Jpn. J. Cancer Res. **89**, 221–227, February 1998

Role of MDM2 Overexpression in Doxorubicin Resistance of Breast Carcinoma

Akio Suzuki, Masakazu Toi,[1] Yutaka Yamamoto, Shigehira Saji, Mariko Muta and Takeshi Tominaga

Department of Surgery, Tokyo Metropolitan Komagome Hospital, 3-18-22 Honkomagome, Bunkyo-ku, Tokyo 113

Several oncoproteins or tumor suppressor gene products have been indicated to be of value as predictors of the *de novo* resistance to cytotoxic agents. In this study, we have investigated the role of MDM2 (murine double minutes) overexpression in doxorubicin resistance of breast cancer. Immunocytochemical analysis demonstrated that MDM2-positive tumors, even with p53-negative phenotype, were significantly more resistant to doxorubicin treatment compared to MDM2-negative tumors. An *in vitro* experimental model using stable *mdm2*-transfected MCF-7 cells carrying wild-type p53 confirmed that the cells become approximately 3-fold more resistant to doxorubicin as a result of MDM2 overexpression, and the wild-type p53 function, such as the induction of $p21^{Waf1}$ following DNA damage, was significantly suppressed. MDM2 overexpression is suggested to be a novel marker for predicting lack of response to doxorubicin treatment in breast cancer patients.

Key words: MDM2 — Doxorubicin — p53 — $p21^{Waf1}$

The *mdm2* oncogene was originally discovered in a spontaneously transformed BALB/c 3T3 mouse cell line that stably maintains double minute chromosomes.[1, 2] The *mdm2* gene product, MDM2, is a nuclear phosphoprotein that is a putative transcription factor, though the genes transcriptionally activated by MDM2 have not yet been identified.[2–4] MDM2 is well known to bind p53 tumor suppressor protein and to inhibit its transcriptional function.[3, 4] On the other hand, the transcription of *mdm2* gene is activated by p53.[5, 6] In addition, MDM2 protein also inhibits the G1-phase blocking effect of retinoblastoma protein, pRB, and stimulates the S-phase-inducing effect of E2F1/DP1 transcription factor, suggesting that MDM2 plays a crucial role in cell cycle control, especially G1/S transition.[7, 8]

MDM2 protein is overexpressed in many types of cancers, including breast cancer.[9–17] In MDM2-overexpressing cancer tissues and cultured cancer cells, multiple-sized *mdm2* transcripts and proteins have been identified.[14, 18–20] In particular, *mdm2* transcriptional variants lacking the p53 binding site, which are frequently detected in high-grade and late-stage tumors, showed transforming ability.[20] These data suggest that MDM2 plays a more important role in epithelial tumor growth than has been believed.

Several oncogene products or tumor suppressor proteins which are involved in the apoptotic pathway have been suggested to be involved in drug resistance mechanisms, because induction of apoptosis has been considered to be central to the efficacy of anti-cancer treatments. For example, in breast cancer, overexpression of c-*erb*B-2 protein or mutation of p53 is reported to be a predictor of doxorubicin (DOX) resistance.[21, 22] Thus, from the viewpoint of drug resistance, particularly DOX resistance, we have examined the correlation between MDM2 protein overexpression detected by immunocytochemical analysis and the clinical response to DOX-containing treatments in advanced breast cancer patients. In addition, in order to investigate the mechanism of MDM2-related drug resistance, we have generated *mdm2*-transfected cells carrying either wild-type p53 or mutant *p53*[23] and examined the alterations of p53 protein and *p53*-regulated gene products, including $p21^{Waf1}$ and Bcl-2, induced by DOX treatment. It is known that p53 protein is elevated by DNA-damaging agents, such as DOX, and stimulates the expressions of *p53*-regulated genes, which are responsible for cell cycle arrest and induction of apoptosis.[24–27] In this study, MDM2 overexpression was significantly associated with DOX resistance both *in vitro* and *in vivo*. Thus, determination and characterization of MDM2 expression may be useful for predicting DOX resistance, not only in breast cancer, but also in other types of epithelial tumors.

MATERIALS AND METHODS

Reagents DOX (adriamycin) was the generous gift of Kyowa Hakko Co. (Tokyo). 5-Fluorouracil was kindly supplied by Mitsui Pharmaceuticals Inc. (Tokyo). Cisplatin (Nippon Kayaku Co., Tokyo), vincristine and 17β-estradiol (E_2, Sigma Chemical Co., St. Louis, MO) were purchased.

Cells and cell culture The charactersistics of the breast cancer cell lines MCF-7 and MDA-MB-231 were as previously reported.[18, 23] MCF-7 cells have been maintained routinely in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS, Gibco BRL), 1n*M* E_2, 100 units/ml penicillin G and 100 μg/ml streptomycin.

[1] To whom correspondence should be addressed.

MDA-MB-231 cells were maintained in DMEM supplemented with 10% FBS (Gibco BRL), 100 units/ml penicillin G and 100 μg/ml streptomycin.

DNA transfection Transfection of plasmids was performed by lipofection using Lipofectin Reagent (Gibco BRL). Plasmids, pCmdm-2 expressing human *mdm2* and control plasmid pCmdm-2as containing *mdm2* cDNA in an antisense orientation, were kindly supplied by Dr. Klaus Roemer.[28] At 48 h post-transfection, transfected cells were diluted and cultured in culture medium containing 600–800 μg/ml G418 (Gibco BRL). G418-containing medium was changed every 3–5 days. After 2–3 weeks, G418-resistant colonies were cloned and maintained in culture medium containing 200 μg/ml G418. The production of MDM2 protein by G418-resistant clones was assessed by western blot analysis, as described below.

Treatment with antitumor agent and preparation of whole cell lysate Cells ($2–3\times10^5$) were plated in ϕ35 mm dishes and incubated for 2 days at 37°C under 5% CO_2. Then, the medium was replaced with fresh medium containing antitumor agent at a desired concentration. Cells were incubated for an appropriate period, then washed twice with ice-cold phosphate-bufferd saline (PBS) and scraped into hot lysis buffer [10 m*M* Tris-HCl, pH 7.5, 1% sodium dodecyl sulfate (SDS)]. Cell lysate was transferred into a microcentrifugation tube and boiled for 5 min. High-molecular-weight DNA was sheared by passage through a 26 gauge needle, insoluble materials were removed by centrifugation and the supernatant was transferred into another tube. Protein in the whole cell lysate was quantitated by using a BCA protein assay kit (Pierce, Rockford, IL).

Western blotting Equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in 7.5 or 15% polyacrylamide gel under reducing conditions, and subsequently electrophoretically transferred to polyvinylidene fluoride (PVDF) membrane (Bio Rad, Hercules, CA). To assess the efficiency of electrophoretic transfer, prestained marker (Rainbow marker, Amersham, Little Chalfont, Buckinghamshire, UK) was used. The blot was incubated for 24 h in blocking buffer (5% skim milk, 0.1% Tween-20 in PBS; PBS-T) at 4°C. After having been washed with PBS-T, the blot was incubated with the respective antibody for 1 h. MDM2 was detected by using 2.5 μg/ml IF2 antibody (Calbiochem, Cambridge, MA), $p21^{Waf1}$ was detected by using 1 μg/ml EA10 antibody (Calbiochem), Bcl-2 was detected by using 2.5 μg/ml bcl-2(Ab-1) antibody (Calbiochem), and p53 was detected by using NLC-p53-BP antibody (Novocastra, Clermont Place, Newcastle, UK) at a dilution of 1:100. The blot was washed with PBS-T, and incubated with horseradish peroxidase-conjugated anti mouse Ig (Amersham) or anti rabbit Ig (Amersham), and specific complexes were detected using the ECL chemiluminescence technique (Amersham) according to manufacturer's recommendations. Relative expression of the protein was analyzed by densitometry using NIH Image (National Institute of Health).

Cell viability The cytotoxic effect of antitumor agents on cells was evaluated by a modified MTT colorimetric assay.[29] Cells were seeded at the appropriate concentration in 96-well microtiter plates (Becton Dickinson, Bedford, MA) and incubated for 24 h at 37°C. Anticancer agent at the desired concentration was added to each well. Triplicate wells were assayed within individual experiments. Cells were exposed to drugs for 6–7 days. Culture medium containing an antitumor agent was changed every 2–3 days. Then, MTT (Research Organics, Inc., Cleveland, OH) in PBS (2 mg/ml) was added to each well. After further incubation for 4 h at 37°C, the supernatant was removed and 0.15 ml of DMSO was added to each well to dissolve precipitates. The absorbance was measured at 570 nm immediately after dissolution.

Immunocytochemical analysis of MDM2, p53 and Bcl-2 MDM2, p53 and Bcl-2 protein expressions were examined by immunocytochemical methods using IF2 anti-MDM2 monoclonal antibody (mdm2 Ab-1: Oncogene Science, Manhasset, NY), anti-p53 monoclonal antibody PAb1801 (Ab-2: Oncogene Science) and anti-Bcl-2 monoclonal antibody (Novocastra Lab., Ltd., Newcastle upon Tyne, UK) in frozen tissues of primary tumors as previously described.[14] According to the manufacturer's recommendations, immunostaining was performed using the avidin-biotin complex immunoperoxidase system (Vector Laboratories, Burlingame, CA). IF2 monoclonal antibody, which is an IgG_{2b} mouse monoclonal antibody raised against human MDM2 protein, recognizes an epitope in the amino-terminal portion of human MDM2 protein, and the anti-p53 antibody recognizes an epitope located between amino acids 32 and 79 of both wild-type and mutant human p53 proteins. Immunoreactivities for all three markers were determined as positive when at least 20% of the tumor cells showed positive stainings (MDM2 and p53, nuclear staining; Bcl-2, cytoplasmic staining). Histological examinations were also performed in slides stained with hematoxylin and eosin (HE).

Patients treated with DOX-containing chemotherapy Fifty recurrent breast cancer patients consecutively treated with DOX-containing regimens in Tokyo Metropolitan Komagome Hospital during 1992 to 1996 were included in this clinical analysis. The background factors are summarized in Table I. Estrogen receptor (ER) and progesterone receptor (PgR) were measured by means of enzymatic immunoassays, and a tumor with more than 5 fmol/mg protein was defined as positive.

Out of 50 cases, 13 were treated with DOX alone, and the remaining 37 were given combination treatments consisting of DOX and cyclophosphamide with or without 5-

fluorouracil. A hormonal agent (medroxyprogesterone acetate)was included in the combination regimen in 4 cases.

Clinical objective response was assessed according to the criteria of the Japanese Breast Cancer Society (based on UICC criteria). Briefly, complete response (CR) was defined as complete disappearance of all metastatic lesions. Partial response (PR) was defined for bidimensional lesions as a reduction in the size of the tumors of at least 50% from the initial measurements, as indicated by the sum of the perpendicular diameters. For patients with bone metastases, PR was defined as marked healing of lytic lesions as visualized on X-ray films. PR required continuity of the improvements for at least 28 days. Progressive disease (PD) was defined as the appearance of new lesions or an increase of ≥25% in the sum of the bidimensional lesions relative to the most favorable previous assessment.

Table I. Characteristics of Patients

	MDM2 (–)	MDM2 (+)	*P* value
Menopause			
pre-	13 (43.3)	12 (60.0)	NS
post-	17 (56.7)	8 (40.0)	
DFI (month)	20.6±13.1	20.5±15.1	NS
Adjuvant therapy			
DOX	1 (3.3)	1 (5.0)	NS
HOR + DOX	3 (10.0)	5 (25.0)	
others	26 (86.7)	14 (70.0)	
Prior therapy			
none	29(96.7)	19 (95.0)	NS
HOR	1 (3.3)	1 (5.0)	
DOX therapy			
alone	10 (33.3)	3 (15.0)	
combination	20 (66.7)	17 (85.0)	NS
Recurrence site			
soft tissue	11 (36.6)	6 (30.0)	
bone	8 (26.7)	6 (30.0)	
lung	5 (16.7)	4 (20.0)	
liver	6 (20.0)	2 (10.0)	NS
others	0	2 (10.0)	
ER			
+	17 (56.7)	7 (35.0)	NS
–	13 (43.3)	13 (65.0)	
PgR			
+	17 (56.7)	8 (40.0)	NS
–	13 (43.3)	12 (60.0)	
p53			
+	12 (40.0)	11 (55.0)	NS
–	18 (60.0)	9 (45.0)	
Bcl-2			
+	14 (46.7)	13 (65.0)	NS
–	16 (53.3)	7 (35.0)	
Survival after DOX treatment (month)	23.3±14.5	13.7±11.9	NS

DFI, disease-free interval; DOX, doxorubicin; HOR, hormone; NS, no significant difference; *P* value was evaluated by use of the χ^2 test.

Statistics The background factor analysis was done by use of the χ^2 test. All these analyses were performed by the Statistical Analysis System package, Beccel Mark-II (Beccel Co., Ltd., Tokyo).

RESULTS

Acquisition of DOX resistance The human MDM2 expression vector pCmdm-2 was transfected into ER-positive MCF-7 cells and ER-negative MDA-MB-231 cells. After stable cloning, MDM2-overexpressing clones, MCF-7/pCmdm-2 and MDA-MB-231/pCmdm-2, were isolated. In the same way, control plasmid pCmdm-2as-transfected clones, MCF-7/pCmdm-2as and MDA-MB-231/pCmdm-2as, were also obtained (Fig. 1).

The cells were subjected to drug sensitivity tests. Three separate tests were performed and the data indicated that MCF-7/pCmdm-2 cells had acquired resistance to DOX (Fig. 2A) and the DOX concentration causing 50% inhibition of the cell growth of MCF-7/pCmdm-2 cells was approximately 3-fold higher than that for MCF-7 or MCF-7/pCmdm-2as cells. This was also seen in other MCF-7/pCmdm-2 and MCF-7/pCmdm-2as clones. MDA-MB-231/pCmdm-2 cells showed no acquisition of resistance to any kind of antitumor agent (Fig. 2B).

Effect of *mdm2* transfection on the expression of *p53* and *p53*-related gene products Alterations of p53, $p21^{Waf1}$, Bcl-2, and MDM2 levels following DOX treatment were examined by western blot analyses (Fig. 3, A, B, C). As shown in Fig. 3, A and C, the induction of p53 and $p21^{Waf1}$ proteins was observed in MCF-7/pCmdm-2, as well as in MCF-7/pCmdm-2as and parental MCF-7 treated with 200 ng/ml of DOX. Although no marked change was

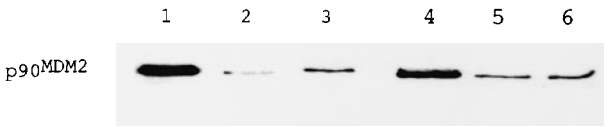

Fig. 1. The $p90^{MDM2}$ protein in the stable mdm2 transfectant. MCF-7/pCmdm-2 (lane 1), MCF-7/pCmdm-2as (lane 2), MCF-7 (lane 3), MDA-MB-231/pCmdm-2 (lane 4), MDA-MB-231/pCmdm-2as (lane 5) and MDA-MB-231 (lane 6) cells were harvested after two-day incubation in the DMEM supplemented with 10% FBS, and total cell lysates were prepared. Proteins (20 μg/lane) were separated in a 7.5% SDS-polyacrylamide gel and transferred onto a PVDF membrane. The membrane was treated with anti-MDM2 antibody (IF-2).

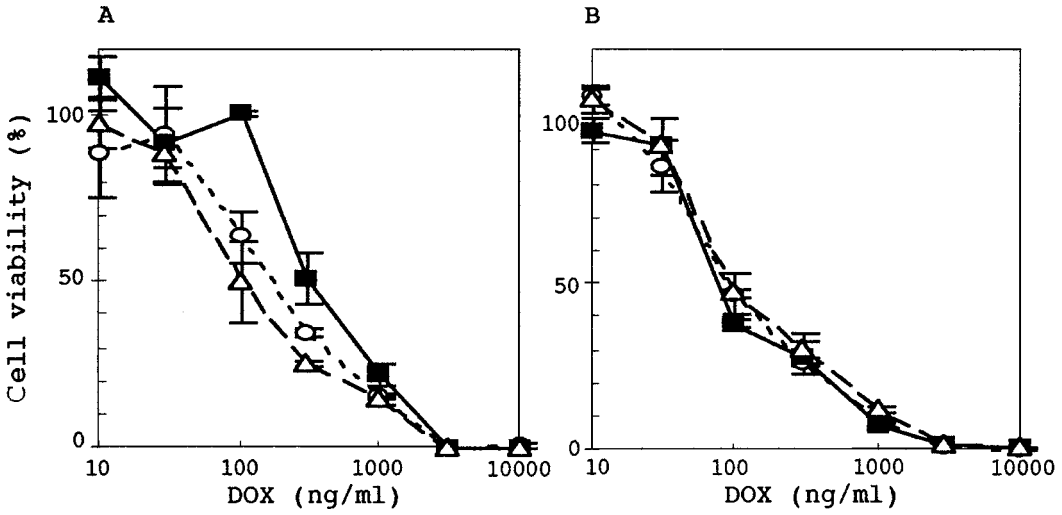

Fig. 2. A, Dose-response curves of MCF-7/pCmdm-2 (■), MCF-7/pCmdm-2as (△) and MCF-7 (○) cells treated with DOX. B, Dose-response curves of MDA-MB-231/pCmdm-2 (■), MDA-MB-231/pCmdm-2as (△) and MDA-MB-231(○) cells treated with DOX. Cell viability was determined by using a modified MTT assay. Values represent the mean±SD.

detected in p53 protein induction levels, the induction of $p21^{Waf1}$ was significantly less in MCF-7/pCmdm-2 cells compared to MCF-7 parental or MCF-7/pCmdm-2as cells (Fig. 3C). In MCF-7 and MCF-7/pCmdm-2as cells, MDM2 levels were increased after exposure to DOX. In contrast, in MCF-7/pCmdm-2 cells, high MDM2 levels were maintained at all times. There were no marked changes in Bcl-2 protein levels after DOX treatment (Fig. 3B). Although the growth of MCF-7/pCmdm-2 and MCF-7/pCmdm-2as cells was dependent on estrogen conditions, the alterations of p53 and $p21^{Waf1}$ levels induced by DOX were independent of estrogen conditions (data not shown).

There were no changes of p53, $p21^{Waf1}$, or MDM2 protein levels in MDA-MB-231/pCmdm-2, MDA-MB-231/pCmdm-2as, or MDA-MB-231 parental cells treated with DOX (data not shown).

Clinical assessments Out of 50 cases, 27 responded to DOX-containing treatments. There was no difference in the response rate between the DOX-alone and the combination treatment groups.

As shown in Table II, MDM2-positive tumors showed a statistically significantly lower CR+PR rate compared to MDM2-negative tumors ($P<0.0001$, χ^2 test). p53-positive tumors also showed a lower response rate compared to p53-negative tumors; however, the difference was statistically marginal ($P=0.052$). As shown in Table III, combination analysis using MDM2 and p53 status demonstrated that 6 out of 10 p53-negative but MDM2-positive tumors were resistant to DOX treatments. In addition, tumors positive for both MDM2 and p53 were very resistant to DOX-containing treatments. Only one case responded among MDM2-positive and p53-positive tumors. The average durations of response were 14.3 months (M), 13.1 M, 5.3 M and 3 M in MDM2(−)/p53(−), (−)/(+), (+)/(−) and (+)/(+), respectively. The former two groups showed significantly longer durations of response compared to the latter two groups ($P<0.05$, *t* test). In the survival analysis, patients with MDM2-positive tumors tended to show earlier death compared to those with MDM2-negative tumors; however, no significant difference was seen.

DISCUSSION

Our results show that overexpression of the oncoprotein MDM2 has the potential to cause resistance to DOX in human breast cancer. Immunocytochemical analysis indicated that MDM2 overexpression is significantly more frequent in DOX-resistant breast cancer tumors compared to DOX-responsive tumors. Aas *et al*. recently reported that mutations in the *p53* gene are significantly associated with lack of response to DOX treatments.[21] Mutated *p53* gene is also known to be involved in the anthracycline resistance mechanism in a mouse sarcoma tumor model.[30] Therefore, the possibility arises that MDM2 overexpression might be related to DOX resistance via the suppression of wild-type p53 function. The IF2 anti-MDM2 antibody used in our immunocytochemical study is known to recognize an epitope in the amino-terminal portion of the human MDM2 protein, which suggests that the MDM2 protein detected should have p53-binding activity.

Many investigations have shown that p53 is involved in multiple central cellular processes, and p53 function is

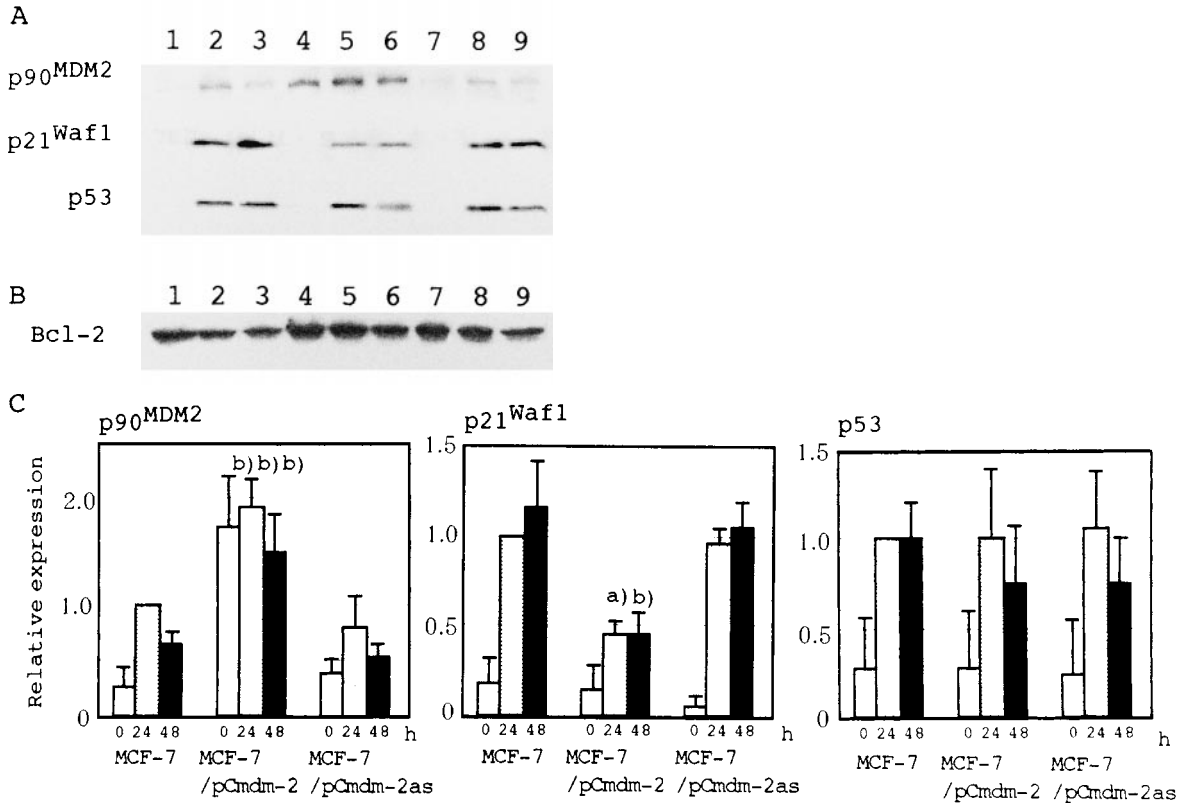

Fig. 3. The effect of MDM2 overexpression on p53, $p21^{Waf1}$, and Bcl-2 in MCF-7/pCmdm-2 (lanes 4–6) MCF-7/pCmdm-2as (lanes 7–9) and MCF-7 (lanes 1–3) cells treated with DOX. A, Western blot analysis shows the expression of p53, $p21^{Waf1}$ and $p90^{MDM2}$. Cells were treated with 200 ng/ml of DOX for 0 (lanes 1, 4, 7), 24 (lanes 2, 5, 8) or 48 (lanes 3, 6, 9) h. The induction of $p21^{Waf1}$ was down-regulated in MCF-7/pCmdm-2. B, Western blot analysis of Bcl-2. Cells were treated with 0 (lanes 1, 4, 7), 200 (lanes 2, 5, 8) or 400 (lanes 3, 6, 9) ng/ml of DOX for 48 h. Bcl-2 protein levels were similar in all conditions examined in all three types of MCF-7 cells. C, Relative expression of $p90^{MDM2}$, $p21^{Waf1}$ and p53 protein. Relative expression represents the ratio of the protein to that in MCF-7 cells treated with DOX for 24 h. Three separate western blot analyses were performed and relative expression was determined by densitometry. Columns and bars show mean±SD. Significant differences from the relative expression of the protein in MCF-7 cells treated with DOX at the corresponding time point: a) $P<0.01$ and b) $P<0.05$ (*t* test).

Table II. Doxorubicin Treatment and MDM2/p53 Status (%)

Response rate	MDM2 (–)	MDM2 (+)		p53 (–)	p53 (+)	
CR+PR	23 (76.7)	4 (20.0)	*	18 (66.7)	9 (39.1)	**
NC+PD	7 (23.3)	16 (80.0)		9 (33.3)	14 (60.9)	

* $\chi^2=15.5$, $P<0.0001$, ** $\chi^2=3.79$, $P=0.052$.

Table III. MDM2/p53 Status and Doxorubicin Treatment (%)

Response rate	MDM2	(–)	(–)	(+)	(+)
	p53	(–)	(+)	(–)	(+)
CR+PR		15 (83.3)	7 (63.6)	4 (40.0)	1 (9.1)
NC+PD		3 (16.7)	4 (36.4)	6 (60.0)	10 (90.9)

$\chi^2=16.37$, $P=0.0010$.

completely inactivated by structural mutation,[24–26, 31–35] which causes progression of tumor malignancy and produces resistance to DNA-damaging agents usually used in cancer chemotherapy and radiotherapeutic approaches. As a model of the tumor with wild-type p53 and overexpression of MDM2 protein, we have established stable MDM2-overexpressing MCF-7 transfectants. With these transfectants, monolayer cell growth experiments using the MTT assay demonstrated that MCF-7/pCmdm-2 cells had acquired approximately 3-fold greater resistance to DOX compared to MCF-7 or MCF-7/pCmdm-2as cells. Western blot analysis indicated that MDM2 overexpres-

sion repressed wild-type p53 functions. In particular, $p21^{Waf1}$ expression caused via the induction of p53 by exposure to DOX was significantly suppressed by the transfection of *mdm2*. Therefore, MDM2 overexpression at a level sufficient to suppress p53 function is thought to diminish cell-cycle arrest and apoptosis induction through p53 by DNA-damaging agents. In accordance with this idea, we found no effect on DOX resistance in *mdm2*-transfected MDA-MB-231 cells, which carry mutated *p53*. These findings seem to be compatible with the clinical results, and suggest the involvement of p53 functional abnormality in DOX resistance.

On the other hand, there was no induction of resistance to cytotoxic agents other than DOX, including 5-fluorouracil, cisplatin, and vincristine, in MCF-7/pCmdm-2 cells (data not shown). Lack of induction of vincristine resistance seems to suggest the irrelevance of the induction of multidrug resistance (MDR) in these cells. This suggestion was also supported by the observation that an MDR-reversing drug, MS-209, was without effect (data not shown). Although there is a report that the *mdm2* gene is related to the expression of the *mdr1* gene in a glioblastoma cell line,[36] we failed to demonstrate any significant alteration of MDR-1 protein in a preliminary analysis. DOX activity might be more dependent on a p53-dependent pathway in breast cancer cells.

In addition to the suppression of p53 function, MDM2 is known to associate with pRB and E2F proteins.[7, 8] Therefore, the interaction of MDM2 and RB or E2F when DOX is added is of particular interest. Recently, in a preliminary study, we found a significant association between MDM2 overexpression and expression of an angiogenic enzyme, thymidine phosphorylase, in human breast cancer tissues.[14] In the present study, double-positive phenotype for p53 and MDM2 was the most potent predictor of DOX resistance. However, little is known about MDM2 transcriptional activities: *mdm2* is a ring-finger family gene.[37, 38] Further study is needed to investigate the role of *mdm2* regulatory genes in DOX resistance.

Several oncoproteins and tumor suppressor gene products, including c-*erb*B-2 and *p53*, have been indicated to be of value for predicting resistance to anticancer agents.[21, 22] The implication of MDM2 in drug resistance, along with the phenotype of p53, should be further investigated.

(Received September 17, 1997/Revised November 25, 1997/ Accepted December 1, 1997)

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