

Changes in Protein Expression in Breast Cancer after Anthracycline-Based Chemotherapy

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Background : Anthracyclines are the standard agents used to treat patients with advanced breast carcinoma. Some molecules are reportedly associated with anthracycline resistance; however, there has been some controversy surrounding these claims. The gain or loss of certain molecules after chemotherapy can explain the discrepancies in the results. **Methods :** We evaluated the expression levels of the estrogen receptor (ER), p53, and bcl-2 in specimens obtained from twenty patients with advanced breast cancer before and after anthracycline-based chemotherapy using immunohistochemistry (IHC). We also examined HER2/neu expression in these specimens using IHC and fluorescence in situ hybridization (FISH) analysis. **Results :** After chemotherapy, one of the twenty cases (5%) showed decreased ER expression, one (5%) showed decreased p53 expression, and one (5%) showed increased bcl-2 expression. IHC and FISH analysis in pre- and post-chemotherapy specimens showed that the expression of HER2/neu changed from equivocal to negative in one case (5%). **Conclusion :** Our results showed that the expression levels of HER2/neu, ER, p53 and bcl-2 remained stable after chemotherapy, although the statistical significance of these results may not be validated due to the small number of cases. We also suggested that the resistance to anthracycline-based chemotherapy might not be associated with the modification of these molecules.

Key Words : Anthracyclines; Carcinoma, ductal, breast; Drug resistance, neoplasm; Neoadjuvant therapy

Many biological factors expressed by the tumor as well as classical pathologic features have great value for the prediction of the prognosis and development of treatment plans for patients with breast cancer. Gene amplification and/or protein overexpression of HER2/neu (also called c-erbB2), which is a member of the growth factor receptors with tyrosine kinase activity, is found in about 25% of breast carcinomas and is associated with negative hormone receptor status, higher nuclear and histological grades, resistance to hormone therapy or nonanthracycline-based chemotherapy and poor outcomes.¹⁻⁵ It is recommended that hormone receptors, such as the estrogen receptor (ER) and progesterone receptor, should be measured on every primary breast cancer in order to help predict the response to hormonal therapy.⁶ Anti-apoptotic factor bcl-2 and pro-apoptotic factor p53 can also influence the characteristics of breast cancer; however, the effects of these proteins on breast cancer remain controversial.⁷ Rolland *et al.*⁸ recently demonstrated that the p53-positive and bcl-2-negative phenotype was associated with a poor prognosis, while neither p53 nor bcl-2 alone had definite

prognostic significance.

Anthracycline-containing regimens are the standard neoadjuvant chemotherapies for patients with locally advanced breast cancer.⁹ Some authors showed that the aberrant expression of certain proteins, including HER2/neu, p53 and hormone receptors, was associated with decreased responsiveness to anthracycline-based chemotherapy in breast carcinoma,^{10,11} while other studies failed to demonstrate the association.^{12,13} These discrepancies may result from the modulation of biological prognostic factors after chemotherapy or the presence of other molecules that may interfere with the action of anti-cancer drugs in some of the cases. Therefore, investigations into the changes in protein expression in breast cancer cells after chemotherapy can aid in elucidating the resistance mechanisms of breast cancer and in the development of new chemotherapeutic agents. We examined the expressions of HER2/neu, ER, p53, and bcl-2 in the tumors before and after chemotherapy with immunohistochemistry (IHC), and we examined the gene amplification of HER2/neu using fluorescence in situ hybridization (FISH).

MATERIALS AND METHODS

Patients and tumors

Twenty women with advanced stages of infiltrating duct carcinoma who visited Seoul National University Hospital between July 2001 and September 2002 were included in this study. The patients' ages ranged from 32 to 63 years (mean 45.9 years). The tumor size of each patient was at least 5 cm on palpation or ultrasonography, and ipsilateral axillary lymph node enlargement was found in every patient at the time of visit. To study the IHC status of the tumor before chemotherapy, three to four pieces of needle biopsy specimens were obtained. Twelve patients (60%) then received two or three cycles of neoadjuvant chemotherapy, which consisted of doxorubicin 60 mg/m² plus cyclophosphamide 600 mg/m² (AC regimen), and seven patients (35%) received two cycles of doxorubicin 60 mg/m² plus docetaxel 60 mg/m² (AD regimen). Patient 20, who had visited the hospital with bilateral breast carcinoma, received a total of 28 cycles of chemotherapy, which consisted of nine cycles of AD, two cycles of paclitaxel, five cycles of docetaxel and twelve cycles of capecitabine. The patients then underwent simple or modified radical mastectomy and axillary lymph node dissection at two to 13 weeks after the end point of the chemotherapy. The resected specimens were delivered into the pathology department, and the pathologists obtained at least three sections from each breast cancer specimen. Both needle biopsy and mastectomy specimens were fixed in 4% buffered formaldehyde and embedded in paraffin.

Immunohistochemical staining (IHC)

Since none of the twenty patients demonstrated complete remission after chemotherapy, we assayed IHC in both pre-chemotherapy needle biopsy specimens (NBS) and post-chemotherapy resected specimens (RS) from all patients. IHC studies of ER, p53, bcl-2 and HER2/neu were performed on the 4- μ m cut sections of both specimens from all patients (Table 1). In brief, slides were rehydrated, washed and treated with the appropriate antigen retrieval method for each antibody. The slides were then immersed in 3% H₂O₂ solution to block endogenous peroxidase activity and incubated with the primary antibodies. After incubation, the slides were stained with peroxidase-labeled streptavidin-biotin complex and counterstained with Meyer's hematoxylin. Positive staining was defined as nuclear staining in 10% or more of the tumor cells for ER and in 50% or more for p53. For bcl-2, cytoplasmic staining in 10% or more of the

tumor cells was defined as positive. Only the membranous staining of tumor cells was scored for the determination of HER2/neu expression, and the intensity was scored as follows: 0 for negative staining, 1+ for weak staining, 2+ for moderate staining and 3+ for strong staining in at least 10% of the tumor cells.

Fluorescent in situ hybridization (FISH)

We used FISH to analyze the NBS and RS of the cases showing altered expression after chemotherapy, except for the changes from 0 to 1+ and vice versa because tumors with HER2/neu IHC score of either 0 or 1+ usually demonstrated a normal HER2/neu gene status and were regarded as negative.¹⁴ PathVysion HER2/neu DNA probe Kit made by Vysis, Inc (IL, USA) was used for dual-color FISH analysis. In brief, the 4- μ m cut slides were deparaffinized, treated with protease solution and denatured in 70% formamide/2 \times SSC. After washing with iced cold ethanol solutions, the slides were hybridized with HER2/neu and chromosome 17 (CEP17) probes and incubated overnight at 37°C. The slides were then washed with wash buffer and counterstained with 4',6-diamidino-2-phenylindole (DAPI). Signals were visualized on a reflected fluorescence system microscope (BX51; BH2-RFL-T3, Olympus, Japan) at 40 \times or 100 \times magnification. Triple-band pass filters (DAPI/Orange/Green, Vysis) were utilized for the detection and enumeration of the HER2/neu probe signal (orange), CEP17 probe signal (green) and DAPI (blue). The scores for each slide were determined by comparing the ratio of the average HER2/neu signal to that of the CEP17 signal in at least 60 cells showing non-overlapping intact nuclei. FISH scores greater than 2.2 were considered to be positive whereas scores less than 1.8 were considered to be negative and scores between 1.8 and 2.2 were considered to be equivocal, according to the guidelines suggested by Wolff *et al.*¹⁴

Determination of the changes between NBS and RS

ER, p53 and bcl-2 were considered to be increased when negative in the NBS and positive in the RS, and decreased when

Table 1. Antibodies used in the immunohistochemical staining

Antibody	Clone	Manufacturer	Antigen retrieval	Dilution
ER	1D5	DAKO, Denmark	microwave	1:50
p53	DO-7	DAKO, Denmark	microwave	1:800
bcl-2	124	DAKO, Denmark	steamer	1:50
HER-2/neu	CB11	DAKO, Denmark	microwave	1:200

ER, estrogen receptor.

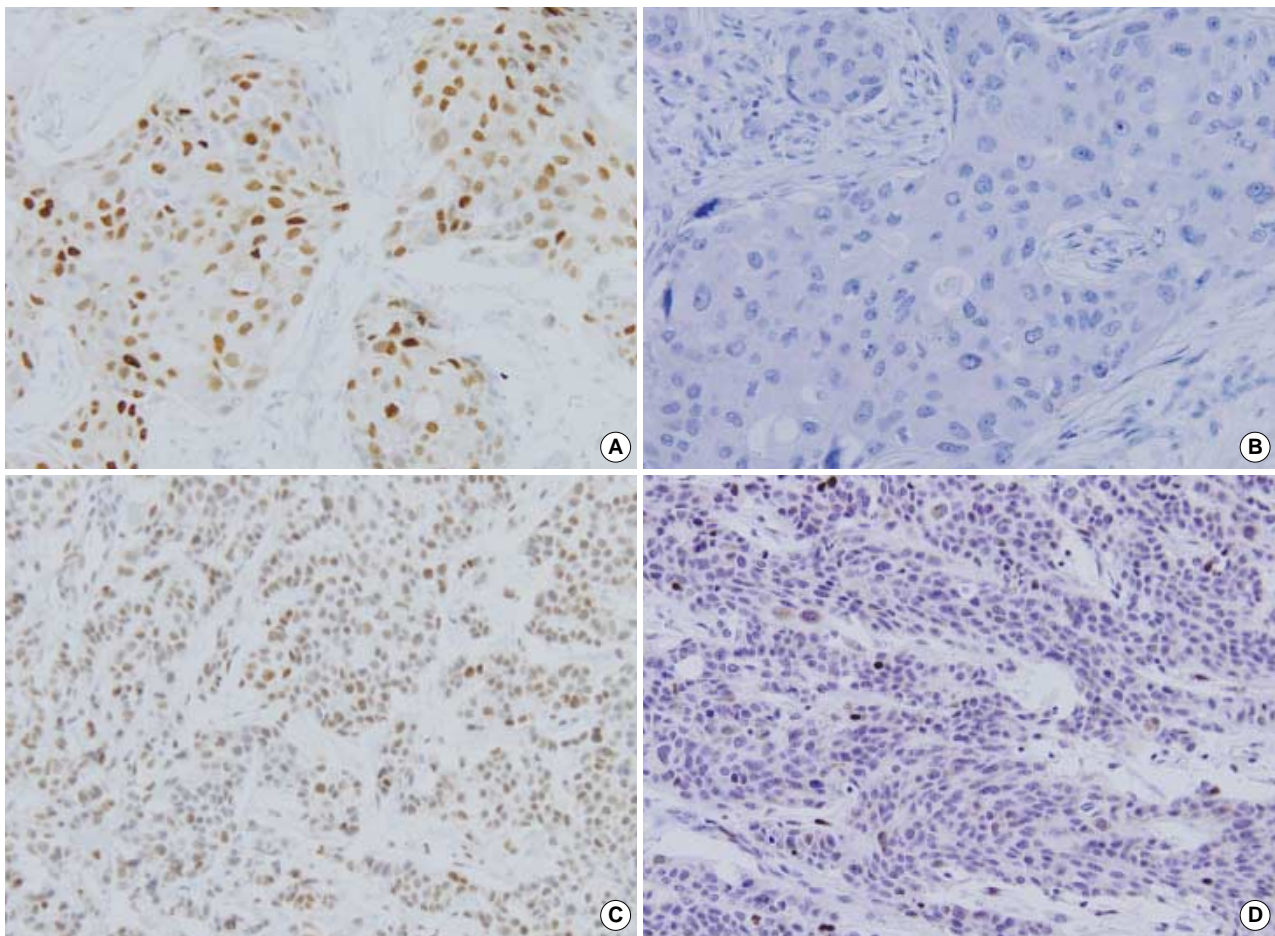


Fig. 1. Photomicrographs of IHC for ER and p53 in the specimens before (A, C) and after (B, D) chemotherapy. In case 2, ER is positive in the NBS (A) and negative in the RS (B). In case 13, p53 is positive in the NBS (C) and negative in the RS (D).

these proteins were positive in the former and negative in the latter. For the FISH assay, changes from negative to equivocal or positive and from equivocal to positive were considered as indicative of increased amplification/expression of HER2/neu and changes from positive to equivocal or negative and from equivocal to negative were indicative of decreased HER2/neu amplification/expression.

RESULTS

Each of the 20 cases showed decreased expression levels of ER (case 2, 5%) and p53 (case 13, 5%) after chemotherapy, respectively (Fig. 1). One case (case 18, 5%) showed increased expression of bcl-2 in the NBS and RS. Two of the 20 cases (case 11 and 12, 10%) showed differential expression of HER2/neu IHC between the NBS and RS. One (case 11) scored 1+ on the NBS and 2+ on the RS, and the other (case 12) scored 2+ on the NBS

and 1+ on the RS. We performed FISH analysis in both the NBS and RS from the two cases, and the FISH scores in the specimens were 1.05 and 1.12 for case 11 and 2.01 and 1.66 for case 12, respectively. Compared to their NBS scores, only one case (case 12) showed altered expression of HER2/neu, which was a decrease from equivocal to negative. No changes in the expression of more than one protein were found in any of the cases. Two of the twelve (17%) cases that received chemotherapy with the AC regimen and two of the seven (28%) cases that were treated with the AD regimen showed changes in the expression of any of the proteins after chemotherapy. Protein expression status was not changed by preoperative chemotherapy in sixteen of the twenty cases (80%).

DISCUSSION

It is important to determine whether the expression of vari-

Table 2. Immunohistochemical results for HER2/neu, ER, p53 and bcl-2 and fluorescence *in situ* hybridization scores for HER2/neu in the pre-chemotherapy needle biopsy specimens and post-chemotherapy resected specimens

Patient No.	Chemotherapy regimen × cycles	HER-2/neu				ER		p53		bcl-2	
		NBS		RS		NBS	RS	NBS	RS	NBS	RS
		IHC	FISH	IHC	FISH						
1	AC × 2	0		0		-	-	-	-	-	-
2	AC × 2	3		3		+	-	+	+	-	-
3	AC × 3	0		0		+	+	-	-	+	+
4	AC × 2	1		1		+	+	-	-	+	+
5	AC × 2	3		3		-	-	+	+	-	-
6	AC × 2	0		1		+	+	-	-	-	-
7	AC × 2	3		3		-	-	-	-	-	-
8	AC × 2	0		1		+	+	-	-	+	+
9	AC × 2	3		3		-	-	+	+	-	-
10	AC × 2	1		1		+	+	-	-	+	+
11	AC × 2	1	1.05	2	1.22	-	-	+	+	+	+
12	AC × 2	2	2.01	1	1.67	+	+	+	+	+	+
13	AD × 2	1		1		+	+	+	-	+	+
14	AD × 2	0		1		+	+	-	-	+	+
15	AD × 2	3		3		-	-	+	+	+	+
16	AD × 2	3		3		-	-	+	+	-	-
17	AD × 2	3		3		-	-	-	-	-	-
18	AD × 2	1		1		-	-	+	+	-	+
19	AD × 2	3		3		-	-	+	+	-	-
20	multiple	3		3		+	+	-	-	+	+

AC, doxorubicin+cyclophosphamide; AD, doxorubicin+docetaxel; ER, estrogen receptor; FISH, fluorescence in situ hybridization; IHC, immunohistochemistry; NBS, pre-chemotherapy needle biopsy specimen; RS, post-chemotherapy resected specimen.

ous proteins in breast carcinomas typically change after chemotherapy. First, modifications or changes in the expression levels of certain proteins after chemotherapy can lead to drug resistance. Second, if changes in certain molecules commonly occur, the assessment of these proteins after chemotherapy may not be an appropriate tool for the prediction of the patient's prognosis. Some studies found that the expression levels of certain molecules were significantly changed after chemotherapy,¹⁵⁻¹⁸ while others were not.¹⁹⁻²⁴ Rasbridge *et al.*¹⁵ noticed that chemotherapy modified the expression of HER2/neu in eleven (36.7%) out of thirty breast carcinomas available for the assay of both pre- and post-chemotherapy specimens. The expression of HER2/neu, however, were assayed by IHC alone in this study. In the following studies, FISH analysis revealed that changes in HER2/neu expression after chemotherapy were observed in 4-13% of patients and were not statistically significant when compared with the expression levels in the control cases.¹⁹⁻²³ Moreover, Vincent-Salomon *et al.*¹⁹ demonstrated that the expression of HER2/neu was stable not only after chemotherapy, but also during the metastatic process.

The expression of p53 was modified after chemotherapy in some studies.¹⁵⁻¹⁷ However, these results were not consistent: p53 expression was significantly increased in two studies,^{15,16} but

decreased in another study.¹⁷ Rasbridge *et al.*¹⁵ demonstrated an increase in p53 expression in 57% of p53-negative tumors and interpreted the gain in p53 as being the accumulation of normal protein due to toxic damage by anticancer drugs and not mutations in the p53 gene. Daidone *et al.*¹⁷ showed that a significant decrease in the number of p53-positive cells was found in 62% of p53-overexpressing tumors, while almost all p53-negative tumors remained unchanged after chemotherapy. Other studies reported that p53 expression was stable after chemotherapy.^{23,24} In contrast to p53, however, a significant change in the expression of the anti-apoptotic factor bcl-2 expression after chemotherapy was not found in these studies.^{15,17} Taucher *et al.*¹⁸ showed that hormone receptor levels decreased after chemotherapy and speculated that the progression to hormone independence was due to the acquisition of the ability to express autocrine growth factors. However, this result was not supported by other studies.^{17,22-24}

Even though there were not enough cases to allow for an analysis of the statistical significance of the data and we did not study patients who did not receive neoadjuvant chemotherapy as control cases, our study did reveal that changes in the expression of HER2/neu, ER, p53 and bcl-2 after chemotherapy were not common. We also suggest that the gain or loss of these molecules

after chemotherapy in a small percentage of cases may have been caused by tumor heterogeneity rather than by chemotherapy. We prudently suggest that the expressions of HER2/neu, ER, p53 and bcl-2 seem to be the early events of carcinogenesis in breast cancer, and that their expression levels remain unchanged after anthracycline-based chemotherapy. We also suggest that the assessment of these molecules before and after chemotherapy is valid. We also suggest that changes in the expression of some other proteins after chemotherapy or the aberrant expression of certain molecules can influence the resistance to chemotherapeutic agents. Climent *et al.*²⁵ revealed that no genomic changes were significantly correlated with recurrence in patients treated with chemotherapy while the deletion of chromosome 11q was independently associated with relapse in those who had not received chemotherapy, suggesting that breast carcinomas with this chromosomal abnormality might benefit from anthracycline-based chemotherapy. Tinari *et al.*²⁶ demonstrated that the expression of topoisomerase II α , which is the target molecule of anthracyclines, was significantly reduced after anthracycline-based chemotherapy in some patients with breast carcinoma and that these changes were strongly related to a poor relapse-free survival. Schroll *et al.*²⁷ showed that elevated tissue inhibitor of metalloproteinases-1 levels in breast carcinoma were significantly associated with a poor response to chemotherapy with either cyclophosphamide/methotrexate/5-fluorouracil or anthracyclines. Harris *et al.*²⁸ also revealed that HER2/neu-overexpressing tumors with a basal-like phenotype or with the expression of insulin-like growth factor-I receptor are more likely to be resistant to the chemotherapy with trastuzumab and vinorelbine.

In summary, our results indicate that the expressions of HER2/neu, ER, p53 and bcl-2 were not significantly changed after chemotherapy. Although the statical importance of these data may not be validated due to the small number of cases and a further study in a larger population is needed, we suggest that it is unlikely that the resistance mechanism against chemotherapy is associated with the changes in the expression levels of HER2/neu, ER, p53 and bcl-2.

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