Eur J Cancer, Vol. 27, No. 2, pp. 155–158, 1991.
Printed in Great Britain

0277–5379/91 $3.00 + 0.00


# Expression of P-glycoprotein in Breast Cancer Tissue and *in vitro* Resistance to Doxorubicin and Vincristine

O. Sanfilippo, E. Ronchi, C. De Marco, G. Di Fronzo and R. Silvestrini

**Expression of P-glycoprotein was evaluated by C219 monoclonal antibody immunoblots in 34 previously untreated and 14 pretreated breast cancers and in benign breast lesions or histologically normal breast glands. P-glycoprotein was not detectable in the few cases of normal or benign tissue. P-glycoprotein was expressed in the 170 kD areas of 29% (10/34) of untreated and 64% (9/14) of previously treated tumours ($P=0.02$). In treated tumours, high intensity expression was observed more frequently than in untreated breast cancer (40% vs. 9%). Moreover, there was a significant association between P-glycoprotein expression and *in vitro* resistance to doxorubicin and vincristine. Simultaneous resistance was observed in all of the P-glycoprotein positive and in only 56% of the P-glycoprotein negative tissues ($P<0.01$). Some aspects of the typical multidrug resistant phenotype, such as P-glycoprotein expression and simultaneous resistance to doxorubicin and vincristine, could be detected in small subsets of breast cancer patients. No relation between P-glycoprotein expression and the type of previous clinical treatment was observed.**
*Eur J Cancer*, Vol. 27, No. 2, pp. 155–158, 1991.

## INTRODUCTION

MANY MECHANISMS of resistance to anthracyclines, antitumour antibiotics and other natural products have been described. Membrane-mediated drug removal by P-glycoprotein or intracellular inactivation via the increased level of glutathione-S-transferase may be important in tumours that originate from tissues involved in detoxification processes, such as digestive tract or kidney neoplasms [1, 2]. Altered activities of topoisomerase II [3] also seem to be important in drug resistance. This kind of resistance has been called atypical multidrug resistance (MDR) since it involves most of the drugs included in MDR but is not related to P-glycoprotein gene amplification, to overexpression or to reduced drug accumulation [4]. As more information about these mechanisms in experimental cell lines has become available, great effort has been devoted to establishing their involvement in natural or acquired clinical MDR. Identification of the main mechanisms of resistance in subsets of patients should help to clarify how the mechanisms explored in experimental systems act in human tumours *in vivo* and to design biologically logical therapies.

The search for P-glycoprotein gene amplification, for overexpression and for increased gene product in human tumour biopsy specimens has revealed the importance of the P-glycoprotein mediated mechanism in digestive tract and kidney tumours [5]. The results for breast cancer are less consistent. The absence of MDR gene amplification or overexpression in a large series of breast cancers seems to exclude the involvement of this mechanism in resistance to doxorubicin [6]. On the contrary, increased P-glycoprotein levels have been observed in tumours *in vitro* or in tumours clinically resistant to doxorubicin [7–9].

In our study, we looked for P-glycoprotein expression in 48 fresh breast cancers by immunoblotting with C219 monoclonal antibody (Mab). P-glycoprotein expression was analysed in relation to pretreatment status and to *in vitro* resistance to doxorubicin and vincristine. Moreover, we attempted to analyse the expression and molecular mass of P-glycoprotein in breast compared with digestive tract cancers.

## MATERIALS AND METHODS

### *Chemicals*

The following solutions were used: phosphate-buffered saline, Tris-HCl buffer with 250 mmol/l sucrose and Tris-saline buffer (TS). Sheep ($^{125}$I)-anti-mouse immunoglobulin (Ig) (whole antibody), [methyl-$^{3}$H]thymidine (18.5 GBq/mmol) and [5-$^{3}$H]uridine (93-T11 GBq/mmol) were obtained from Amersham International. Bovine serum albumin fraction V and Nonidet P40 were purchased from Sigma. Unlabelled C219 and CH$^{R}$C5 cell membrane preparations were obtained from Cis Diagnostici (Santhia, Italy). All other products were analytical grade.

### *Cell lines and human tissues*

The CH$^{R}$C5 cell line was used as positive control. The MCF7 breast cancer line was used as a negative control and its MCF7/Dx subline, which is permanently 50 times more resistant to doxorubicin without *in vitro* treatment, as a second tissue-specific positive control. P-glycoprotein expression was looked for in surgical biopsy samples from 34 previously untreated and 14 previously treated primary breast cancers. Among the latter, 5 had been treated with 3 cycles of cyclophosphamide-methotrexate-5-fluorouracil (CMF), 7 with 3–4 cycles of 5-fluorouracil/doxorubicin/cyclophosphamide (FAC), 1 with 6 cycles of 4'-iododoxorubicin and 1 with high-dose chemotherapy including doxorubicin. Some mammary glands from microscopically nor-

Correspondence to R. Silvestrini.
O. Sanfilippo, E. Ronchi, C. De Marco and R. Silvestrini are at the Oncologia Sperimentale C, Via Venezian 1, 20133 Milano; and G. Di Fronzo is at the Centro di Studio sulla Patologia Cellulare del CNR, Milano, Italy.
Presented in part at the annual meeting of the EORTC Clonogenic Assay Screening Study Group, June 1990, Glasgow.
Revised 7 Nov. 1990; accepted 19 Nov. 1990.

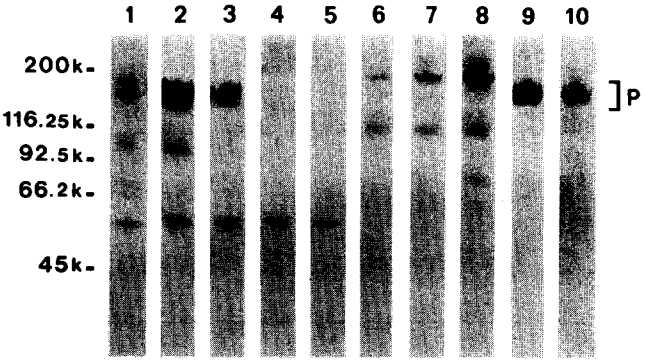


**Fig. 1. Representative western blot of purified membranes from cell lines and fresh human tissues. Lanes 1 and 2 = 1 μg and 15 μg membrane proteins from CH$^R$C5 cell line; lanes 3 and 4 = 100 μg proteins from MCF7/Dx and MCF7 cell lines; lanes 5–8 = 100 μg proteins from P-glycoprotein negative, weakly positive, strongly positive and maximally positive breast cancers, respectively; and lanes 9 and 10 = 100 μg protein from liver and colon cancers. P = P-glycoprotein.**

mal peritumoral tissue and benign mammary lesions were also analysed. In addition, 12 previously untreated intestinal and 4 primary liver cancers were studied.

Representative samples were examined histologically to assure the predominance of tumour cells over stromal cells in the samples used for P-glycoprotein analysis.

*P-glycoprotein detection*

Purified membrane preparations were obtained from cell lines and solid tissues [10]. Membrane proteins (100 μg from human tissues and MCF7 lines and 1–15μg from CH$^R$C5 line) were separated by sodium dodecyl sulphate/polyacrylamide gel electrophoresis and transferred to nitrocellulose by electroblot. Western blotting was done as described [10] with a slight modification: after transferring the proteins, the nitrocellulose sheets were probed with murine C219 against P-glycoprotein and directly labelled after washes with ($^{125}$I)-antimouse Ig (300–500 × $10^3$ cpm/ml in TS) for 1.5 h at room temperature with rocking. This modification provides higher specificity of apparent labelling and increased sensitivity. The threshold of 0.74 absorbance units (AU) (by densitometric analysis of autoradiograms), corresponding to 1 μg CH$^R$C5 membrane proteins and to the lowest limit of linearity of the concentration absorbance plot, was used as a cut-off for strong P-glycoprotein positivity.

*In vitro resistance*

The sensitivity of the tumours to inhibition by doxorubicin and vincristine was evaluated by a metabolic assay [11]. Fragments of about 1 mm$^3$ sampled from different areas of the tumour were treated *in vitro* with the drugs for 3 h. At the end of the second hour, $^3$H-thymidine was added. Drug activity was expressed as the ability to inhibit the incorporation of labelled precursor. A tumour was considered to be sensitive when the inhibition was greater than 20%, which represents the median coefficient of variation of incorporation in untreated samples [11].

*In vitro* chemosensitivity to doxorubicin and/or vincristine was measured for 35 breast cancers in a total of 68 assays.

*Statistical analysis*

The significance of differences between rates of P-glycoprotein positive and negative tumours for the different groups was analysed by Fisher's exact test. The Spearman test for unpaired samples was used to analyse the ranking of absorbance values in the different groups. The association between P-glycoprotein expression and resistance to one or both drugs was tested by the kappa test [12].

*Table 1. Expression of P-glycoprotein in breast tissues**

| | C219 Mab reaction† | |
|---|---|---|
| | Positive | Strong‡ |
| Healthy gland and benign lesions ($n$=5) | 0 | 0 |
| Untreated cancers ($n$=34) | 10 | 2 (0.76–0.85) |
| Pretreated cancers ($n$=14) | 9 | 6 (0.74–2.53) |

*For untreated vs. pretreated cancers, $P$=0.02 and $P$=0.004 for positive and strong reaction, respectively.
†Evaluated for 100 μg cell membrane protein.
‡AU (range). Strong = equal to or higher than the 0.74 AU for 1 μg CH$^R$C5 cell membrane protein.

## RESULTS

Immunoreaction to C219 Mab was analysed in mammary tissues of different clinical status, including microscopically healthy tissue from cancer patients, benign lesions and both untreated and previously treated breast cancers. Representative blots of fresh human breast cancers with different banding intensities are shown in Fig. 1.

As shown in Table 1, there were no positive reactions in the 5 samples from healthy glands or benign lesions. There was an appreciable 170 kD band in about 30% of previously untreated tumours and in more than 60% of pretreated tumours. Moreover, quantitative analysis of the absorbance of 170 kD bands showed different frequencies of strong reactions in the two groups: 2 of 10 untreated and 6 of 9 pretreated cancers had strongly positive bands. The frequency of positive tumours and the 170 kD absorbance were significantly higher in previously treated than in untreated tumours, whether or not the treatment had included doxorubicin or other drugs known to be involved in MDR.

We compared the expression of P-glycoprotein in previously untreated breast cancers with that in previously untreated colorectal and liver tumours. Qualitative analysis showed smaller P-glycoprotein molecular mass, in the 145 kD area, for digestive tract tumours than in breast cancer (Fig. 1). Moreover, in intestinal tumours, P-glycoprotein was expressed in 75% of cases, but generally with low intensity (Table 2). All liver

*Table 2. Expression of P-glycoprotein in tumours from different organs**

| | C219 Mab reaction | | |
|---|---|---|---|
| | Positive | Strong | Median AU (range) |
| Breast ($n$=34) | 10 | 2 | 0.46 (0.24–0.85) |
| Gut ($n$=12) | 9 | 2 | 0.30 (0.21–2.15) |
| Liver ($n$=4) | 4 | 3 | 3.08 (0.34–4.80) |

*For breast vs. gut and liver, $P$<0.01 for positive and strong reaction.

tumours were positive with strong reactions in 3 cases (Table 2). Overall, the frequency of P-glycoprotein positivity was significantly higher for digestive tract cancers than for breast cancers.

*P-glycoprotein expression and in vitro resistance to doxorubicin and vincristine*

For 35 breast cancers, P-glycoprotein expression was analysed in relation to resistance to doxorubicin and vincristine, defined as inability of these drugs to inhibit significantly $^3$H-thymidine incorporation into DNA. Inhibition of nucleic acid precursor incorporation has proven to be important as an indicator of clinical responsiveness for these drugs [11].

*In vitro* sensitivity to doxorubicin or vincristine was always accompanied by lack of P-glycoprotein expression. Conversely, P-glycoprotein was detected in only about one-third of tumours resistant to doxorubicin (10/27) or to vincristine (10/29) (Fig. 2).

Simultaneous resistance to both drugs was observed in 68% of cases and was found in all 10 tumours that expressed P-glycoprotein and in 14 of the 25 P-glycoprotein negative tumours ($P<0.01$). Tumours that were sensitive to either drug did not express P-glycoprotein (Table 3).

*Table 3. Expression of P-glycoprotein and in vitro resistance to doxorubicin and vincristine in breast cancer*

| C219 Mab reaction | *In vitro* resistance to | |
|---|---|---|
| | Both drugs | Either drug |
| Positive | 10 | 0 |
| Negative | 14 | 11 |

$P<0.01$.

## DISCUSSION

The involvement of MDR amplification or the expression of its coded P-glycoprotein in natural or acquired clinical resistance has been widely investigated [13, 14]. Low to intermediate expression of MDR gene has been seen by blotting analysis [5] and confirmed by immunocytochemical staining with different monoclonal antibodies in untreated breast cancer [7, 8]. Enhanced expression of the gene has been consistently observed in tumours clinically or *in vitro* resistant to doxorubicin [7–9, 15].

We chose to analyse the gene product because of experimental evidence of the direct involvement of the glycoprotein on drug efflux, which is associated with the MDR phenotype, and because of the lack of a direct relation between gene amplification or overexpression and P-glycoprotein levels in cell lines [16].

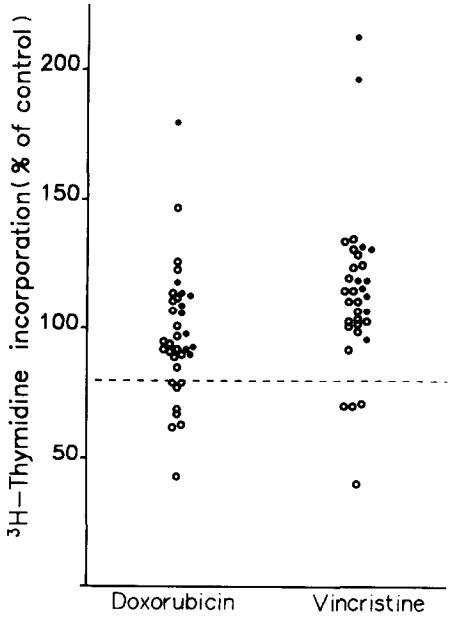


**Fig. 2. *In vitro* sensitivity to drugs in P-glycoprotein positive or negative breast cancers. Broken line indicates cut-off inhibition for sensitivity. Closed symbols = P-glycoprotein positive and open symbols = P-glycoprotein negative tumours.**

The molecular mass of the C219-reacting antigen was consistently 170 kD in breast cancer and 145 kD in digestive tract tumours. This difference could be due to a different protein glycosylation in the two tumours. P-glycoprotein was detectable in about one third of previously untreated tumours, though generally at low levels. In fact, positivity of the 170 kD band only occasionally exceeded the lowest level of sensitivity of the method.

P-glycoprotein expression was detected in a significantly higher percentage of breast cancers, and even more so following chemotherapy, perhaps due to selection of initially drug-resistant cells. These results are in agreement with those reported by other investigators who used immunocytochemical [7, 8] or northern blotting [9], whereas they are not in agreement with the findings for a large series of breast cancers obtained with several blotting methods [6]. The amount of the tumour samples used for western blotting and the degree of cell membrane purification could explain some of the disagreement.

Expression was enhanced whether or not the clinical protocols had included drugs known to be involved in MDR. This indicates that enhancement of P-glycoprotein expression is not related to specific selectivity processes in our series. However, it must be emphasised that the treatment of our series was only moderately intensive compared with the highly intensive treatment needed to evoke specific selection processes in experimental systems.

Moreover, since C219 Mab cannot distinguish between the MDR1 and MDR2 gene products [17], we cannot draw any definite conclusion about which is the major gene in the series we studied and, hence, about the involvement of the P-glycoprotein in functional or in more specifically MDR-related mechanisms.

Our results showed that expression of P-glycoprotein was always associated with *in vitro* resistance to doxorubicin and vincristine. Conversely, not all P-glycoprotein negative tumours were drug sensitive, and therefore P-glycoprotein expression should not be considered to be the only requisite for simultaneous resistance to MDR involved drugs, as demonstrated in experimental systems [1–4].

However, identification through P-glycoprotein detection of a subgroup of previously untreated breast cancers that are resistant to MDR drugs should be very important for designing therapeutic regimens on a biological basis. One such clinical application arises from the results of Dalton *et al.* [18] on the antagonising effects of verapamil on MDR in multiple myeloma and also since many well characterised, perhaps less toxic,

substances that can modulate the MDR phenotype have become available [19, 20].

Long follow-up might also increase our knowledge of the prognostic importance of P-glycoprotein positivity in breast cancer, as already demonstrated by Chan *et al.* [21] for soft tissue sarcoma of childhood.


1. Kramer RA, Zakher J, Kim G. Role of glutathione redox cycle in acquired and de novo multidrug resistance. *Science* 1988, **241**, 694–697.
2. Gottesman MM. Multidrug resistance during chemical carcinogenesis: a mechanism revealed. *J Natl Cancer Inst* 1988, **17**, 1352–1353.
3. Wolverton JS, Danks MK, Schmidt CA, Bect WT. Genetic characterization of the multidrug-resistant phenotype of VM-26-resistant human leukemic cells. *Cancer Res* 1989, **49**, 2422–2426.
4. Beck WT, Cirtain MC, Danks MK, *et al.* Pharmacological, molecular and cytogenetic analysis of "atypical" multidrug-resistant human leukemic cells. *Cancer Res* 1987, **47**, 5455–5460.
5. Goldstein LJ, Galski H, Fojo A, *et al.* Expression of a multidrug resistance gene in human cancers. *J Natl Cancer Inst* 1989, **81**, 116–124.
6. Merkel DE, Fuqua SAW, Tandon AK, Hill SM, Buzdar AU, McGuire WL. Electrophoretic analysis of 248 clinical breast cancer specimens for gene amplification. *J Clin Oncol* 1989, **7**, 1129–1136.
7. Salmon SE, Grogan TM, Miller T, Scheper R, Dalton WS. Prediction of doxorubicin resistance in *in vitro* in myeloma, lymphoma, and breast cancer by P-glycoprotein staining. *J Natl Cancer Inst* 1989, **81**, 696–701.
8. Schneider J, Bak M, Efferth TH, Kaufmann M, Mattern J, Volm M. P-glycoprotein expression in treated and untreated human breast cancer. *Br J Cancer* 1989, **60**, 815–818.
9. Keith WN, Stallard S, Brown R. Expression of mdr1 and gst-$\pi$ in human breast tumours: comparison to *in vitro* chemosensitivity. *Br J Cancer* 1990, **61**, 712–716.
10. Ronchi E, Sanfilippo O, Di Fronzo G, *et al.* Detection of the 170 kDa P-glycoprotein in neoplastic and normal tissues. *Tumori* 1989, **75**, 542–546.
11. Daidone MG, Silvestrini R, Sanfilippo O, Zaffaroni N, Varini M, De Lena M. Reliability of an *in vitro* short-term assay to predict the drug sensitivity of human breast cancer. *Cancer* 1985, **56**, 450–456.
12. Flyss JL. *Statistical Methods for Rates and Proportions*. Wiley Series in Probability and Mathematical Statistics, New York, Wiley, 1973.
13. Juranka PF, Zastawny RL, Ling V. P-glycoprotein: multidrug-resistance and a superfamily of membrane-associated transport proteins. *FASEB* 1989, **3**, 2583–2592.
14. Deuchars KL, Ling V. P-glycoprotein and multidrug resistance in cancer chemotherapy. *Semin Oncol* 1989, **16**, 156–165.
15. Gottesman MM, Goldstein LJ, Buggerman, *et al.* Molecular diagnosis of multidrug resistance. *Cancer Cells* 1989, **7**, 75–80.
16. Bradley G, Naik M, Ling V. P-glycoprotein expression in multidrug-resistant human ovarian carcinoma cell lines. *Cancer Res* 1989, **49**, 2790–2796.
17. Georges E, Bradley G, Gariepy J, *et al.* Detection of P-glycoprotein isoforms by gene specific monoclonal antibodies. *Proc Natl Acad Sci USA* 1990, **87**, 152–156.
18. Dalton WS, Grogan TM, Meetzer PS, *et al.* Drug-resistance in multiple myeloma and non-Hodgkin's lymphoma: detection of P-glycoprotein and potential circumvention by addition of verapamil to chemotherapy. *J Clin Oncol* 1989, **7**, 415–424.
19. Zamora JM, Pearce HL, Beck WT. Physical-chemical properties showed by compounds that modulate multidrug resistance in human leukemic cells. *Mol Pharmacol* 1988, **33**, 454–462.
20. Friche E, Buhl Jensen P, Roed H, Skovsgaard T, Nissen NI. *In vitro* circumvention of anthracycline-resistance in Ehrlich ascites tumour by anthracycline analogues. *Biochem Pharmacol* 1990, **39**, 1721–1726.
21. Chan HSL, Thorner PS, Haddad G, Ling V. Immunohistochemical detection of P-glycoprotein: prognostic correlation in soft tissue sarcoma of childhood. *J Clin Oncol* 1990, **8**, 689–704.


**Acknowledgements**—We thank Dr S. Catania from Ospedale V. Buzzi, Milan for supplying all the tumour material, R. Motta for technical assistance and R. Ballarini for manuscript preparation.

*Eur J Cancer, Vol. 27, No. 2, pp. 158–161, 1991.*
*Printed in Great Britain*

*0277–5379/91 $3.00 + 0.00*


# Polymorphic Oxidation of Debrisoquine in Lung Cancer Patients

**Julio Benítez, José M. Ladero, Carlos Jara, Juan A. Carrillo, Jesús Cobaleda, Adrián Llerena, Emilio Vargas and Juan J. Muñoz**

**Oxidative polymorphism of debrisoquine (DBQ) was assessed in 84 patients (81 male) with histologically proven bronchogenic carcinoma and in 143 healthy male smokers. 80 (95%) patients and 133 (93%) controls, with a metabolic ratio (MR) below 12.6, were classified as extensive metabolisers of DBQ (no significant difference between patients and controls). Only 1 of the 73 patients with epidermoid or microcytic carcinomas was classified as a poor metaboliser (PM) ($P = 0.031$ compared with controls). 63 patients (75%) and 110 controls (77%) showed a very fast oxidative rate, with MR values under 1 (not significant). The EM phenotype of DBQ might be a secondary genetic risk factor for developing bronchogenic carcinoma in male smokers.**
*Eur J Cancer*, Vol. 27, No. 2, pp. 158–161, 1991.

## INTRODUCTION

ONLY A small percentage of smokers eventually develop lung cancer. This peculiar susceptibility could be due to a genetic predisposition based upon differences in enzymatic activation (or inactivation) of carcinogens, a possibility first suggested by Kellerman *et al.* [1] when they detected a higher inducibility of aryl-hydrocarbon hydroxylase in lymphocytes of patients with bronchogenic carcinoma than in smoker controls. This finding has not been universally accepted [2], but some years later Idle *et al.* [3] found an excess of extensive metabolisers (EM) of debrisoquine (DBQ) among patients with various types of cancer compared with controls. The same group reported a tendency towards higher rates of DBQ hydroxylation in two different series of lung cancer patients than in the general population