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Although clinical development of drug resistance during treatment of breast cancer has been apparent for at least two decades, little is known about the mechanisms involved. Multidrug resistance (mdr) mediated by MDR1 gene expression is one of multiple factors identified which may be associated with declining therapeutic efficacy of anticancer drugs after failure of an initial therapy. This research program evaluated MDR1 expression in clinical specimens and demonstrated its lack of a relationship to age, menopausal status, stage, hormone receptors, site of disease, and prior treatment. Through use of clearly defined laboratory methods and specimens from women in defined clinical treatment protocols, MDR1 expression was shown to be unrelated to a history of chemotherapy drug treatment in either the neoadjuvant or adjuvant setting. Although MDR1 expression was identified in breast tumors using reverse transcriptase PCR techniques, this expression was found to be restricted to lymphoid cells in the breast tumor stroma and was not present in the breast carcinoma cells.

As tumor tissue quantity permitted, other molecular alterations were also assessed in the same breast cancer specimens for correlations with responsiveness to treatment. Expression of p53 tumor suppressor gene, HER-2/*neu* oncogene and an mdr-associated protein, p7, were evaluated. Overexpression of HER-2/*neu* was correlated with responsiveness to Taxol chemotherapy but expression of p53 was not correlated to responsiveness to Taxol chemotherapy.

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ANNUAL REPORT
TABLE OF CONTENTS

PAGE NUMBERS

INTRODUCTION.

The systemic treatment of breast cancer has been hampered by the eventual emergence of drug-resistant populations, in spite of initial responses in the majority of patients. In addition to anthracyclines, alkylating agents, flouropyrimidines and other antimetabolites, in the past 5 years the taxanes have emerged as drugs with major antitumor activity both in the absence and the presence of previous treatment. Since paclitaxel is an excellent substrate of the Pgp-efflux pump encoded by the multidrug resistance gene MDR1 and its role in breast cancer treatment is undergoing extensive evaluation, trials with this drug represented an excellent opportunity to delineate the importance of this drug-resistance mechanism in this disease. Therefore, the grant was structured to focus on determinations of Pgp before and after treatment with anthracycline-containing regimens, and on clinical trials of paclitaxel with drugs known to reverse Pgp action and restore sensitivity. These MDR1-reversal drugs have included calcium-channel blockers, steroid hormone-related derivatives, cyclosporins, and a number of other drug classes. The four tasks of the grant included 1) delineating the distribution of Pgp in various subsets of patients with breast cancer (premenopausal, postmenopausal, stage related, hormone receptor related, 2) developing a paclitaxel plus a resistance reversal drug regimen in the treatment of advanced breast cancer, 3) performing a randomized clinical trial to assess the strategy of adding a multidrug resistance reversal to paclitaxel, and 4) investigating new methods to identify and reverse multidrug resistance.

The identification of the circumstances surrounding the occurrence of multidrug resistance in breast cancer has become even more important since the grant funding began in October 1994. Two of the drug classes that are key components in the current systematic treatment of breast cancer, the taxanes and the anthracyclines have been the focus of much clinical work, both in the adjuvant and in the advanced disease setting. Cooperative group trials are investigating adjuvant doxurubin as sequentially versus combined with cyclophosphamide in high risk patients with 0 to 3 lymph nodes involved (SWOG-9313), or in arms with escalating doses, with or without paclitaxel for node positive disease (SWOG-9410, intergroup study). In advanced breast cancer, combinations of doxorubicin and paclitaxel are being compared to either drug alone (Intergroup study, coordinator Dr. George Sledge). While awaiting the results of this study, pilot studies have indicated not only striking activity, but also the likelihood of pharmacokinetic interactions between the two drugs with the potential of leading to higher AUCs of both doxorubicin and its metabolite, doxorubicinol. Presumably, in part related to this interaction, a propensity to enhanced cardiotoxicity has been described (1-2). The pharmacokinetic interactions are likely to occur because both doxorubicin and paclitaxel are excellent substrates for MDR1-P-glycoprotein (Pgp). In the development of future therapeutic strategies it is highly desirable that the circumstances surrounding the expression of MDR1 in these cancers be well characterized.

During the grant period there have been some administrative changes in the conduct of the research effort. Dr. Franco Muggia, Principal Investigator during years 01 - 03, accepted a position at New York University (NYU) and suggested that the co-Investigator of the grant, Dr. Michael Press, become the Principal Investigator during the remaining grant period. Dr. Muggia's clinical investigations related to this grant would continue at NYU under a subcontract from the University of Southern California (USC) to complete the clinical investigations. Prior to leaving USC to become director of NYU Breast Cancer Research Program, Dr. Muggia held a series of meetings with participants in this research effort, including the current principal investigator, Michael Press, personnel involved in the laboratory studies (Dr. Xiaowei Yang), personnel involved in the randomized clinical study that was undergoing final changes prior to activation and submission to the U.S. Army Medical Research and Materiel Command's Human Use Office (Dr. Darcy Spicer, and Dr. Susan Groshen, biostatistician in the project). The purpose of these meetings was to review research progress and expedite an orderly transition in the administration of these on-going investigations.

EXPERIMENTAL METHODS AND PROCEDURES.

Our progress during the period of funding is summarized below. The report covers work conducted in the laboratory component of the study to characterize expression of the multi-drug resistance gene (*MDR-1*) and other genes as potential predictors of responsiveness to taxol chemotherapy.

Laboratory Studies.

Multi-Drug Resistance Gene 1 Expression. P-glycoprotein (Pgp)-associated multidrug resistance (MDR) is related to intrinsic and acquired cross resistance to anthracyclines, vinca alkaloids and other antineoplastic antibiotics. Expression of *MDR1* is widely considered to play an important role in conferring resistance to adjuvant chemotherapy in women with breast tumor cells in women with disseminated disease, although data supporting this view is, at best, conflicting. The expression of *MDR1* gene and its gene product, P-glycoprotein, was investigated in primary and advanced breast cancers to assess the role of P-glycoprotein in determining responsiveness to adjuvant chemotherapy. Expression was assessed by immunohistochemistry, reverse transcription-polymerase chain reaction (RT-PCR), Northern Blot and Western Blot. *MDR1* mRNA was detected in forty percent of the breast cancers tested by RT-PCR with 40 cycles of PCR amplification. When reducing the PCR amplification cycles to 28, the *MDR1* gene expression signal disappeared from breast cancers of the highest expressers, however, known *MDR1* positive control normal tissues, such as adrenal, kidney, and liver continued to show an expression product. Western and Northern blots failed to demonstrate the *MDR1* gene product, P-glycoprotein, in these breast cancers. In contrast, physiological levels of P-glycoprotein was clearly detected in normal adrenal, kidney and liver by these techniques. Immunohistochemistry confirmed that breast carcinoma cells lacked P-gycoprotein expression; however, interstitial mononuclear cells, morphologically consistent with lymphocytes or macrohpages did show immunostaining in some of these breast tumors. *MDR1* gene expression identified by RT-PCR was not correlated either with response to paclitaxel therapy (29 patients evaluable, P=0.34, Fisher's exact test) or overall survival (32 breast cancer patients with clinical follow-up information, P=0.336, logrank). In conclusion, P-glycoprotein was not expressed in breast carcinoma cells at significant levels although it was expressed in stomal lymphocytes or macrophages. These results suggest that P-glycoprotein does not play a significant role in multidrug resistance of breast cancer. The findings have been accepted for publication in *Laboratory Investigation* (Yang et al., 1999).

Other Genes. We decided that there was a need to explore other mechanisms of drug resistance in order to evaluate the manner in which cancer cells become resistant to drugs. Because of our access to the tissue resource provided by the clinical trials funded by this grant we were able to begin pilot studies of other genes which might be correlated with cytotoxic drug resistance in breast cancer. The genes initially investigated were the p53 tumor suppressor gene, a small molecular weight protein (p7) which is overexpressed in human drug-resistant breast and ovarian cancer cell lines and HER-2/*neu* oncogene.

P53 TUMOR SUPPRESSOR. Analysis of p53 tumor suppressor gene overexpression was analyzed by immunohistochemistry in breast cancers from women entered in the clinical trial of paclitaxel treatment for advanced breast cancer (protocol no. 1B-92-3) (48 patients). Breast tumor tissue was available from forty specimens for analysis of p53. Seventeen of the 40 (43%) breast carcinomas showed p53 overexpression. p53 overexpression in the breast carcinoma cells did not show a correlation with overall survival of the women ($p = 0.31$) or responsiveness to treatment ($p = 0.86$) (see Table below for comparison with HER-2/*neu*).

ANALYSIS OF P7 EXPRESSION. P7 was initially discovered in ovarian carcinoma cell lines following exposure to vinblastine or adriamycin treatment *in vitro* (3-6). A similar cell line expressing high levels of p7 was obtained with MCF-7 human breast cancer cells following treatment with doxorubicin and vinblastine chemotherapeutics. The expression of p7 was preliminarily investigated in three groups of women with breast cancer: 1.) frozen primary breast cancers from the USC Breast

Tumor and Tissue Bank and obtained from women with known clinical follow-up histories (25 patients), 2.) women entered in a clinical trial of 5-fluorouracil and radiation therapy for locally advanced breast cancer (protocol no. 1B-93-3) (38 patients) and 3.) breast cancers from women prior to entry in a clinical trial of paclitaxel treatment for advanced breast cancer (protocol no. 1B-92-3) (48 patients).

P7 expression in breast cancer specimens. Among primary, untreated breast cancers p7 was expressed in six of 25 carcinomas (24%) by immunohistochemistry with 1D7 monoclonal antibody. P7 was localized to the cell membrane and cytoplasm of tumor cells. No immunostaining was found in normal breast ductal or lobular epithelium, stromal cells, endothelial cells of blood vessels, lymphocytes or macrophages. In this small cohort of cases p7 expression was not related to histologic grade, tumor size, lymph node involvement, presence of distant metastases at diagnosis, estrogen receptor status, HER-2/*neu* oncogene expression or P53 tumor suppressor protein expression. p7 was correlated with stage, however this did not reach statistical significance ($p = 0.08$) probably because of the small number of cases in this preliminary study. p7 was correlated with expression of progesterone receptor ($p = 0.035$) and recurrent disease during follow-up ($p = 0.003$) (see Table). These preliminary results appeared to merit additional studies.

Is P7 a prognostic marker and/or as a predictor of responsiveness to conventional therapies in breast cancer? P7 expression was evaluated in 38 women who accrued to an institutional multimodality protocol of continuous infusion 5-fluorouracil (FU) and concomitant radiation therapy for potentially resectable, locally advanced breast cancer. In this experimental protocol, biopsy of breast carcinomas was performed prior to neoadjuvant therapy and resection of the mass was performed after therapy. Neoadjuvant treatment involved pre-operative 5-FU (200mg/m^2) administration followed by a second incisional biopsy of the remaining breast tumor and then radiotherapy (50 Gy) to the breast and regional lymph nodes followed, finally, by resection of the remaining breast tumor mass. In these women P7 expression was increased following 5-FU treatment and radiation therapy. Overall, expression of P7 protein was increased from 21% of cases showing P7 expression to 44% after 5-FU and radiation therapy treatments. Only one breast cancer which had expression of P7 in less than 5% of tumor cells showed a loss of expression following treatment. The change in expression approached statistical significance ($p = 0.06$) even though the number of cases evaluated was small.

Table 1. Association of p7 Expression with Other Prognostic Factors.

Patient group	No. of patients	No of P7 positive case (%)	P value[1]
Total	25	6 (24)	
Age			
<50 yr	10	3 (30)	0.566
≥50 yr	15	3 (20)	
Tumor size			
<3 cm	6	1 (17)	1
≥3 cm	19	5 (26)	
Histologic grade			
1-2	8	2 (25)	1
3	17	4 (24)	
Stage			
T1/T2	15	1 (7)	0.08
T3/T4	7	3 (43)	
Unknown	3		
Regional node involvement			
positive	16	5 (31)	0.62
negative	8	1 (13)	
Unknown	1		
Distant metastasis			
positive	16	5 (31)	0.13
negative	8	1 (13)	
Unknown	1		
ER status			
positive	9	3 (33)	0.27
negative	12	1 (8)	
Unknown	4		
PR status			
positive	10	4 (40)	0.035
negative	11	0 (0)	
Unknown	4		
P53			
positive	5	2 (40)	0.54
negative	12	2 (17)	
Unknown	8		
HER-2/*neu*			
positive	8	1 (13)	0.59
negative	10	3 (30)	
Unknown	7		
Clinical outcome[2]			
AWD	11	6 (55)	**0.003**
NED	14	0 (0)	

[1]Fisher's Exact Test (2-Tail).

[2]AWD: Alive with disease; NED: No evidence of disease.

p7 expression was also characterized in our study of tumor markers as predictors of responsiveness to taxol chemotherapy, clinical trial (1B-92-3). We found a significant correlation between p7 expression in these breast cancer and both overall survival and responsiveness to treatment. After a median follow-up of 7 months, 9 of 9 patients (100%) whose breast cancers expressed p7 died from tumor progression, compared with 23 of 40 patients (55.7%) whose breast cancer did not express p7 (p = 0.017).

Among 37 patients, evaluable for response, there were four complete responders (CRs) (10.9%) and 11 partial responders (PRs) (32.4%), for an overall response rate of 43.3%. p53 expression was also investigated but in these cases but only P7 protein was significantly correlated with the response of advanced breast cancer patients to paclitaxal therapy (P=0.032). Fourteen of the 15 patients (93%) lacking P7 expression were responders to paclitaxol treatment whereas only 9 of 22 (40.9%) with P7 expression responded.

Our preliminary studies in different cohorts of breast cancer patients, suggested that P7 protein might have clinical utility for predicting tumor progression and response to neoadjuvant therapy.

HER-2/*neu* AS A PREDICTOR OF RESPONSIVENESS TO PACLITAXEL CHEMOTHERAPY. The potential association of molecular genetic alterations with responsiveness to paclitaxel chemotherapy was evaluated in a pilot study of 40 women with metastatic breast carcinoma. All participants in the paclitaxel clinical trial had disseminated breast carcinoma and consented to a pre-treatment tissue biopsy to confirm the diagnosis and to provide tissue for analysis of molecular genetic alterations. HER-2/*neu* was analyzed in this cohort and the preliminary findings are summarized below. HER-2/*neu* oncoprotein was evaluated by immunohistochemistry according to methods described elsewhere in detail (Yang et al., 1999).

In our pilot study the 17 women whose metastatic breast carcinoma <u>showed</u> HER-2/*neu* overexpression had a <u>longer</u> overall survival than the 23 women whose metastatic breast carcinoma lacked HER-2/*neu* .

Summary of the Association of HER-2/*neu* and P53 with Survival in Women with Metastatic Breast Cancer Treated with Taxol Chemotherapy.

	Number	Relative Risk	p-value
P53-negative	23	1.00	0.31
P53-positive	17	1.44	
HER-2/*neu* low expression	23	1.89	0.07
HER-2/*neu* overexpression	17	1.00	

The improved survival among women whose metastatic breast carcinoma had HER-2/*neu* overexpression is paradoxical. Since HER-2/*neu* overexpression is associated with a <u>worse</u> clinical outcome in both node-negative and node-positive breast cancer, it would have been considered promising if the HER-2/*neu* overexpressing breast cancers had had a survival probability similar to that of the HER-2/*neu* low expression group. However, the women with this poor prognostic marker (HER-2/*neu* overexpression) actually had a higher probability of survival after paclitaxel chemotherapy which was apparent within the first 10 months after treatment (see Figure below). In the small number of cases characterized this did not achieve statistical significance ($p = 0.07$) but did show a strong trend. The survival curves of women with and without HER-2/*neu* overexpression were clearly separated throughout the study period, an indication that the results are likely to achieve significance with a larger sample size. We expect to be able to resolve this issue in the near future with analysis of more patients entered in the trial.

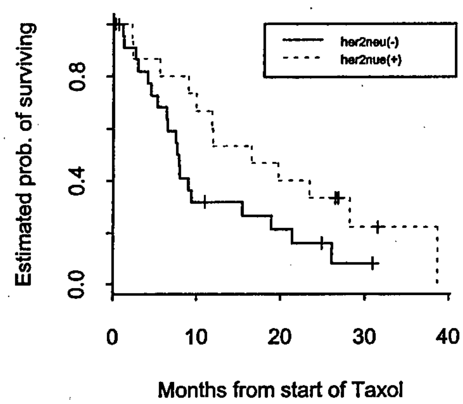

Figure: Comparison of the estimated probability of survival among women treated with Paclitaxel Chemotherapy whose metastatic breast cancer had (- - -) or did not have (——) HER-2/*neu* overexpression. Please note the separation and reversal of the survival curves which persists throughout the follow-up period.

Clinical Studies.

Protocol #1B-93-8. This clinical study, previously approved by U.S. Army Medical Research and Materiel Command's Human Use Office, was closed for accrual in January 1997. The protocol accrued eight patients into the study, with the last patient being accrued in February 1996, and going off study in May 1996. Table 1 provides the outcome of these patients and comments on their course. In common to many other investigators, the major problems encountered in treating patients in this protocol, were practical issues in relation to continuos infusion paclitaxel exceeding 24 hours. Patients 1 and 4 manifested several episodes of catheter sepsis, and patient 2 went off study because of catheter fracture, and eventually had a chest wall abscess in relation to a retained catheter fragment. This experience led us to explore and develop a protocol to test resistance-reversal strategies employing short-infusion paclitaxel, since a randomized trial employing infusions was not likely to be feasible.

Table 1. Patient demographics and outcome (protocol #1B-93-8)

Patient Number	On Study (months)	Response/Evaluability	Comments
1	5	Stable soft tissue	catheter sepsis
2	16	Stable bone/imp marker	catheter fracture
3	2	Refused therapy/NE	
4	5	partial response/lung	catheter sepsis
5	2	Refused therapy/NE	
6	6	Partial response/skin	
7	1	skin progression/NE	started other therapy
8	3	stable skin	started other therapy

NE=not evaluable

The slow accrual in this study was partly related to changes in the way paclitaxel was usually administered from infusions to short (1 to 3 hours infusions), and partly due to the reluctance in placing central venous catheters in patients at the Los Angeles County Hospital when the episodes of catheter sepsis first became manifest. Possible factors in the high incidence of infections may include the insolubility of paclitaxel leading to not infrequent visible particulate matter, and the predisposition to have some neutropenia. No toxicity was clearly attributable to the high doses of magestrol acetate given by suspension over 6 days. Patient #2, in fact, tolerated the intermittent doses on the drug without the usual weight gain that is seen with this drug. Also, no thromboembolic phenomena were evident. It is noteworthy that two objective responses and one long improvement in bone disease and markers were seen among 5 evaluable patients, inpatients who had already paclitaxel and had shown progressive disease on this drug. This might be related to schedule and not necessarily to resistance reversal since Seidman et al. did report responses to paclitaxel by 96 h infusions, following failure of shorter infusions. However, in ovarian cancer long infusions were ineffective after failure of the shorter schedules (Markman M, personal communications). After the last two patients opted to terminate treatment early, it was decide to close the protocol as it would never lead to a practical, randomized clinical study.

Protocol #1B-95-4. When it became evident that the long term infusion protocol was impractical, we initiated efforts to select a resistance reversal agent that could be given with a short infusion of paclitaxel. Also, we considered practical issues of great importance if one were to be able to mount a randomized study of paclitaxel with and without a resistance reversal agent. We selected PSC-833 as the most promising agent to reverse MDR1, and were successful in obtaining the drug from Sandoz, now Novartis. The protocol underwent several drafts in order to comply with their requirements and seek collaborations with the City of Hope, UC Davis, and Bayside Hospital in Toronto. Except for the City of Hope, others remain to work out their protocols. In the meantime, the rationale for the study changed somewhat from aiming to reverse drug resistance, to perhaps inhibit the emergence of MDR. This new rationale is based on the findings by the laboratory of Braninmir Sikic (Beketic-Oreskovic, et al. JNCI 1995; 87:1593-1602) that PSC-833 can inhibit the emergence of MDR in cell lines exposed to doxurubicin, with mutants eventually emerging but the decreased topoisomerase II-alpha rather than MDR1.

Seventeen patients have been accrued to this study as summarized in the attached table. Ten subjects have entered arm 2 (Paclitaxel + PSC833) and 7 subjects on arm 1 (Paclitaxel alone). Preliminary evaluation of response indicates 4 partial responses, 2 in each arm. The median time on study is 133 days, and 5 subjects continue on study.

No.	On-study Date	Off-study Date	Study Site	On-study Days	Arm	Response	Comments
1	8/19/96		COH	414	2		
2	12/10/96	02/12/97	COH	64	1		
3	8/13/97		COH	55	2		
4	8/18/97		COH	50	2		
5	8/22/96	06/20/97	USC	302	1	PR	
6	10/9/96	10/11/96	USC	2	1	Inevaluable	
7	10/29/96	09/04/97	USC	310	1		
8	11/12/96	12/23/96	USC	41	2		
9	12/10/96	02/25/97	USC	77	1		
10	12/16/96	03/17/97	USC	91	2		
11	1/3/97	08/11/97	USC	220	1		
12	1/17/97	04/17/97	USC	90	2		
13	1/21/97	07/02/97	USC	162	2	PR	
14	3/3/97		USC	218	2	PR	
15	3/10/97	06/03/97	USC	85	1	PR	
16	4/30/97	05/21/97	USC	21	2	Inevaluable	
17	8/12/97		USC	56	2		

The study, submitted to the U.S. Army Medical Research and Materiel Command's Human Use Office, has gone through additional revisions to increase accrual since there is considerable competition for patients. The current format is one of a randomized phase II study, and the major objective is to verify response rates. If feasible, biopsy material will seek to identify baseline tumor characteristics with respect to MDR1, and also whether treatment with paclitaxel leads to MDR1 overexpression. The last question is also being addressed in specimens obtained from other trials using paclitaxel.

Other related clinical studies. Investigators at USC (Dr. Silvia Formenti) and New York University (NYU, Drs. Franco Muggia and Matthew Volm) are collaborating in a study of neoadjuvant paclitaxel for locally advanced breast cancer, under grant support from the California Breast Cancer research Program. NYU has recruited 11 patients into this study and has stored specimens, being batched for subsequent determinations in the laboratory of Dr. Michael Press. Some specimens were sent earlier, but technical difficulties prompted storage of the specimens until they can be safely sent. Among the specimens, we have post treatment samples that will allow us to answer the question about the presence of P-glycoprotein after treatment with paclitaxel. Also, through collaboration with Dr. Sikic's laboratory we plan to assess the presence of beta-tubulin isoforms that are considered to predict for paclitaxel sensitivity.

Finally, at NYU, work with the Nuclear Medicine Department (Dr. Elissa Kramer) has permitted the measurement of 99Tecnetium sestamibi clearances. Fast clearances have been related to the presence of P-glycoprotein, but may also reflect the function of other ATP binding cassette proteins, such as MRP. The study of such clearance is associated with assessment of P-glycoprotein, MRP, and other markers of resistance may prove of interest, particularly since earlier studies have claimed a correlation of altered clearances with response to treatment with anthracyclines in breast cancer.

CONCLUSIONS.

Taxol chemotherapy was effective in prolonging the survival of some patients in the clinical trial, however, neither P-glycoprotein nor p53 expression in the breast cancer specimens was significantly correlated with responsiveness to drug treatment. A recently described small molecular weight protein, p7, was correlated with responsiveness to treatment and longer overall survival suggesting that this protein merits further investigation in our breast cancer clinical trials. HER-2/neu overexpression did show a correlation with responsiveness to paclitaxel chemotherapy.

REFERENCES:

1. Muggia FM. Managing breast cancer in an outpatients setting. Breast Cancer Res & Treat 21: 27-34, 1992.
2. Harris J, Veronesi U, Henderson IC. Breast cancer. New Eng J Med, 1993.
3. Yang X, and Page M. An Mr. 7 KDa membrane protein overexpressed in human multidrug-resistant ovarian cancer cells. Cancer Lett. 88: 171-178, 1995.
4. Yang X, and Page M. Growth regulation of multidrug resistant ovarian cancer cells by 1D7 monoclonal antibody. Anticancer Res. 16: 273-276, 1996.
5. Yang X, and Page M. A Mr. 7 kDa protein is present in both plasma and nuclear membranes in multidrug-resistant ovarian cancer cells. Oncol Rep. 3: 181-182, 1996.
6. Yang X, and Press M. Unpublished observations.
7. Yang X, Uziely B, Groshen S, Lukas J, Israel V, russell C, Formenti S, Dunnington G, Muggia F, Press MF. MDR1 Gene expression in primary and advanced breast cancer. Lab. Invest. (in press), 1999.
8. Yang X, Groshen S, formenti S, Press MF. P7 antigen expression in breast cancer. (in preparation), 1999.

Bibliography of published papers and manuscripts:

Yang X, Uziely B, Groshen S, Lukas J, Israel V, Russell C, Dunnington G, Formenti S, Muggia F, Press M. MDR1 gene expression in primary and advanced breast cancer. Lab. Invest. 79: (in press for March), 1999.

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MDR1 Gene Expression in Primary and Advanced Breast Cancer

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SUMMARY: P-glycoprotein (Pgp)-associated multidrug resistance (MDR) is related to intrinsic and acquired cross resistance to anthracyclines, vinca alkaloids, and other antineoplastic antibiotics. Expression of *MDR1* is widely considered to play an important role in conferring resistance to adjuvant chemotherapy in women with breast tumor cells in women with disseminated disease, although data supporting this view is, at best, conflicting. The expression of *MDR1* gene and its gene product, P-glycoprotein, was investigated in primary and advanced breast cancers to assess the role of P-glycoprotein in determining responsiveness to adjuvant chemotherapy. Expression was assessed by immunohistochemistry, reverse transcription-PCR (RT-PCR), Northern Blot and Western Blot. *MDR1* mRNA was detected in 40% of the breast cancers tested by RT-PCR with 40 cycles of PCR amplification. When reducing the PCR amplification cycles to 28, the *MDR1* gene expression signal disappeared from breast cancers of the highest expressers; however, known *MDR1* positive control normal tissues, such as adrenal, kidney, and liver continued to show an expression product. Western and Northern blots failed to demonstrate the *MDR1* gene product, P-glycoprotein, in these breast cancers. In contrast, physiologic levels of P-glycoprotein was clearly detected in normal adrenal, kidney, and liver by these techniques. Immunohistochemistry confirmed that breast carcinoma cells lacked P-glycoprotein expression; however, interstitial mononuclear cells, morphologically consistent with lymphocytes or macrophages did show immunostaining in some of these breast tumors. *MDR1* gene expression identified by RT-PCR was not correlated either with response to paclitaxel therapy (29 patients able to be evaluated, $p = 0.34$, Fisher Exact Test) or overall survival (32 breast cancer patients with clinical follow-up information, $p = 0.336$, log rank). In conclusion, P-glycoprotein was not expressed in breast carcinoma cells at significant levels, although it was expressed in stomal lymphocytes or macrophages. These results suggest that P-glycoprotein does not play a significant role in multidrug resistance of breast cancer. (*Lab Invest 1999, 79:1–000*).

Resistance to multiple chemotherapeutic agents is a common problem after treatment of breast cancer patients with standard cytotoxic chemotherapy regimens. The conventional wisdom is that drug resistance predominantly is due to drug efflux mechanisms, especially P-glycoprotein. P-glycoprotein decreases intracellular concentrations of cytotoxic drugs such as anthracyclines, vinca alkaloids, epipodophyllotoxins, and paclitaxel in in vitro model systems by pumping these agents out of tumor cells against a concentration gradient and, therefore, has been considered to be a major factor in clinical multidrug resistance observed in a wide variety of human cancers including breast cancer (Biedler and Riehn, 1970; Endicott and Ling, 1989; Kane et al, 1990).

The P-glycoprotein gene, *MDR1*, has been cloned and sequenced (Chen et al, 1986). Overexpression of P-glycoprotein is generally mediated by elevated levels of *MDR1* mRNA with or without concomitant amplification of the *MDR1* gene. The degree of *MDR1* gene expression and P-glycoprotein production correlates with the degree of drug resistance in in vitro model systems (Bradley et al, 1989; Kartner et al, 1983). This has been demonstrated both with cell lines grown in different concentrations of cytotoxic drug during stepwise selection and with revertant cell lines that have lost expression of P-glycoprotein and become more susceptible to the drug (Ling et al, 1983; Yang and Page, 1996). *MDR1* mRNA and P-glycoprotein is normally found in the adrenal gland, liver (bile canaliculi), kidney (proximal convoluted tubules), and colon and jejunum (apical brush border of epithelial cells) where it plays a role in excreting various biological toxins (Fojo et al, 1987; Sugawara et al, 1988). Several kinds of cancer, including acute lymphoblastic leukemia (Brophy et al, 1994), acute myelogenous leukemia (Leith et al, 1995; Marie et al, 1991; Pirker et al, 1991), neuroblastoma (Chan et al,

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1991), and pediatric soft-tissue sarcomas (Chan et al, 1990), have been shown to express P-glycoprotein. However, published reports provide conflicting information regarding the expression of P-glycoprotein in human breast carcinomas, and it is unclear whether P-glycoprotein plays a significant role in the drug resistance observed in breast cancer patients. The current study was carried out to assess ~~MDR1 gene and~~ *MDR1* gene expression in human breast cancers and to evaluate expression as a predictor of responsiveness to paclitaxel chemotherapy.

Results

T1 A total of 112 breast cancer specimens were analyzed in this investigation (Table 1). However, because of limited tissue sample size only representative samples were analyzed by RT-PCR and Northern hybridization and immunoblot for gene expression. Of the 112 specimens, 106 specimens in the entire cohort were analyzed by immunohistochemistry. Six cases from the metastatic breast cancer specimens were analyzed only by RT-PCR (Table 2). T2

Testing of P-Glycoprotein Antibodies

T3 Several commercially available anti–P-glycoprotein monoclonal antibodies (Table 3) were compared by immunohistochemical staining of P-glycoprotein–positive normal human tissues (adrenal gland, kidney, and liver), and multidrug-resistant cell lines (myeloma 8226/40 and an ovarian cancer cell line OVCAR4/ADR100). JSB-1, C219, and C494 react with intracellular epitopes of P-glycoprotein, whereas MRK16, UIC2, and 4E3 recognize external epitopes of P-glycoprotein. Although all of the antibodies showed immunostaining in each of *MDR1*-expressing tissues or cells, the intensity of this staining varied with the antibody and tissue type (Table 3). JSB-1 and MRK16 were selected as the antibodies of choice for this study because they provided strong staining across the spectrum of positive-control tissues (Table 3). Frozen tissue sections were prepared with each of four different fixatives (acetone, 4% formaldehyde, picric acid-paraformaldehyde, and 95% ethanol) and immunostained with anti–P-glycoprotein monoclonal antibodies (JSB-1 and MRK16). Immunostaining for P-glycoprotein was observed with all fixatives when JSB-1 was used; however, weaker immunostaining was observed with 4% formaldehyde and 95% alcohol fixation than was observed with acetone or picric acid-paraformaldehyde fixation. Immunostaining with MRK16 was similar with 4% formaldehyde, acetone, and picric acid-paraformaldehyde; however, 95% alcohol resulted in substantially reduced immunostaining. For this study, frozen tissue was fixed with acetone for JSB-1 immunostaining and 4% formaldehyde for MRK16 antibody.

Immunohistochemical Analysis of P-Glycoprotein Expression

Except in a few lymphocytes or monocytes in some samples, immunostaining for P-glycoprotein was not observed in breast carcinomas analyzed from any of the 106 breast cancer specimens, including 39 specimens of primary breast carcinoma from patients who had no prior systemic therapy and 73 specimens from patients with locally advanced or metastatic breast carcinoma, 49 of which from patients who had been treated previously with doxorubicin-containing regimen chemotherapy, 8 of which from patients who had been previously treated with 5-fluorouracil and radiation therapy or other chemotherapy, and 16 of which from patients who had locally advanced disease but had not been previously treated (Table 2). P-glycoprotein membrane staining was clearly recognized in normal adrenal cortex, proximal tubules of the kidney, and bile canaliculi of normal liver, which demonstrate normal physiologic levels of expression of P-glycoprotein (Fig. 1). Doxorubicin-resistant myeloma cell lines, 8226/40 and doxorubicin-resistant ovarian cancer cell line, OVCAR 4/ADR 100 showed strong immunostaining for P-glycoprotein as expected for cell lines with known P-glycoprotein overexpression. To demonstrate that background, nonspecific staining could be obtained at antibody concentrations above the determined optimal concentrations, at approximately 2-fold to 3-fold higher concentrations of P-glycoprotein antibody (16 to 24 μg/ml and 10 to 15 μg/ml, respectively, with JSB1 and MRK16 antibodies (see "Materials and Methods") stained all the cellular components (stromal, skin, and tumor cells), but membrane staining was not observed. Other membrane proteins such as HER-2/*neu* could be clearly demonstrated in some of these same breast cancers, indicating adequate preservation of membrane protein components (data not shown). F1

Table 1. History of Patient Treatment for Various Types of Breast Carcinoma Specimens

Extent of breast cancer at biopsy	No prior treatment	Prior treatment with doxorubicin	Prior other treatment	Totals
Advanced/metastatic	0	49	5[1]	54[1]
Locally advanced disease	16	0	3[2]	19[2]
Primary breast cancer	39	0	0	39
Total	55	49	8[1,2]	112[1,2]

[1] For one patient, specimens of both primary and metastatic disease were analyzed.

[2] Two specimens were obtained from three patients. In each patient, one specimen was obtained prior to any treatment and the other specimen was obtained after 5-FU treatment and radiation therapy.

Table 2. Summary of Specimens Used for Various Analyses

Extent of breast cancer at biopsy	Immunohistochemistry	MDR 1 mRNA by RT-PCR	MDR 1 mRNA by Northern blot	Western blot analysis of Pgp
Advanced/metastatic	48	33	5	2
Locally advanced disease	19	0	0	0
Primary breast cancer	39	0	1	2
Total	106	33	6	4

Table 3. Comparison of Immunohistochemical Detection of P-Glycoprotein with Six Different Antibodies

	Antibodies					
Tissue/cell type	JSB-1	MRK16	UIC2	C219	C494	4E3
Normal adrenal	3+	3+	3+	2+	2+	3+
Normal kidney	3+	3+	3+	1+	1+	2+
Normal liver	2+	1+	1+	3+	3+	1+
OVCAR4/ADR100	3+	3+	3+	1+	1+	3+

Analysis of P-Glycoprotein Expression by Western Blot

To confirm our immunohistochemistry result P-glycoprotein expression in breast cancer was analyzed by Western blot. P-glycoprotein was not detected by immunoblot analysis in advanced or primary breast cancers, although *MDR1* gene expression had been demonstrated by prolonged RT-PCR amplification. Control OVCAR4/ADR cells, normal adrenal, kidney, and liver did demonstrate P-glycoprotein by immunoblot in all cases (Fig. 2). F2

Analysis of MDR1 mRNA Expression by Reverse Transcriptase PCR

Total RNA from 33 recurrent or metastatic breast cancer specimens were analyzed by the reverse transcriptase PCR (RT-PCR) method. *MDR1* mRNA was observed in 13 of these 33 cases (40%) as an RT-PCR product of 157 bp when the PCR was amplified through 40 cycles. Known *MDR1*-positive control specimens, including normal adrenal cortex, kidney and liver, and a tumor cell line, either a multidrug-resistant myeloma cell line, 8226/40, or ovarian cancer cells (OVCAR 4/ADR 100), also showed the expected RT-PCR product of 157 bp. The control gene (β2m) product (120 bp) was detected in all breast cancer samples, confirming that mRNA in these samples was intact (Fig. 3). F3

When the RT-PCR product was amplified through the more standard 28 cycles, the 157 bp *MDR1* was not detected in breast cancers, whereas the product was recognized in total RNA from multidrug-resistant ovarian cancer cell line OVCAR4/ADR, normal adrenal gland, kidney, and liver. The control β_2m product showed an equal level of expression among these breast cancers and control tissues and cell lines (Fig. 4). F4 These results indicate that *MDR1* mRNA was present in breast cancer specimens, but at a low level in comparison with physiologic levels of *MDR1* mRNA expression found in normal adrenal, kidney, and liver. The identity of the PCR products was confirmed by DNA sequence analysis (data not shown).

Analysis of MDR1 mRNA by Northern Blot

Six breast cancer total RNA were analyzed by Northern hybridization. Of these six, four had *MDR1* expression by RT-PCR, one lacked expression by RT-PCR, and one was not analyzed by RT-PCR. Two specimens, a primary and a metastatic tumors, were from the same patient with advanced breast cancer (Table 4). T4 *MDR1* transcript was not detectable in any of these breast tumors by Northern blot analysis in contrast to a 4.6-kb *MDR1* gene transcript identified in the multidrug-resistant ovarian cancer cell line OVCAR4/ADR100 (Fig. 5). F5

Association between MDR1 mRNA Expression by RT-PCR and Clinical Data

To assess whether the low levels of P-glycoprotein found by RT-PCR in some breast cancers correlated with responsiveness to treatment, expression was compared with treatment response to paclitaxel, to previous doxorubicin therapy and survival. With regard to response to paclitaxel, 27% of women (3 of 11 patients) who were RT-PCR positive responded to the treatment compared with 11% of women (2 of 18 patients) who were RT-PCR negative ($p = 0.34$, Fisher Exact Test). Ten of twelve women (83%) who were RT-PCR positive had a history of previous doxorubicin therapy compared with 2 of 12 women (17%) who were RT-PCR positive had a history of the therapy ($p = 0.133$, Fisher Exact Test). The time since doxorubicin treatment and total prior dose were also not correlated with the gene expression (32 patients) ($p = 0.89$ and $p = 0.45$, respectively, median tests). Expression correlated with neither tumor progression nor overall survival during a median follow-up period of 2.6 years for these metastatic breast cancers ($p = 0.242$ and $p = 0.336$, log rank).

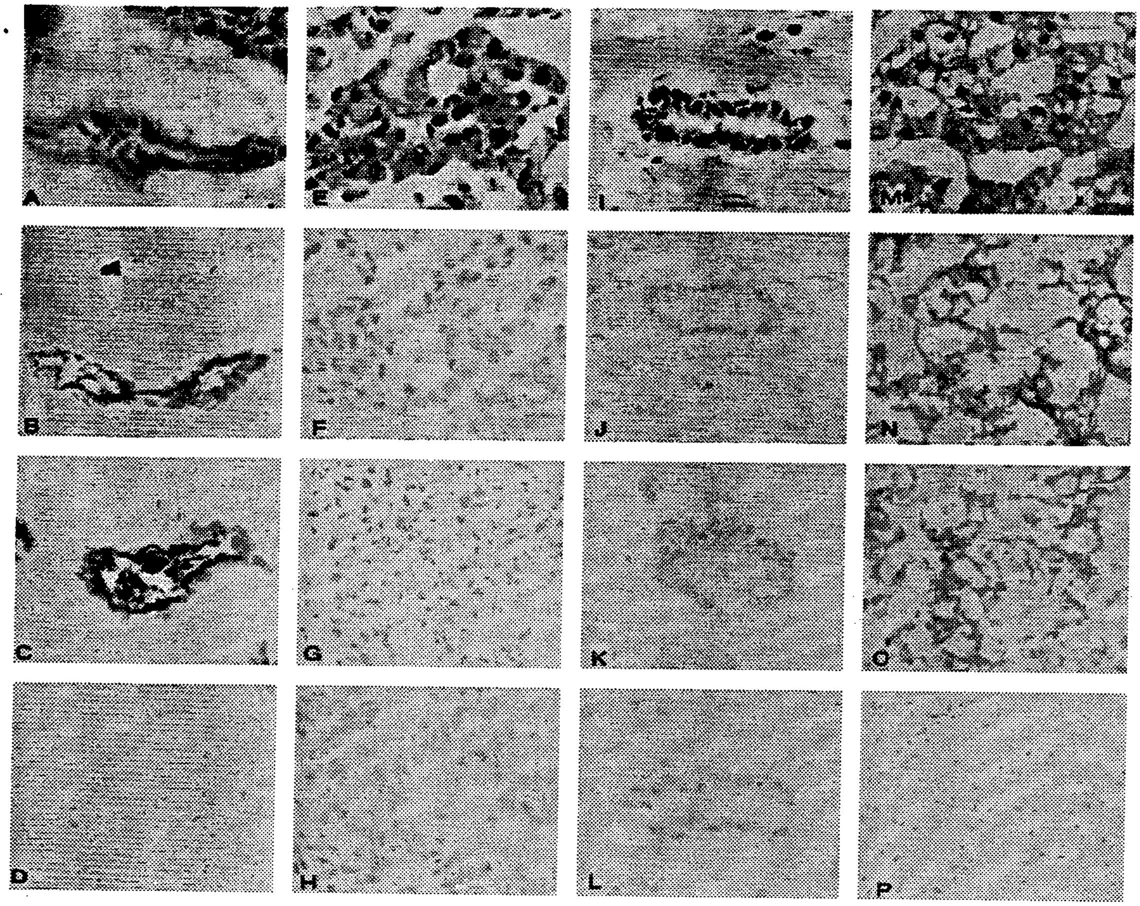

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Figure 1.

Immunohistochemical staining for P-glycoprotein in normal, positive-control kidney (A–D), in two breast cancers (E–L) and in normal control adrenal gland (M–P). A, Photomicrograph of normal renal cortex stained with hematoxylin and eosin histologic stains. B, Immunohistochemical staining for P-glycoprotein with MRK16 antibody shows immunolocalization of P-glycoprotein in renal cortical epithelial cells, especially the luminal membrane. C, Immunohistochemical staining for P-glycoprotein with JSB-1 antibody also shows immunolocalization of P-glycoprotein in renal cortical epithelial cells, especially the luminal membrane. D, Substitution of a mouse normal IgG antibody for JSB-1/MRK16 anti–P-glycoprotein antibody provides a negative control demonstrating no immunostaining of the renal cortex. E and I, Photomicrographs of two breast cancers stained with hematoxylin and eosin histologic stains. F and J, No immunohistochemical staining is identified in breast cancers incubated with MRK16 anti–P-glycoprotein antibody. G and K.) No immunohistochemical staining is identified in breast cancers incubated with JSB-1 anti–P-glycoprotein antibody. H and L, Negative control breast cancer tissue sections incubated with normal IgG antibody instead of anti–P-glycoprotein antibody demonstrate no immunostaining as observed in F, G, J, and K. M, Photomicrograph of normal adrenal cortex stained with hematoxylin and eosin histologic stains. N, Immunohistochemical staining for P-glycoprotein with MRK16 antibody shows immunolocalization of P-glycoprotein in adrenal cortical cells. O, Immunohistochemical staining for P-glycoprotein with JSB-1 antibody also shows immunolocalization of P-glycoprotein in adrenal cortical cells. P, Substitution of a mouse normal IgG antibody for JSB-1/MRK16 anti–P-glycoprotein antibody provides a negative control demonstrating no immunostaining of the adrenal cortex. Similar results were obtained for other positive control specimens including bile canaliculi of normal liver, a doxorubicin-resistant myeloma cell line, 8226/6 and 8226/40, and a doxorubicin-resistant ovarian cancer cell line, OVCAR4/ADR 100 (not illustrated). Original magnifications of all photomicrographs, ×400.

Discussion

A variety of solid and hematopoietic malignancies have been observed to develop resistance to "natural product" anticancer agents after an initial period of responsiveness. Because this observed clinical multidrug resistance corresponds with the known mechanism of action of the *MDR1* gene product P-glycoprotein, there has been an expectation that expression of P-glycoprotein is responsible for this resistance in many types of cancer. Although P-glycoprotein expression is associated with resistance to increasing concentrations of various chemotherapeutic agents in vitro for several human tumor cell lines, laboratory confirmation of this association in clinical specimens has proven to be inconsistent.

Because of contradictory findings and perceived difficulties in detecting P-glycoprotein, an international workshop was convened to promote the standardization of approaches to *MDR1* and P-glycoprotein detection in clinical specimens (Beck et al, 1996). At this workshop, it was agreed that detection of P-glycoprotein and *MDR1* mRNA is more reliable in leukemia and lymphomas than in solid tumors (Marie et al, 1991; Pirker et al, 1991). Several recommendations for detection of P-glycoprotein in clinical specimens were made (Beck et al, 1996). These recommendations included the use of more than one P-glycoprotein antibody for immunohistochemical studies and well-characterized positive controls. Frozen specimens were considered preferable to paraffin-embedded tissues because the potential for tissue fixation processing artifacts was minimized and

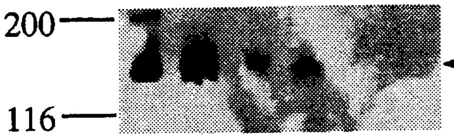

Figure 2.
Western Blot analysis of P-glycoprotein in extracts from tissues and control cell line by anti-P-glycoprotein antibody, C219. P-glycoprotein is identified as a 170-kDa band in an ovarian cancer cell line (OVCAR4/ADR) (lane 1), normal adrenal gland (lane 2), normal kidney (lane 3), and normal liver (lane 4). P-glycoprotein is not identified in metastatic breast cancers (lanes 5 and 6) or primary breast cancers (not shown). Cross-reactivity with a 200-kDa protein band corresponding to myosin is observed with the C219 antibody (lane 1).

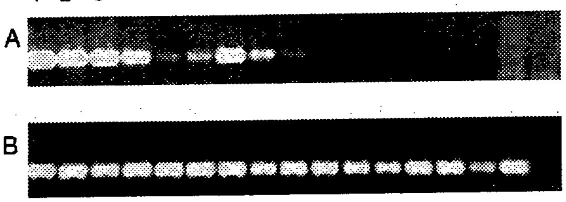

Figure 3.
Reverse transcription-PCR (RT-PCR) analysis of *MDR1* (A) and β2-microglobulin (B) gene expression with 40 cycles of PCR amplification. A, A 157-bp corresponding to the *MDR1* gene product is identified in RNA from normal human adrenal cortex (lane 1), kidney (lane 2), liver (lane 3), and metastatic breast cancers (lanes 4 to 16). A negative control (lane 17) lacking RNA demonstrates no reaction product. B, The 120-bp β2-microglobulin product confirms that intact mRNA is present in each of the samples.

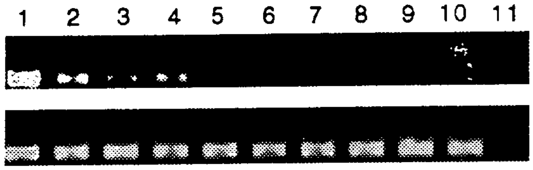

Figure 4.
RT-PCR analysis of *MDR1* (A) and β_2-microglobulin (B) gene expression with 28 cycles of PCR amplification. A, OVCAR4/ADR cell line (lane 1), normal adrenal gland (lane 2), kidney (lane 3) and liver (lane 4) demonstrated an *MDR1* gene product, but the product was no longer identified in breast cancers (lanes 5 to 10). B, In contrast, β_2m gene RT-PCR products were equally present among the above tissues and cell line. A negative control (lane 11) lacking RNA demonstrated no reaction product.

the need for antigen retrieval was eliminated. It was also considered highly desirable to use a second or third confirmatory method, such as RT-PCR, Northern hybridization, or Western blot to support immunohistochemical findings. The current study of breast cancer meets all of these recommendations.

To determine optimal conditions for immunohistochemistry in this study, several P-glycoprotein antibodies were tested with different tissue fixatives by using known positive control, frozen tissues, and cell lines to demonstrate the appropriateness of immunohistochemical assay conditions. Limiting dilution of the antibodies eliminated background staining and established the range for specific staining. To demonstrate the artifactual background staining that results from use of primary antibodies at a high concentration above the optimum determined by limiting dilutions, antibody concentrations from 2-fold to 3-fold higher than the optimum were evaluated. These high concentrations of primary antibody gave different false "positive" rates of immunohistochemical staining. When MRK 16 was used at 1:20 dilution positivity was reported in 72% (Hegewisch-Becker et al, 1998); but with a dilution of 1:100, 14% low expression was reported (De La Torre et al, 1994). In our study 1:100 dilution (5 μg/ml) of MRK 16 at an optimum demonstrated no specific membrane staining. At higher concentrations of MRK 16 (1:50 dilution, 10 μg/ml; 1:25 dilution, 20 μg/ml) all cellular components were stained. Because no membrane staining was identified in breast carcinoma cells at the dilutional optimum for MRK16 antibody and because nonspecific staining at all cellular compartments was observed at a higher antibody concentrations, the immunostaining was considered as to be high background staining at the tissue section. Therefore, an inappropriate increase in antibody concentration can lead to false-positive results. Optimal working concentrations of antibodies should be determined before use in any cohort study. Western blot confirmed our immunohistochemical results at the protein level, which is consistent with findings of other groups (Merkel et al, 1989). P-glycoprotein expression in tissue sections has been most frequently characterized by using the C219 antibody (De La Torre et al, 1994; Dixon et al, 1992; Filipits et al, 1996; Gregorcyk et al, 1996; He et al, 1995; Ro et al, 1990; Sanfillippo et al, 1991; Schneider et al, 1989; Veneroni et al, 1994). However, this antibody cross-reacts with the HER-2/*neu* protein because it contains a peptide sequence that is homologous to the epitope of P-glycoprotein recognized by C219 (Chan et al, 1991; Chan and Ling, 1997; Liu et al, 1997). This may result in misinterpretation of HER-2/*neu*–expressing tumor as P-glycoprotein–expressing carcinoma. C219 also cross-reacts with the heavy chain of myosin, which may account for some additional differences in immunostaining interpretations (Thiebaut et al, 1989). Our conclusion that P-glycoprotein is not expressed in breast carcinoma cells is supported by functional evaluation of P-glycoprotein efflux pump in breast cancer cells (Hegewisch-Becker et al, 1998). CD8+ and the CD+ lymphocytes exhibited the pump activity by rhodamine 123 (Rh123) efflux assay, but no such activity was detectable in a single breast carcinoma case.

RT-PCR is a putative sensitive technique in assessing *MDR1* gene expression in tumor specimens. Our result is consistent with findings of others that *MDR1* mRNA was detected in some breast carcinomas. By comparing the *MDR1* gene expression level in breast cancer with the physiologic level of expression of *MDR1* gene in normal control tissues, we demonstrated that a relative low level of the *MDR1* gene, which might come from contaminating lymphocytes, is expressed in breast cancer. By using a novel method for separating breast carcinoma cells from stromal and inflammatory cells, Maas et al demon-

Table 4. Treatment Information and RT-PCR Results for Patients Analyzed by Northern Blot

Category	Prior doxorubicin therapy	Months since doxorubicin therapy	Response to paclitaxel therapy	RT-PCR
1 Advanced breast cancer	Yes	9	Minor response	+
2 Advanced breast cancer	Yes	43	Progressive disease	−
3 Advanced breast cancer	Yes	7	Stable disease	+
4 Advanced breast cancer[2]	No	—	Complete response	+
5 Advanced breast cancer[3]	No	—	Complete response	+
6 Primary breast cancer	No	—	—	N/A

[1] Tumors 4 and 5 were from the same patient.
[2] Specimen was taken from the primary site.
[3] Specimen was taken from the metastatic site in the neck.

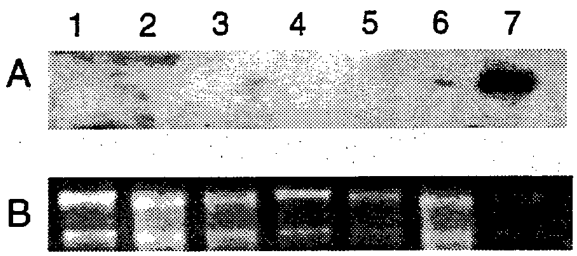

Figure 5.
Northern blot analysis of *MDR1* gene expression in breast cancer. A, MDR1 gene product is not identified in metastatic (lanes 1 to 5) or primary (lane 6) breast cancers, whereas, MDR1 mRNA was identified in a known positive control cell line (OVCAR4/ADR) (lane 7). B, Equal amount of loading (10 μg of RNA/lane) was demonstrated by ethidium bromide-stained 28S and 18S rRNA separated by 1% formaldehyde-agarose gel.

strated that *MDR1* expression in metastatic breast carcinoma was associated with lymphoid cells not the carcinoma cells (Maas et al, 1995). The investigation carried out by Hegewisch-Becker et al is in concordance with the RT-PCR results of Maas et al, ie, that *MDR1* expression is correlated with the percentage of contaminating lymphocytes in the breast tumor.

P-Glycoprotein is expressed in normal human tissues, including lymphoid/myeloid cells in the bone marrow and peripheral blood (Chaudhary et al, 1992; Drach et al, 1992; Klimecki et al, 1994), epithelial cells of bile ducts and bile canaliculi, kidney, and gastrointestinal tract (Sugawara et al, 1988). The presence of P-glycoprotein on the surface epithelium of normal secretary organs is consistent with a role in the transport of exogenous toxins and endogenous hormones. The physiologic level of expression of P-glycoprotein, which functions as an efflux pump removing certain types of "natural product" toxic agents from the interior of the cell, can readily be detected in these normal tissues by standard immunologic techniques. This finding raises the question of whether or not low or barely detectable levels of P-glycoprotein expression in breast cancer have a significant function and clinical relevance in breast cancer.

Although a few MDR phenotypes are associated with gene amplification, the majority appear to be classic genetic mutations (Roninson et al, 1984). This finding is supported by evidence derived from fluctuation tests (Goldie and Coldman, 1985). Fluctuation tests have been performed by using several drugs (eg, hydroxyurea, asparaginase, and doxorubicin) and have established mutation followed by selection as the major mechanism for the generation of drug-resistant mutants. Fluctuation tests demonstrate that mutations conferring drug resistance occur in tumor cells independent of the presence of an anticancer drug. Thus, in the case of resistance arising by means of mutation, the role played by the drug is to provide selective pressure in favor of the resistant cells by killing the sensitive cells (Woodhouse and Ferry, 1995). Both MDR1/P-glycoprotein–related and non–MDR1/P-glycoprotein–related drugs have no effect on mutations unless they are mutagens (Tannock, 1987). In other words, induction of P-glycoprotein expression by MRD1/P-glycoprotein-related treatment regimen is not expected if no P-glycoprotein–positive cells are present in the tumor sample. In the present study, a history of doxorubicin treatment was associated with a low level of P-glycoprotein expression detected only with RT-PCR analysis of the metastatic breast cancer. This low level of expression is probably due to contaminating lymphocytes in the tissue specimen. The expression was not correlated with subsequent responsiveness to paclitaxel therapy or overall survival.

The role of P-glycoprotein-associated multidrug resistance in breast cancer has been controversial. Our results demonstrate that P-glycoprotein is not expressed in human breast carcinoma cells and, therefore, does not play a role in resistance to adjuvant chemotherapy.

In conclusion, development of drug resistance in vivo is considered to be far more complicated than development of drug resistance in vitro and a variety of different mechanisms for drug resistance in addition to drug efflux mechanisms should be considered. Other mechanisms such as expression of topoisomerase II (Loflin and Zwelling, 1994), expression of selected oncogenes (eg, HER-2/*neu*) (Muss et al, 1994; Tsai et al, 1993), glutathione metabolism, intracellular stress-related proteins, and apoptotic cell death (p53 mutations) (Lowe et al, 1994) should be investigated as possible sources of drug resistance in breast cancer.

Materials and Methods

Breast Cancer Specimens and Normal Control Tissues

One hundred twelve frozen tissue specimens of primary or metastatic breast carcinoma were used for this investigation. Breast carcinoma tissues were immediately embedded in Optimal Cutting Tissue Compound (Tissue Tech Laboratories, Elk Hart, Indiana) and snap-frozen in liquid nitrogen before storage at −80° C in the USC Breast Tumor and Tissue Bank. Normal human adrenal gland, kidney, and liver also obtained from the USC Breast Tumor and Tissue Bank were used as P-glycoprotein–positive control tissues.

Protocols Protocols

Clinical ~~Information~~

The expression of *MDR 1* gene was investigated in three groups of women with breast cancer: (1) those entered in a clinical trial of paclitaxel for treatment of recurrent disease after chemotherapy (49 of 53 patients [53 patients with 54 specimens]) had received prior chemotherapy regimens containing doxorubicin (protocol 1B-92-3), (2) women in a neoadjuvant trial of 5-fluorouracil and radiation therapy to treat locally advanced disease (protocol 1B-93-3) (16 patients with 19 specimens), and (3) primary breast cancers obtained as frozen archival specimens with no history of prior chemotherapy were obtained from our tissue bank with known follow-up histories (39 patients) (Table 2).

Protocol 1B-92-3 consisted of two sequential phase II studies of two dose-schedules of paclitaxel and was begun under sponsorship of the Cancer Therapy Evaluation Program, National Cancer Institute. It was completed in April of 1996. Twenty-eight patients received paclitaxel given as 24-hour infusion (135 mg/m^2), and 25 patients received 3-hour infusion (150–225 mg/m^2). All patients (median age, 52 years; range, 26 to 77 years) had histologically proven metastatic breast cancer, with tumor accessible for biopsy and failure of one or more prior standard chemotherapy treatments. Other requirements included performance status of 2 or better (Zubrod scale); satisfactory hematologic, renal, and hepatic functions; adequate interval from preceding therapies; negative pregnancy test if applicable; and signed informed consent.

Specimens were also obtained from 16 women (median age, 54 years; range, 32 to 69 years) who accrued to a multimodality protocol of continuous infusion 5-fluorouracil and concomitant radiation therapy for potentially resectable, locally advanced breast cancer (1B-93-3). Women who were entered in this clinical trial had locally advanced breast cancer. Nine of these 16 patients had positive involvement of axillary lymph nodes, but without distant metastasis. In this experimental protocol, biopsy of the breast mass was obtained before neoadjuvant therapy and resection of the mass was performed after therapy. Neoadjuvant treatment involved preoperative 5-fluorouracil (200 mg/m^2) administration during radiotherapy (50 Gy) to the breast and regional lymph nodes.

Finally, 39 frozen primary breast cancers from the USC Breast Tumor and Tissue Bank were analyzed. None of these women (median age, 56 years; range, 30 to 86 years) received any therapy before removal of the breast carcinoma specimen. Seven of the breast cancers were less than 2.0 cm in diameter, 26 were 2.0 to 5.0 cm in diameter, and 6 were more than 5.0 cm in diameter. Twenty-two women with breast cancer had no involvement of axillary lymph nodes, 11 women had axillary lymph node metastases, and the status of axillary nodes was not available for six women.

Detection of MDR1 mRNA by RT-PCR

MDR1 mRNA was characterized by RT-PCR of total RNA prepared from frozen tissues. Total RNA was prepared from frozen tissue sections by the TRIZOL method (GIBCO BRL) as recommended by the manufacturer. RNA yield was determined by Spectrophotometry at 260 nm. cDNA was synthesized with 2.5 μg of total RNA and 250 ng of poly(A) oligo (dT)15 primer (Promega), in 50 μl of a solution containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 500 μM each of the dNTP, and 500 units of Moloney murine leukemia virus reverse transcriptase (GIBCO BRL, Grand Island, New York). After 1 hour at 40° C, the reaction was stopped by heating to 95° C for 5 minutes to inactivate the reverse transcriptase. cDNA was stored at −20° C until used. AQ: B

PCR was carried out with cDNA derived from 100 ng of RNA, 2 units of *Taq* DNA polymerase and reaction kits (Promega) in a final volume of 50 μl. Each cycle of PCR included 1 minute of denaturation at 94° C, 1 minute of primer annealing at 55° C, and 2 minutes of extension/synthesis at 72° C. PCR primers were synthesized by using an Applied Biosystems DNA synthesizer (model 394) at Norris Comprehensive Cancer Center, USC. β2-Microglobulin (β2m) was selected as a control to demonstrate intactness of the RNA and suitable PCR primers were synthesized. Sense primer 5′-ACCCCCACTGAAAAAGATGA-3′, corresponding to β2m genomic DNA nucleotides 1544–1563, and antisense primer 5′-ATCTTCAAACCTCCATGATG-3′ corresponding to genomic DNA nucleotide sequence 2253–2262 and 3508–3517, which encompasses the 3′ side of the nucleotides of exon 3 and the 5′ side of the nucleotides of exon 4 (Gussow et al, 1987). *MDR1* sense (5′-CCCATCATTGCAATAGCAGG-3′) and antisense (5′-GTTCAAACTTCTGCTCCTGA-3′) primers were used to amplify an *MDR1* cDNA segment (157 bp) from nucleotide 2596 to 2752 (Noonan et al, 1990). Each primer was added at 50 pmol of concentration per reaction. The PCR amplifications of *MDR1* and β2-microglobulin were carried out in separate tubes. PCR products were separated on 2% agarose gel. Electrophoresis was performed (45 mM Tris-borate buffer, 1 mM EDTA) at 100 V for 2 hours. Gels were stained with ethidium bromide, examined on a ultraviolet transilluminator (Gel Documentation System, ULTRA-LUM), and photographed. Negative controls were prepared with water instead of cDNA. AQ: C

Detection of MDR1 mRNA by Northern Blot

Total RNA was extracted from the same specimens as used for RT-PCR. Ten micrograms of RNA for each sample was loaded, electrophoresed, and transferred to Hybond-N+ nylon nucleic acid transfer membrane (Amersham, Cleveland, Ohio). Equal loading and integrity of RNA were confirmed by ethidium bromide staining of the formaldehyde-denatured 1% agarose gel. The blot was hybridized with a ^{32}P-dCTP labeled 4.15-kb P-glycoprotein cDNA probe (*Xba*I restriction digest 4.15 KB sequence from pGEM-3Zf (-) Xba vector) (ATCC, Rockville, Maryland) for 2 hours in Rapid-hyb buffer (Amersham) and exposed to x-ray film for 3 days.

Immunohistochemical Characterization of P-Glycoprotein Expression

A series of commercially available P-glycoprotein antibodies were compared for immunostaining sensitivity and specificity by using control tissues and cell lines. These antibodies were JSB1 (Signet, Boston, Massachusetts), MRK16 (Kamiya Biomedical Co., Tukwila, Washington), UIC2 (Immunotech, Inc., Westbrook, Maine), C219 (Signet), C494 (Signet), and 4E3 (Signet). Fixation conditions were determined by testing each of the antibodies in P-glycoprotein-expressing tissues fixed with formaldehyde (4%), paraformaldehyde-picric acid (Press et al, 1985), acetone, and 95% ethanol. Optimal concentrations were determined by serial dilution of each antibody (8 μg/ml and 5 μg/ml for JSB1 and MRK16, respectively).

The peroxidase antiperoxidase immunohistochemical technique was used to localize P-glycoprotein in tissue sections. Frozen tissue sections (6 μm) were thaw-mounted on glass slides, fixed in acetone at room temperature for 10 minutes, washed in phosphate buffered saline (PBS) and treated with 0.5% H_2O_2-PBS for 15 minutes to inactivate endogenous peroxidase activity, washed twice with PBS, and treated with 10% normal rabbit serum in PBS to block nonspecific binding sites. Monoclonal mouse P-glycoprotein antibodies, JSB1 and MRK16, were applied to serial tissue sections for 1 hour at a concentration of 8 μg/ml and 5 μg/ml, respectively, in PBS containing 10% rabbit serum after rotating with normal IgG coated beads overnight to reduce nonspecific, background staining. The antibodies and concentrations were chosen after testing with positive control tissue and cell lines to select the most sensitive antibodies for use (see "Results"). A secondary rabbit anti-mouse IgG (anti-H+L chains, ZYMED Inc., S. San Francisco, California) bridging antibody (1:50 dilution, 30 minutes) and a tertiary mouse peroxidase antiperoxidase antibody (1:50 dilution, 30 minutes) (Sternberger Laboratories, Inc.) was used to label the primary antibodies. After each antibody incubation, the tissue sections were washed in excess PBS three times for 3 minutes each. Each of the antibodies were adsorbed with human IgG-coated latex beads to reduce nonspecific background staining as described elsewhere (Press and Greene, 1984).

AQ: D

Diaminobenzidine-H_2O_2 solution (Abbott Laboratories, Inc., Abbott Park, Illinois) was applied to the sections and reacted for 10 minutes to identify the sites of immunoprecipitate formation. Specimens were then washed in PBS, counterstained with ethyl green, mounted in Permount, and cover-slipped.

Negative controls for each sample were performed as above but with normal mouse isotype control immunoglobulins (ZYMED) instead of primary antibody. Doxorubicin-resistant myeloma cell lines 8226/6 and 40; ovarian cancer cell line OVCAR4/ADR100; and normal adrenal gland, kidney, and liver were used as positive control samples assayed with each group of tumor samples. Doxorubicin-resistant myeloma cell lines 8226/Dox 6 and 40 were provided by Dr. T. M. Grogan, University of Arizona. Another doxorubicin-resistant subline, OVCAR 4/ADR 100, was generated by stepwise selection in escalating concentrations of doxorubicin (Yang and Page, 1995). These cell lines were continuously maintained in the presence of doxorubicin (6 ng/ml, 40 ng/ml, and 100 ng/ml, respectively). They were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (GIBCO BRL), 2 mM L-glutamine, 100 U of penicillin, and 100 μg of streptomycin at 37° C in a humidified atmosphere of 5% CO_2. All slides were evaluated by two of the authors without knowledge of the clinical data. Immunohistochemistry was scored on a four-point basis: 0 = no staining, 1+ = weak staining, 2+ = moderate staining, and 3+ = strong staining; the percentage of positive tumor cells in each category was recorded.

Western Blot Analysis of P-Glycoprotein Expression

Breast cancer and normal control tissues were homogenized with homogenizer in ice-cold lysis buffer (50 mM Tris-HCl, pH 8.0, containing 0.5% CHAPS, 150 mM NaCl, 2 mM $MgCl_2$, 1 mM PMSF, and 5 mM EDTA). Protein concentrations were measured by spectrophotometry. The same amount of protein was applied on each lane. After electrophoresis on a 7.5% homogeneous gel, the proteins were transferred to nitrocellulose paper by using PhastSystem transferring Unit (25 mAV for 9 Vh) (Pharmacia Biotech). Nitrocellulose was washed briefly with 0.05% Tween 20-phosphate buffered saline (PBST) containing 10% rabbit serum in PBST solution. The blot was then incubated at room temperature for 2 hours with 10 μg/ml of the anti–P-glycoprotein antibody C219 in PBS containing 10% rabbit serum. After washing with PBST, the paper was incubated with peroxidase-labeled rabbit anti-mouse Ig (DACO, A/S Denmark) at a 1:500 dilution for 1 hour at room temperature and thereafter washed extensively with PBST. The immunoreactive proteins were detected by ECL as recommended by the manufacturer.

AQ: F

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References

Beck WT, Grogan TM, Willman CL, Cordon-Cardo C, Parham DM, Kuttesch JF, Andreeff M, Bates SE, Berard CW, Boyett JM, Brophy NA, Broxterman HJ, Chan HSL, Dalton WS, Dietel M, Fojo AT, Gascoyne RD, Head D, Houghton PJ, Srivastava DK, Lehnert M, Leith CP, Paietta E, Pavelic ZP, Rimsza L, Roninson IB, Sikic BI, Twentyman PR, Warnke R, and Weinstein R (1996). Methods to detect P-glycoprotein-associated multidrug resistance in patients' tumors: Consensus recommendations. Cancer Res 56:3010–3020.

Biedler JL and Riehn H (1970). Cellular resistance to actinomycin D in Chinese hamster ovary cells in vitro: Cross resistance, radiographic, and cytogenetic studies. Cancer Res 30:1174–1184.

Bradley G, Naik M, and Ling V (1989). P-glycoprotein expression in multidrug-resistant human ovarian carcinoma cell lines. Cancer Res 49:2790–2796.

Brophy N, Marie J, Rojas V, Warnke R, McFall P, Smith S, and Sikic B (1994). Mdr1 gene expression in childhood acute lymphoblastic leukemias and lymphomas: A critical evaluation by four techniques. Leukemia 8:327–335.

Chan HSL and Ling V (1997). Anti–P-glycoprotein antibody C219 cross-reactivity with c-erbB2 protein: Diagnostic and clinical implications. J Natl Cancer Inst 89:1473–1476.

Chan H, Thorner P, Haddad G, and Ling V (1990). Immunohistochemical detection of P-glycoprotein: Prognostic correlation in soft tissue sarcoma of childhood. J Clin Oncol 8:689–704.

Chan H, Haddad G, Thorner P, DeBoer G, Lin Y, Ondrusek N, Yeger H, and Ling V (1991). P-glycoprotein expression as a predictor of the outcome of therapy for neuroblastoma. N Engl J Med 325:1608–1614.

Chaudhary PM, Mechetner EB, and Roninson IB (1992). Expression and activity of the multidrug resistance P-glycoprotein in human peripheral blood lymphocytes. Blood 11:2735–2739.

Chen CJ, Chin JE, Ueda K, Clark DP, Pastan I, Gottesman MM, and Roninson IB (1986). Internal duplication and homology with bacterial transport proteins in the mdr1 (P-glycoprotein) gene from multidrug-resistant human cells. Cell 47:381–389.

De La Torre M, Larsson R, Nygren P, Lindgren A, and Bergh J (1994). Expression of the multidrug-resistance gene product in untreated human breast cancer and its relationship to prognostic markers. Acta Oncol 33:773–777.

Dixon AR, Bell J, Ellis IO, Elston CW, and Blamey RW (1992). P-glycoprotein expression in locally advanced breast cancer treated by neoadjuvant chemotherapy. Br J Cancer 66:537–541.

Drach D, Shourong Z, Drach J, Mahaderia R, Gattringer C, Huber H, and Andreeff M (1992). Subpopulations of normal peripheral blood and bone marrow cells express a functional multidrug resistant phenotype. Blood 80:2729–2734.

Endicott J and Ling V (1989). The biochemistry of P-glycoprotein-mediated multidrug resistance. Annu Rev Biochem 58:137–171.

Filipits M, Suchomel R, Dekan G, Haider K, Valdimarsson G, Depisch D, and Pirker R (1996). MRP and MDR1 gene expression in primary breast carcinomas. Clin Cancer Res 2:1231–1237.

Fojo AT, Ueda K, Slamon DJ, Poplack DG, Gottesman MM, and Pastan I (1987). Expression of a multidrug-resistance gene in human tumors and tissues. Proc Natl Acad Sci USA 84:256–269.

Goldie JH and Coldman AJ (1985). Genetic instability in the development of drug resistance. Semin Oncol 12:222–230.

Gregorcyk S, Kang Y, Brandt D, Kolm P, Singer G, and Perry R (1996). P-glycoprotein expression as a predictor of breast cancer recurrence. Ann Surg Oncol 3:8–14.

Gussow D, Rein R, Ginjaar E, Hochstenbach F, Seemann G, Kottman A, and Plough FL (1987). The human beta 2-microglobulin gene. Primary structure and definition of the transcriptional unit. J Immunol 139:3132–3138.

He L, Hao C, Lin B, Wang Y, and Gao F (1995). P-glycoprotein expression in primary breast cancer. Clin Med Sci J 10:12–15.

Hegewisch-Becker S, Staib F, Loning T, Pichlmeier U, Kroger N, Reymann A, and Hossfeld D (1998). No evidence of significant activity of the multidrug resistance gene product in primary human breast cancer. Ann Oncol 85–93. AQ: H G,

Kane SE, Pastan I, and Gottesman MM (1990). Genetic basis of multidrug resistance of tumor cells. Bioenerg Biomembr 22:593–618.

Kartner N, Riordan JR, and Ling V (1983). Cell surface P-glycoprotein is associated with multidrug resistance in mammalian cell line. Science 221:1285–1288.

Klimecki WT, Futscher BW, Grogan TM, and Dalton WS (1994). P-glycoprotein expression and function in circulating blood cells from normal volunteers. Blood 83:2451–2458.

Leith C, Chen I, Kopecky K, Appelbaum F, Head D, Godwin J, Weick J, and Willman C (1995). Correlation of multidrug resistance (MDR1) protein expression with functional dye/drug efflux in acute myeloid leukemia by multiparameter flow cytometry: Identification of discordant MDR-/efflux+ and MDR+/efflux- cases. Blood 86:2329–2342.

Ling V, Kartner N, Sudo T, Siminovitch L, and Riordan JR (1983). Multidrug-resistance phenotype in Chinese hamster ovary cells. Cancer Treat Rep 67:869–874.

Liu B, Sun D, Xia W, Hung M-C, and Yu D (1997). Cross-reactivity of C219 anti-p170 mdr1 antibody with p185 c-erb2 in breast cancer cells: cautions on evaluating p170 mdr-1. J Natl Cancer Inst 89:1524–1529.

Loflin P and Zwelling L (1994). Topoisomerase II and the path of apoptosis. Contemp Oncol 11:46–57.

Lowe S, Bodis S, McClatchey A, Remington L, Ruley H, Fisher D, Housman D, and Jacks T (1994). p53 status and the efficacy of cancer therapy in vivo. Science 266:807–810.

Maas R, Bruning P, AJ B, B T, and HL. P (1995). Immunomagnetic purification of human breast carcinoma cells allows tumor-specific detection of multidrug resistance gene 1-mRNA by reverse transcriptase polymerase chain reaction in fine-needle aspirates. Lab Invest 72:760–764. AQ: I

Marie J, Zittoun R, and Sikic B (1991). Multidrug resistance (MDR1) gene expression in adult acute leukemias: Correlations with treatment outcome and in vitro drug sensitivity. Blood 78:586–592.

Merkel DE, Fuqua AW, Tandon AK, Hill SM, Buzdar AU, and McGuire WL (1989). Electrophoretic analysis of 248 clinical breast cancer specimens for P-glycoprotein expression or gene amplification. J Clin Oncol 7:1129–1136.

AQ: J Muss H, AD T, Berry D, Kute T, Liu E, Koerner F, Cirrincione C, Budman D, Wood W, Barcos M, and Henderson I (1994). *Cerb* B-2 expression and response to adjuvant therapy in women with node-positive early breast cancer. N Engl J Med 330:1260–1266.

Noonan KE, Beck C, Holzmayer TA, Chin JE, Wunder JS, Andrulis IL, Gazdar AF, Willman CL, Griffith B, Von Hoff DD, and Roninson IB (1990). Quantitative analysis of *MDR1* (multidrug resistance) gene expression in human tumors by polymerase chain reaction. Proc Natl Acad Sci USA 87: 7160–7164.

Pirker R, Wallner J, Geissler K, Linkesch W, Haas O, Bettelheim P, Hopfner M, Scherrer R, Valent P, Havelec L, Ludwig H, and Lechner K (1991). MDR1 gene expression and treatment outcome in acute myeloid leukemia. J Natl Cancer Inst 83:708–712.

Press MF and Greene GL (1984). An immunocytochemical method for demonstrating estrogen receptor in human uterus using monoclonal antibodies to human estrophilin. Lab Invest 50:480–486.

Press MF, Nousek-Goebl NA, and Greene GL (1985). Immunoelectron microscopic localization of estrogen receptor with monoclonal antibodies. J Histochem Cytochem 33:915–924.

Ro J, Sahin A, Ro JY, Fritsche H, Hortobagyi G, and Blick M (1990). Immunohistochemical analysis of P-glycoprotein expression correlated with chemotherapy resistance in locally advanced breast cancer. Hum Pathol 21:787–791.

Roninson IB, Abelson HT, Husman DE, Howell N, and Varshavsky A (1984). Amplification of specific DNA sequences correlates with multidrug resistance in Chinese hamster cells. Nature 309:626–628.

Sanfillippo O, Ronchi E, DeMarco C, Di-Fronzo G, and Silvestrini R (1991). Expression of P-glycoprotein in breast cancer tissue and in vitro resistance to doxorubicin and vincristine. Eur J Cancer 27:155–158.

Schneider J, Bak M, Efferth T, Kaufmann M, Mattern J, and Volm M (1989). P-glycoprotein expression in treated and untreated human breast cancer. Br J Cancer 60:815–818.

Sugawara I, Kataoka I, Morishita Y, Hamada H, Tsuruo T, Itoyama S, and Mori S (1988). Tissue distribution of P-glycoprotein encoded by a multidrug-resistant gene as revealed by a monoclonal antibody, MRK-16. Cancer Res 48:1926–1929.

Tannock IF (1987). Experimental chemotherapy. In: Tannock IF and Hill RP, editors. The basic science of oncology. New York: McGraw-Hill, Inc., 308–325.

Thiebaut F, Tsuruo T, Hamada H, Gottesman MM, Pastan I, and Willingham MC (1989). Immunohistochemical localisation in normal tissues of different epitopes in the multidrug transport protein P170: Evidence for localisation in brain capillaries and cross-reactivity of one antibody with a muscle protein. J Histochem Cytochem 37:159–164.

Tsai C-M, Chang K-T, Perng R-P, Mitsudomi T, Chen M-H, Kadoyama C, and AF G (1993). Correlation of intrinsic chemoresistance of non-small-cell lung cancer cell lines with HER-2/neu gene expression but not with ras gene mutations. J. Natl. Cancer Inst. 85:897–901. AQ: K

Veneroni S, Zaffaroni N, Daidone M, Benini E, Villa R, and Silvestrini R (1994). Expression of P-glycoprotein and in vitro or in vivo resistance to doxorubicin and cisplatin in breast and ovarian cancers. Eur J Cancer 30A:1002–1007.

Woodhouse J and Ferry D (1995). The genetic basis of resistance to cancer chemotherapy. Ann Med 27:157–167.

Yang X and Page M (1995). An Mr 7-kDa membrane protein overexpressed in human multidrug-resistant ovarian cancer cells. Cancer Lett 88:171–178.

Yang X and Page M (1996). Growth regulation of multidrug resistant ovarian cancer cells by 1D7 monoclonal antibody. Anticancer Res 16:273–276.

P7 ANTIGEN EXPRESSION IN BREAST CANCER[1]

by

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[4]The abbreviations used are: CHAPS, 3[3-cholamidopropyl) dimethyl-ammonio]-1-propanesulfonate; ECL, enhanced chemiluminecence; Mab, monoclonal antibody; PBS, phosphate buffered saline; PBST, 0.05% Tween 20 phosphate buffered saline; SDS, sodium dodecylsulfate.

Abstract

A novel 7 kDa glycoprotein (P7), originally identified in multidrug resistant ovarian and breast cancer cell lines, was expressed in breast cancer specimens as demonstrated by immunohistochemistry and Western blot analyses using a P7 specific monoclonal antibody, 1D7. P7 was expressed in breast cancer cells, but not other cells in the breast tissue. Its expression was significantly associated with the presence of distant metastasis and residual disease at the time of diagnosis in women with primary breast cancer (P=0.027, Fisher's exact test). P7 expression was also associated with patient survival in women without evidence of distant metastases or residual disease at diagnosis although this association did not reach statistical significance (p=0.06, logrank test). P7 expression was found in 13.3% of women with locally advanced breast cancer before 5-fluorouracil and radiation therapy and in 53.3% following chemotherapy (p = 0.016). Our preliminary results demonstrated that a novel 7 kDa protein had increased expression in human breast cancer following chemotherapy and radiation therapy suggesting that it could play a role in the drug resistant phenotype and in tumor progression. The specific expression of P7 on breast cancer cells makes it a potential target for novel therapies.

Introduction

Breast cancer is a major cause of morbidity and mortality in women in the United States [1]. The process of tumor progression has been extensively studied, but research has not yet effected significant change in understanding the process or in controlling tumor progression. There is a continuing need to identify new molecules that play a role in tumor development and progression.

P7 antigen, recognized by a monoclonal antibody, 1D7, was first identified in cell lines [2]. Its expression in ovarian cancer cell lines was augmented following exposure to vinblastine or adriamycin treatment *in vitro*. A similar cell line expressing high levels of P7 has been obtained from MCF-7 human breast cancer cells following treatment by doxorubicin and vinblastine [3]. P7 expression was not found in other drug-resistant human cell lines such as vinblastine-resistant CEM leukemia cells (CEM/VBL 300), and intrinsic MDR colon cancer cells, HCT15. Therefore, it appears that P7 does not represent a generalized multidrug-resistant phenotype. Previous results showed that the small molecular weight protein is involved in the proliferation of P7-positive tumor cells since Mab 1D7 specifically inhibited the growth of these cells [4]. In this study we evaluated P7 expression in human breast cancer specimens and assessed its association with disease progression.

Materials and Methods

Patient Information and Breast Cancer Specimens. The expression of P7 was investigated in two groups of women with breast cancer. The first group consisted of 64 women diagnosed with breast cancer at Los Angeles County General Hospital between 1992 and 1996 who had surgery by either modified radical mastectomy (38 patients) or lumpectomy (26 patients). None of these patients received chemotherapy or radiation treatment prior to the surgery or biopsy. The median age of the 64 patients was 53 years (range, 34 to 83 years). The median clinical follow-up was 1.94 years, and ranged from 0.38 to 3.24 years after initial surgery. Frozen specimens which were consecutively entered in the USC Breast Tissue and Tumor Bank were analyzed. The second group of women with breast cancer consisted of 15 women entered in a neoadjuvant trial of 5-fluorouracil and radiation therapy to treat locally advanced disease (protocol no. 1B-93-3). In this experimental protocol, biopsy of the breast mass was obtained prior to neoadjuvant therapy and resection of the mass was performed after therapy. Neoadjuvant treatment involved pre-operative 5-fluorouracil (200 mg/m^2) administration during radiotherapy (50 Gy) to the breast and regional lymph nodes. Eight of these 15 women had involvement of axillary lymph nodes by tumor but without evidence of distant metastasis. Breast carcinoma tissue from each case was immediately frozen and stored in liquid nitrogen. The women had a median age of 50 years (range: 31 - 64 years).

P7-positive ovarian cancer cells, OVCAR 4/ADR, were cultured as described elsewhere and used as a positive control for both immunohistochemistry and Western immunoblot [2].

1D7 Monoclonal Antibody. The production of 1D7 monoclonal antibody, which recognizes P7, has been previously described [2]. 1D7 antibody was used either as hybridoma supernatant or as purified immunoglobulin by precipitation with ammonium sulfate and DEAE-matrix chromatography.

Immunohistochemical Detection of P7 in Breast Cancer. The peroxidase anti-peroxidase immunohistochemical technique [5] was used to localize P7 in breast cancer specimens. Cryostat sections (6 μm) were prepared and fixed with 4% formaldehyde at room temperature for 5 minutes. The sections were then washed in phosphate buffered saline (PBS) and treated with 0.5% H_2O_2-PBS for 15 minutes to inactivate the endogenous peroxidase activity. 1D7 antibody (1: 5 dilution of hybridoma supernatant or 5 μg/ml of purified antibody) was applied to the sections for 1 h in 10% rabbit serum PBS. Specimens were washed in PBS, 3 times for 3 minutes each. Rabbit anti-mouse IgG bridging antibody (anti-H+L chains, ZYMED) diluted 1: 50 in 10% rabbit serum PBS was applied to the tissue section for 30 minutes. After washing mouse peroxidase antiperoxidase antibody (1: 50 dilution) was applied for 30 minutes. DAB solution (3',3-diamenobenzidine) was used to identify the sites of immunoprecipitate formation. The preparations were counterstained with ethyl green, mounted in Permount and cover-slipped. Negative controls for each sample were performed as above but with normal mouse isotype control immunoglobulins (ZYMED, S. San Francisco, CA) instead of primary antibody. The histology of each specimen was confirmed by review of one section stained with hematoxylin and eosin. All slides were evaluated by two of the authors without knowledge of the clinical data. Pattern of staining was described as either focal or diffuse when more than 20% of tumor cells were stained. Intensity of staining was scored as: 0 = no staining, 1+ = weak staining, 2+ = moderate staining, and 3+ = strong staining.

Western Blot Analysis of P7. Breast cancer tissues (about 200mg) were homogenized with a motor-driven homogenizer in 0.5 ml of ice-cold lysis buffer (50 mM This-HCL, pH 8.0 containing 0.5% CHAPS, 150 mM NaCl, 2 mM $MgCl_2$, 2 mM PMSF). The homogenates were centrifuged at 10,000g for 20 min at 4°C. Supernatants were measured by spectrophotometry at OD280 and stored at -20°C until used. Methanol-extracted proteins were similarly prepared with the following changes: breast cancer tissues were homogenized in 0.5 ml methanol and centrifuged at 10,000g for 20 min at 4°C. The supernatants were evaporated in a SpeedVac (Savant, Farmingnale, NY) until a volume of 50 µl remained. Proteins were solubilized with SDS-containing sample buffer in the presence or absence of β-mercaptoethanol. Electrophoresis was carried out using 8-25% gradient gels. After electrophoresis, the proteins were transferred to nitrocellulose paper using the PhastSystem transfer unit set at 25mAV for 9 Vh (Pharmacia, Piscataway, New York). All subsequent steps were carried out at room temperature. Nitrocellulose was washed briefly with 0.05% Tween 20 phosphate buffered saline (PBST) and blocked with 10% rabbit serum in PBST solution for one hour. The blot was then incubated for 1 h with 10 µg/ml of the monoclonal antibody 1D7 in PBS containing 10% rabbit serum PBS. After washing with PBST, the nitrocellulose blot was incubated for 1 h with peroxidase-labeled rabbit anti-mouse immunoglobulins (DACO, A/S, Denmark) diluted 1:500 in 10% rabbit serum PBS and thereafter washed extensively with PBST. The immunoreactive proteins were detected by the ECL method as recommended by the manufacturer or by revealing the band with DAB solution.

Statistical Analysis. Fisher's exact test (2 tailed) and the logrank test were used to assess the association of P7 expression with breast cancer metastasis and residual disease at diagnosis, survival, or other clinical factors. For the purpose of analysis, specimens in which, at least, 20% of tumor cells were stained (diffuse staining pattern) were considered to be positive in primary breast cancer. McNemar's test was used to assess P7 expression in locally advanced breast cancer prior to and after 5-fluorouracil therapy including focal and diffuse staining patterns.

Results

Staining Intensity Differences with Various Fixatives. Tissue sections demonstrated substantial differences in 1D7 antibody immunoreactivity with different fixatives. The strongest immunostaining was identified with either 4% formaldehyde or with acetone fixation. No antibody immunoreactivity was identified with methanol or ethanol fixation (95%). Only weak staining was seen with 50% ethanol fixation. The number of tumor cells stained with picric acid-paraformaldehyde fixation was similar to that obtained with formaldehyde and acetone fixation but the intensity of staining in individual cells was not as strong as formaldehyde fixation which was used for the immunohistochemical staining described in Materials and Methods.

P7 Expression in Primary Breast Cancer. Among the primary, untreated breast cancers, P7 was identified in 21 of 64 (33%) specimens by immunohistochemistry using P7 specific monoclonal antibody, 1D7, including both the focal (9%, 6 of 64) and diffuse pattern (23%, 15 of 64) (Figure 1A and B). The P7 protein was localized in both the cytoplasm and plasma membrane of tumor cells of infiltrating ductal and lobular carcinomas. No immunostaining was found in adjacent normal breast ductal or lobular epithelium, stromal cells, endothelial cells of blood vessels, lymphocytes or macrophages (Figure 1C). These results indicated that P7 antigen is predominantly expressed in breast cancer cells.

Association of P7 Expression with Prognostic Factors and Clinical Outcome in Primary Breast Cancer. P7 expression was significantly associated with distant metastasis or residual disease among 64 breast cancer patients at diagnosis (P=0.027, Fisher's exact test) (Table 1). This result indicated that P7 expression was associated with extent of disease at the time of diagnosis. P7 expression also had an association with the survival of breast cancer patients who had no evidence of distant metastasis and residual disease at the time of diagnosis (55 patients, P=0.0624, logrank test) although this association did not reach formal statistical significance. P7 expression in this small cohort was not significantly associated with histologic grade, tumor size, lymph node involvement, estrogen or progesterone receptor status, Her-2/*neu* oncogene expression or P53 tumor suppressor protein expression (Table 1).

P7 Expression in Locally Advanced Breast Cancer before and after Radiation and 5-Fluorouracil Therapy. In order to test whether P7 expression may be augmented after chemotherapy, 2 sequential frozen specimens, one before and one after neoadjuvant chemotherapy (see Materials and Methods) were obtained from 15 women and analyzed for P7 expression. In one of these women, P7 expression was dramatically augmented following radiation and 5-fluorouracil therapy in terms of staining intensity and the percentage of tumor cells stained (Figure 1D and E). In six of these locally advanced breast cancers in which the first sample was completely negative for P7, the specimen obtained after radiation and chemotherapy was positive for P7 expression. Overall, expression of P7 protein was identified in only two (2/15, 13.3%) breast cancers prior to 5-Fluorouracil and radiation therapy but P7 expression was identified in 8 (8/15, 53.3%) breast cancers and in a higher percentage of tumor cells per positive case after the therapies (Table 2). The change in expression was statistically significant (p=0.016, McNemar's test) even though the number of cases evaluated was small.

Western Blot Analysis of P7 Expression. To confirm that the immunohistochemical staining seen with monoclonal antibody, 1D7 was specific for P7, proteins were extracted from breast cancer tissues and subjected to Western blot analysis. Both detergent solublized breast tissue lysates (Figure 2A) and methanol-extracted materials (Figure 2B) were analyzed. An ovarian cancer cell line, OVCAR4/ADR, which overexpresses P7, was used as positive control. A *M* r 7,000 Dalton band was detected in samples from both the positive control and from breast cancers that stained positive for P7 expression by immunohistochemistry. As shown in Figure 2A, the 7 kDa band was relatively weak in breast cancer samples compared with positive control cell lines, and the signal in one case was missing in detergent-treated lysates. However, the 7 kDa bands were clearly observed in all methanol-extracted samples.

Discussion

A novel *M* r 7 kDa glycoprotein was detected in breast cancer specimens by immunohistochemistry and Western blot analyses. Twenty-one of 58 invasive ductal and lobular carcinomas were positive with 1D7, including twenty of ductal type and one of mixed ductal and lobular histology. The other breast tumors consisted of six other cases having varied histologies (mucinous, colloid, and medullary carcinomas) known to be associated with a good prognosis. None of these more favorable breast carcinoma histologic types showed any immunostaining with Mab 1D7. P7 antigen expression in primary breast cancer was associated with the presence of distant metastasis and residual disease at the time of diagnosis in this study. Therefore, P7 antigen expression in these breast cancer cells was correlated with tumor progression. Since previous results have shown that P7 is involved in the proliferation of P7-positive cells, this molecule may play a role in disease progression.

P7 expression was augmented following radiation and 5-fluorouracil therapy as revealed by immunohistochemistry for both staining intensity and percentage of positive tumor cells. The expression was increased from 13% of breast cancers to 53% of breast cancers following chemotherapy and radiation therapy. What role, if any, that this increase in P7 expression after neoadjuvant treatment might play for tumor cells needs further exploration. Because only the tumor cells expressed P7, this protein could be a potential target for immunotherapy or other targeted therapies.

Western immunoblot analysis was used to confirm immunohistochemical findings. Although P7 was identified by Western immunoblot the level of detection varied slightly with the solubilizing agent. With detergent lysates of breast cancer tissue, a relatively weak signal was identified in P7 expressing specimens. In one immunostain-positive case, the 7 kDa band was not detected by analysis of detergent-treated lysate, while it was detected in methanol or ethanol extracted tissue.

indicating that P7 antigen may specifically or non-specifically attach to a or some detergent non-soluble components of the breast tissue. Tissue sections which were fixed with methanol or ethanol showed a loose of immunostaining with 1D7 antibody suggesting that either these solvents destroyed the epitope recognized by the antibody or these hydroxy-containing solvents extracted the antigen. The Western immunoblot results demonstrated that P7 was extracted by these organic solvents.

This is the first report that the 7 kDa glycoprotein recognized by 1D7 was expressed in breast cancer tissue. Further study of the novel 7 kDa protein is warranted because its expression in breast cancer cells may provide a better understanding of the mechanisms of breast cancer cell proliferation as well as a potential new therapeutic target in treatment of breast cancer.

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References

1. World, Health Organization: World Health Statistics. Annuals, 1987-1991. Geneva: p. WHO.
2. Yang, X. and M. Page, An Mr 7-kDa membrane protein overexpressed in human multidrug-resistant ovarian cancer cells. Cancer Lett., 1995. *88*: p. 171-178.
3. Yang, X. and M. Page, Unpublished results. 1995.
4. Yang, X. and M. Page, Growth regulation of multidrug resistant ovarian cancer cells by 1D7 monoclonal antibody. Anticancer Res., 1996. *16*: 273-276.
5. Press, M., Nousek-Goebl, N., King, W., Herbst, A., G. Greene, Immunohistochemical assessment of estrogen receptor distribution in the human endometrium throughout the menstrual cycle. Laboratory Invest., 1984. *51*: 495-504.

Table 1. *Association of P7 Expression (diffuse pattern) with Baseline Clinical Characteristics among 64 Primary Breast Cancer Patients*

Factors	No. of Patients	No. of P7 Positive Case (%)	p-value[a]
Total	64	15 (23.4)	
Distant Metastasis or Residual Disease			0.027
No	55	10 (18.2)	
Yes	9	5 (55.6)	
Tumor Size			1.00
<3cm	26	6 (23.1)	
≥3cm	38	9 (23.7)	
Histologic Grade			0.514
2	15	5 (33.3)	
3	41	10 (24.4)	
Unknown	8	0	
Lymph Nodes			1.000
Negative	36	8 (22.2)	
Positive	26	6 (23.1)	
Unknown	2	1	
Stage			0.279
I/II	43	7 (16.3)	
III/IV	15	5 (33.3)	
Unknown	6	3	
Histologic Diagnosis			0.322
IDC+LC	58	15 (25.9)	
Other	6	0 (0)	
Her 2/Neu			0.346
<++	41	11 (26.8)	
≥++	21	3 (14.3)	
Unknown	2	1	
P53			1.000
Negative	50	12 (24.0)	
Positive	10	2 (20.0)	
Unknown	4	1	
ER			0.419
Negative	19	2 (10.5)	
Positive	22	5 (22.7)	
Unknown	23	8	
PR			0.419
Negative	19	2 (10.5)	
Positive	22	5 (22.7)	
Unknown	23	8	

[a]Based on Fisher's exact test.

Table 2. *P7 Expression before and after Radiation and 5-FU Therapy among 16 Patients in Locally Advanced Breast Cancer*

Patients	P7 Expression (Intensity and Percentage)	
	Before	After
No. 1	++, 10	++, 10
No. 2	-, 0	-, 0
No. 3	-, 0	-, 0
No. 4	-, 0	+++, 80
No. 5	-, 0	-, 0
No. 6	-, 0	-, 0
No. 7	++, 40	+++, 100
No. 8	-, 0	++, 20
No. 9	-, 0	-, 0
No. 10	-, 0	+++, 100
No. 11	-, 0	++, 2
No. 12	-, 0	-, 0
No. 13	-, 0	+, 40
No. 14	-, 0	-, 0
No. 15	-, 0	++, 80

Figure Legend.

Figure 1. Immunohistochemical analysis of P7 antigen expression in primary breast cancer (A, B and C) and locally advanced breast cancer (D and F). P7 was detected on the cell membranes and in the cytoplasm of breast carcinoma cells by peroxidase antiperoxidase immunohistochemistry using monoclonal antibody, 1D7 (A and B). Only breast cancer cells show immunostaining, not other cells in the breast tissue section, including stromal cells, lymphocytes and/or macrophages (Original magnification, X100; B, original magnification X200). Benign breast epithelium including ducts and lobules (C) showed no P7 immunostaining. P7 expression was increased in breast cancer cells with regard to both staining intensity and percentage of tumor cells stained when immunostained before and after radiation therapy and 5-fluorouracil chemotherapy were compared in women with locally advanced breast cancer, indicating increased P7 expression following treatment (D and E, before and after, respectively). Original magnifications, X200.

Figure 2. Western blot analysis of P7 expression in primary breast cancer with detergent-treated breast tissue lysates (A) and methanol-extracted proteins (B). A. Both ovarian cancer cells (lane 1) and primary breast cancer (lane 2) showed the 7 kDa band, but one P7 positive breast cancer demonstrated by immunohistochemistry did not show the 7 kDa band (lane 3). B. Biotinylated protein markers (lane 1) were run on the same gel with both ovarian cancer cells (lane 2) and primary breast cancers (lanes 3 and 4). Note the presence of the 7 kDa band in all lanes, including the case (lane 4) that lacked a 7 kDa band in detergent lysate. Arrow indicates the 7 kDa band.