Changes of Topoisomerase IIα Expression in Breast Tumors after Neoadjuvant Chemotherapy Predicts Relapse-Free Survival

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Abstract

Purpose: To assess the value of changes in the expression of topoisomerase IIα (TopoII) and the proto-oncogene erbB-2 (HER-2) as predictors of relapse-free survival in women with operable breast cancer treated with anthracycline-based neoadjuvant chemotherapy.

Patients and Methods: Seventy-seven patients with primary breast cancer who had undergone neoadjuvant anthracycline-based chemotherapy were included in the present study. TopoII and HER-2 were measured by immunohistochemistry in prechemotherapy and postchemotherapy (at the time of surgery) tumor specimens, and the value of their changes as predictors of relapse-free survival were evaluated by Kaplan-Meier and Cox proportional hazard regression analyses.

Results: Neoadjuvant chemotherapy resulted in a significant reduction in the percentage of cells expressing TopoII ($P < 0.0001$). No significant change was observed for HER-2. TopoII and HER-2 expression before chemotherapy predicted tumor response to treatment. Changes in TopoII expression after chemotherapy were strongly associated with a poor relapse-free survival ($P < 0.0001$) in a Cox multivariate analysis adjusted for other clinicopathologic prognostic factors.

Conclusion: Changes in TopoII expression after anthracycline-based neoadjuvant chemotherapy is an independent predictor of a poor relapse-free survival in patients with breast cancer. Tumor cells displaying an increased TopoII expression after treatment may be responsible for relapses, and may, therefore, define a group of patients with anthracycline-resistant breast cancer.

Breast cancer is the most common malignancy among women in the Western world. The majority of women present with disease localized to the breast with or without axillary lymph node involvement (1). Despite radical surgery, >50% of surgically treated patients eventually relapse. The introduction of adjuvant treatment (endocrine, chemotherapy, and radiotherapy) has resulted in a reduction in mortality, with a 25% survival improvement at 10-year follow-up (2). Metastatic disease, however, remains incurable, with patients becoming progressively less sensitive to systemic therapy.

Clinical studies have shown that the use of neoadjuvant (primary) chemotherapy for patients with locally advanced breast cancer could increase surgical resectability rates, and response to therapy is correlated with the patient's ultimate disease-free survival (3–6). In addition, significant volume reduction in tumors after neoadjuvant chemotherapy may permit subsequent, successful breast-conserving surgical treatment (7–10). A unique advantage of primary chemotherapy is the possibility to take serial measurements of the primary tumor, thus allowing the *in vivo* assessment of factors predictive of sensitivity to treatment (11). Moreover, changes in the expression of these molecular markers, together with tumor response, may allow for early prediction of relapse and survival.

Anthracycline-based regimens are frequently used in the neoadjuvant setting. These drugs act by several mechanisms, but interaction with the nuclear enzyme topoisomerase IIα (TopoII) seems to be the most prominent one. TopoII reduces DNA twisting and super-coiling, allowing selected regions of DNA to untangle and thus engage in transcription, replication, or repair processes (12, 13). Previous studies have reported variable results regarding TopoII expression and response to anthracycline-based chemotherapy in breast cancer (reviewed in ref. 14). It has been suggested that high concentrations of TopoII may be associated with features of poor nuclear differentiation, high proliferation, and absence of steroid hormone receptors (15), and studies using cell lines have shown that cells with high amounts of the enzyme might be more sensitive to TopoII poisons (16, 17).

HER-2 is overexpressed in 20% to 25% of breast cancers (18, 19). This 185-kDa transmembrane glycoprotein with intrinsic tyrosine kinase activity is encoded by the HER-2/*neu* proto-oncogene and is involved in the reception of growth factors of the epidermal growth factor family. HER-2 amplification/overexpression has been established as an important

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independent predictor of poor prognosis in early stage breast cancer (20). Additionally, amplification/overexpression of HER-2 has been generally viewed as an indicator of improved outcome after anthracycline-based therapy (21, 22). Because of its location in the same amplicon on chromosome 17, the gene encoding for TopoII has been found frequently coamplified with that of HER-2, which leads to the overexpression of its protein product (23, 24) and, possibly, to a greater sensitivity to anthracyclines (24).

With these considerations in mind, we decided to investigate the prognostic role of changes in TopoII and HER-2 expression assessed by immunohistochemistry in tumor specimens obtained before and after exposure to an anthracycline-based neoadjuvant chemotherapy in a series of consecutive patients with operable breast cancer.

Patients and Methods

Ninety-three consecutive patients with breast cancer treated at the Department of Oncology, University Hospital of Chieti between January 1999 and August 2003, with anthracycline-based primary chemotherapy were considered. Patients with inflammatory carcinoma, metastasis at presentation or within 6 months from surgery, and those for whom paraffin-embedded tissue from the pretreatment biopsy was insufficient to allow a pathologic diagnosis and evaluation of biological variables, were excluded. According to these criteria, a total of 77 patients were selected for the present study. Median age was 46.1 years (range, 25.5-73.7). The pretreatment staging work-up included a complete history and physical examination, complete blood count with differential and platelet counts, blood chemistry analysis, electrocardiography, bilateral mammography or breast ultrasonography, chest X-ray, abdominal computed tomography or abdominal ultrasonography, and bone scan. Tumor diameters were measured before the start of chemotherapy during physical exams or on radiograms, and ranged from 20 to 120 mm. Pathologic diagnosis was done in all cases on core needle biopsy specimens obtained before treatment. Routine pathologic evaluation included tumor grade, immunohistochemical assessment of estrogen (ER) and progesterone receptors (PgR), and Ki67. Patients received fluorouracil (600 mg/m^2), epirubicin (100 mg/m^2), and cyclophosphamide (600 mg/m^2) every 3 weeks. All patients completed at least 12 weeks of treatment before surgery. Mastectomy was considered necessary if the tumor was located within 2 cm from the nipple or was too large in comparison to breast size. Otherwise, breast-conserving surgery was done with complete surgical excision. The median number of excised axillary lymph nodes was 10. Radiotherapy was administered according to local institutional practice after both chemotherapy and definitive surgery. Estrogen and progesterone receptors and Ki67 were reassessed by immunohistochemistry on the surgical specimens.

Median follow-up was 27.4 months (range, 8.4-67 months). During the first 2 years after treatment, patients had a complete check-up (physical examination, bilateral mammography, blood chemistry, CEA and CA 15-3, chest X-ray) every 3 months, and every 6 months thereafter. A bone scan was requested every 6 months. At any time, if the patient exhibited elevated liver function tests, an ultrasound of the liver was obtained. The clinicopathologic characteristics of the study population are listed in Table 1.

The response to treatment was pathologically assessed after tumor excision and axillary lymph node resection. A pathologic complete response (pCR) was defined as the absence of residual invasive tumor in the breast or axillary lymph nodes as evaluated by H&E staining. Minimal Residual Disease (MRD) was defined as no gross tumor and microscopic invasive carcinoma present in two or fewer high-power fields. A partial response (PR) was defined as a >50% reduction of all the measurable lesions in the pathologic specimen compared with the clinical measurements taken at diagnosis. A <25% increase or decrease of the lesions was regarded as a stable disease (SD). An increase of >25% was defined as progression disease (PD). Tumor response was defined as pCR or MRD.

Table 1. Patient characteristics

Age	
Mean ± SD	46.8 ± 9.38
Median	46
Range	25.5-73.7
Tumor size (cm)	
2-5	58 of 77 (75%)
>5	19 of 77 (25%)
ER	
≤10%	29 of 77 (38%)
>10%	45 of 77 (62%)
PgR	
≤10%	42 of 77 (55%)
>10%	32 of 77 (45%)
Tumor grade*	
1	14 of 75 (19%)
2	38 of 75 (51%)
3	23 of 75 (30%)
HER-2	
Negative	57 of 77 (74%)
Positive	20 of 77 (26%)
Ki67 (percentage of positive cells) †	
Mean ± SD	36.0 ± 27.4
Median	30
Range	2-95
TopoII (percentage of positive cells) †	
Mean ± SD	15.4 ± 17.5
Median	8.5
Range	1-73
Response to treatment ‡	
pCR	3 of 77 (4%)
MRD	15 of 77 (19%)
PR	39 of 77 (51%)
SD	20 of 77 (26%)
Follow-up (mo)	
Median	27.4
Range	8.4-67
Relapse	
Local	5 of 77 (6%)
Distant metastasis	

*Tumor grade was assessable in 75 of 77 cases.
† Values represent percentage of immunostained cells based on a total count of 200 cells.
‡ Overall response rate was 74% (pCR, MRD, and PR); 23.4% of patients achieved pCR or MRD.

Immunohistochemistry. Immunohistochemistry was done on paired tumor samples taken from the pretreatment biopsies and surgical specimens. All samples were formalin-fixed and paraffin-embedded. Monoclonal antibody Ki-S1 (Chemicon Int., Temecula, CA) was used to detect TopoII by a peroxidase-streptavidin-biotin technique. Slides were deparaffinized in Histoclear, rehydrated and treated with 3% H_2O_2 in methanol for 10 minutes to block endogenous peroxidase activity. Sections were immersed in 10 mmol/L sodium citrate buffer (pH 6.0), subjected to heat-induced epitope retrieval in a microwave oven for 15

minutes, and then cooled for 20 minutes. The Vectastain ABC peroxidase kit (Vector Laboratories, Burlingame, CA) was used to detect the antigens. Endogenous biotin was saturated by a biotin blocking kit (Vector Laboratories). Negative controls were obtained by omitting the primary antibodies. Immunostained sections were evaluated by two independent pathologists who had no prior knowledge of the clinicopathologic variables. Each pathologist counted at least 200 cells within randomly selected and outlined areas on each slide, and the percentage of immunostained cells was determined. Disagreement between the pathologists was noted for <8% of samples. In these cases, consensus was reached by a joint re-evaluation of the slide.

HER-2 status, assessed by Hercept test (Dako, Glostrup, Denmark), was scored on a scale of 0 to 3+, according to the Dako scoring system: score 0, no or up to 10% membrane staining; score 1+, partial and/or faint membrane staining in >10% of tumor cells; score 2+, weak to moderate complete membrane staining in >10% of tumor cells; score 3+, strong complete membrane staining in >10% of tumor cells. Only tumors with a score of 3+, and those with a score of 2+ in which an amplification of the HER-2 gene was confirmed by fluorescent *in situ* hybridization, were considered as overexpressing HER-2.

Statistical analysis. For statistical analyses, age, clinical tumor size, ER, PgR, TopoII, and Ki67 were used as continuous variables, tumor grades (1-2 versus 3), HER-2 (negative versus positive), and response-to-treatment (pCR + MRD versus partial response + significant decrease) were categorical variables. Changes in the expression of TopoII and Ki67 were calculated by comparing the pretreatment with the posttreatment percentage of positive cells, and was coded in two additional dichotomous variables. In particular, 0 indicated no variation, a reduction or an increase of ≤50%, and 1 an increase of >50% in the percentage of positive tumor cells. The variations in the expression of HER-2 were recorded in a dichotomous variable, and coded as 0 (no variation or negativization) or 1 (positivization). Pretreatment characteristics predictive of response were identified by a multivariate logistic regression. Relapse-free survival was calculated from the date of surgery to the date of a documented relapse or the date of the last control. The significance of the various markers as predictors of relapse-free survival was analyzed by means of a univariate proportional hazard model. Relapse-free survival curves were calculated according to the Kaplan-Meier method. Only those variables found to predict relapse-free survival at the univariate analysis, were considered for Cox multivariate analysis with a stepwise variable selection. All the statistical analyses were done using BMDP Statistical Software (Los Angeles, CA).

Results

Clinical outcome. Seventy-seven patients who received primary anthracycline-based chemotherapy have been included in the study. After the administration of a median of four courses of chemotherapy (range, three to six), the overall response (pCR, MRD, and PR) to primary chemotherapy was 74% (56 of 77 patients). A tumor response (pCR or MRD) was obtained in 23.4% of the cases (18 of 77 patients). No PD under treatment was observed. Conservative breast surgery, mainly quadrantectomy, was done in 45 patients (57% of the total), whereas mastectomy was required in the other 32 cases. The median number of excised axillary lymph nodes was 10.

During the follow-up period (median of 27.4 months; range, 8.4-67 months), 15 relapses were observed (5 local recurrences and 10 distant metastases). In relapsing patients, relapse-free survival ranged from 9.5 to 37.6 months (median of 20 months).

Effect of neoadjuvant chemotherapy on TopoII and HER-2 expression. Paired tumor samples taken before and after chemotherapy from the same patients were analyzed for TopoII and HER-2 expression (Fig. 1). After primary chemotherapy, the percentage of cells expressing TopoII was significantly reduced (pretreatment versus posttreatment median value: 8.5% versus 1%; $P < 0.001$ by Wilcoxon signed-rank test). HER-2 was found to be positive before and after chemotherapy in 26% and 20.8% of the patients, respectively (P = not significant). When changes in the expression of these two markers in response to chemotherapy were assessed case-wise, an increase of >50% in the percentage of positive cells was observed in 20 of 74 evaluable cases for TopoII, whereas the HER-2 score became negative in 7 of 70 evaluable cases. No correlation was observed between TopoII and HER-2 expression either before or after treatment. Before treatment, a significant correlation was found only between TopoII and Ki67 expression (Spearman's $\rho = 0.72$, $P < 0.001$). The correlation was lost when the posttreatment expression levels were considered.

No statistically significant difference in the pretreatment percentage of TopoII-positive cells or HER-2 score was observed between patients in whom these two markers were unchanged/reduced or increased after treatment. Tumors displaying increased TopoII staining after chemotherapy were less likely to have responded by pathologic criteria (5%) than those with unchanged or decreased expression (35%; $P = 0.055$ by Fisher's exact test). There were no significant changes in the expression of ER, PgR, and Ki67 evaluated before and after chemotherapy.

Univariate and multivariate analysis of response and relapse-free survival. By logistic regression, a significant predictive influence on the likelihood of response was found for pretreatment TopoII and HER-2. In our population, the odds of achieving a tumor response (pCR and MRD) to chemotherapy increases by a factor of 1.05 [or by 5%; 95% confidence interval (CI), 1.01-1.08; $P < 0.001$] for each unit increase in the percentage of TopoII-immunostained cells, and is 5.28-fold

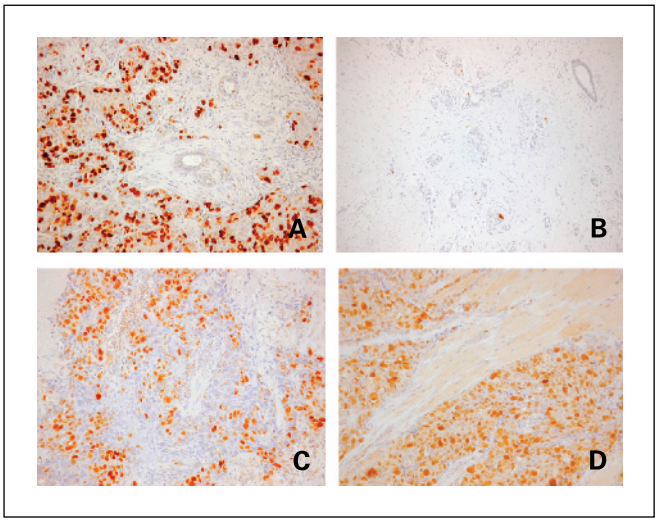

Fig. 1. TopoII immunohistochemistry. Pretreatment biopsies (*A* and *C*) and breast tumor specimens collected at surgery (*B* and *D*) showing a decrease (*A* and *B*) or no-change/increase (*C* and *D*) of TopoII immunostaining. Sections were counterstained with hematoxylin (*blue*). Samples are taken from same patients before and after fluorouracil, epirubicin, and cyclophosphamide chemotherapy (magnification, ×150).

Table 2. Clinicopathologic characteristics associated with response to treatment by logistic regression analysis

Variable*	Odds ratio (95% CI)	*P*
Age	0.99 (0.90-1.08)	0.80
Tumor size	0.88 (0.61-1.28)	0.52
ER	0.98 (0.95-1.01)	0.23
PgR	1.00 (0.98-1.03)	0.54
Tumor grade (1-2 versus 3)	1.34 (0.96-6.30)	0.18
HER-2 (negative versus positive)	5.28 (1.57-19.6)	0.008
Ki67	0.97 (0.93-1.02)	0.24
TopoII	1.05 (1.01-1.08)	0.001

*The following parameters were used as continuous variables: age, tumor size, ER, PgR, Ki67, and TopoII.

higher in HER-2-positive than in HER-2-negative patients (95% CI, 1.57-19.6; $P = 0.008$). None of the other variables included in the logistic model (age, clinical tumor size, ER and PgR status, tumor grade, and Ki67) reached statistical significance (Table 2). When an artificial interaction term TopoII*HER-2 was included in the logistic regression, the predictive model was not improved.

Using univariate Cox regression analysis, there was a significant correlation between relapse-free survival and changes in the percentage of TopoII-positive cells. The 67-month relapse-free survival rate was 86% (95% CI, 75-98%; median not reached) in patients with stable or reduced TopoII levels after neoadjuvant treatment ($n = 20$) versus 29% (95% CI, 3-54.5%; median, 35.6 months) in those with increased levels ($n = 54$; $P < 0.0001$) as evidenced by Kaplan-Meier analysis (Fig. 2). In particular, 10 of 20 patients with increased levels of TopoII after primary chemotherapy relapsed, whereas only five relapses were recorded among the 54 patients with unchanged or reduced levels.

By univariate analysis, a significant association with relapse-free survival was also found for tumor response, pretreatment ER and PgR status, clinical tumor size, and number of positive lymph nodes assessed at surgery.

A Cox multivariate analysis of relapse-free survival was conducted using the clinical features that showed statistical significance at univariate analysis. This identified changes in TopoII levels (relative risk, 11.2; 95% CI, 2.52-49.84; $P < 0.001$) and clinical tumor size at diagnosis (relative risk, 1.35; 95% CI, 1.13-1.59; $P < 0.001$) as the only independent prognostic factors (Table 3).

Discussion

In the present study, using immunohistochemistry, we evaluated the expression of TopoII and HER-2 in tumor samples taken from the pretreatment biopsies and surgical specimens in a homogeneous group of breast cancer patients all receiving the same anthracycline-based regimen as primary chemotherapy. Baseline and treatment-induced changes in the expression of these markers were analyzed in relation to response to treatment and relapse-free survival.

Pretreatment levels of TopoII and HER-2 were found to individually act as independent predictors of response to treatment. These results argue in favor of the concept that sensitivity of tumor cells to TopoII poisons is proportional to their enzyme content (12). Several studies have addressed the hypothesis that the expression of these markers in breast cancer might influence the response to primary treatment with anthracyclines (25–30), often with contradictory results. For instance, in a study by MacGrogan et al. (25), the response to neoadjuvant chemotherapy was found to correlate with the immunohistochemical expression of TopoII, but not with that of HER-2. Similar results were reported by Martin-Richard et al. (29). In another study (26), a favorable response to chemotherapy regimens containing anthracyclines and taxanes was more frequently observed in patients with tumors displaying either TopoII overexpression or gene amplification, and in those displaying HER-2 amplification. Park et al. (27) reported a good response to doxorubicin in breast cancers with coamplification of HER-2 and TopoII and in HER-2-amplified tumors without TopoII amplification. In other studies, neither TopoII or HER-2 were found to correlate with response (28, 30).

The contrasting results regarding TopoII may be explained, at least in part, by the different cytotoxic protocols used (i.e., only anthracycline-based or including drugs, such as taxanes, which may influence the activity of TopoII; ref. 31). The diverse tumor size at diagnosis (small versus large tumors) among the different studies may represent another explanation because response to chemotherapy seems to be increased in smaller tumors (8). Finally, it should be emphasized that a lack of standardization of the TopoII assay renders the results of the different studies hard to compare.

Concerning the predictive value of HER-2, all the above-mentioned studies failed to show any significant correlation with the response to treatment when the marker was assayed by immunohistochemistry. Mechanistically, it has been suggested that the predictive value of HER-2 is not direct, but rather dependent on the TopoII gene status. In fact, it has been reported that the TopoII gene is frequently coamplified with

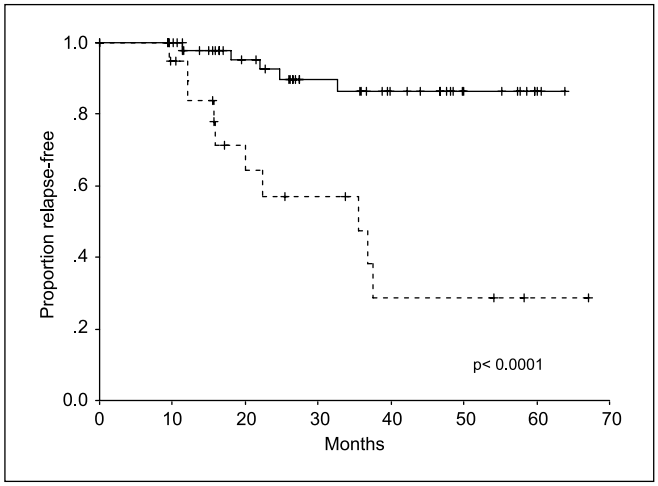

Fig. 2. Relapse-free survival rate according to variations in the percentage of TopoII-positive cells after primary chemotherapy (*continuous line,* TopoII unchanged or reduced; *dashed line,* TopoII increased). +, censored observations.

Table 3. Cox multivariate analysis of relapse-free survival

Variable	Relative risk (95% CI)	*P*
ER	0.89 (0.99-1.02)	0.8
PgR	0.97 (0.95-1)	0.06
Tumor size	1.35 (1.13-1.59)	0.001
Tumor response	1.30 (0.12-13.5)	0.8
TopoII changes	11.20 (2.52-49.84)	0.001

HER-2 in breast cancer, due to the close proximity of the two genes on chromosome 17 (23, 24). In addition, because a substantial incidence of TopoII deletions have been described (24, 32), benefit from anthracycline-based therapy could be limited to those patients with HER-2-amplified breast cancer displaying a concomitant amplification of TopoII (27). Nevertheless, several studies have shown a greater benefit of anthracycline-based chemotherapy in patients with breast cancer overexpressing HER-2 (21, 22, 33).

In line with the proliferation-specific expression of TopoII, we observed a significant correlation between pretreatment levels of TopoII and those of Ki67, as reported by others (34, 35). However, in our series, Ki67 failed to show any value as a predictor of response to treatment in multivariate logistic regression. A similar lack of correlation between Ki67 and response to chemotherapy has been observed by others (11, 25, 36, 37). As suggested elsewhere (25), the effect of TopoII on tumor response to chemotherapy might not be simply explained by the dependency of its expression during proliferation, which is known to increase the sensitivity to anthracyclines (12).

Only few studies have addressed the significance of changes in biological marker expression (such as proliferation indices, proliferating cell nuclear antigen, expression of *P-gp*, and epidermal growth factor receptor) as a consequence of primary chemotherapy in breast cancer (29, 30, 38–40). In two of these studies (29, 30), changes in TopoII expression were found to correlate with response to treatment, but a consistent picture correlating these changes to prognosis has not yet emerged. Here, we show for the first time, that changes in the expression of TopoII are of prognostic value in patients with operable breast cancer receiving anthracycline-based chemotherapy prior to surgery. Patients with increased TopoII after chemotherapy have a substantially worse relapse-free survival as compared with those with unchanged or decreased levels of the marker. Changes of TopoII observed as a consequence of therapy do not seem to be dependent on the percentage of cells expressing the protein at baseline. In fact, we found no significant difference in the pretreatment levels of TopoII between patients with increased and those with unchanged or decreased levels of this marker after chemotherapy. However, tumors displaying increased TopoII staining after chemotherapy were less likely to have responded to treatment than those with unchanged or decreased expression. This may explain why tumor response did not retain an independent prognostic value at multivariate analysis adjusted for changes in TopoII expression.

These observations are consistent with the hypothesis that tumor cells displaying enhanced TopoII expression after anthracycline-based chemotherapy had become resistant to these drugs (12). Besides the classical multidrug resistance linked to efflux pumps in the cell membrane and to the decreased levels of TopoII protein (resulting from down-regulation of transcription, increased degradation, or allele deletion), several other mechanisms of resistance to TopoII poisons have been described. The altered TopoII multidrug resistance may be dependent on mutations resulting in altered drug-DNA-protein interactions or ATP binding, alterations in the ratio of isoenzymes, or altered enzymatic function by posttranslational modification of the phosphorylation status of the protein (reviewed in refs. 41, 42). Additionally, because the effect of anthracyclines is partly due to free radical–induced mechanisms, it has been suggested that an increase in the intracellular glutathione levels could induce resistance to these drugs (43), although no clinical study has shown this mechanism in breast cancer patients. On the contrary, several reports have documented that increased levels of glutathione play a key role in the multidrug resistance–associated protein 1–mediated resistance (44, 45). Finally, loss of the mismatch repair protein, MLH1, has also been reported as being associated with resistance to TopoII poisons (46, 47). The existence of these different mechanisms of resistance to anthracyclines allows us to hypothesize that the increased percentage of cells expressing the protein after treatment might reflect the clonal expansion of resistant cells expressing TopoII before treatment, caused by the selective pressure of anthracyclines. Although a formal demonstration is not provided in our study, it is likely that the emergence of these clones is responsible for the observed relapses.

In summary, our results provide, for the first time, clear evidence that a reduced or stable expression of TopoII after anthracycline-based chemotherapy is an important predictor of longer relapse-free survival in patients with operable breast cancer. Nevertheless, ours must be seen as hypothesis-generating results because of the fairly limited number of patients and the retrospective nature of the study. Large prospective studies are warranted to validate these observations. If changes in TopoII are confirmed as being associated with clinical outcome in operable breast cancer, then this opens up a number of possibilities for improving treatment results, including the use of agents that may be active in cells resistant to TopoII poisons or that may be capable of reversal of resistance.

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