

Original article

Prognostic relevance of P-glycoprotein expression in breast cancer

S. C. Linn,¹ G. Giaccone,¹ P. J. van Diest,² W. M. D. Blokhuis,¹ P. van der Valk,²
C. K. van Kalken,¹ C. M. Kuiper,¹ H. M. Pinedo¹ & J. P. A. Baak³

¹Department of Medical Oncology and ²Pathology, Free University Hospital, Amsterdam, The Netherlands

Summary

Background: P-glycoprotein (Pgp) expression has been reported to be associated with a poor prognosis in some malignancies such as neuroblastoma, soft tissue sarcoma and acute myeloid leukemia. The prognostic role of Pgp expression in breast cancer is still unclear. We investigated the expression of Pgp in primary and metastatic breast cancer tissues in relation to patient characteristics and treatment outcome.

Patients and methods: Pgp expression was evaluated in 92 primary and 12 metastatic breast cancers by the use of immunohisto/cytochemistry with three monoclonal antibodies (MAbs) (JSB-1, C219, MRK16), and an RNase protection assay. Follow-up information was available for 77 primary breast cancer patients (median follow-up 42 months; range 2-63 months).

Results: Concordance among the anti-Pgp MAbs varied, the highest being between JSB-1 and MRK16 (71%; $p=0.002$). Pgp expression was more frequent in metastatic disease (58%) than in primary breast cancer (29%) (JSB-1; $p=0.055$). Pgp expression as assessed with JSB-1 (univariate

analysis; $p < 0.05$) was associated with shorter overall survival (OS). Nineteen (21%) primary breast cancers had Pgp expression in fibroblasts in desmoplastic stroma and this did not correlate with Pgp expression in the tumor. The combination of Pgp-positive tumor cells and Pgp-expressing fibroblasts was the strongest prognostic factor for OS by multivariate analysis. Subgroup analysis suggested that Pgp expression was associated with a shorter OS in tamoxifen-treated patients, but not in those who received chemotherapy (most often CMF).

Conclusions: Pgp expression in tumor cells, and especially when accompanied by Pgp expression in fibroblasts in desmoplastic stroma, has prognostic value in primary breast cancer patients and is likely to be a marker of a more malignant phenotype. Pgp expression of tumor cells might play a role in tamoxifen resistance. These findings may have important implications for the treatment of breast cancer patients, and warrant further prospective investigation in a larger patient population.

Key words: MDR1, breast cancer, fibroblast, tamoxifen

Introduction

Breast cancer is the leading cause of death among women in the Western world, where, despite radical surgery, about 35% of affected women die of it [1]. Although adjuvant hormonal and chemotherapy treatments have been demonstrated to improve survival [2], they have adverse side effects [3], so only patients at high risk for relapse should be treated. The most important prognostic factor for relapse-free and overall survival is the axillary lymph node status, which is used to select patients to be given adjuvant therapy [1]. Two-thirds of all patients presenting with breast cancer do not have axillary lymph node involvement [3]; nevertheless, 30% of them will eventually relapse [3]. Other prognostic factors such as tumor diameter, histologic type, histologic and nuclear grade, estrogen receptor (ER) and progesterone receptor (PR) status, may be used to select, within the node-negative group, patients at higher risk for relapse [3]. Despite the use of adjuvant treatment, around 50% of all patients will eventually relapse [2].

It is thought that the presence or the development of cell clones which are resistant to drugs used as adjuvant treatment are responsible for the treatment failure. One of the most extensively studied mechanisms of drug resistance is multidrug resistance (MDR). MDR confers cellular cross-resistance to natural-product anticancer drugs (anthracyclines, epipodophyllotoxins, vinca alkaloids, actinomycin D, colchicine and paclitaxel) in vitro and is associated with overexpression of Pgp, a plasma membrane molecule of 170-180 kD, encoded by the MDR1 gene [4]. Pgp acts as an energy-dependent drug efflux pump, decreasing the intracellular drug accumulation [4]. An interesting feature of Pgp-mediated MDR is that it can be reversed in vitro and also in vivo by several resistance-modifying agents [4]. Moreover, Pgp expression has been related to an adverse prognosis in several malignancies such as sarcoma [5], neuroblastoma [6], and acute myeloid leukemia [7].

From a limited number of studies there is evidence that Pgp might play a role in resistance to cytotoxic drugs used in breast cancer [8-11]. A correlation has been observed between Pgp expression in breast cancer

cells obtained from patients and in vitro resistance to doxorubicin [8–10]. Furthermore, a high Pgp expression in 17 locally advanced breast cancer patients was associated with a lack of response to neoadjuvant chemotherapy (doxorubicin, vincristine, cyclophosphamide and 5-fluorouracil) and a shorter disease-free survival (DFS) [11].

In this study we investigated the expression of Pgp in more than 100 patients with breast cancer, in relation to the patient characteristics and treatment outcome.

Patients and methods

Ninety-two patients with primary breast cancer and 12 patients with metastatic disease, diagnosed between 1983 and 1989, were treated at the Free University Hospital, Amsterdam, or in other Dutch institutions participating in a multicenter morphometric mammary carcinoma project [12].

Immunohisto/cytochemistry

Specimens were obtained at the time of primary surgery or from metastatic sites, and patient material was centralized at the Department of Pathology, Free University Hospital, Amsterdam. Tissue samples and cytospin preparations of effusions were stored at -70°C until analysis. Cryostat sections ($5\ \mu\text{m}$) and cytospin preparations were air-dried and fixed in acetone at room temperature prior to staining. For assessment of Pgp expression, a panel of three anti-Pgp MAbs (C219, JSB-1, and MRK-16), directed against different epitopes of the P-gp molecule, was used. C219 (IgG_{2b}), kindly provided by Centocor (Tongeren, Belgium), was used at a working dilution of 1:100. JSB-1 (IgG₁), raised in our laboratory [13], was used at a dilution of 1:50. MRK-16 (IgG_{2a}), provided by Dr. T. Tsuruo, Tokyo, Japan, was used at a dilution of 1:400 [14]. Immunohisto/cytochemistry was performed with an avidin-biotin complex immunoperoxidase method (Vectastain[®], Vector Laboratories, Inc., Burlingame, CA, U.S.A.) as described earlier [14]. Negative control slides were run in parallel, omitting the primary antibody or substituting an irrelevant mouse myeloma IgG MAb for it. In addition, cytospin preparations of a sensitive human epidermoid carcinoma cell line, KB3-1, and of the multidrug-resistant human lung cancer cell line, SW-1573/2R160, served as negative and positive controls, respectively. Samples were scored for each MAb separately by two independent investigators (SCL, PvdV), blinded to clinical outcome. One of three distinct staining patterns was observed in all the samples examined: all tumor cells negative; occasional staining of single cells (<5% positive cells); numerous positive tumor cells ($\geq 20\%$) throughout the section. Only one case was observed with 10% positive tumor cells. The same staining pattern has been described by others [15]. Considering the observed staining pattern of tumor cells, and in order to minimize the risk of a false positivity, samples were considered positive for each MAb only if $\geq 20\%$ of tumor cells were stained. This cut-off value has also been reported by other investigators [6, 15]. Interestingly, in a number of samples, desmoplastic stroma (i.e., fibroblasts and fibroblast-like cells only, with the exclusion of other cell types present in desmoplastic stroma) stained with anti-Pgp MAbs. Staining of desmoplastic stroma followed a staining pattern similar to the one observed in tumors: no staining; occasional staining of single cells (<5% of cells); numerous ($\geq 20\%$) fibroblasts and/or fibroblast-like cells positive. Desmoplastic stroma was considered Pgp-positive if $\geq 20\%$ of fibroblasts and/or fibroblast-like cells stained with at least two MAbs.

RNAse protection assay

RNAse protection assay was performed in selected samples. Frozen tumor specimens were pulverized in a microdismembrator and total cellular RNA was extracted by homogenization in guanidine isothiocyanate followed by ultracentrifugation on a cesium chloride gradient [16]. RNAse protection was performed as described earlier [17]. Briefly, $10\ \mu\text{g}$ of total RNA was hybridized with a [³²P]CTP-labeled anti-sense RNA probe, specific for MDR1-mRNA, which was obtained by transcription of a 301 nucleotide cDNA fragment (positions 3500–3801) with SP6 RNA polymerase. A probe for γ -actin was included as an internal control for determination of RNA loading. The hybridized probe was visualized by autoradiography after electrophoresis through a denaturing 6% acrylamide gel, followed by autoradiography. MDR1 expression levels of patient material were compared with known expression levels of the KB cell lines (KB3-1, KB8, KB8-5) by densitometry.

Assessment of mitotic activity

Mitotic activity index (MAI) was defined as the total number of mitotic figures in 10 consecutive fields ($400\times$ magnification) in the most cellular invasive peripheral area of the tumor, the count starting at the spot with the highest density of mitotic figures [18].

Statistics

Survival analysis was performed according to Kaplan–Meier [19]. Overall survival time was defined as the time between date of operation and date of last follow-up or death of recurrent disease. Disease-free survival was defined as the time between date of operation and date of last follow-up or date of first recurrence. For continuous variables, the cut-off point was either the median value (tumor diameter, age) or based on other studies (MAI, Pgp) [18, 6, 15].

Differences between survival curves were analyzed using the Mantel–Cox test [20, 21]. Multivariate analysis of prognostic variables was performed with the Cox regression model [21]. For assessment of possible correlations between staining results and other breast cancer variables Fisher's exact test (2-tail) was used. Tests were carried out with the BMDP statistical package (Los Angeles, CA) [18].

Results

Patient characteristics are summarized in Table 1. Twenty-four (19%) primary breast cancer samples were not evaluable for Pgp expression, 21 of them because of poor preservation and 3 because they contained no tumor cells. Only 40% (12) of specimens obtained from metastatic sites were evaluable for Pgp expression, whereas eighteen samples were not evaluable due to poor preservation or absence of tumor cells.

Staining was observed in 34/104 samples (33%) with JSB-1, in 30/104 cases (29%) with MRK16, and in only 11/104 tumors (11%) using C219. Concordance among different MAbs was highest between JSB-1 and MRK16 (71%; $p=0.002$). C219-positive samples, with one exception, always stained with at least one other MAb, supporting its reactivity with the MDR1 and not the MDR3 isoform of Pgp, despite its lower sensitivity.

Pgp-expressing tumor cells (*tumor Pgp expression refers to positivity with JSB-1, unless otherwise stated*)

Table 1. Patient characteristics.^a

	Primary breast cancer	Metastatic disease
Number of patients evaluable for Pgp expression/total number	92/116	12/39
Median age (range), years	56 (26-84)	64 (48-79)
Premenopausal status	39	1 ^b
Postmenopausal status	53 ^b	9
Median tumor size (range), cm	2.5 (1.2-8.0)	2.5 (2.0-8.0)
Axillary lymph node status		
Negative	40	3
1-3 positive	26	2
>3 positive	26	4
Unknown	0	3
Primary tumor histology		
Ductal	89	6
Lobular	2	1
Tubular	1	0
Mixed ductal/lobular	0	2
Unknown	0	3

^a Characteristics are of 104 patients whose tumors were evaluable for Pgp staining.

^b In two cases the menopausal status was not known, and 13 patients of ≥ 50 years of age were considered postmenopausal (Early Breast Cancer Trialists' Collaborative Group, 1992 [2]).

were more often found in metastatic (58%) than in primary (29%) breast cancer ($p = 0.055$).

Pgp-positive staining in desmoplastic stroma was observed in 19 samples (21%) of primary breast cancer, and in 5 cases was accompanied by Pgp expression in tumor cells (Fig. 1), whereas Pgp staining was restricted to desmoplastic stroma only in the remaining 14 samples.

RNAse protection assay in 13 selected samples showed that MDR1 gene expression varied between the level present in KB3-1 and KB8-5 cells, and staining of $\geq 20\%$ of tumor cells corresponded approximately to an MDR1 expression level of at least KB8 cells (not shown) [22]. Pgp-positive staining in desmoplastic stroma in the absence of staining with any of the three MAbs in tumor cells and in normal mammary gland was supported by RNAse protection assay experiments, and the level was comparable to the expression of KB8 cells (not shown), strongly suggesting that MDR1/Pgp is specifically expressed in desmoplastic stroma in a subset of breast cancers.

Expression of Pgp in primary breast cancer cells and other prognostic factors

Information on Pgp expression was available for 92 primary breast cancer patients, while information on other variables was not uniformly available. Pgp expression was observed significantly more often in tumors of premenopausal women than in breast cancers of postmenopausal women ($p < 0.05$). Pgp expression was significantly more often observed in tumors with a low MAI ($p < 0.05$). Pgp-positive primary breast tumors were more frequently associated with the pres-

ence of more than 3 positive axillary lymph nodes (10/24, 42%) than were Pgp-negative primary tumors (15/66, 23%), although this difference did not reach statistical significance. No relationship was found between Pgp expression and differentiation grade, or ER/PR status.

Pgp expression and survival

Follow-up data with a median of 42 months (range 2-63 months) were available from 77 patients (84%) with primary breast cancer. In Table 3, DFS and OS at 3 years are given for known prognostic features and Pgp expression. By univariate analysis, among the strongest prognostic factors for OS were MAI, lymph node status, Pgp expression (Fig. 2), and, interestingly, the combination of Pgp-expressing tumor cells, surrounded by Pgp-expressing desmoplastic stroma (Pgp⁺ tumor & Pgp⁺ desmoplastic stroma). Strong prognostic

Table 2. Survival of the primary breast cancer group (n = 77) by univariate analysis.

Variable	N	Disease free survival at 3 years (%)	p	Overall survival at 3 years (%)	p
Pgp expression tumor					
<20% ^a	54	77	NS ^b	93	0.047
$\geq 20\%$	23	77		75	
Pgp ⁺ tumor & Pgp ⁺ desmoplastic stroma					
No	73	80	0.0003	92	0.0000
Yes	4	25		25	
Lymph node status					
≤ 3 positive	56	90	0.0000	93	0.013
>3 positive	21	43		73	
Mitotic activity index					
≤ 10	46	86	0.0014	98	0.0010
>10	23	59		63	
Unknown	8	73		88	
Tumor diameter					
≤ 2.5 cm	49	82	0.044	92	0.062
>2.5 cm	28	69		80	
Differentiation grade					
Well diff.	2	100		100	
Moderately diff.	29	82	NS	89	NS
Poorly diff.	43	71		86	
Unknown	3	100		100	
Age					
≤ 55 years	38	70	NS	84	NS
>55 years	39	84		92	
Menopausal status					
Pre	34	66	NS	82	NS
Post	43	85		92	

^a % of staining tumor cells.

^b NS = not significant.

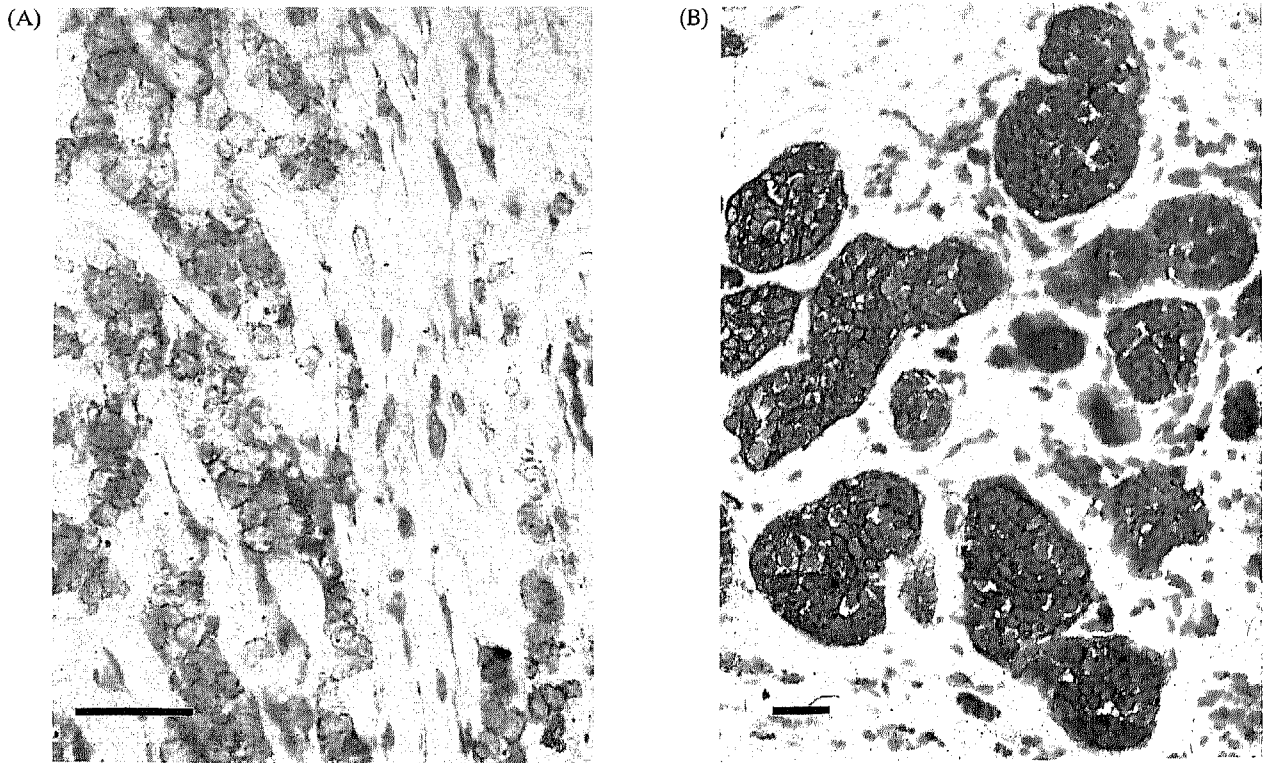


Fig. 1. (A) Staining of tumor cells and fibroblasts in desmoplastic stroma in a primary breast cancer sample (MRK16). (B) Another primary breast cancer sample with staining of tumor cells only (MRK16). The solid bar represents 45 μ m.

factors for DFS by univariate analysis were lymph node status, Pgp⁺ tumor & Pgp⁺ desmoplastic stroma, MAI and tumor diameter.

Multivariate survival analysis for OS pointed to the combination of Pgp⁺ tumor & Pgp⁺ desmoplastic stroma as the strongest prognostic factor ($p = 0.002$).

The influence of Pgp expression on survival was studied in a group of patients treated with tamoxifen ($n = 20$) or chemotherapy ($n = 18$). These patients had been treated in the adjuvant setting ($n = 27$) and/or in advanced disease ($n = 11$). The chemotherapy-treated group consisted of 18 patients: 13 had received CMF (cyclophosphamide, methotrexate, 5-fluorouracil) (11 adjuvant, 2 in advanced disease), and 5 patients had been treated with an anthracycline-containing regimen (1 neoadjuvant therapy, 4 in advanced disease). The tamoxifen-treated group consisted of 20 patients (15 adjuvant, 5 in advanced disease). Six patients received tamoxifen (3 adjuvant, 3 advanced disease) and chemotherapy (5 an anthracycline-containing regimen, 1 CMF) and have been included in both treatment groups. Although the number of patients is small and the type of analysis could be misleading [23], interestingly, tumor Pgp positivity was related to an adverse prognosis in the group of tamoxifen-treated patients (15 Pgp-negative patients with a 85% 3-year OS, and 5 Pgp-positive patients with a 27% 3-year OS; $p = 0.019$), while this was not the case for the chemotherapy-treated group (10 Pgp-negative patients with a 67% 3-year OS, and 8 Pgp-positive patients with a 50% 3-year OS; $p =$ not significant).

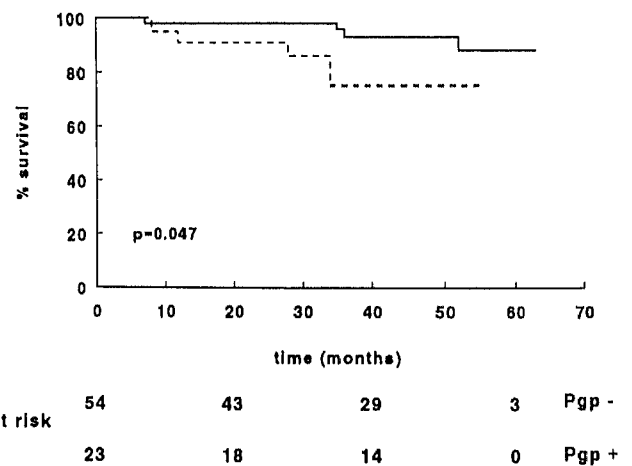


Fig. 2. Survival curves of the group of primary breast cancer patients and tumor Pgp expression (JSB-1 staining). Solid line represents patients with Pgp-negative tumors. Dashed line represents patients which Pgp-positive tumors.

To verify whether the patients in the tamoxifen-treated group were representative of the general type of breast cancer patients, the effect of ER positivity on overall survival was also studied. Supportive evidence was obtained: although ER receptor status was only available from 14 patients in the tamoxifen-treated group, a trend was present for ER positivity to be associated with longer OS (3 ER-negative patients with a 33% 3-year OS, and 11 ER-positive patients with a 78% 3-year OS; $p = 0.13$). As no correlation was pre-

sent between Pgp expression and ER status for the subgroup of tamoxifen-treated patients ($p=0.76$), it seems unlikely that ER status influenced this subgroup analysis.

Discussion

Several studies have demonstrated expression of MDR1/Pgp in chemotherapy-treated as well as in untreated primary breast cancer, using different detection techniques [8–11, 15, 24–26]. Differences in reactivity with the MAbs used in our study have also been found by other investigators [27, 28], and may be partly due to the use of a uniform procedure for cellular preparation, fixation and staining for all MAbs [27, 28]. Alternatively, there may be differences among the MAbs in cross-reactivity with other cellular epitopes [27], or each MAb may recognize different configurations of Pgp [29, 30]. C219 recognizes a highly conserved peptide sequence present in all known isoforms of Pgp [31], and therefore was expected to be most often positive, but with the staining procedure applied C219 appeared the least sensitive MAb of the panel used in our study.

In the present study Pgp expression was associated with a shorter OS. It is conceivable that Pgp expression could represent a marker of a more malignant phenotype, and/or that patients with Pgp positive tumors are more resistant to treatment with MDR-related drugs. In line with the former hypothesis, Pgp was more frequently expressed in premenopausal than in postmenopausal breast cancer. A trend was found for Pgp-expressing tumor cells in the primary tumor to be related to tumor cell infiltration in over three axillary lymph nodes, which might suggest an increased metastatic potential, as has been reported for colon cancer [32]. Furthermore, Pgp expression was more often found in metastatic than in primary breast cancer.

Unlike results reported for renal cell cancer and colon cancer [5, 29], differentiation grade in breast cancer was not related to Pgp expression in our study.

Interestingly, a relationship between Pgp expression in the tumor and a low MAI was found. A high mitotic activity has been associated with a poor prognosis [18]. A low MAI, however, might negatively influence the results of chemotherapy.

MDR1 gene expression has been investigated by others with an RNA slot blot technique in 67 primary breast cancers, and no relationship was found between known prognostic factors and MDR1 gene expression [26]. However, as MDR1/Pgp can be expressed in normal mammary gland [14, 15, 24, 33], this might confound the results obtained by RNA slot blot technique. Furthermore, in this study MDR1 gene expression was not analyzed in relation to DFS/OS.

Pgp has been reported to be expressed in several normal human tissues [14, 33, 34], notably epithelial cells with excretory/secretory functions (kidney, liver, colon), but also in endothelial cells at several blood-

tissue barrier sites (brain, testis) [33], in the secretory and gestational endometrium [35], in placental trophoblasts [33], and in the adrenal gland, predominantly in the cortex [33]. It was recently shown that Pgp is expressed in natural killer cells, lymphocytes, granulocytes, monocytes, and in a minority of CD34+ haematopoietic stem cells [36]. The pattern of distribution of Pgp in normal humans suggests that its physiological role is to protect cells against xenobiotics and endogenous toxins, but in fact little is known about the normal function of Pgp. A steroid-transporting role has been suggested in the gravid uterus [35], and the adrenal [37], and an active transport of cortisol, aldosterone and dexamethasone has been demonstrated in porcine cells transfected with human *MDR1* cDNA isolated from the human adrenal gland [37]. The finding that Pgp is not always expressed in normal mammary epithelial cells [24] raises the possibility that Pgp expression in this organ may also be hormonally regulated, as in the endometrium [35]. Pgp expression might be involved in the preparation of the breast for lactation, which is mediated by progesterone.

Nineteen primary breast cancer specimens had Pgp expression in desmoplastic stroma cells. The combination of Pgp⁺ tumor with Pgp⁺ desmoplastic stroma was the strongest prognostic factor of a shorter OS by multivariate analysis. The combination of Pgp expression in tumor cells and desmoplastic stroma may identify a subgroup of very aggressive tumors with increased metastatic potential. Stromal-epithelial communication can play an important role in these tumors [38]. It has been hypothesized that Pgp might play a role in the transport of basic fibroblast growth factor (bFGF) over the cell membrane [39]. bFGF has been associated with growth stimulation and angiogenesis [38]. Recently, tumor microvessel density was reported to be an independent prognostic indicator for DFS and OS in lymph node-negative breast cancer patients [40]. Patients with tumors with a high microvessel density were at a significantly higher risk to develop visceral, bone and soft tissue metastases [40].

The finding that Pgp expression was associated with a shorter OS in the tamoxifen-treated group, but not in the chemotherapy-treated group might have therapeutic implications. Tamoxifen is known to have MDR-reversing potential [41], while none of the compounds of the CMF regimen and only the anthracyclines of the FAC/FEC (5-fluorouracil, (epi-)adriamycin, cyclophosphamide) regimens play a role in MDR. If Pgp expression is related to the lack of benefit from tamoxifen therapy, this might obviously influence the choice of adjuvant therapy in a patient with a Pgp-expressing primary tumor. These results, however, need to be prospectively confirmed in a larger number of patients.

In conclusion, Pgp expression in tumor cells, and especially, when accompanied by Pgp expression in desmoplastic stroma, appears to have prognostic value in primary breast cancer patients and is probably a marker of a more malignant phenotype. Furthermore,

Pgp expression might play a role in tamoxifen resistance. These findings may have important implications for the choice of systemic treatment. Additional prospective studies of Pgp expression of both tumor and desmoplastic stroma in breast cancer patients, particularly in those undergoing adjuvant treatment, are warranted.

Acknowledgments

This study was partially supported by a research grant from the Bristol-Myers Squibb company. Sabine C. Linn is a recipient of a Margot Mattheijssen-van der Voort fellowship.

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Received 15 March 1995; accepted 16 May 1995.

Correspondence to:

Giuseppe Giaccone, M.D.
Department of Medical Oncology
Free University Hospital
P.O. Box 7057, 1007 MB Amsterdam
The Netherlands