View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by DSpace at VU

Int. J. Cancer: **71,** 787–795 (1997)
© 1997 Wiley-Liss, Inc.

Publication of the International Union Against Cancer
Publication de l'Union Internationale Contre le Cancer

EXPRESSION OF DRUG RESISTANCE PROTEINS IN BREAST CANCER, IN RELATION TO CHEMOTHERAPY

Sabine C. LINN[1], Herbert M. PINEDO[1], Jannette VAN ARK-OTTE[1], Paul VAN DER VALK[2], Klaas HOEKMAN[1], Aafke H. HONKOOP[1], Jan B. VERMORKEN[1] and Giuseppe GIACCONE[1,*]

[1]*Department of Medical Oncology, Free University Hospital, Amsterdam, The Netherlands*
[2]*Department of Pathology, Free University Hospital, Amsterdam, The Netherlands*

Drug resistance plays an important role in chemotherapy failure in breast cancer. We studied the expression of MDR1, MRP, LRP, DNA topoisomerases, p53 and Ki-67 in different groups of breast cancer patients in relation to chemotherapy. Tissues from 6 normal breasts and 20 primary operable, 40 locally advanced and 10 anthracycline-resistant metastatic breast cancers were assessed. Sequential samples of the same patient were available from 17 patients with locally advanced breast cancer undergoing neo-adjuvant chemotherapy and in 7 metastatic patients undergoing paclitaxel treatment. Protein expression was investigated by immunohistochemistry. Significantly higher protein expression was observed for Pgp, Ki-67 and p53 in the locally advanced breast cancers than in primary operable breast cancers. No other significant differences in protein expression were found among the 3 breast cancer groups. Expression of none of the markers that could be assessed (Pgp, MRP, LRP, p53 and Ki-67) in locally advanced breast cancer had predictive value for pathological response. Interestingly, after chemotherapy a significant decrease in percentage of Ki-67 positive tumor cells was observed, whereas the other markers did not vary substantially. Furthermore, considering all breast cancer samples, a cumulative dose of doxorubicin >400 mg/m² inversely correlated with Ki-67 positivity. However, 2 patients with a pathological complete remission had only 5–10% Ki67-positive tumor cells before chemotherapy, indicating that Ki67 negativity itself is not responsible for chemoresistance. In conclusion, none of the known proteins related to multidrug resistance predicted response to chemotherapy in breast cancer, and resistant clones left behind generally had a low proliferation rate. *Int. J. Cancer* 71:787–795, 1997.
© 1997 Wiley-Liss, Inc.

Breast cancer is the most common malignancy among women in the Western world (Harris *et al.,* 1993). Chemotherapy represents a major treatment modality of various stages of this disease. Despite radical surgery, approximately 50% of resected patients eventually relapse. In these patients adjuvant systemic treatment (chemotherapy and hormone therapy) prolongs long-term survival (Early Breast Cancer Trialists' Collaborative Group, 1992); nevertheless, 35% of patients die from the disease. When surgery is not feasible or is too mutilating, such as in locally advanced breast cancer, neo-adjuvant chemotherapy can achieve high remission rates (70–90% clinical response rate) and allow adequate local control afterwards (generally surgery followed by radiation); the 5-year survival for stage IIIB patients remains, however, below 50% (Harris *et al.,* 1993). For patients who develop distant metastases, first-line combination chemotherapy is generally effective, with a 50–70% response rate (Porrka *et al.,* 1994). Response rates decline to 20–30% in second-line chemotherapy, and all metastatic patients eventually die from resistant disease.

The presence *ab initio* or the development of cell clones which are resistant to drugs is thought to be responsible for the failure of chemotherapy. Little is known about the causes of drug resistance in breast cancer. Most available studies investigated the role of Pgp in this disease. A correlation has been observed between Pgp expression in breast cancer cells obtained from patients and *in vitro* resistance to doxorubicin (Sanfilippo *et al.,* 1991; Keith *et al.,* 1990; Salmon *et al.,* 1989). Furthermore, high Pgp expression in 17 locally advanced breast cancer patients was associated with the lack of response to neo-adjuvant chemotherapy (doxorubicin, vincristine, cyclophosphamide and 5-fluorouracil) and a shorter disease-free survival (Verelle *et al.,* 1991).

Pgp acts as an energy-dependent drug efflux pump (van Kalken *et al.,* 1991), and belongs to the ATP-binding cassette transporter superfamily, like the more recently identified MRP (Cole *et al.,* 1992). Like Pgp, MRP can also function as a plasma membrane drug-efflux pump (Zaman *et al.,* 1994), and its overexpression has been associated with increased ATP-dependent glutathione S-conjugate transport (Müller *et al.,* 1994). Patterns of cross-resistance *in vitro* vary slightly between Pgp and MRP overexpressing cells, and interestingly, paclitaxel does not appear to be a substrate for MRP (Zaman *et al.,* 1994; Müller *et al.,* 1994).

Other mechanisms have been found to cause similar multidrug resistance patterns in cancer cell lines. In several resistant cell lines selected by exposure to MDR drugs, the overexpression of a new protein has been discovered, which is independent of Pgp expression. This protein has been called LRP (lung resistance protein), originally indentified in a lung cancer multidrug-resistant line (Scheper *et al.,* 1993). The *LRP* gene maps proximal to the *MRP* gene on the short arm of chromosome 16, and its product has been demonstrated to be the major human vault protein (Scheffer *et al.,* 1995). Given the cellular localization of the vaults in multi-subunit organelles, associated with cytoplasmic vesicle structures and nuclear pore complexes, a function in nucleo-cytoplasmic transport of a wide variety of substrates has been suggested (Scheffer *et al.,* 1995).

DNA topoisomerase II, being the target of anthracyclines and epipodophyllotoxins, can also be involved in causing MDR: decreased topo IIα expression, decreased activity or an altered function of the enzyme can lead to a MDR multidrug-resistant phenotype *in vitro,* that is distinct from the classical MDR phenotype (Giaccone, 1994).

It has been suggested that mutant p53 may be responsible for broad resistance to cytotoxic drugs, as wild-type p53 is required for the efficient activation of apoptosis following treatment with anticancer drugs (Lowe *et al.,* 1993, 1994). The *p53* tumor suppressor gene regulates genomic stability (Greenblatt *et al.,* 1994), and inactivation of its product by several mechanisms, including point mutation, gene deletion, sequestration by mdm-2 and binding to proteins encoded by DNA tumor viruses, is at

---

Abbreviations: Abs, antibodies; AML, acute myelogenous leukemia; APAAP, alkaline phosphatase/monoclonal anti-alkaline phosphatase; ATP, adenosine triphosphate; G-CSF, granulocyte colony-stimulating factor; IHC, immunohistochemistry; LRP, lung resistance protein; mAb, monoclonal antibody; MDR, multidrug resistance; MRP, multidrug resistance-associated protein; PBS, phosphate-buffered saline; Pgp, P-glycoprotein; topo, topoisomerase.

*Correspondence to: Department of Medical Oncology, Free University Hospital, P.O. Box 7057, 1007 MB Amsterdam, The Netherlands. Fax: +31-20 4444355.

Received 6 December 1996

TABLE I – ANTIBODY CHARACTERISTICS

Ab	Directed against	Vendor/donation	Mono/polyclonal Ab	Host species	Ab class	Dilution
JSB-1	Pgp (MDR1)	Gift of Dr. R.J. Scheper (Amsterdam, The Netherlands)	Monoclonal	Mouse	IgG_1	1:50
C219	Pgp (MDR1/3)	ITK diagnostics, Uithoorn, The Netherlands	Monoclonal	Mouse	IgG_{2b}	1:100
MRPr1[1]	MRP	Gift of Dr. M.J. Flens (Amsterdam, The Netherlands)	Monoclonal	Rat	IgG_{2a}	1:100 (N_2) 1:200 (paraffin)
LRP56[2]	LRP	Gift of Dr. R.J. Scheper	Monoclonal	Mouse	IgG_{2b}	1:500 (N_2) 1:100 (paraffin)
DO-7	p53	Dako, Glostrup, Denmark	Monoclonal	Mouse	IgG_{2b}	1:500
Ki-67	Ki-67 antigen	Dako, Glostrup, Denmark	Monoclonal	Mouse	IgG_1	1:10 (N_2)
Ki-67	Ki-67 antigen	Dako, Glostrup, Denmark	Polyclonal	Rabbit		1:100 (paraffin)
6G2[3]	Topo IIα	Gift of Dr. G. Astaldi-Ricotti (Pavia, Italy)	Monoclonal	Mouse	IgG_1	1:50
8F8[3]	Topo IIβ	Gift of Dr. G. Astaldi-Ricotti	Monoclonal	Mouse	IgG_{2a}	1:25
6B5[3]	Topo I	Gift of Dr. G. Astaldi-Ricotti	Monoclonal	Mouse	IgG_{2a}	1:25

[1]Flens *et al.* (1994).–[2]Scheper *et al.* (1993).–[3]Negri *et al.* (1992).

present the most common event identified in human cancers. In breast cancer, p53 is often mutated (Greenblatt *et al.,* 1994).

Our study was undertaken to investigate the expression of 8 proteins, a number of which are involved in broad resistance to drugs, in different groups of patients with breast cancer. In particular, we compared protein expression levels in sequential samples of locally advanced breast cancer patients undergoing neo-adjuvant chemotherapy, and in serial biopsies of anthracycline-resistant, metastatic breast cancer patients undergoing chemotherapy with paclitaxel.

MATERIAL AND METHODS

Patient material

Normal breast tissue and tumors from 3 groups of breast cancer patients were used in our study: primary operable, locally advanced and metastatic anthracycline-resistant. Normal breast tissue was obtained from 6 women who underwent cosmetic surgery. Twenty primary operable breast cancers were snap-frozen in liquid nitrogen during operation and stored at −70°C until use.

Locally advanced breast cancer. Material was obtained from 40 locally advanced breast cancer patients, enrolled in a trial of high-dose neo-adjuvant chemotherapy consisting of doxorubicin (90–100 mg/m^2) and cyclophosphamide (1 g/m^2) on day 1, followed by 250 µg/m^2/day GM-CSF s.c. or i.v. on days 2–11. Cycles were repeated every 3 weeks (20 patients had 6 cycles, 14 patients 5 cycles, 4 patients 4 cycles, 1 patient 3 cycles and 1 patient 1 cycle). Patients who responded to the treatment underwent mastectomy and radiotherapy (4,005 cGy in 15 fractions) (Hoekman *et al.,* 1991). Of 40 patients entered into the study, 17 had adequate material for evaluation before and after neo-adjuvant chemotherapy. Most tumor tissues before and after neo-adjuvant chemotherapy were formalin-fixed and paraffin-embedded. Furthermore, for 14 patients only mastectomy material after chemotherapy was available because they were referred from other institutes and had only a diagnostic cytologic needle aspiration before chemotherapy. In 9 cases only tumor material biopsied before chemotherapy was assessable, because 1 patient received only radiotherapy after neo-adjuvant chemotherapy, 7 patients had a pathological complete remission of the primary tumor (3 patients had only a few tumor cells left in the axillary lymph nodes) and 1 patient did not have enough tumor cells left to evaluate staining results.

Anthracycline-resistant breast cancer. Tumor tissue was obtained from 10 anthracycline-resistant patients, who participated in a study of high-dose paclitaxel (3 hr infusion of 250 mg/m^2 every 3 weeks, with G-CSF s.c. days 2–19) (Vermorken *et al.,* 1995). Anthracycline-resistance was defined as progression on an anthracycline-containing regimen, having received at least 2 cycles. All patients gave informed consent to have skin metastases biopsied within 2 weeks prior to the first paclitaxel cycle and again directly after progression on paclitaxel. One patient who did not fulfill the definition of anthracycline-resistance was, however, entered into the study because she had received the maximum cumulative dose of anthracyclines. This patient and 6 others in this group had sequential sampling before and after paclitaxel therapy. Tissues were snap-frozen in liquid nitrogen and stored at −70°C until analysis. Furthermore, paraffin-embedded tumor tissues derived from the primary tumors of 8 patients in this group were also collected and analyzed.

Immunohistochemistry

Expression of Pgp, MRP, LRP, p53, Ki-67, topoI, topo II-α and topo II-β was assessed by IHC. Cryostat sections (4 µm) and cytospin preparations were fixed in acetone or 4% paraformaldehyde plus 5% acetic acid (topo II-α) at room temperature. Formalin-fixed, paraffin-embedded tissue was processed with an antigen retrieval technique (Shi *et al.,* 1991) in the case of Pgp, p53 and Ki-67 staining. Characteristics of the Abs are summarized in Table I. Immunohisto/cytochemistry was performed with an avidin-biotin complex immuno-peroxidase method (Vectastain, Vector, Burlingame, CA), as described by van der Valk *et al.* (1990). Endogenous biotin was blocked with streptavidin (0.1% in PBS) and d-biotin (0.01% in PBS) (Sigma, St. Louis, MO), or by the APAAP method (Dakopatts, Copenhagen, Denmark) (Moir *et al.,* 1983). Slides were incubated with the primary Abs for 1 hr at room temperature or overnight at 4°C (LRP56 (paraffin), DO-7). PBS was used for washing steps, with the exception of LRP56 staining on formalin-fixed, paraffin-embedded tissues; these slides were pre-incubated and rinsed in PBS with 0.03% casein (Merck, Darmstadt, Germany) and 0.05% Tween 20 (Genfarma, Maarssen, The Netherlands) as described elsewhere (Tacha and McKinney, 1992). For these slides, one extra step was included with mouse-anti-biotin (Boehringer, Mannheim, Germany), dilution 1:200. For detection of Pgp in formalin-fixed, paraffin-embedded tissues the second and third steps of the avidin-biotin complex immunoperoxidase method were repeated. Slides were developed in 0.05% 3,3′-diaminobenzidine tetrahydrochloride dihydrate (Sigma) with 0.02% H_2O_2 in PBS, or in new fuchsine (APAAP). Negative control slides were run in parallel, omitting the primary antibody or substituting it with an irrelevant mouse myeloma IgG MAb, isotype-matched for JSB-1, MRPr1, DO-7 and 6G2.

The cell lines KB3-1, KB-Ch^R-8-5 and 8226DOX4, described in detail elsewhere (Linn *et al.,* 1994), served as negative and positive controls for Pgp staining in all experiments. Other positive controls included the GLC4/ADR human lung cancer cell line (Zijlstra *et al.,* 1987) for MRP (Flens *et al.,* 1994), the non-Pgp MDR SW-1573/2R120 doxorubicin-resistant subline (Scheper *et al.,* 1993) for LRP56 and the NCI-H322 and NCI-H187 human lung cancer cell lines for topo I, topo IIα and topo IIβ. Normal tonsil tissue and a colorectal cancer specimen served as positive controls

for Ki-67 and p53, respectively. Specimens were independently scored for each MAb separately by two of us, both blinded to clinical outcome.

By prior agreement, samples were considered Pgp-positive if ≥20% of tumor cells were stained. This cutoff point was based on previous studies (Linn *et al.,* 1995, 1996; Schneider *et al.,* 1989; Chan *et al.,* 1991). For LRP56 the following staining pattern was observed: all tumor cells negative, occasional staining of single tumor cells (≤5%) and a substantial part of tumor cells positive (≥10%). Tumor samples were therefore considered positive for LRP56 if ≥10% tumor cells were stained, as also described previously (Izquierdo *et al.,* 1995). For MRP, p53, Ki-67, topo I, topo IIα and topo IIβ, the median expression in the whole group of breast cancer specimens examined was pre-specified as cutoff points: 60% for MRP, 1 cell nucleus/slide for p53, 10% for Ki-67, 60% for topo I, 20% for topo IIα and 80% for topo IIβ.

Statistics

Linear regression analysis and Spearman's correlation test were used to assess correlation between different protein expression values. Possible correlations between protein expression and clinico-pathological parameters were assessed by Fisher's exact test (two-tailed). For continuous variables, the cutoff point was either the median value (MRP, p53, Ki-67, topo I, topo IIα and topo IIβ) or dictated by the staining patterns observed (Pgp and LRP). For some proteins the cutoff point was similar to those used in other studies (Pgp: Linn *et al.,* 1995; Schneider *et al.,* 1989; Chan *et al.,* 1991; Linn *et al.,* 1996; LRP: Izquierdo *et al.,* 1995; p53: Allred *et al.,* 1993; Thor *et al.,* 1992; Ki-67: Railo *et al.,* 1993). The Wilcoxon test was used to assess changes in protein expression levels of tumors sampled sequentially before and after chemotherapy. Survival analysis was performed according to Kaplan and Meier (1958). For locally advanced cancers, overall survival time was defined as the time between date of start of neo-adjuvant chemotherapy and date of last follow-up or death of recurrent disease. Differences between survival curves were analyzed using the Mantel-Cox test (Mantel, 1966; Cox, 1972). Tests were carried out with the BMDP statistical package (Los Angeles, CA).

TABLE II – PATIENT CHARACTERISTICS IN DIFFERENT BREAST CANCER GROUPS

	Primary operable	Locally advanced	Metastatic anthracycline-resistant
N	20	40	10
Median age (range), years	63 (44–86)	47 (26–63)	53 (43–71)
Clinical stage			
I-II	18		
III	2	40	
IV			10
Axillary lymph node status			
0	12	16[1]	2
1–3	7	7	1
>3	1	15	5
Unknown		2[2]	2[3]
Mean tumor diameter (range), cm[4]	2.9 (1.0–8.5[5])	0 (0–8)[1]	4.0 (1.5–7.0)
Primary tumor histological type			
Ductal	19	33	9
Lobular		6	1
Colloid	1		
Unclassified		1[6]	
Differentiation grade			
Well-differentiated		2	
Moderately differentiated	5	8	2
Poorly differentiated	14	28	8
Unclassified	1[7]	2[8]	
Median follow-up (months)	36	20	52

[1]Pathologic examination after neo-adjuvant chemotherapy. Clinical evaluation before neo-adjuvant chemotherapy: N_0, 4; N_1, 13; N_2, 23; T_2, 2, T_3, 18, T_4, 20 patients.–[2]One patient had a tumor embolus in the axilla; one received radiotherapy (mastectomy not performed).–[3]No axillary lymph node dissection.–[4]Diameter at pathologic examination.–[5]8.5 cm was a pathologic estimation of lymphangitis carcinomatosa.–[6]Not enough tumor cells in subclavicular lymph node biopsy for histologic classification, and pathologic complete remission after chemotherapy.–[7]Mucinous carcinoma.–[8]In 2 cases, not enough tumor cells were evaluable for differentiation grade.

RESULTS

Patient characteristics are summarized in Table II. Median age in the locally advanced and metastatic breast cancer patients was significantly lower than in the group of operable breast cancers ($p = 0.009$).

Concordance between C219 and JSB-1 staining was 76% (n = 93; $p < 0.0001$). In our hands, staining sensitivity was higher with JSB-1, and because, unlike C219, JSB-1 is MDR1 specific, Pgp positivity was further defined as staining of ≥20% tumor cells with JSB-1.

Formalin-fixed, paraffin-embedded material was unsuitable for IHC study of topoisomerase expression with the topo MAbs available. Most tumor material from the locally advanced breast cancer group was formalin-fixed and paraffin-embedded. Therefore, information on topoisomerase expression was scarce in this group (Table III). For many locally advanced breast cancers, frozen tissue sampling after chemotherapy was impossible, because only microscopic tumor cells were left, which could not be localized at macroscopic evaluation.

Protein expression in normal breast tissue

Generally, expression of drug resistance proteins was low or undetectable in normal breast tissue. Nevertheless, LRP was expressed in epithelial cells of all samples and in stromal cells of 5/6 samples examined, MRP was found in epithelial cells of 3/6 samples, but not in stroma, and topo IIβ was expressed in nucleoli of virtually all cell types observed.

Differential protein expression in different breast cancer groups

In Table III, the expression of all 8 proteins tested is summarized for the 3 groups of breast cancer examined. In locally advanced breast cancer before chemotherapy, Pgp ($p = 0.0003$), p53 ($p = 0.04$) and Ki-67 ($p = 0.02$) were significantly more often expressed than in primary operable breast cancer, suggesting that they may be markers of a more aggressive phenotype.

Remarkably, none of the 9 metastatic, anthracycline-resistant patients was Pgp-positive.

Interestingly, MRP expression was more frequently observed in primary operable breast cancer than in more advanced stages of breast cancer. High frequency of LRP expression was found in all breast cancer groups examined. Frequency of topo I, topo IIα and topo IIβ expression was similar in primary operable (chemotherapy naive) and chemotherapy-treated breast cancer groups.

Figures 1 and 2 show typical staining results of all proteins assessed with IHC.

Predictive value of protein expression for response

Overall clinical response rate in the locally advanced breast cancer group was 98% (19 complete remissions, 20 partial remissions, 1 stable disease). Four patients had a pathological complete remission, and three had a pathological complete remis-

TABLE III – FREQUENCY OF EXPRESSION OF DRUG RESISTANCE PROTEINS IN DIFFERENT BREAST CANCER GROUPS

Protein	Operable primary (N = 20)	Locally advanced (N = 40)		Metastatic, anthracycline-resistant (number positive/ number analyzed)	Median expression (all breast cancers) % (range)
		Before CT (number positive/ number analyzed)	After CT (number positive/ number analyzed)		
Pgp	2[2] (10%)	16/25 (64%)	17/30 (57%)	0/9	3 (0–100)
MRP	16 (80%)	2/10 (20%)	9/16 (56%)	1/5	60 (0–100)
LRP	15 (75%)	12/17 (71%)	18/26 (69%)	6/8	65 (0–100)
p53	6 (30%)	17/26 (65%)	16/31 (52%)	1/5	0 (0–90)
Ki-67	7 (35%)	18/25 (72%)	6/31 (19%)	5/8	10 (0–80)
Topoisomerase I	10 (50%)	ND[1]	1/5	3/8	60 (0–100)
Topoisomerase IIα	9 (45%)	ND	2/4	3/8	20 (0–80)
Topoisomerase IIβ	9 (45%)	ND	2/6	6/9	80 (0–100)

[1]ND, not done.–[2]Number of cases positive by definition, as described in Material and Methods.

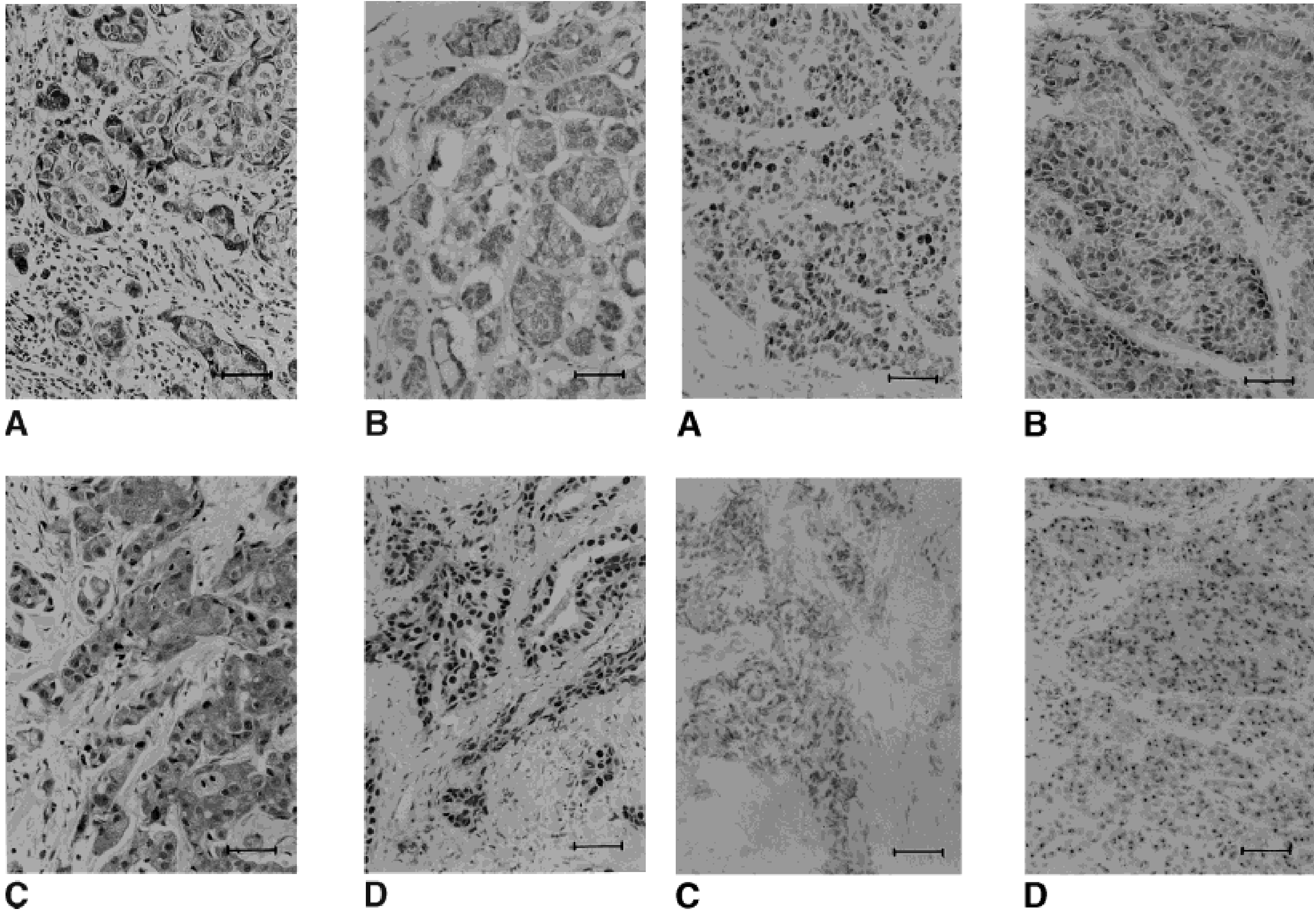


FIGURE 1 – Results of IHC on formalin-fixed, paraffin-embedded material. Bars: 100 μm. *(a)* MRP; cytoplasmic and membranous staining. *(b)* Pgp (JSB-1); cytoplasmic and membranous staining. *(c)* LRP; primarily granular cytoplasmatic staining pattern, and to a lesser extent membranous. *(d)* p53; mainly nuclear staining.

FIGURE 2 – Results of IHC on frozen tumor specimens. Bars: 100 μm. *(a)* Ki-67; nuclear staining was observed in tumor cells, mononuclear infiltrating cells and occasionally in normal breast epithelial cells. *(b)* Topoisomerase IIα; staining was generally restricted to tumor cell nuclei, but in some tumor cells specific cytoplasmic staining was also observed, with and without nuclear staining. *(c)* Topoisomerase I; staining was restricted to epithelial tumor cell nuclei. *(d)* Topoisomerase IIβ; a typical nucleolar staining pattern was expressed in virtually all cell types present.

sion of the primary tumor, with only a few tumor cells left in axillary lymph nodes.

None of the 9 metastatic, anthracycline-resistant patients showed a response on paclitaxel (Vermorken *et al.,* 1995).

Protein expression levels measured before neo-adjuvant chemotherapy (Pgp, MRP, LRP, Ki-67 and p53) or before paclitaxel treatment (Pgp, MRP, LRP, p53, Ki-67, topo I, topo IIα and topo IIβ) had no predictive value for clinical or pathological response (data not shown). Of the 4 patients with a pathological complete remission on high-dose doxorubicin/cyclophosphamide, 3 patients had high Pgp expression (≥50%), 2 patients had high p53 expression (≥50%) and 2 patients had low Ki-67 expression (≤10%) of the primary tumor. This finding suggests that neither high Pgp and/or p53 expression, nor low Ki-67 expression, is an obstacle to tumor cell kill with chemotherapy.

Changes in protein expression after chemotherapy

Locally advanced breast cancer. For 17 patients, tumor material was available at 2 time points during treatment: a biopsy was taken before the start of chemotherapy, and tumor specimens were obtained at the time of mastectomy, after the last chemotherapy cycle. Protein expression levels before the start of chemotherapy were compared with the expression values seen after completion of chemotherapy (4–6 cycles). Figure 3 summarizes results of protein expression in these samples. After chemotherapy, the percentage of Pgp-, p53- and LRP-positive tumor cells did not significantly vary. Interestingly, however, a significant decrease in percentage of Ki-67-positive tumor cells was observed after chemotherapy ($p = 0.009$), suggesting that tumor cells survive chemotherapy more readily when out of the cell cycle.

Metastatic anthracycline-resistant breast cancer. No significant change of expression levels of any of the proteins studied in at least 6 patients (Pgp, LRP, Ki-67 and topo I, IIα and IIβ) was observed comparing samples taken before the start of paclitaxel with biopsies obtained after progression on paclitaxel. However, a trend was present for LRP ($p = 0.06$) and Ki-67 expression ($p = 0.09$) to decrease after progression on paclitaxel chemotherapy (data not shown).

Protein expression and clinico-pathological features

We studied the correlations between protein expression and clinico-pathologic parameters (age, clinical stage, TNM-classification, tumor histology and differentiation grade) in 54 untreated breast cancers (20 operable, 26 locally advanced and 8 primaries of the anthracycline-resistant group). Pgp expression was more often observed in advanced stages of breast cancer (stage I, 3/8; IIA, 0/8; IIB, 0/4; IIIA, 9/17; IIIB, 11/15; $p = 0.004$). Furthermore, Pgp expression was associated with a larger tumor diameter (n = 53; p = 0.04), as reported previously (Linn *et al.,* 1996), suggesting that Pgp is a marker of an aggressive phenotype.

Ki-67 positivity was associated with younger age (<52 years, 20/26 positive; ≥52 years, 10/27 positive; $p = 0.005$), confirming its association with a malignant phenotype, as younger age is related to worse prognosis in breast cancer.

Protein expression and doxorubicin cumulative dose

Patient samples obtained after the administration of >400 mg/m^2 doxorubicin cumulative dose (100% dose of epirubicin equals 66% dose of doxorubicin) were less often Ki-67-positive than tumor samples taken after administration of ≤400 mg/m^2 (6/28 *vs.* 35/64 Ki-67-positive; $p = 0.003$; linear regression n = 92; $r = -0.25$; $p = 0.02$). No association was found between expression values of any of the other proteins and cumulative dose of doxorubicin administered. This result suggests that breast cancer cells survive doxorubicin-based chemotherapy more readily when they are in a dormant state.

Co-expression of p53 and Pgp and survival

In the locally advanced breast cancer group, the presence in the mastectomy specimen of both Pgp-positive and p53-positive tumor cells was associated with a shorter survival, as reported before in a smaller series (Pgp + /p53 + patients (n = 11) with 2-year survival of 0% *vs.* the other locally advanced breast cancer patients (n = 15) with 2-year survival of 90%; $p = 0.0007$) (Linn *et al.,* 1996).

Relations between expression levels of different proteins in tumors

Pgp expression was confirmed to be associated with nuclear p53 accumulation (n = 93; $r = 0.34$; $p < 0.001$), as previously observed (Linn *et al.,* 1996).

Ki-67 positivity correlated with topo IIα (n = 39; $r = 0.42$; $p = 0.008$) and less strongly with topo IIβ (n = 41; $r = 0.31$; $p = 0.05$), supporting the notion that topo II is involved in proliferation (Giaccone *et al.,* 1995; Kaufmann *et al.,* 1994; D'Andrea *et al.,* 1994). A correlation was also found between topo IIα and topo IIβ expression (n = 39; $r = 0.38$; $p = 0.02$). Furthermore, an association was present between topo I and both topo II-isotypes (IIα, n = 38; $r = 0.33$; $p = 0.04$; IIβ, n = 38; $r = 0.41$; $p = 0.01$). All correlation coefficients were, however, relatively small, which indicates that these associations are far from being complete.

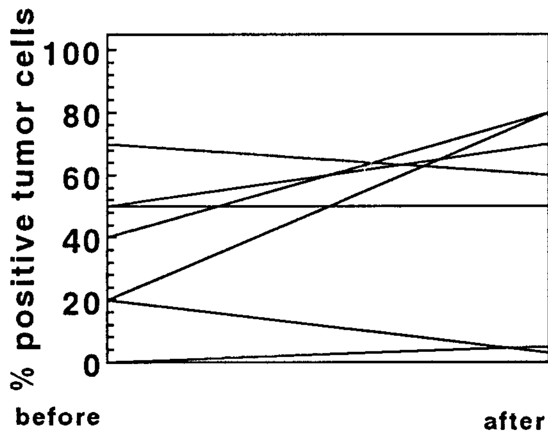
DISCUSSION

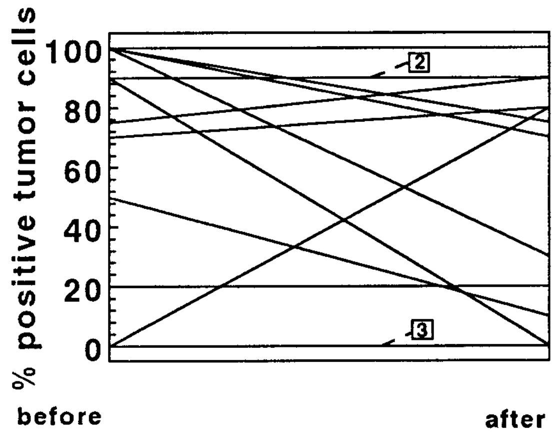
Breast cancer is an interesting clinical model to investigate protein expression and changes induced by systemic treatment. In particular, patients with large tumors are nowadays usually treated by neo-adjuvant chemotherapy and then by surgery, providing sufficient tumor material for such studies. Furthermore, the frequent cutaneous localizations of metastases also provides a relatively easy access for biological material.

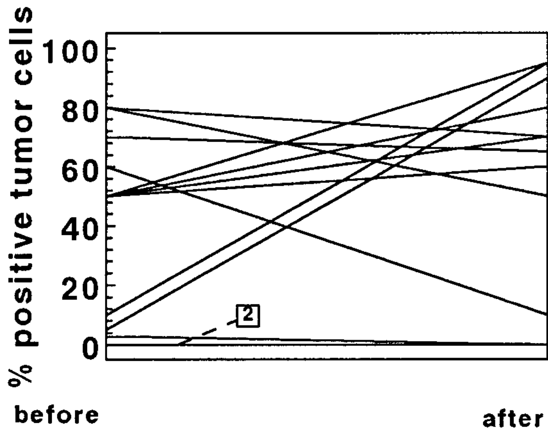
In a pilot study we had used both IHC and RNase protection assay to assess expression of MDR1, MRP and topo IIα in patient samples. However, there was no good concordance between the two methods, which is probably due to the fact that the RNase protection assay is a bulk tissue technique. Information on histology is lost, while with IHC one can distinguish which cell types show protein expression. Furthermore, the tumor-to-stroma ratio varies considerably among samples, as does the protein expression within stromal components (Linn *et al.,* 1995). These facts make a bulk tissue technique less suitable for studies on clinical material. Therefore, we used only IHC in this study.

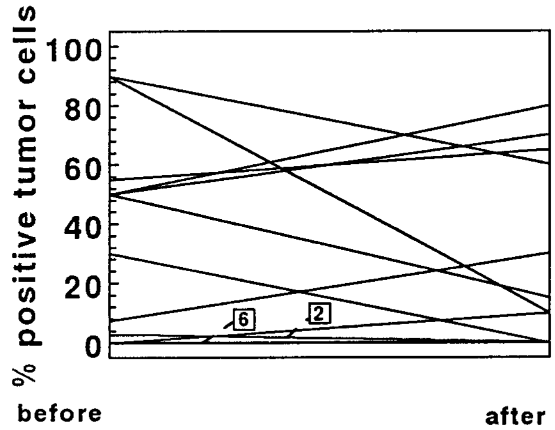
We studied the expression of several drug resistance markers in different breast cancer groups and in normal breast tissue. Interestingly, the locally advanced breast cancers, which were more aggressive than the smaller tumors, had significantly higher expression of Pgp, p53 and Ki-67. In addition, in chemotherapy-naive patients, Ki-67 positivity was associated with younger age. The fact that these proteins have already been identified as markers of poor prognosis in breast cancer (Verelle *et al.,* 1991; Greenblatt *et al.,* 1994; Linn *et al.,* 1995, 1996; Allred *et al.,* 1993; Thor *et al.,* 1992; Railo *et al.,* 1993; Wintzer *et al.,* 1991), as well as in several other cancer types, supports their association with tumor aggressiveness. A significant correlation between increased Pgp expression and poor prognosis has been observed in osteosarcoma (Baldini *et al.,* 1995). Interestingly, the presence of Pgp was unrelated to the response of tumors to chemotherapy. Lack of correlation between expression of drug resistance proteins and chemotherapy response was also observed in our study. This suggests that the role of these markers in tumor progression may be independent from their putative role in drug resistance (Pinedo and Giaccone, 1995). It is difficult to determine the exact contribution of these markers to clinical drug resistance, as most chemotherapeutic regimens consist of a combination of anticancer drugs with at least one drug not involved in the MDR-phenotype.

Although 16/25 locally advanced breast cancer patients were Pgp-positive before chemotherapy in our study, Pgp did not have predictive value regarding pathologic response. This might be in contrast with results reported by others (Verelle *et al.,* 1991), who subdivided a group of 17 Pgp-positive, locally advanced breast cancer patients into 2 subgroups, depending on staining intensity. The subgroup with strong Pgp-positivity appeared to be associated with chemoresistance and shorter survival. However, Verelle *et al.* (1991) related Pgp expression only to clinical response, and not to pathologic response. Furthermore, we did not judge staining intensity, as differences in staining intensity in our series were only minimal, and easily attributable to experimental variability. Interestingly, some patients who achieved a pathological complete remission had high Pgp expression and nuclear p53 accumulation in pretreatment biopsies, indicating that expression of these proteins is no obstacle to tumor cell kill with a regimen containing moderately high doses of doxorubicin and cyclophosphamide. In addition, we did not find a correlation between Pgp expression and









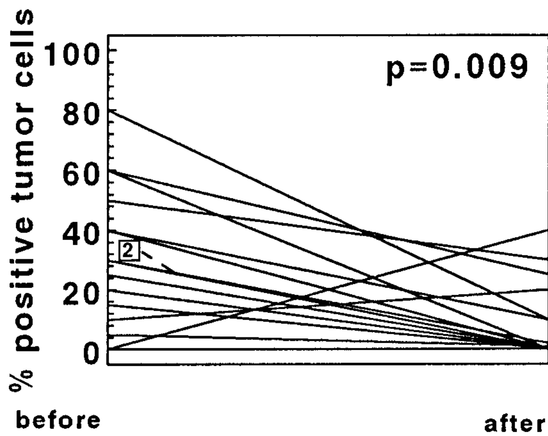


FIGURE 3 – Changes in protein expression in tumors of locally advanced breast cancer patients, sampled before and after neo-adjuvant chemotherapy. While the median tumor diameter decreased from >5 cm (clinical evaluation) to microscopic tumor cell rests (pathologic examination) after chemotherapy, the expression of Pgp, p53 and LRP did not consistently vary. In contrast, a significant decrease in the percentage of Ki-67-positive tumor cells was observed (n = 16). Whenever one line represents more than one patient, the number of patients is indicated next to the corresponding line in the graph.

cumulative doxorubicin dose, in contrast with findings reported in myeloma (Grogan *et al.,* 1993). In the myeloma study, samples were obtained for clinical decision making, *e.g.,* at the moment of therapy failure, and this approach might well have biased the results, as more malignant tumors may require higher doses of chemotherapy and may earlier receive a doxorubicin-containing regimen. However, the observed differences may relate to the different tumor types studied (breast *vs.* multiple myeloma).

Goldie and Coldman (1979) hypothesized that tumor cells are born chemosensitive, but that drug resistance occurs spontaneously and rapidly, with the likelihood of a resistant cell clone being present increasing markedly between 10^4–10^6 tumor cells (De Vita, 1991). In locally advanced breast cancers, which consist of approximately 10^{10}–10^{11} cells, one would then expect resistant tumor cell clones to be already present at the moment of diagnosis. While these resistant cells would only be a minority of the tumor burden before chemotherapy, they would be the only tumor cells left after effective chemotherapy. Based on this hypothesis we studied protein expression in sequential samples from the same patient before and after chemotherapy. The only significant change in protein expression we observed was a decrease in Ki-67. This might indicate the selection of dormant tumor cells by chemotherapy. Nevertheless, the dormant state of tumor cells itself is probably not responsible for drug resistance, as demonstrated in the 2 patients with low Ki-67 expression before chemotherapy who achieved a pathological complete remission. Another hypothesis is that a low proliferation rate is energetically favorable for survival, and therefore resistant tumor cells might stop cycling whenever exposed to chemotherapy. This is also in line with our finding that in the whole group of breast cancers examined, Ki-67 expression correlated inversely with a high cumulative dose of doxorubicin (>400 mg/m^2; $p = 0.003$). While in our series Ki-67 expression did not predict chemotherapy response, Remvikos *et al.* (1989) found a correlation between high cell cycle S-phase fractions in breast cancers and response to a doxorubicin-containing regimen. The difference with our results may be explained by the fact that Remvikos *et al.* (1989) used clinical response data, instead of pathologic response data, that Ki-67 not only stains cells in S-phase, but also in late G_1, M and G_2 phases (Railo *et al.,* 1993) and that different techniques were used. A few other groups studied kinetic parameters by means of repeated fine-needle aspirates and DNA flow cytometry in breast cancer patients undergoing neo-adjuvant chemotherapy (O'Reilly *et al.,* 1992; Spyratos *et al.,* 1992). These studies did not report an increase of tumor cells in the G_0-phase after chemotherapy. The difference with our results may again be due to different techniques, including histology *vs.* cytology.

A number of strategies have been proposed to kill quiescent tumor cells; one of them is late intensification treatment (Norton and Simon, 1977), which appeared to be of benefit in diffuse aggressive lymphomas (De Vita, 1991). The addition of alkylating agents, which at high doses can also kill non-proliferating cells, might be crucial. Another strategy is hormonal-induced expansion of the growth fraction of a tumor, followed by chemotherapy (Conte *et al.,* 1985; Fabian *et al.,* 1994). Preliminary results in breast cancer seemed promising, with an increase in cycling cells after diethylstilbestrol administration and an early decrease in proliferating cells after chemotherapy (Conte *et al.,* 1985). Nine years later, however, a carefully designed study, including monitoring of estradiol blood levels, failed to demonstrate a survival benefit of this strategy (Fabian *et al.,* 1994). Several reasons for the lack of effect were proposed. Patients with a relatively high baseline growth fraction had only a small absolute increase in proliferative index after estrogen treatment, which did not yield improved responses (Fabian *et al.,* 1994). The authors suggested restricting future efforts in hormonal recruitment to patients with a low baseline proliferative index. Another assumption for the lack of success of hormonal recruitment was a supposedly high MDR1 expression level in locally advanced breast cancer before chemotherapy (Verelle *et al.,* 1991).

We also studied protein expression in a small group of anthracycline-resistant, metastatic breast cancer patients undergoing paclitaxel treatment. In this group we saw no responders to paclitaxel. Remarkably, none of the 9 anthracycline-resistant patients was Pgp-positive. Furthermore, topo IIα expression was comparable to that in the primary operable breast cancer group, rendering decreased topo IIα expression also an unlikely explanation for anthracycline resistance in this group. However, with the technique used we cannot exclude decreased topo IIα activity or an altered gene as playing a role in anthracycline resistance.

We found a correlation between topo IIα and β in breast cancer, albeit weaker than reported for AML (Kaufmann *et al.,* 1994), and stronger than observed in lung cancer (Giaccone *et al.,* 1995), indicating possible differences between tumor types. In the AML study, topo IIα and topo IIβ content appeared relatively constant over time in 20 paired samples from AML patients obtained at diagnosis and relapse (Kaufmann *et al.,* 1994). In line with these results, we found relatively constant topo IIα and topo IIβ expression values in different breast cancer groups, and no consistent change in expression in 7 sequentially sampled metastatic breast cancer patients undergoing paclitaxel treatment. As in our study, a marked cell-to-cell heterogeneity of topo IIα expression was observed in AML, using immunocytochemistry. In our study, Ki-67 positivity correlated with topo IIα ($p = 0.008$), and less strongly with topo IIβ expression ($p = 0.05$), which corresponds with results reported by others, who studied Ki-67, topo IIα and topo IIβ expression in several human tumors (D'Andrea *et al.,* 1994). For AML (Kaufmann *et al.,* 1994) and lung cancer (Giaccone *et al.,* 1995), topo IIα, but not topo IIβ, has been suggested to serve as a proliferation marker.

Topo I expression was virtually undetectable in normal breast tissue, while it was expressed in 50% of primary operable breast cancers, which may explain some of the activity of topo I inhibitors in breast cancer observed during phase I trials (Burris *et al.,* 1992). Furthermore, a correlation between topo I and both topo II isoforms was found in breast cancer, which might be of interest for future treatment designs, including topo I and topo II inhibitors.

In conclusion, it is feasible to serially study tumor resistance markers in breast cancer patients undergoing neo-adjuvant chemotherapy. Our results suggest that only a quiescent tumor cell status, as assessed with Ki-67, but not Pgp, LRP or p53 expression, might play some role in drug resistance of breast cancer. Independent of their putative role in drug resistance, Ki-67, Pgp and p53 may be involved in tumor progression. None of the markers studied reliably predicted tumor response. New treatment strategies directed against those quiescent tumor cells, that are not killed by the available drugs, are awaited. High-dose chemotherapy is being tested and may provide a valid approach in patients with small tumor burden. Surgery after neo-adjuvant chemotherapy remains definitely recommended for optimal local control in locally advanced breast cancer patients.

ACKNOWLEDGEMENTS

We thank Mrs. Tadema for expert technical assistance and Dr. G. Zaman of the Netherlands Cancer Institute for kindly providing *MRP* cDNA. S.C.L. was the recipient of a Margot Mattheijssen-van der Voort fellowship.

REFERENCES

Allred, D.C., Clark, G.M., Elledge, R., Fuqua, S.A.W., Brown, R.W., Chamness, G.C., Osborne, C.K. and McGuire, W.L., Association of p53 protein expression with tumor cell proliferation rate and clinical outcome in node-negative breast cancer. *J. nat. Cancer Inst.,* **85,** 200–206 (1993).

Baldini, N., Scotlandi, K., Barbanti-Brodano, G., Manara, M.C., Maurici, D., Bacci, G., Bertoni, F., Picci, P., Sottili, S., Campanacci, M. and Serra, M., Expression of P-glycoprotein in high-grade osteosarcomas in relation to clinical outcome. *N. Engl. J. Med.,* **333,** 1380–1385 (1995).

BURRIS, H.A., ROTHEMBERG, M.L., KUHN, J.G. and VON HOFF, D.D., Clinical trials with the topoisomerase I inhibitors. *Semin. Oncol.,* **19,** 663–669 (1992).

CHAN, H.S.L., HADDAD, G., THORMER, P.S., DEBOER, G., LIN, Y.P., ONDRUSEK, N., YEGER, H. and LING, V., P-glycoprotein expression as a predictor of the outcome of therapy for neuroblastoma. *N. Engl. J. Med.,* **325,** 1608–1614 (1991).

COLE, S.P.C., BHARDWAJ, G., GERLACH, J.H., MACKIE, J.E., GRANT, C.E., ALMQUIST, K.C., STEWART, A.J., KURZ, E.U., DUNCAN, A.M.V. and DEELEY, R.G., Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line. *Science,* **258,** 1650–1654 (1992).

CONTE, P.F., FRASCHINI, G., ALAMA, A., NICOLIN, A., CORSARO, E., CANAVESE, G., ROSSO, R. and DREWINKO, B., Chemotherapy following estrogen-induced expansion of the growth fraction of human breast cancer. *Cancer Res.,* **45,** 5926–5930 (1985).

COX, D.R., Regression models and life tables. *J. Roy. Stat. Soc. B.,* **34,** 187–220 (1972).

D'ANDREA, M.R., FARBER, P.A. and FOGLESONG, P.D., Immunohistochemical detection of DNA topoisomerases IIα and IIβ compared with detection of Ki67, a marker of cellular proliferation, in human tumors. *Appl. Immunohistochem.,* **2,** 177–185 (1994).

DE VITA, V.T., JR., The influence of information on drug resistance on protocol design. *Ann. Oncol.,* **2,** 93–106 (1991).

EARLY BREAST CANCER TRIALISTS' COLLABORATIVE GROUP, Systemic treatment of early breast cancer by hormonal, cytotoxic, or immune therapy: 133 randomised trials involving 31,000 recurrences and 24,000 deaths among 75,000 women. *Lancet,* **339,** 1–15, 71–84 (1992).

FABIAN, C.J., KIMLER, B.F., MCKITTRICK, R., PARK, C.H., LIN, F., KRISHNAN, L., JEWELL, W.R., OSBORNE, C.K., MARTINO, S., HUTCHINS, L.F., LEONG, L.A. and GREEN, S., Recruitment with high physiological doses of estradiol preceding chemotherapy: flow cytometric and therapeutic results in women with locally advanced breast cancer. A Southwest Oncology Group study. *Cancer Res.,* **54,** 5357–5362 (1994).

FLENS, M.J., IZQUIERDO, M.A., SCHEFFER, G.L., FRITZ, J.M., MEIJER, C.J.L.M., SCHEPER, R.J. and ZAMAN, G.J.R., Immunochemical detection of the multidrug resistance-associated protein MRP in human multidrug-resistant tumor cells by monoclonal antibodies. *Cancer Res.,* **54,** 4557–4563 (1994).

GIACCONE, G., DNA topoisomerases and topoisomerase inhibitors. *Pathol. Biol.,* **42,** 346–352 (1994).

GIACCONE, G., VAN ARK-OTTE, J., SCAGLIOTTI, G., CAPRANICO, G., VAN DER VALK, P., RUBIO, G., DALESIO, O., LOPEZ, R., ZUNINO, F., WALBOOMERS, J. and PINEDO, H.M., Differential expression of DNA topoisomerases in non-small cell lung cancer and normal lung. *Biochim. biophys. Acta,* **1264,** 337–346 (1995).

GOLDIE, J.H. and COLDMAN, A.J., A mathematical model for relating the drug sensitivity of tumors to their spontaneous mutation rate. *Cancer Treat. Rep.,* **63,** 1727–1735 (1979).

GREENBLATT, M.S., BENNETT, W.P., HOLLSTEIN, M. and HARRIS, C.C., Mutations in the *p53* tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. *Cancer Res.,* **54,** 4855–4878 (1994).

GROGAN, T.M., SPIER, C.M., SALMON, S.E., MATZNER, M., RYBSKI, J., WEINSTEIN, R.S., SCHEPER, R.J. and DALTON, W.S., P-glycoprotein expression in human plasma cell myeloma: correlation with prior chemotherapy. *Blood,* **81,** 490–495 (1993).

HARRIS, J.R., MORROW, M. and BONADONNA, G., Cancer of the breast. *In:* V.T. De Vita, Jr., S. Hellman and S. Rosenberg (eds.), *Cancer: principles and practice of oncology,* pp. 1264–1332, Lippincott, Philadelphia (1993).

HOEKMAN, K., WAGSTAFF, J., VAN GROENINGEN, C.J., VERMORKEN, J.B., BOVEN, E. and PINEDO, H.M., Effects of recombinant human granulocyte-macrophage colony-stimulating factor on myelosuppression induced by multiple cycles of high-dose chemotherapy in patients with advanced breast cancer. *J. nat. Cancer Inst.,* **83,** 1546–1553 (1991).

IZQUIERDO, M.A. and 12 OTHERS, Drug resistance-related marker Lrp for prediction of response to chemotherapy and prognoses in advanced ovarian carcinoma. *J. nat. Cancer Inst.,* **87,** 1230–1237 (1995).

KAPLAN, E. and MEIER, P., Non parametric estimation from incomplete observations. *J. Amer. Stat. Assoc.,* **53,** 457–481 (1958).

KAUFMANN, S.H., KARP, J.E., JONES, R.J., MILLER, C.B., SCHNEIDER, E., ZWELLING, L.A., COWAN, K., WENDEL, K. and BURKE, P.J., Topoisomerase II levels and drug sensitivity in adult acute myelogenous leukemia. *Blood,* **83,** 517–530 (1994).

KEITH, W.N., STALLARD, S. and BROWN, R., Expression of *mdr*1 and *gst*-π in human breast tumors: comparison to *in vitro* chemosensitivity. *Brit. J. Cancer,* **61,** 712–716 (1990).

LINN, S.C., VAN KALKEN, C.K., VAN TELLINGEN, O., VAN DER VALK, P., VAN GROENINGEN, C.J., KUIPER, C.M., PINEDO, H.M. and GIACCONE, G., Clinical and pharmacologic study of multidrug resistance reversal with vinblastine and bepridil. *J. clin. Oncol.,* **12,** 812–819 (1994).

LINN, S.C., GIACCONE, G., VAN DIEST, P.J., BLOKHUIS, W.M.D., VAN DER VALK, P., VAN KALKEN, C.K., KUIPER, C.M., PINEDO, H.M. and BAAK, J.P.A., Prognostic relevance of P-glycoprotein expression in breast cancer. *Ann. Oncol.,* **6,** 679–685 (1995).

LINN, S.C., HONKOOP, A.H., HOEKMAN, K., VAN DER VALK, P., PINEDO, H.M. and GIACCONE, G., p53 and P-glycoprotein are often coexpressed and are associated with poor prognosis in breast cancer. *Brit. J. Cancer,* **74,** 63–68 (1996).

LOWE, S.W., BODIS, S., MCCLATCHEY, A., REMINGTON, L., RULEY, H.E., FISHER, D.E., HOUSMAN, D.E. and JACKS, T., *p53* status and the efficacy of cancer therapy *in vivo. Science,* **266,** 807–810 (1994).

LOWE, S.W., RULEY, H.E., JACKS, T. and HOUSMAN, D.E., p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. *Cell,* **74,** 957–967 (1993).

MANTEL, N., Evaluation of survival data and two new rank order statistics arising in its consideration. *Cancer Chemother. Rep.,* **50,** 163–170 (1966).

MOIR, D.J., GHOSH, A.K., ABDULAZIZ, Z., KNIGHT, P.M. and MASON, D.Y., Immunoenzymatic staining of haematological samples with monoclonal antibodies. *Brit. J. Haematol.,* **55,** 395–410 (1983).

MÜLLER, M., MEIJER, C., ZAMAN, G.J.R., BORST, P., SCHEPER, R.J., MULDER, N.H., DE VRIES, E.G.E. and JANSEN, P.L.M., Overexpression of the gene encoding the multidrug resistance-associated protein results in increased ATP-dependent glutathione *S*-conjugate transport. *Proc. nat. Acad. Sci. (Wash.),* **91,** 13033–13037 (1994).

NEGRI, C., CHIESA, R., CERINO, A., BASTAGNO, M., SALA, C., ZINI, M., MARALDI, N.M. and ASTALDI-RICOTTI, G.C.B., Monoclonal antibodies to human DNA topoisomerase I and the two isoforms of DNA topoisomerases: 170- and 180-kDa isozymes. *Exp. Cell Res.,* **200,** 452–459 (1992).

NORTON, L. and SIMON, R., Tumor size sensitivity to therapy and the design of treatment schedules. *Cancer Treat. Rep.,* **61,** 1307–1317 (1977).

O'REILLY, S.M., CAMPLEJOHN, R.S., RUBENS, R.D. and RICHARDS, M.A., DNA flow cytometry and response to preoperative chemotherapy for primary breast cancer. *Europ. J. Cancer,* **28,** 681–683 (1992).

PINEDO, H.M. and GIACCONE, G., P-glycoprotein—A marker of cancer-cell behavior. *N. Engl. J. Med.,* **333,** 1417–1419 (1995).

PORKKA, K., BLOMQVIST, C., RISSANEN, P., ELOMAA, I. and PYRHÖNEN, S., Salvage therapies in women who fail to respond to first-line treatment with fluorouracil, epirubicin, and cyclophosphamide for advanced breast cancer. *J. clin. Oncol.,* **12,** 1639–1647 (1994).

RAILO, M., NORDLING, S., VON BOGUSLAWSKY, K., LEIVONEN, M., KYLLÖNEN, L. and VON SMITTEN, K., Prognostic value of Ki-67 immunolabelling in primary operable breast cancer. *Brit. J. Cancer,* **68,** 579–583 (1993).

REMVIKOS, Y., BEUZEBOC, P., ZAJDELA, A., VOILLEMOT, N., MAGDELENAT, H. and POUILLART, P., Correlation of pretreatment proliferative activity of breast cancer with the response to cytotoxic chemotherapy. *J. nat. Cancer Inst.,* **81,** 1383–1387 (1989).

SALMON, S.E., GROGAN, T.M., MILLER, T., SCHEPER, R. and DALTON, W.S., Prediction of doxorubicin resistance *in vitro* in myeloma, lymphoma, and breast cancer by P-glycoprotein. *J. nat. Cancer Inst.,* **81,** 696–701 (1989).

SANFILIPPO, O., RONCHI, E., DE MARCO, C., DI FRONZO, G. and SILVESTRINI, R., Expression of P-glycoprotein in breast cancer tissue and *in vitro* resistance to doxorubicin and vincristine. *Europ. J. Cancer,* **27,** 155–158 (1991).

SCHEFFER, G.L., WIJNGAARD, P.L.J., FLENS, M.J., IZQUIERDO, M.A., SLOVAK, M.L., PINEDO, H.M., MEIJER, C.J.L.M., CLEVERS, H.C. and SCHEPER, R.J., The drug resistance-related protein LRP is the major vault protein. *Nature (Med.),* **1,** 578–582 (1995).

SCHEPER, R.J., BROXTERMAN, H.J., SCHEFFER, G.L., KAAIJK, P., DALTON, W.S., VAN HEIJNINGEN, T.H.M., VAN KALKEN, C.K., SLOVAK, M.L., DE VRIES, E.G.E., VAN DER VALK, P., MEIJER, C.J.L.M. and PINEDO, H.M., Overexpression of a M_r 110,000 vesicular protein in non-P-glycoprotein-mediated multidrug resistance. *Cancer Res.,* **53,** 1475–1479 (1993).

SCHNEIDER, J., BAK, M., EFFERTH, T.H., KAUFMANN, M., MATTERN, J. and VOLM, M., P-glycoprotein expression in treated and untreated human breast cancer. *Brit. J. Cancer,* **60,** 815–818 (1989).

SHI, S.-R., KEY, M.E. and KALRA, K.L., Antigen retrieval in formalin-fixed, paraffin-embedded tissues. An enhancement method for immunohistochemical staining based on microwave oven heating of tissue sections. *J. Histochem. Cytochem.,* **39,** 741–748 (1991).

SPYRATOS, F., BRIFFOD, M., TUBIANA-HULIN, M., ANDRIEU, C., MAYRAS, C., PALLUD, C., LASRY, S. and ROUËSSE, J., Sequential cytopunctures during

preoperative chemotherapy for primary breast carcinoma. *Cancer,* **69,** 470–475 (1992).

TACHA, D.E. and MCKINNEY, L., Casein reduces nonspecific background staining in immunolabeling techniques. *J. Histotechnol.,* **15,** 127–132 (1992).

THOR, A.D., MOORE, D.H., II, EDGERTON, S.M., KAWASAKI, E.S., REIHSAUS, E., LYNCH, H.T., MARCUS, J.N., SCHWARTZ, L., CHEN, L.C., MAYALL, B.H. and SMITH, H., Accumulation of p53 tumor suppressor gene protein: an independent marker of prognosis in breast cancers. *J. nat. Cancer Inst.,* **84,** 845–855 (1992).

VAN DER VALK, P., VAN KALKEN, C.K., KETELAARS, H., BROXTERMAN, H.J., SCHEFFER, G.L., KUIPER, C.M., TSURUO, T., LANKELMA, J., MEIJER, C.J.L.M., PINEDO, H.M. and SCHEPER, R.J., Distribution of multi-drug resistance-associated P-glycoprotein in normal and neoplastic human tissues. *Ann. Oncol.,* **1,** 56–64 (1990).

VAN KALKEN, C.K., PINEDO, H.M. and GIACCONE, G., Multidrug resistance from the clinical point of view. *Europ. J. Cancer,* **27,** 1481–1486 (1991).

VERELLE, P., MEISSONNIER, F., FONCK, Y., FEILLEL, V., DIONET, C., KWIATKOWSKI, F., PLAGNE, R. and CHASSAGNE, J., Clinical relevance of immunohistochemical detection of multidrug resistance of P-glycoprotein in breast carcinoma. *J. nat. Cancer Inst.,* **83,** 111–116 (1991).

VERMORKEN, J.B., TEN BOKKEL HUININK, W., MANDJES, I.A.M., POSTMA, T.J., HUIZING, M.T., HEIMANS, J.J., BEIJNEN, J.H., BIERHORST, F., WINOGRAD, B. and PINEDO, H.M., High-dose paclitaxel with granulocyte colony-stimulating factor in patients with advanced breast cancer refractory to anthracycline therapy: a European Cancer Center trial. *Semin. Oncol.,* **22,** 16–22 (1995).

WINTZER, H.-O., ZIPFEL, I., SCHULTE-MÖNTING, J., HELLERICH, U. and VON KLEIST, S., Ki-67 immunostaining in human breast tumors and its relationship to prognosis. *Cancer,* **67,** 421–428 (1991).

ZAMAN, G.J.R., FLENS, M.J., VAN LEUSDEN, M.R., DE HAAS, M., MÜLDER, H.S., LANKELMA, J., PINEDO, H.M., SCHEPER, R.J., BAAS, F., BROXTERMAN, H.J. and BORST, P., The human multidrug resistance-associated protein MRP is a plasma membrane drug-efflux pump. *Proc. nat. Acad. Sci. (Wash.),* **91,** 8822–8826 (1994).

ZIJLSTRA, J.G., DE VRIES, E.G.E. and MULDER, N.H., Multifactorial drug resistance in an adriamycin-resistant human small-cell lung-carcinoma cell line. *Cancer Res.,* **47,** 1780–1784 (1987).