAA 557
T-plastin:   708 TT--C-- 714
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B

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dd78      :     1 AGGTGACCGTAGAAGATGAACGGACTTTAGTCAGAAGGAACAACACTTTC 50
HUMTFPA:   598 -T-------------------------------------------------- 591
dd78      :    51 CTAAGCCTCCGGGATGTTTTTGGCAAGGACTTAATTTATACACTTTATTA 100
HUMTFPA:   648 -------------------------------------------------- 641
dd78      :   101 GAAAGAAAACAGCCAAAACAAACACTAATGAGTTTTTGATTGATGTGGAA 150
HUMTFPA:   698 ----------------------------------------------------T 691
dd78      :   151 AAAAAAAAAA 160
HUMTFPA:   748 ---GG-G--- 757
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Fig. 2. Nucleotide sequences of clones dd72 (A) and dd78 (B) aligned with the corresponding regions of human T-plastin and human tissue factor (TF) genes, respectively. Dashes represent identical residues, flanking primer sequences are underlined, and numbering of T-plastin [37] and tissue factor [38] sequences is as previously described.

get topoisomerases I and II (etoposide and camptothecin, respectively) as T24/DDP10(vector)-1, -2 and T24/DDP10(AS)-3 cells (data not shown).

4. Discussion

We have shown that the T-plastin gene is overexpressed in cisplatin-resistant cell lines derived from T24 bladder cancer, PC-3 prostatic cancer, and KB head and neck cancer cells. In addition, the results of our antisense experiments indicated a functional relation between the extent of T-plastin expression and the extent of cisplatin resistance. The higher cisplatin resistance of T24/DDP10(AS)-1 and -2 cells relative to T24(vector) cells suggests either a role for other mechanisms, such as overexpression of topoisomerase I [16], in the resistance phenotype of T24/DDP10 cells, or inadequate suppression of T-plastin function by antisense experiments. Both plastins and fimbrins, families of actin-binding proteins, are differentially expressed in normal tissues and malignant tumors [32,37]. The L isoform of plastin is expressed in hematopoietic cells and tumor cells of nonhematopoietic origin, whereas the T isoform is present in all other normal cells [39]. Arpin et al. [33] showed that overproduction of T- and L-plastins induces cell rounding and a concomitant reorganization of actin fibers in both fibroblast-like and polarized epithelial cell lines. T-plastin induces shape changes in microvilli and remains associated with microvillar actin filaments, whereas L-plastin remains associated with microfilaments, suggesting functional differences between L- and T-plastin isoforms [34].

Plastin-associated actin might affect the intracellular distribution of cisplatin. Bauman et al. [40] showed that mitoxan-

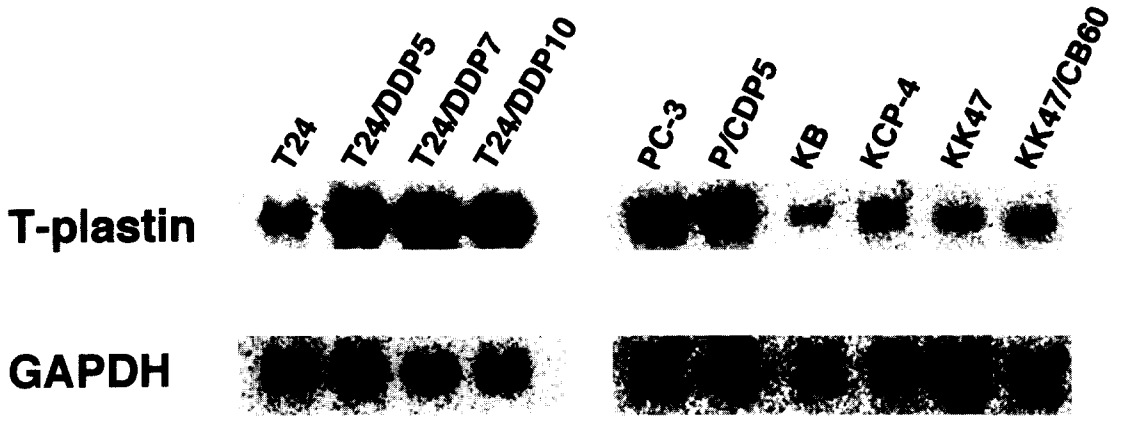

Fig. 3. Northern blot analysis of total RNA from the indicated cisplatin-resistant and parental cell lines with a dd72(T-plastin) probe. The bands corresponding to T-plastin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA, the latter of which was used as a control, are indicated.

tron binds to cytokeratins, which are components of intermediate filaments, and that forced expression of cytokeratins 8 and 18 increased mitoxantron resistance without affecting intracellular drug accumulation, suggesting that the cytokeratin network may influence the intracellular drug distribution in such a manner that the nuclear targets of the drug are spared. Thus, cisplatin may be similarly trapped in the cytoplasm by plastin-associated actin. Alternatively, cisplatin-mediated binding of actin to DNA [41] may be decreased by overexpression of plastin. Moreover, reduction of intracellular accumulation of cisplatin might induce reduced cisplatin-DNA adduct in P/CDP5 cells, because difference in the amount of DNA interstrand cross-links between P/CDP5 and its parental strain PC3 are less than difference of cisplatin accumulation between the two cell lines ([15] and see below). Alternatively a reorganization of actin filaments associated with plastin overexpression might affect the expression of genes important in cisplatin resistance or DNA repair. Actin has been shown to modulate DNA replication, transcription, and repair [42–44].

Overexpression of plastin might also affect the activity of the transporter that mediates cisplatin efflux. Recently, it has been proposed that cisplatin could be transported out of the cell after conjugated with glutathione [18] and a putative corresponding pump has been isolated and identified as human cMOAT [19]. Overexpression of the human cMOAT mRNA was observed in all three cisplatin-resistant cell lines with increased expression of T-plastin, T24/DDP10, P/CDP5 and KCP-4, but not in KK47/CB60 without increased expression of T-plastin [19]. The actin-based cytoskeleton regulates the activity of many ion channels and transport proteins [45]. For example, phallacidin, a mushroom toxin that stabilizes actin, inhibits cyclic AMP-stimulated Cl^- secretion thought to be mediated by the cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel in a human colonic epithelial cell line [46]. Moreover, CFTR possesses three domains that are homologous to the actin-binding domains of gelsolin and villin, and cytochalasin D increases CFTR-mediated ATP currents [47,48]. On the other hand, Christen et al. [49] detected a difference in microtubule arrangements between a cisplatin-resistant cell line and the corresponding parental human ovarian cancer cells. Treatment of human ovarian cancer cells with forskolin, an activator of adenylate cyclase, or with 3-isobutyl-1-methylxanthine, a phosphodiesterase inhibitor, induced phosphorylation of β-tubulin and increased intracellular accumulation of cisplatin [49]. We observed increased expression of T-plastin in cisplatin-resistant cell lines (derived from parental T24, PC-3, and KB cells) that show decreased intracellular accumulation of the drug [13,15,16]. However, T-plastin expression was not increased in cisplatin-resistant KK47/CB60 cells, which do not show decreased cisplatin accumulation relative to the parental KK47 cells [17]. Thus, overexpression

Table 2
Characteristics of dd clones expressed differentially between cisplatin resistant and sensitive cell line

dd Clone	PCR product size (bp)	Parental: resistant line[a]	Relative mRNA increase[b]	mRNA size (kb)[c]	Identity
dd21	150	PC3: P/CDP5	3.0 ± 0.62	4.2	Human mitochondrial D loop region (100%)
dd39	300	T24: T24/DDP10	4.8 ± 0.72	4.0	None
dd47	200	KK47: CB60	0.3 ± 0.02	2.5	gb: T68184 liver cDNA library (100%)[d]
dd59	300	PC3: P/CDP5	3.1 ± 0.79	3.3	*E. coli* acetylglucosamine deaminase-like
dd69	400	PC3: P/CDP5	16.8 ± 1.08	3.5	gb: H14661 infant brain cDNA library (100%)[e]
dd72	550	T24: T24/DDP10	12.1 ± 1.41	3.7	T-plastin (100%)
dd78	160	T24: T24/DDP10	5.8 ± 1.59	3.4	Human tissue factor (100%)
dd87	550	T24: T24/DDP10	11.9 ± 0.79	3.7	T-plastin (100%)

[a]Parental cell lines and their resistant counterparts from which dd clone was isolated.
[b]Relative mRNA increase for each clone was determined by Northern blot analysis (Fig. 1); those of each parental cell line were normalized as 1.0. The relative values were the average of more than two independent assays, and the two analyses showed 20% variation from the average. S.D. is given after ±.
[c]Approximate mRNA sizes were calculated from Fig. 1 by using ribosomal RNA as a standard.
[d]These sequences have been deposited in Genbank by the WASH U-Merk random sequencing project without functional information.

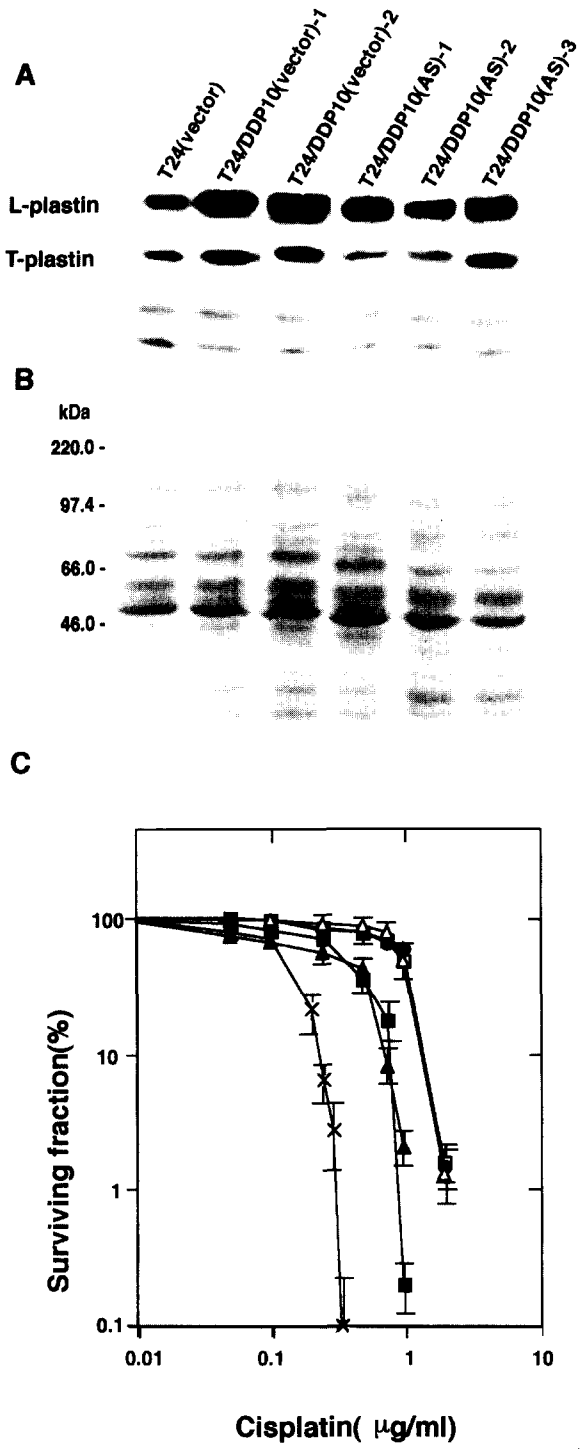

Fig. 4. A: Immunoblot analysis of extracts of T24 and T24/DDP10 cells transfected with the expression vector pRC/CMV alone [T24(vector), T24/DDP10(vector)-1 and -2, respectively] or of T24/DDP10 cells transfected with the vector containing T-plastin cDNA in the antisense orientation [T24/DDP10(AS)-1, -2 and -3]. Blots were probed with antibodies specific for T- or L-plastin as indicated. Two nonspecific bands, the intensities of which are identical in all six cell lines, are apparent below the T-plastin band. B: Coomassie blue-stained gel before blotting demonstrating equal sample loading among lanes. Molecular size standards are indicated in kilodaltons. C: Dose-response relations for the cytotoxic effect of cisplatin on T24(vector) (×), T24/DDP10(vector)-1 (□), -2 (△), T24/DDP10(AS)-1 (▲), -2 (■) and -3 (●) cells. Data are means of triplicate dishes, with 100% corresponding to the colony number for each cell line incubated in the absence of drug. Bars, S.D.

of T-plastin might contribute to a decreased cellular accumulation of cisplatin through modulation of the transporter activity.

We also showed that the abundance of human tissue factor mRNA in T24/DDP10 cells was approximately six times that in the parental T24 cells. Both exogenously added and endogenously generated oxygen free radicals induce tissue factor expression in vascular endothelial cells [50]. Cisplatin generates oxygen free radicals [51], and tissue factor might thus be induced by treatment with cisplatin. Selection for drug resistance to cisplatin in bladder cancer cells may generate oxygen free radicals, and thereby result in constitutive expression of tissue factor.

Genes encoding topoisomerase I and thioredoxin which are known to be overexpressed in P/CDP5 and/or T24/DDP10 [16,17], respectively were not isolated in this study. This is because either we used limited number of primer sets and estimated coverage was 30–60% of total mRNA species expressed, or such genes did not match with arbitrary primers used under even low stringent conditions. Nucleotide sequence alignment analysis between each primers used in this study and either topoisomerase I or thioredoxin revealed that expected size of PCR products in differential display was either smaller than 100 bp or larger than 1 kb, which range DNA could not be recovered from in our system. It would be necessary to use more primer sets for complete coverage of every mRNA species. With all these limitations, the differential gene expression detected in our study between cisplatin-resistant and parental cell lines is consistent with the notion that resistance to this drug is mediated by multiple mechanisms. Further investigations are required to elucidate the roles of these differentially expressed genes in development of the resistance phenotype.

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References

[1] Loehner, P.J. and Einhorn, L.H. (1984) Ann. Intern. Med. 100, 704–713.
[2] Thew, K.D. (1994) Cancer Res. 54, 4313–4320.
[3] Chu, G. (1990) J. Biol. Chem. 269, 787–790.
[4] Miyazaki, M., Kohno, K., Saburi, Y., Matsuo, K., Ono, M., Kuwano, M., Tsuchida, S., Sato, K., Sakai, M. and Muramatsu, M. (1990) Biochem. Biophys. Res. Commun. 166, 1358–1364.
[5] Saburi, Y., Nakagawa, M., Ono, M., Sakai, M., Muramatsu, M., Kohno, K. and Kuwano, M. (1989) Cancer Res. 49, 7020–7025.
[6] Yokomizo, A., Kohno, K., Wada, M., Ono, M., Morrow, C.S., Cowan, K.H. and Kuwano, M. (1995) J. Biol. Chem. 270, 19451–19457.
[7] Kasahara, K., Fujiwara, Y., Nishio, K., Ohmori, T., Sugimoto, Y., Komiya, K., Matsuda, T. and Saijo, N. (1991) Cancer Res. 51, 3237–3242.
[8] Kelley, S.L., Basu, A., Teicher, B.A., Hacker, M.P., Hamer, D.H. and Lazo, J.S. (1988) Science 241, 1813–1815.
[9] Eastman, A. and Schulte, N. (1988) Biochemistry 27, 4730–4734.
[10] Masuda, H., Ozols, R.F., Lai, G.M., Fojo, A., Rothenberg, M. and Hamilton, T.C. (1988) Cancer Res. 48, 5713–5716.
[11] Sheibani, N., Jennerwein, M.M. and Eastman, A. (1989) Biochemistry 28, 3120–3124.
[12] Andrews, P.A., Velury, S.C. and Howell, S.B. (1988) Cancer Res. 48, 68–73.
[13] Fujii, R., Mutoh, M., Niwa, K., Yamada, K., Aikou, T., Naka-

gawa, M., Kuwano, M. and Akiyama, S. (1994) Jpn. J. Cancer Res. 85, 426–433.
[14] Hromas, R.A., North, J.A. and Burns, C.P. (1992) Cancer Lett. 1, 278–283.
[15] Nakagawa, M., Nomura, Y., Kohno, K., Ono, M., Mizoguchi, H., Ogata, J. and Kuwano, M. (1993) J. Urol. 150, 1970–1973.
[16] Kotoh, S., Naito, S., Yokomizo, A., Kumazawa, J., Asakuno, K., Kohno, K. and Kuwano, M. (1994) Cancer Res. 54, 3248–3252.
[17] Yokomizo, A., Ono, M., Nanri, H., Makino, Y., Ohga, T., Wada, M., Okamoto, T., Yodoi, J., Kuwano, M. and Kohno, K. (1995) Cancer Res. 55, 4293–4296.
[18] Ishikawa, T. and Ali-Osman, F. (1993) J. Biol. Chem. 268, 20116–20125.
[19] Taniguchi, K., Wada, M., Kohno, K., Nakamura, T., Kawabe, T., Kawakami, M., Kagotani, K., Okumura, K., Akiyama, S., Kuwano, M. (1996) Cancer Res. 56, 4124–4129.
[20] Gros, P.B., Neriah, Y.B., Croop, J.M. and Housman, D.E. (1986) Nature 323, 728–731.
[21] Lincke, C.R., van der Bliek, A.M., Schuurhuis, G.J., van der Velde-Koerts, T., Smit, J.J. and Borst, P. (1990) Cancer Res. 50, 1779–1785.
[22] Riordan, J.R., Deuchars, K., Kartner, N., Alon, N., Trent, J. and Ling, V. (1985) Nature 316, 817–819.
[23] Ueda, K., Cardarelli, C., Gottesman, M.M. and Pastan, I. (1987) Proc. Natl. Acad. Sci. USA 84, 3004–3008.
[24] Cole, S.P., Bhardwaj, G., Gerlach, J.H., Mackie J.E., Grant, C.E., Almquist, K.C., Stewart, A.J., Kurz, E.U., Duncan, A.M. and Deeley, R.G. (1992) Science 258, 1650–1654.
[25] Cole, S.P., Sparks, K.E., Fraser, K., Loe, D.W., Grant, C.E., Wilson, G.M. and Deeley, R.G. (1994) Cancer Res. 54, 5902–5910.
[26] Jones, K.W., Chevrette, M., Shapero, M.H. and Fournier, R.E. (1992) Nature Genet. 1, 278–283.
[27] Spiegelman, B.M. and Ginty, C.A. (1983) Cell 35, 657–666.
[28] Liang, P., Averboukh, L., Keyomarsi, K., Sager, R. and Pardee, A.B. (1992) Cancer Res. 52, 6966–6968.
[29] Liang, P. and Pardee, A. (1992) Science 257, 967–971.
[30] Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. 162, 156–159.
[31] Feinberg, A.P. and Vogelstein, B. (1983) Biochem. Biophys. Res. Commun. 111, 47–54.
[32] Lin, C.S., Aebersold, R.H. and Leavitt, J. (1990) Mol. Cell. Biol. 10, 1818–1821.
[33] Lin, C.S., Shen, W., Chen, Z.P., Tu, Y.H. and Matsudaira, P. (1994) Mol. Cell. Biol. 14, 2457–2467.
[34] Arpin, M., Friederich, E., Algrain, M., Vernel, F. and Louvard, D.(1995) J. Cell Biol. 127, 1995–2008.
[35] Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K. and Watson, J. (1989) Molecular Biology of the Cell, Garland, New York.
[36] Peri, K.G. and Waygood, E.B. (1988) Biochemistry 27, 6054–6061.
[37] Lin, C.S., Aebersold, R.H., Kent, S.B., Varma, M. and Leavitt, J. (1988) Mol. Cell. Biol. 8, 4659–4668.
[38] Spicer, E.K., Horton, R., Bloem, L., Bach, R., Williams, K.R., Guha, A., Kraus, J., Lin, T.C., Nemerson, Y. and Konigsberg, W.H. (1987) Proc. Natl. Acad. Sci. USA 84, 5148–5152.
[39] Lin, C.S., Park, T., Chen, Z.P. and Leavitt, J. (1993) J. Biol. Chem. 268, 2781–2792.
[40] Bauman, P.A., Dalton, W.S., Anderson, J.M. and Cress, A.E. (1994) Proc. Natl. Acad. Sci. USA 91, 5311–5314.
[41] Miller, C.A., 3d, Cohen, M.D. and Costa, M. (1991) Carcinogenesis 12, 269–276.
[42] Egly, J.M., Miyamoto, N.G., Moncollin, V. and Chambon, P. (19840) EMBO J. 3, 2363–2371.
[43] Rungger, D., Huber, J.P. and Turler, H. (1979) Cell Biol. Int. Rep. 3, 183–188.
[44] Scheer, U., Hinssen, H., Franke, W.W. and Jockusch, B.M. (1984) Cell 39, 111–122.
[45] Matthews, J.B., Awtrey, C.S. and Madara, J.L. (1992) J. Clin. Invest. 90, 1608–1613.
[46] Mills, J.W., Schwiebert, E.M. and Stanton, B.A. (1994) Curr. Opin. Nephrol. Hypertens. 3, 529–534.
[47] Prat, A.G., Jacson, G.R., Jr., Ausiello, D.A. and Cantiello, H.F. (1994) J. Gen. Physiol. 104, 38–39.
[48] Xiao, Y.-F., O'Riordan, C.R. and Cantiello, H.F. (1994) J. Gen. Physiol. 104, 37–38.
[49] Christen, R.D., Jekunen, A.P., Jones, J.A., Thiebaut, F. Shalinsky, D.R. and Howell, S.B. (1993) J. Clin. Invest. 92, 431–40.
[50] Golino, G., Ragni, M., Cirillo, P., Avvedimento, V.E., Feliciello, A., Esposito, N., Scognamiglio, A., Trimarco, B., Iaccarino, G., Condorelli, M., Chiariello, M. and Ambrosio, G. (1996) Nature Med. 2, 35–40.
[51] Torii, Y., Mutoh, M., Saito, H. and Matsuki N. (1993) Eur. J. Pharmacol. 248, 131–135.