Amplification of the *TOP2A* Gene Does Not Predict High Levels of Topoisomerase II Alpha Protein in Human Breast Tumor Samples

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Recent clinical trials have suggested that patients whose breast tumors overexpress *HER2* may derive particular benefit from anthracycline-containing chemotherapy compared to that without anthracycline. It has been proposed that the *HER2* gene amplification reported in these tumors might mask an underlying *TOP2A* gene amplification that occurs frequently and concurrently with *HER2* amplification. Topoisomerase II alpha, encoded by *TOP2A*, is a direct molecular target of anthracycline drug action and is potentially useful as a predictive marker of response to anthracycline therapy for breast cancer. In this study, we examined whether *TOP2A* gene amplification is an appropriate marker for identifying breast tumors expressing high levels of topoisomerase II alpha. We determined topoisomerase II alpha protein expression by immunohistochemistry in 81 human breast tumors in relation to *HER2* and *TOP2A* gene copy numbers analyzed by fluorescence in situ hybridization, histologic grade, cell proliferation fraction measured by MIB-1 expression, and *HER2* protein expression determined by immunohistochemistry. The results showed no correlation between *TOP2A* gene copy number and topoisomerase II alpha protein expression levels in breast tumors, in contrast to the analogous situation for *HER2* gene amplification and *HER2* immunohistochemistry. Our results suggest that *TOP2A* gene amplification in breast tumors does not predict high expression of topoisomerase II alpha protein. © 2004 Wiley-Liss, Inc.

INTRODUCTION

At present, anthracycline drugs are widely used in adjuvant therapy for breast cancer. Adjuvant chemotherapy containing anthracycline in conjunction with cyclophosphamide and fluorouracil has been shown to be superior to combination therapy with cyclophosphamide, methotrexate, and fluorouracil (Levine et al., 1998; McCarthy and Swain, 2000; Adlard and Dodwell, 2001). However, anthracyclines are associated with serious side effects, including an increased risk of acute leukemia and cardiac damage (Kellner, 2002; Nabholtz, 2002). To optimize the use of anthracycline drugs, molecular markers that identify breast tumors most likely to be susceptible to them are needed.

Several recent clinical trials have suggested that patients whose tumors overexpress *HER2* may derive particular benefit from anthracycline-containing therapy compared to that without anthracycline (Paik et al., 1998, 2000; Thor et al., 1998). *HER2* is on chromosome arm 17q, where amplification and rearrangements involving numerous genes, including the retinoic acid receptor α, *GRB7*, and, notably, *TOP2A* are common in tumor cells of the breast (Keith et al., 1993; Murphy et al., 1995; Bieche et al., 1996; Jarvinen et al., 1996, 1999; Barlund et al., 1997; Plummer et al., 1997; Luoh, 2002) and other tissues (Janoueix-Lerosey, 2000). Whereas the *HER2* protein product has no known mechanistic link to the action of anthracycline drugs, topoisomerase II alpha, encoded by *TOP2A*, is a direct molecular target of anthracycline drug action and so has attracted attention because it is potentially useful as a predictive marker for response to anthracycline therapy for breast cancer. Because *HER2* amplification was the molecular marker chosen for analysis in the clinical studies evaluating response to anthracycline-containing therapy, it is possible that *HER2* gene amplification in breast tumors showing increased sensitivity to anthracycline therapy coincided with and masked concurrent *TOP2A*

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TABLE 1. Immunohistochemistry Methods

Antigen recognized	Antibody name/clone	Manufacturer/ distributor of antibody	Dilution of antibody used	Antibody retrieval method	Detection system used
c-erb B2 (*HER2*)	NCL CB11	Novocastra, Vector Laboratories (Canada), Burlington, Ontario, Canada	1/200	None	Ultra HRP detection system, ID Labs, Inc. London, Ontario, Canada
c-erb B2 (*HER2*)	A0485 polyclonal	Dako Diagnostics Mississauga, Ontario, Canada	1/750	None	Ultra HRP detection system
Topoisomerase II alpha	Ki-S1	Dako Diagnostics Mississauga, Ontario, Canada	1/200	Citrate buffer, pH 6, microwaved to 115°C for 12 min.	Vector Elite ABC kit, Vector Laboratories (Canada), Burlington, Ontario, Canada
Ki-67	MIB-1	Dako Diagnostics Mississauga, Ontario, Canada	1/600	Citrate buffer, pH 6, microwaved to 115°C for 12 min.	Ultra HRP detection system

gene amplification, which was not analyzed. It has been proposed that *HER2* amplification in these tumors may actually be a marker of underlying *TOP2A* amplification (Lynch et al., 1997; Jarvinen and Liu, 2000; Di Leo et al., 2001, 2002a, 2002b). Coamplification and overexpression of *TOP2A* represent a relatively simple mechanism to explain the observed anthracycline sensitivity in a subgroup of patients. However, it has not been demonstrated that *TOP2A* gene amplification is an appropriate marker for identifying breast tumors expressing high levels of topoisomerase II alpha. The accurate identification of such topoisomerase II alpha–expressing tumors is an important prerequisite for any study designed to assess whether such tumors are sensitive to anthracycline therapy.

MATERIALS AND METHODS

Selection of Tumors

Formalin-fixed, paraffin-embedded tumor blocks from invasive breast tumors received for clinical diagnosis between 1998 and 2002 were selected from the Mount Sinai Department of Pathology archives and were used anonymously. Hematoxylin- and eosin-stained slides representative of each tumor block were selected and evaluated for the presence of appropriate invasive tumor cells and reviewed for combined histologic grade using the Elston–Ellis modification of the Scarff–Bloom–Richardson grading system (Elston and Ellis, 1991). Tumor sample selection was made to include approximately equal numbers of both grade III and grade II *HER2*-positive and *HER2*-negative cases. Four-micron sections were cut from each tumor block and used in series for immunohistochemical staining for topoisomerase II alpha, *HER2*, and MIB-1 and for fluorescence in situ hybridization (FISH) to assess the gene copy numbers of *HER2* and *TOP2A*. MIB-1 is an antibody that recognizes an epitope of Ki-67 in paraffin-embedded tissues (Cattoretti, 1992). Ki-67 is a protein associated with the dense fibrillary component of the nucleolus, one of three regions defined by electron microscopy that is necessary for producing ribosomes (Brown, 2002). The precise function of Ki-67 is unknown, but its expression increases during DNA synthesis to peak in the G2 and M phases of the cell cycle and is strongly related to other measures of proliferation (Spyratos, 2002). High-level expression of Ki-67 in breast tumors has statistically significant correlation with poor clinical outcome (Brown, 2002).

Immunohistochemical staining was carried out using the conditions described in Table 1. Microwave antigen retrieval was carried out in a Micromed T/T Mega Microwave Processing Lab Station (ESBE Scientific, Markham, Ontario, Canada). Slides were developed with diaminobenzidine tetrahydrochloride and counterstained in Mayer's hematoxylin. Control slides of spleen and thymus were used as positive controls for topoisomerase II alpha and MIB-1 staining. A positive control of a breast tumor with previously identified *HER2* amplification was used as a positive control for *HER2* immunohistochemistry. Normal breast epithelium accompanying tumor samples was used as an internal negative control.

Immunohistochemical staining results for the various antibodies were evaluated independently of each other and without knowledge of the results of FISH analysis.

Interpretation and Scoring of Immunohistochemistry

HER2 immunohistochemistry was scored according to the method of Allred (1998), in which a score between 1 and 5 represents the percentage of cells with positive staining and is added to a score between 1 and 3 representing staining intensity, giving a maximum total score of 8. Based on a technical validation performed in our laboratory, a final score of 5 or greater was categorized as positive for *HER2* protein overexpression (O'Malley, 2001).

Topoisomerase II alpha and MIB-1 were scored for the percentage of positive cells seen in five groups of 100 tumor cells. The average percentage in 500 tumor cells was recorded without a designated cutoff value.

Fluorescence In Situ Hybridization

Fluorescence in situ hybridization (FISH) preparations were made from 4-μm tissue sections from the tumor sample blocks cut in series and mounted in the same orientation as the sections used for immunohistochemistry. The FISH probes used were the PathVysion *HER2* DNA probe kit and the LSI *TOP2A*/CEP17 probe kit, both from Vysis (Downers Grove, IL). Slides were prepared according to the manufacturer's instructions for paraffin sections. Slides were analyzed under a Leica DMBRX epifluorescence microscope equipped with filters for the separate detection of DAPI, spectrum green, and spectrum orange and with a triple-band-pass filter for the simultaneous detection of the three signals. Images were captured by a charge-coupled device camera using software from Applied Imaging (Santa Clara, CA).

The number of signals representing the copy number of the gene of interest (*TOP2A* or *HER2*) and the number of chromosome 17 centromeres present in each cell were recorded for each of 60 tumor cells per sample. The ratio of the signal of interest per chromosome 17 centromere was calculated for each sample. Samples in which more than 50% of the cells counted had more than 2 signals for chromosome 17 were categorized as polysomic for chromosome 17, and samples in which more than 50% had 1 signal for chromosome 17 were categorized as monosomic for chromosome 17. Infiltrating lymphocytes and stromal cells accompanying the tumor cells served as internal normal controls for probe hybridization.

Statistical Analysis

Statistical analysis was based on linear regression and analysis of variance (ANOVA) using a square root (post hoc) transformation of the percentages for topoisomerase II alpha and MIB-1 staining, which were approximately normally distributed when a square root transformation was used. Generalized linear modeling (McCullach and Nelder, 1989) with the link function being the power of 2 was used so that functional relations between variables could be expressed relative to the untransformed variables. All results are stated according to the original rather than the square-root scale.

RESULTS

Description of Breast Tumors in the Study Group

All tumors were graded by the study pathologist (F.O'M.) by use of the Elston–Ellis modification of the Scarff–Bloom–Richardson histological grading system (Elston and Ellis, 1991). There were 52 histologic grade III breast tumors, of which 29 were *HER2*-positive and 23 were *HER2*-negative; there were 29 grade II breast tumors, of which 8 were *HER2*-positive and 21 were *HER2*-negative. Seventy-eight tumors were invasive ductal carcinomas of no special type, two tumors were invasive lobular carcinomas, and one tumor was a colloid carcinoma.

HER2 FISH and Immunohistochemistry Results

HER2 FISH results were concordant with *HER2* immunohistochemistry results in 80 of 81 (98.8%) samples. All positive immunohistochemistry results were concordant with *HER2* gene amplification (ratio of *HER2* gene to chromosome 17 ≥ 2); one sample had negative immunohistochemistry results for *HER2* despite a FISH ratio of 3.72 and was interpreted as having *HER2* gene amplification with a false-negative immunohistochemistry result. Thus, in this article, *HER2*-positive status is used to describe tumors with both positive immunohistochemistry results and a greater than twofold amplification of the *HER2* gene.

The average *HER2* gene signal number per cell ranged from 1.3 to 40.2. Five samples had fewer *HER2* signals per cell than signals for chromosome 17, that is, a ratio of *HER2* gene to chromosome 17 of < 0.8. These five samples were negative for *HER2* protein overexpression.

TABLE 2. Polysomy of Chromosome 17 in Breast Tumors

Number of CEP17 (green) signals counted per cell	Tumor sample categorized as:	Number of tumors with this result: Evaluation using *HER2* PathVysion DNA probe kit	Number of tumors with this result: Evaluation using *TOP2A* LSI DNA probe kit
1	Monosomy 17	5	5
2	Disomy 17	52	56
>2	Polysomy 17	24	20

TABLE 3. Results of FISH Analysis for Gene Amplification of *HER2* and *TOP2A*

		HER2			
		Deleted	No amplification	Amplified	Total
TOP2A[a]	Deleted	2	0	5	7
	No amplification	3	36	21	60
	Amplified	0	3[a]	11	14
	Total	5	39	37	81

[a]*TOP2A:* CEP17 ratio exceeds 2, but these tumors are not highly amplified.

Polysomy of Chromosome 17

The number of chromosome 17 signals per cell was evaluated in two separate FISH analyses: once in conjunction with *HER2* and again with *TOP2A* (see Table 2), demonstrating that the results of assessment of signal counts are reproducible. In four samples, whose mean chromosome 17 signal count ranged from 2.02 to 3.37, the number of cells with two CEP17 signals was greater than 50% in the first assessment, but less than 50% in the second assessment. These discordant results resulted from the heterogeneity within the tumor sample rather than from gross discrepancies in signal counts.

TOP2A Copy Number by FISH Analysis

The ratio of *TOP2A* gene signal to chromosome 17 centromere signal was calculated by counting the number of red signals per cell (*TOP2A*) and dividing that number by the number of green signals (CEP17) in 60 tumor cells (see Fig. 1A,B). In 60 of 81 samples (74%), the ratio of *TOP2A* gene signal to chromosome 17 centromere signal per cell was approximately equal to 1 ($0.8 < \text{ratio} < 2.0$). In 14 samples (17%), there was amplification of the *TOP2A* gene, that is, the ratio of *TOP2A* gene signal to chromosome 17 centromere signal per cell was ≥ 2, with the mean number of *TOP2A* signals per cell ranging from 2.18 to 14.45 (see Fig. 1A). When the results of FISH analysis for *HER2* copy number and *TOP2A* gene copy were compared, most combinations of concurrent amplification, deletion, and normal copy number were observed (see Table 3). Seven samples had deletion of *TOP2A* gene copy number compared to chromosome 17 centromere copy number, that is, a ratio < 0.8. Two of these seven tumor samples showed deletion of both *TOP2A* and *HER2* relative to the copy number of the chromosome 17 centromere (see Fig. 2), but the remaining five were amplified for *HER2*.

Immunohistochemistry Results for Topoisomerase II Alpha and MIB-1

Positive nuclear staining for topoisomerase II alpha in 500 invasive breast tumor cells counted per sample ranged from 1.6% to 71.8%. Positive nuclear staining for MIB-1 ranged from 0.2% to 81.6%.

Results of Statistical Analysis

Controlling for histologic grade, there was no statistically significant difference between the *HER2*-positive and *HER2*-negative tumor groups for the mean percentage of cells expressing topoisomerase II alpha (19.2% versus 17.3%, $P = 0.37$) or the MIB-1 antigen (23.4% versus 22.3%, $P = 0.73$).

For all tumors, there was a strong association between topoisomerase II alpha and MIB-1 expression, with a correlation coefficient of 0.843 ($n = 81$, $P < .0001$; Fig. 3).

There were significant differences between the grade II and grade III groups for expression levels of both topoisomerase II alpha (10.7% in grade II versus 25.8% in grade III, $P < .0001$) and MIB-1

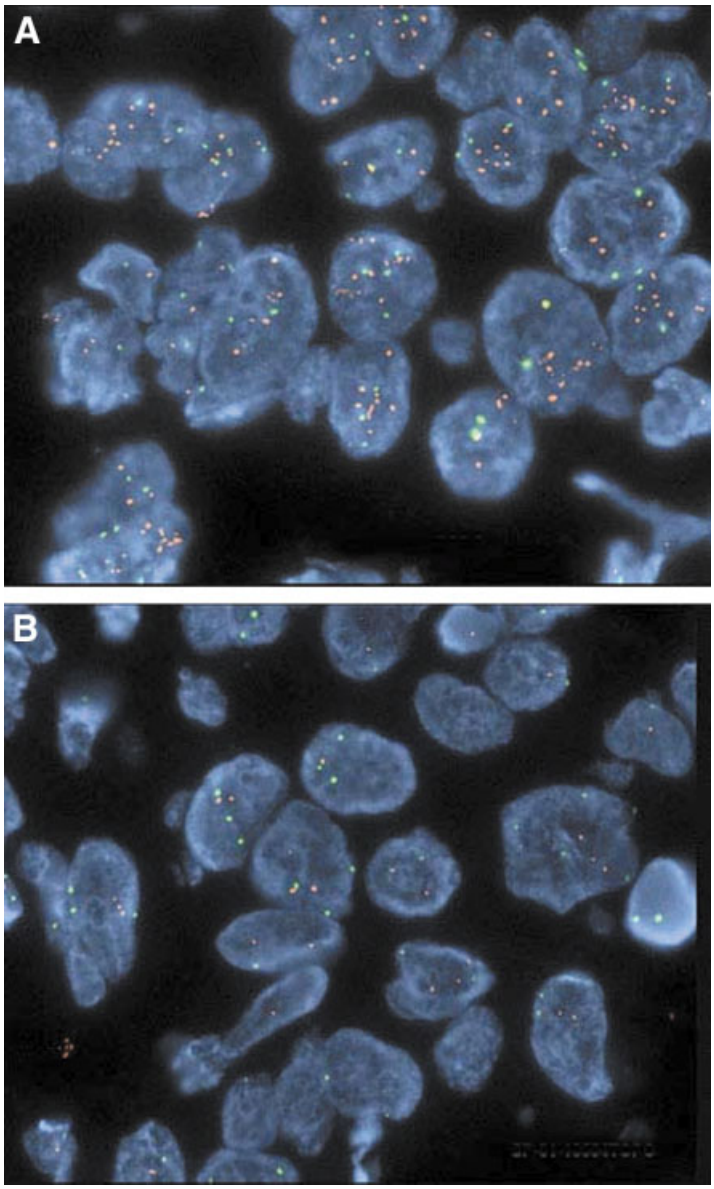

Figure 1. A FISH preparation on formalin-fixed, paraffin-embedded sections of breast tumor showing hybridization of probe for *TOP2A* (red signals) and the centromere of chromosome 17 (CEP17, green signals; LSI *TOP2A*/CEP17, Vysis, Downers Grove, IL). (A) Tumor with amplification of the *TOP2A* gene, with FISH results indicating *TOP2A*/CEP17 = 15.45/2.50, ratio = 5.78 in 60 cells. A corresponding serial section stained for topoisomerase II alpha immunohistochemistry had a mean score of 29.8% positive cells. (B) Tumor with no amplification of *TOP2A,* with FISH results indicating *TOP2A*/CEP17 = 2.95/2.40, ratio = 1.35 in 60 cells. A corresponding serial section stained with topoisomerase II alpha immunohistochemistry had a mean score of 55.8% positive cells.

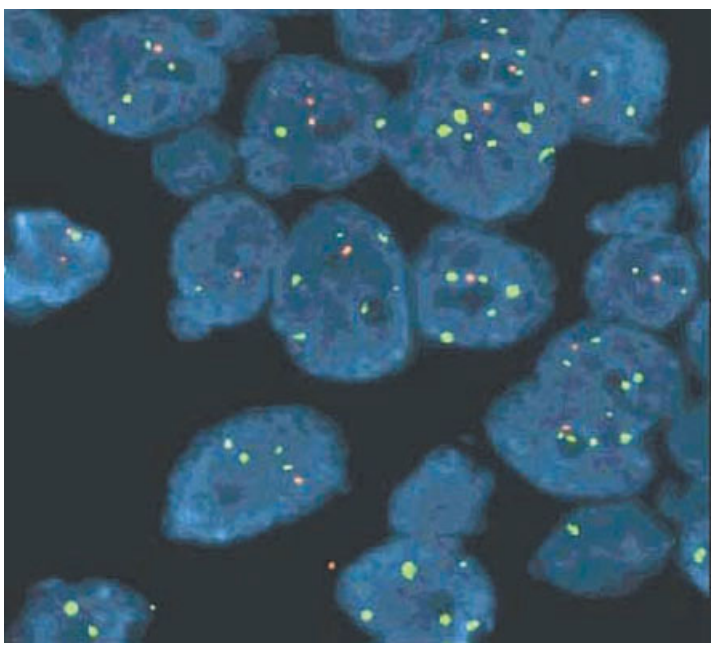

Figure 2. A FISH preparation on formalin-fixed, paraffin-embedded section of breast tumor showing hybridization of probe for *TOP2A* (red signals) and the centromere of chromosome 17 (CEP17, green signals; LSI *TOP2A*/CEP17, Vysis, Downers Grove, IL). This tumor had deletion of the *TOP2A* gene copy number relative to the copy number of chromosome 17 centromeres, with the FISH results indicating *TOP2A*/CEP17 = 2.03/4.85, ratio = 0.43 in 60 cells. A corresponding serial section stained for topoisomerase II alpha immunohistochemistry had a mean score of 36.8% positive cells.

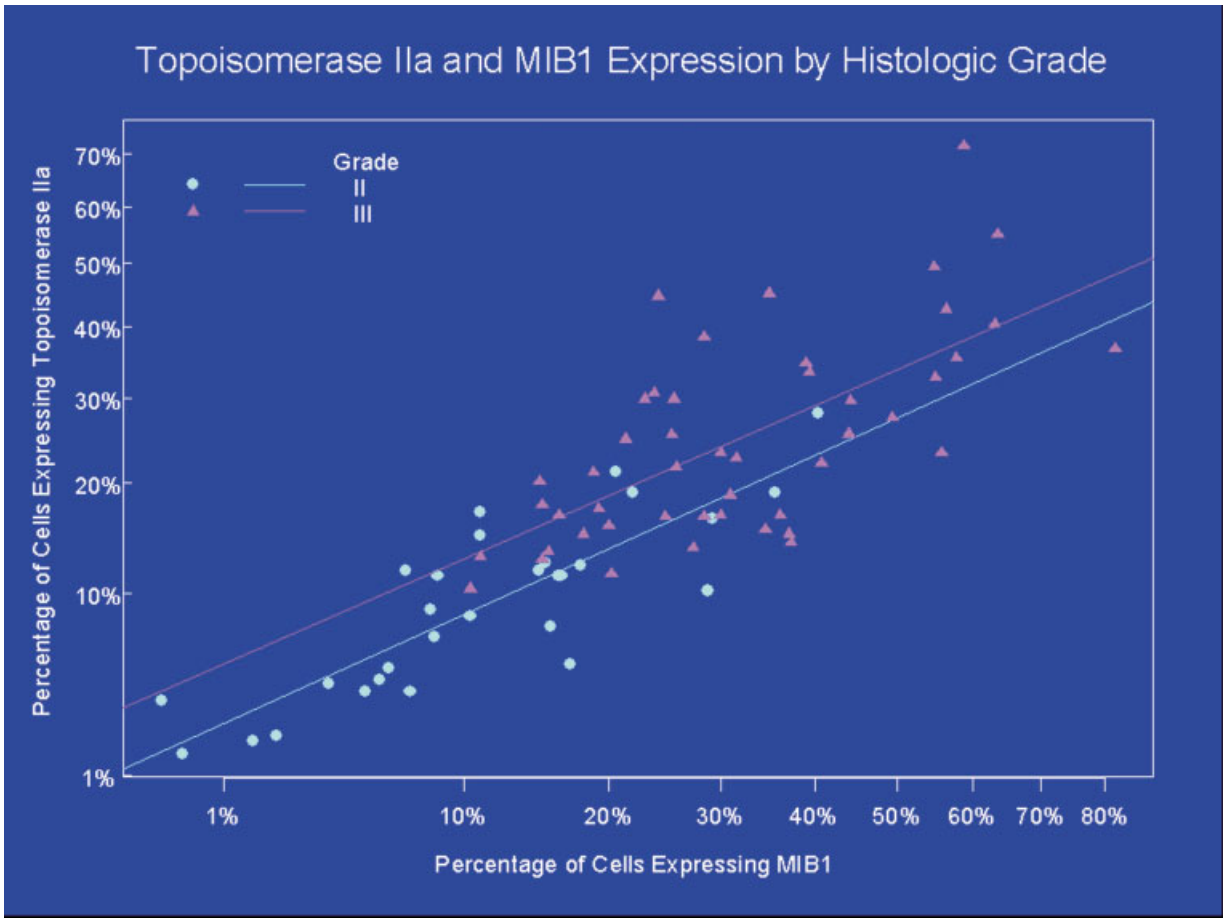

Figure 3. Percentage of tumor cells positive for topoisomerase II alpha immunohistochemical staining versus percentage of cells positive for MIB-1 immunohistochemical staining in breast tumors grouped according to histological grade.

(12.6% versus 33.1%, $P < .0001$) after controlling for *HER2* status (see Figs. 4 and 5). There was no evidence that the relationship between topoisomerase II alpha and MIB-1 expression differed between the grade II and grade III tumor groups (test for heterogeneity of slope, $P = 0.83$).

DISCUSSION

There was no evidence of a difference ($P = 0.43$) in the mean topoisomerase II alpha protein expression level for tumor samples with *TOP2A* amplification (mean 22.1% immunohistochemistry-positive, $n = 14$) versus no *TOP2A* amplification (mean 19.0% immunohistochemistry-positive, $n = 67$). There was only weak evidence of a difference between topoisomerase II alpha levels in tumors with concurrent *HER2* and *TOP2A* amplification ($n = 11$) compared to tumors with no gene amplification of either *HER2* or *TOP2A* (27.5% versus 17.8%, $n = 39$, $P = .07$).

In the current study, we examined whether a relationship between *TOP2A* gene amplification and topoisomerase II alpha protein expression could be detected in clinical breast tumor samples. We looked for evidence that *TOP2A* gene amplification detected by FISH analysis in breast tumor cells resulted in high levels of topoisomerase II alpha protein expression detectable by immunohistochemistry. An important goal in the current clinical treatment of breast cancer patients is to predict which patients are most likely to derive benefit from anthracycline therapy, justifying its risk of cardiotoxic side effects. It has been speculated that *TOP2A* gene amplification could be useful as a marker to identify the tumors of patients likely to respond to anthracycline, analogous to the use of *HER2* gene amplification to identify tumors of patients likely to respond to herceptin.

Our results suggest that *TOP2A* gene amplification does not predict high topoisomerase II alpha

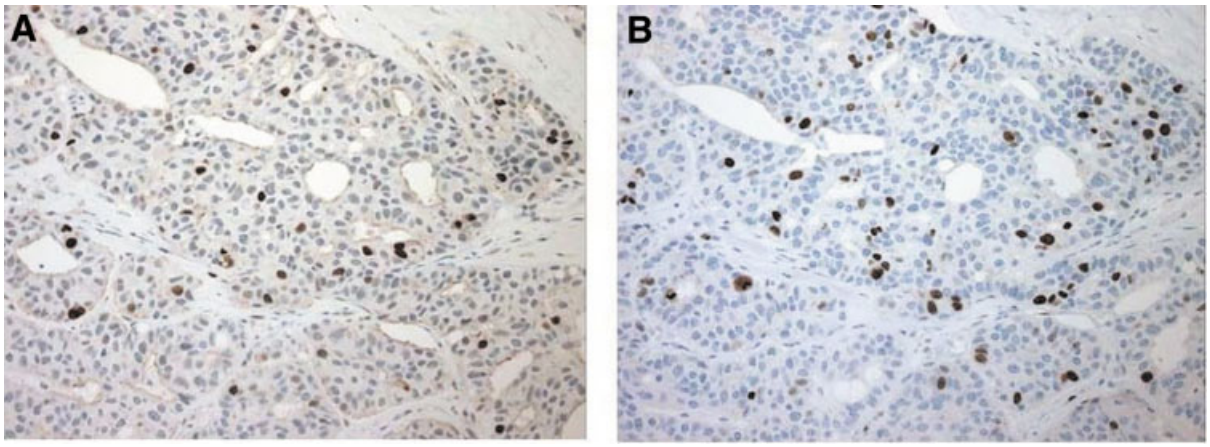

Figure 4. Immunohistochemical staining on formalin-fixed, paraffin-embedded serial sections of histological grade-II breast tumor showing (A) 5.4% positive cells for topoisomerase II alpha and (B) 17% positive cells for MIB-1. The corresponding FISH results for this tumor were: *HER2*/CEP17 = 3.82/3.22, ratio = 1.19; *TOP2A*/CEP17 = 4.00/2.83, ratio = 1.41.

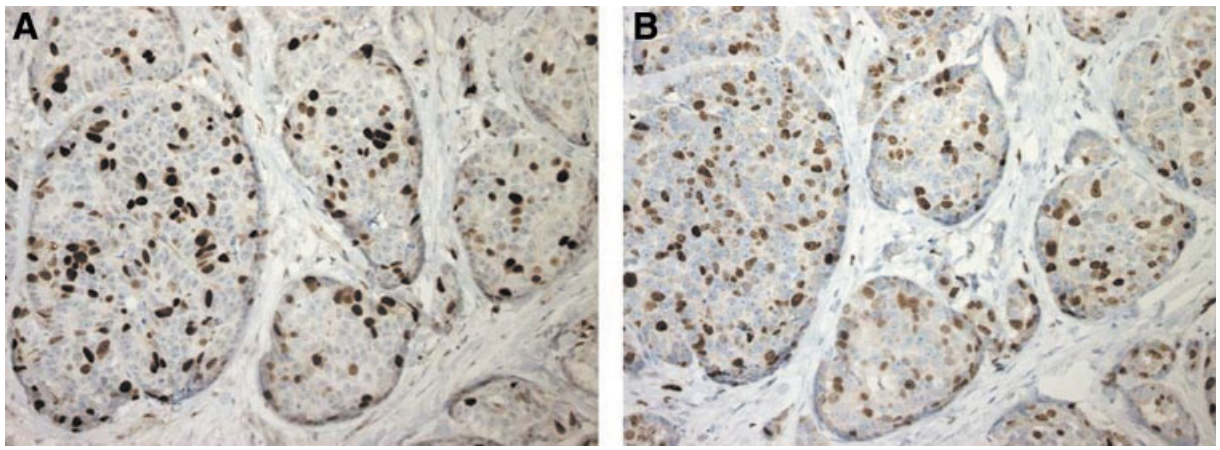

Figure 5. Immunohistochemical staining on formalin-fixed, paraffin-embedded serial sections of histological grade-III breast tumor showing (A) 34.8% positive cells for topoisomerase II alpha and (B) 39.0% positive cells for MIB-1. The corresponding FISH results for this tumor were: *HER2*/CEP17 = 5.72/3.42, ratio = 1.67; *TOP2A*/CEP17 = 6.00/3.55, ratio = 1.71.

protein expression. Rather, we found that topoisomerase II alpha expression is high in high-grade breast tumors regardless of *TOP2A* gene status. In all samples, regardless of grade or *HER2* status, there was a strong correlation between topoisomerase II alpha expression and MIB-1 expression. High topoisomerase II alpha expression has been widely reported in high-grade tumors of many tissue tumor types. It correlates with increased DNA content and seems to be a general feature of rapidly proliferating cells to facilitate their frequent cell divisions (Costa et al., 2000; Miettinen et al., 2000; Nakopoulou et al., 2000; Willman and Holden, 2000; Korshunov and Golanov, 2001; Brustmann and Naude, 2002).

Our study group of breast tumors was selected to include those with and without *HER2* amplification. Although this selection indicates that our cohort does not contain the same representation of tumors as would appear consecutively in the general population, it enabled us to assess the association between amplification of *HER2*, *TOP2A*, and topoisomerase II alpha protein expression. The highest expression levels of topoisomerase II alpha were found in the samples in our study group with polysomy of chromosome 17 but no obvious amplification of *TOP2A*. Tumors with amplification of *TOP2A* did not show evidence of greater expression of topoisomerase II alpha than did other tumors.

In primary breast tumors, *HER2* amplification under certain conditions endows tumor cells with a growth advantage, allowing tumor expansion. Whereas *HER2* appears to be important during mammalian development (Troyer, 2001), it has no well-described, independent function in differentiated cells. Regulation of transcription of the *HER2* gene is linked to the estrogen receptor pathway through AP-2 (Perissi et al., 2000; Vernimmen et al., 2003), and translation of *HER2* may be blocked in normal breast cells (Child et al., 1999). Uncontrolled *HER2* expression is oncogenic; its aberrant functioning results from its extreme abundance on the cell surface, enabling it to crosslink and activate pathways of signal transduction in the absence of a ligand or as a partner with other HER protein family members in heterodimers (Yarden, 2001).

In contrast, topoisomerase II alpha is an enzyme essential to cell function as a structural component of both mitotic chromosomes and the nuclear matrix. Its catalytic function, which creates topologic changes in DNA, is required during replication, transcription, and chromosome segregation (Burden and Osheroff, 1998; Wang, 2002). Unlike the expression of *HER2*, topoisomerase II alpha expression is highly regulated at the level of transcription and translation (Isaacs et al., 1996, 1998), so that gene amplification may not dramatically alter the level of topoisomerase II alpha expressed in tumor cells. Our results may reflect the fundamental differences between the roles of *HER2* and topoisomerase II alpha in the cell. *TOP2A* expression is regulated through inverted CCAAT box motifs (Adachi et al., 2000), which bind p53 (Isaacs et al., 1996; Sandri et al., 1996; Wang et al., 1997; Yoon et al., 1999) and coordinate transcription with the state of cell cycling. The *TOP2A* promoter responds to increased Ras activity associated with cell proliferation (Woessner et al., 1990; Isaacs et al., 1998; Chen et al., 1999; Stacey et al., 2000) At the mRNA level, topoisomerase II alpha is regulated through untranslated 3′ sequences (Goswami et al., 2000) that govern the protein's half-life and its location in either the nuclear or cytoplasmic compartments of the cell. Topoisomerase II alpha expression is part of the larger response that a cell undergoes when committing to division and increases with cell proliferation as an effect rather than a cause.

Our results do not support the suggestion that *HER2* has acted as a surrogate marker for *TOP2A* amplification in the clinical studies in which patients with *HER2* amplification in their tumors showed a better overall response to anthracycline-containing chemotherapy (Jarvinen et al., 2000; Di Leo et al., 2002a, 2002b). In contrast to the amplification of *HER2* in breast tumors, which is tightly associated with *HER2* overexpression, we found that amplification of *TOP2A* is not strongly associated with high levels of topoisomerase II alpha expression in human breast tumors.

Cell proliferation is routinely assessed during the assignment of breast tumor grade through mitotic counts per high-power field of view and is one component of histologic grade. Topoisomerase II alpha expression levels in our study were related even more strongly to histologic grade than to mitotic index (data not shown). It may be pertinent that the analysis of topoisomerase II alpha expression levels can provide information not only about the number of cells undergoing mitosis at any one time but also about the fraction of cells cycling in a tumor sample (Lynch et al., 1997) and the time spent in the G2/M phase (Rudolph et al., 2001; Villman et al., 2002). The cycling activity of a tumor and its DNA ploidy may have separate and different implications for a tumor's response to chemotherapy (Bagwell et al., 2001).

Topoisomerase II alpha levels, if accurately determined, may be predictive of anthracycline response in breast tumor patients, especially when used in combination with other breast tumor markers. In this study, we found that high levels of topoisomerase II alpha were present in high-grade breast tumors and strongly correlated with expression of MIB-1 antigen. The results of this study suggest that topoisomerase II alpha levels are not predicted well by *TOP2A* gene copy number.

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