

The Value of Immunohistochemical Detection of P-Glycoprotein in Breast Cancer Before and After Induction Chemotherapy

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We have studied the patterns of P-glycoprotein expression before and after 3 cycles of induction chemotherapy (5-fluorouracil, adriamycin and cyclophosphamide) using immunohistochemically stained paraffin-embedded specimen of 28 patients with locally advanced breast cancer. The frequency of P-glycoprotein expression in untreated breast cancer turned out to be very low: only one out of 28 untreated, biopsy specimen at the time of diagnosis was positive. The frequency of P-glycoprotein expression was markedly increased from 9.1% before chemotherapy to 63.6% after induction chemotherapy ($p=0.006$). After 3 cycles of induction chemotherapy, 25 patients had obtained clinical response to chemotherapy (4, CR; 21, PR). Eleven out of 25 tumors (44%) showing clinical response and all three tumors (100%) with minimal response have expressed P-glycoprotein. One out of 6 patients (16.7%) with microscopic residual tumor seen in mastectomy specimen expressed P-glycoprotein, whereas 13 of 22 patients (59.1%) with gross residual tumor showed the presence of P-glycoprotein ($p=0.08$). The frequency of intrinsic P-glycoprotein expression in untreated breast cancer was quite low, but approximately half of the patients do acquire P-glycoprotein expression during the cycles of induction chemotherapy. Therefore, the results suggest that the immunohistochemical detection of P-glycoprotein on residual tumor cells after induction chemotherapy can predict acquired drug resistance in breast cancer.

Key Words: P-glycoprotein, immunohistochemical stain

In locally advanced breast cancer, the clinical and pathological responses to induction chemotherapy seem to be correlated well with the prognosis of the patients (Hortobagyi *et al.* 1988; McCready *et al.* 1989) Development of resistance to chemotherapeutic agents would be a major problem in the

treatment of breast cancer, and one of the mechanism associated with chemoresistance might be the expression of the P-glycoprotein encoded by the multidrug resistance (MDR1) gene (Gerlach *et al.* 1986). P-glycoprotein has been thought to be an efflux pump that enforces hydrophobic drugs toward outside the cells (Kartner *et al.* 1983; Fojo *et al.* 1985).

Immunohistochemical detection using monoclonal antibody for P-glycoprotein-like protein has been considered a more sensitive method than Southern, Northern or Western blot analysis because even very low numbers of positive cells or a lower grade of expression could be readily recognize (Chan *et al.* 1988; Merkel *et al.* 1989). In some tumors, the expression of P-glycoprotein at initial

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presentation was highly correlated with refractoriness to chemotherapy (Dalton et al. 1989; Chan et al. 199). However, the P-glycoprotein expression was not common in the untreated breast cancer cell line or tumor when it was tested by immunohistochemistry using various kinds of monoclonal antibodies (Sugawara et al. 1988; Schneider et al. 1989; van der Valk et al. 1990) or Northern blot (Goldstein et al. 1989; Merkel et al. 1989). Therefore, pretreatment detection of P-glycoprotein has not been available as a tool for predicting response to chemotherapy. Recently, there were two reports of extraordinarily high incidence of P-glycoprotein involved in untreated breast cancer specimen, which might suggest the value of assessment of pretreatment P-glycoprotein expression for the prediction of treatment failure (Ro et al. 1990; Verrelle et al. 1991). Still, there remains a question of whether the significant number of pretreatment tumors already had intrinsic P-glycoprotein positive cells which could have had a negative influence on response to chemotherapy. In addition to intrinsic P-glycoprotein, the role of acquired P-glycoprotein after treatment in breast cancer has not been fully studied yet.

Therefore, we have attempted immunohistochemical staining to detect P-glycoprotein expression in paraffin-embedded tumor specimen from 28 locally advanced breast cancers employing monoclonal antibody JSB-1 to compare the frequency of P-glycoprotein expression before and after chemotherapy, and we attempt also to evaluate the influence of the acquired P-glycoprotein on drug response.

PATIENTS AND METHODS

Patients and Treatment

Twenty-eight female patients with locally advanced breast cancer who had entered the multimodality treatment program from January 1983 to December 1989 at Yonsei Cancer Center, Yonsei University College of Medicine, Seoul, Korea, were included in this study. The median age of the patients was 49 years (range; 30 to 71 years) and all tumors were infiltrating ductal carcinoma. Eight patients were stage IIIA, and 20 were IIIB according to the AJC (American Joint Committee on Cancer) staging system revised in 1988.

Chemotherapy and Evaluation of Drug Response

The patients were treated with 3 cycles of induc-

tion chemotherapy consisting of 5-fluorouracil, adriamycin and cyclophosphamide (FAC); modified radical mastectomy; radiotherapy; and 6 cycles of FAC maintenance chemotherapy. Following induction chemotherapy, patients were evaluated in terms of tumor response to chemotherapy by clinical examination and mammography. A complete response (CR) was defined as the total resolution of tumor; a partial response (PR) as a $\geq 50\%$ regression of the maximum diameter of tumor; a minimal response (MR) as a $\leq 50\%$ regression of tumor; and a progressive disease (prog) as an increase in the maximal diameter of the tumor. We have reviewed pathology specimens of the patients in order to assess the degree of tumor reduction after induction chemotherapy (Feldman et al. 1986). Complete response by pathologic criteria was defined as the absence of any gross or microscopic evidence of residual tumor. Gross residual tumor indicates the macroscopic evidence of tumor under gross inspection of the mastectomized specimen. Microscopic residual tumor was defined as having only microscopic evidence of tumor cells without gross residual tumor. These assessments were done regardless of the clinical response.

Immunohistochemical Staining Procedure

Twenty-eight tumor specimens were obtained at surgery. In addition to these, 11 specimens were obtained at the time of biopsy prior to chemotherapy. The tumor specimens were formalin-fixed and embedded in paraffin following the routine method.

Immunohistochemical staining was done by the Avidin-Biotinyl Peroxidase technique on formalin-fixed, paraffin-embedded tissue sections. The sections were placed in a 37°C dry oven overnight, deparaffinized in xylene and rehydrated in ethanol serially. Endogenous peroxidase activity was blocked with 50 mL methanol with 3% hydrogen peroxide. Slides were then incubated with 3% horse serum (Vector Laboratories, Burlingame, CA) at 37°C for 20 minutes, then incubated with primary antibody at 37°C for 30 minutes and washed in phosphate buffer saline (PBS). Murine monoclonal antibody specific for P-glycoprotein, JSB-1 (Sanbio, Am Uden, Holland) was used as the primary antibody (Sheper et al. 1988). Biotinylated secondary antiserum were dropped on slides, which were incubated at 37°C for 30 minutes and washed in PBS. The slides were then incubated with Vector Elite Avidin-Biotin Complex (Vector) at room temperature for 30 minutes and washed in PBS. Diaminobenzidine

(DAB) was used as a peroxidase substrate for color development. The slides were incubated in DAB 1 mg/mL, 45 mL PBS and 3% hydrogen peroxide for 2 to 7 minutes, counterstained with aqueous hematoxyline for 2 minutes, washed in PBS and tap water. Multidrug-resistant SNU-C4 and KB-8-5 cells and multidrug-sensitive KB-3-1 cells were used as positive and negative control (Fojo *et al.* 1987; Park *et al.* 1990). Grades of expression of P-glycoprotein were described arbitrarily according to the percentage of positive cells against the total population of cells (1⁺; less than 25% of positive cells, 2⁺; 25-50 %, 3⁺; more than 50%).

Statistical Analysis

The comparison of the frequency of P-glycoprotein expression before and after chemotherapy, and the association between P-glycoprotein expression and tumor response were determined with the Yates modified chi-square test (Yates 1958).

RESULTS

Incidence of P-glycoprotein Expression in Untreated and Treated Breast Tumors

One of 11 biopsied tumors (9.1%) prior to chemotherapy was positive for P-glycoprotein expression. The frequency of P-glycoprotein expression was markedly increased from 9.1% before chemotherapy to 63.6% after induction chemotherapy. The difference in the incidence of P-glycoprotein expression between untreated and treated tumors was statistically significant (Table 1). Fourteen of 26 tumor samples obtained at mastectomy after 3 cycles of induction chemotherapy demonstrated P-glycoprotein expression (five cases were 3⁺, three

were 2⁺, six cases showed 1⁺). There was regional heterogeneity in the presence and the degree of P-glycoprotein expression of tumor, and a remarkable expression of P-glycoprotein in tumor cells was demonstrated after 3 cycles of induction chemotherapy (Fig. 1).

Evaluation of P-glycoprotein Expression After Chemotherapy Correlated with Clinical and Pathologic Response to Chemotherapy

After 3 cycles of induction chemotherapy, 4 patients showed clinical CR including two pathologically proven CR, and 21 showed PR. Three patients showed MR to chemotherapy. One of four patients obtaining clinical CR and 10 of 21 patients showing PR (44%) had expressed P-glycoprotein. By contrast, all three tumors (100%) showed MR expressed P-glycoprotein (Table 2). When P-glycoprotein expression was evaluated in terms of pathologic response, one of six tumors (16.7%) had microscopic residual tumor and thirteen of twenty-two tumors (59.1%) expressed P-glycoprotein (Table 3).

DISCUSSION

A study to determine whether pretreatment P-glycoprotein detection could predict the response to chemotherapy has been tried in breast cancer. In leukemia and multiple myeloma, intrinsic or acquired P-glycoprotein expression has shown to be significantly correlated with chemoresistance (Ma *et al.* 1987; Dalton *et al.* 1989; Salmon *et al.* 1989). In addition to these hematologic malignancies, the prognostic value of P-glycoprotein expression has been reported in some solid tumors (Bell *et al.* 1985; Bourhis *et al.* 1989; Chan 1990). Thence, it is

Table 1. The frequency of positive P-glycoprotein expression on residual cancer cells of patients with locally advanced breast cancer

	No. of positive P-glycoprotein expressions / total (%)		
	Pre-chemotherapy	Post-chemotherapy	P-value
Breast cancer-locally advanced			
With biopsied specimen	1/11 (9.1)	7/11 (63.6)	0.006
Without biopsied specimen		7/15 (46.7)	
Total	1/11 (9.1)	14/26 (53.8)	0.02

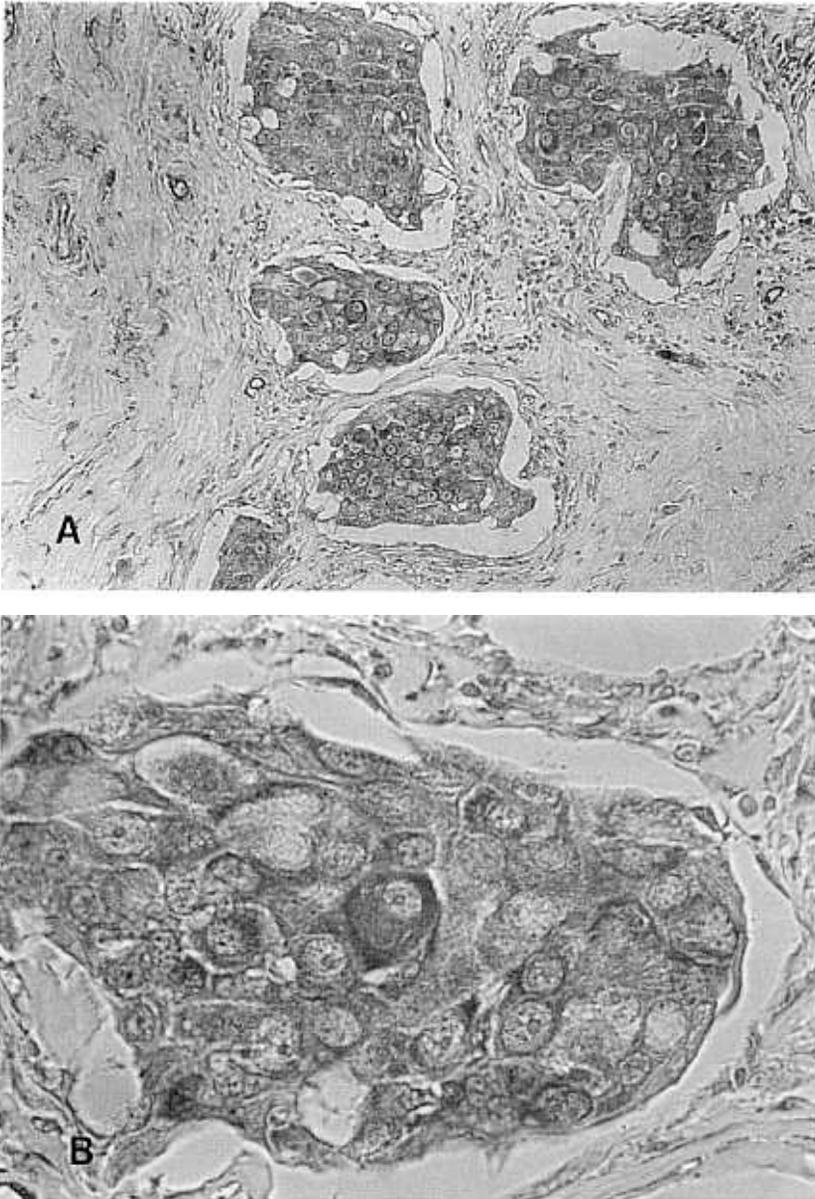


Fig. 1. Immunohistochemical staining using monoclonal antibody JSB-1. Heterogeneous P-glycoprotein expression is identified on breast cancer cells of mastectomy specimen after 3 cycles of induction chemotherapy (A, 100 \times ; B, 400 \times).

commonly accepted that measurement of the level of P-glycoprotein expression could be a valuable tool for guiding chemotherapy and for predicting prognosis of certain malignancies. The electrophoretic methods such as Northern and Western blotting in detecting P-glycoprotein expression with tiny tissue samples containing very small number of P-

glycoprotein expressing tumor cells were proved not to be satisfactory (Merkel et al. 1989). Therefore immunohistochemical detection of P-glycoprotein using monoclonal antibodies is now widely accepted for detecting even a single P-glycoprotein expressing cell or a low level of expression which could not have been detected by electrophoretic

Table 2. Incidence of P-glycoprotein expression after 3 cycles of induction chemotherapy by clinical responses

Clinical response	No. of tumors	No. of positive P-glycoprotein (%)
CR	4*	1(25)
PR	21	10(47.2)
MR	3	3(100)

CR=complete response; PR=partial response, MR=minimal response.

*Two of four tumors achieved pathologic CR.

Table 3. Incidence of P-glycoprotein expression after 3 cycles of induction chemotherapy by pathologic response

Amount of residual tumor	No. of tumors	P-glycoprotein expression	
		Negative	Positive (%)
Total	28	14	14(50)
Microscopic	6*	5	1(16.7)
Gross	22	9	13(59.1)**

* Two samples had no residual tumor cells within mastectomy specimen

** P-value was 0.08 as comparing microscopic versus gross residual groups.

analysis. But despite the higher sensitivity of immunohistochemistry, most of the published data using various monoclonal antibodies (C219, MRK 16, or JSB-1) suggest that P-glycoprotein expression in primary breast tumor was not a common phenomena (Sugawara *et al.* 1988; Schneider *et al.* 1989; van der Valk *et al.* 1990). Recently, two papers have reported detection of P-glycoprotein expressing cells in most of locally advanced breast cancer samples prior to chemotherapy by immunohistochemistry using C219 and C494 (Ro *et al.* 1990; Verrelle *et al.* 1991). On that basis, the authors of these papers suggest that pretreatment measurement of P-glycoprotein expression could be useful for predicting response to induction chemotherapy in breast cancer. However, there are some considerations regarding interpretation of immunohistochemical staining in terms of its false positive and/or false negative results. The cross reactivity of monoclonal antibodies with normal structures of tis-

issues and with MDR2 isoform of P-glycoprotein should be taken into account. The monoclonal antibody JSB-1 used in our study recognized a highly conserved epitope on the same narrow cytoplasmic domain of P-glycoprotein close to the C219 binding but did not discriminate between isoforms of P-glycoprotein (Sheper *et al.* 1988; Weinstein *et al.* 1990). We also experienced difficulties in interpreting the patterns of positivity when extremely small number of cells were weakly positive for P-glycoprotein and in the cases of homogenous staining of cytoplasm. In such cases the tumors were categorized negative for P-glycoprotein.

We have found distinct P-glycoprotein expression in only 1 out of 11 untreated tumor (9.1%) samples, among which seven (63.6%) tumors have expressed P-glycoprotein after 3 cycles of induction chemotherapy. These results would indicate that far more than half of the tumors have expressed P-glycoprotein with chemotherapy including adriamycin. Since the frequency of appearance of P-glycoprotein in breast cancers prior to chemotherapy has been very low, we could not affirm any clinical value in predicting the response to chemotherapy. In leukemia and multiple myeloma, acquired P-glycoprotein expression occurring in the course of chemotherapy is known to be significantly correlated with the development of chemoresistance (Ma *et al.* 1987; Dalton *et al.* 1989). However, the meaning of acquired P-glycoprotein expression in breast cancer occurring during the cycles of induction chemotherapy has not been fully determined yet. As a result we have observed that the frequency of P-glycoprotein expression was far less common in tumors achieving CR and PR than in those which showed MR. P-glycoprotein expression was more prominent in gross residual tumors than in microscopic residual tumors seen at the time of surgery even though significant statistical difference was not achieved in such a limited number of tumor samples ($p=0.08$). Just inferring from the data, one can consider that induction chemotherapy would induce P-glycoprotein expression in metastatic tumor cells as well as in primary tumor cells which could affect a patient's prognosis. However, so far, there has been no difference in recurrence rate and length of recurrence-free interval during the median follow-up of 24 months in terms of P-glycoprotein expression (data not shown).

We have observed that more than half of the breast cancers in our study have shown the appearance of P-glycoprotein expression following 3 cycles of induction chemotherapy. Therefore we suggest

that the immunohistochemical detection of P-glycoprotein in surgical specimen would be an available tool, in part, to predict the acquired chemoresistance in breast cancer.

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