Biomarkers, Genomics, Proteomics, and Gene Regulation

Assessment of Topoisomerase II α Status in Breast Cancer by Quantitative PCR, Gene Expression Microarrays, Immunohistochemistry, and Fluorescence *in Situ* Hybridization

Atocha Romero,* Miguel Martín,†
Maggie C.U. Cheang,‡
José Antonio López García-Asenjo,§ Belén Oliva,¶
Xiaping He,‡ Miguel de la Hoya,*
Jose Ángel García Sáenz,*
Manuel Arroyo Fernández,‖ Eduardo Díaz Rubio,*
Charles M. Perou,‡ and Trinidad Caldés Llopis*

From the Departments of Medical Oncology* and Laboratory Medicine,‖ Hospital Clínico San Carlos, Madrid, Spain; the Department of Medical Oncology,† Hospital General Universitario Gregorio Marañón, Madrid, Spain; the Department of Genetics and Pathology,‡ Lineberger Comprehensive Cancer Center, Chapel Hill, North Carolina; the Department of Pathology,§ Hospital Universitario Príncipe de Asturias, Alcalá de Henares, Spain; and the Agencia Española de Medicamentos y Productos Sanitarios,¶ Ministry of Health, Madrid, Spain

Anthracyclines are frequently used for the treatment of breast cancer and topoisomerase II alpha (*TOP2A*) is considered to be the molecular target. Numerous studies have evaluated the predictive value of *TOP2A* using different methodological approaches and inconsistent results have been reported. Indeed, the correlation between techniques for the assessment of *TOP2A* status has not been well evaluated. In this study, we determined *TOP2A* status in 61 breast tumor samples by real-time PCR, DNA microarrays, immunohistochemistry (IHC), and fluorescence *in situ* hybridization (FISH), and then evaluated these results with clinical-pathological features and breast cancer intrinsic subtypes. First, we observed a statistical significant correlation of *TOP2A* gene expression between real-time PCR and microarrays (Pearson coefficient, 0.816; *P* < 0.001), and both predicted *TOP2A* IHC results fairly well (area under the curve > 0.74). In contrast, poor agreement between FISH and IHC data was observed (k: 0.134). Secondly, *TOP2A* expression was found significantly associated with cell proliferation, and with the highly proliferative Luminal B, Her2-enriched and Basal-like

intrinsic subtypes. In conclusion, *TOP2A* expression in breast cancer was associated with high proliferation and aggressive tumor subtypes and appears to be independent of its amplification status. All of these features should be taken into consideration when assessing the predictive value of *TOP2A* for anthracycline-based chemotherapy. *(Am J Pathol 2011, 178:1453–1460; DOI: 10.1016/j.ajpath.2010.12.042)*

Anthracyclines are some of the most powerful agents available for the treatment of breast cancer and are frequently used in the adjuvant setting. However, anthracyclines are associated with severe adverse effects, such as cardiac toxicity[1] and bone marrow dysfunction, including acute leukemia and myelodysplasia.[2] Therefore, there is a need to identify the subset of patients who might benefit from these drugs.

The mechanism of action of these compounds is related to the inhibition of the nuclear enzyme DNA topoisomerase II (Topo II),[3] although other antitumor mechanisms have also been described.[4] Topoisomerase II is an important enzyme for cell division because it releases torsional stress in double-stranded DNA by inducing transient breaks that are then subsequently resealed.[5] The catalytic activity of Topo II in mammalian cells is mediated by two isoforms [ie, Topo II α (*TOP2A*) and Topo II β].[6] *In vitro* studies[7–10] have suggested that sensitivity to Topo II inhibitors is dependent

on the expression level of *TOP2A* in target cancer cells. Cells with a low concentration of TOP2A protein are less sensitive to Topo II–inhibiting drugs versus cells containing a high concentration of TOP2A. Interestingly, the *TOP2A* gene itself is frequently found coamplified with v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/ glioblastoma derived oncogene homolog (avian) (*ERBB2*) in human breast cancer cells,[11] and several studies[12–16] have shown that anthracycline-containing regimens improve survival in women with *ERBB2*-positive breast cancer.

Numerous retrospective studies have investigated the predictive value of *TOP2A* using different methods. Some of these approaches have focused on determining *TOP2A* expression,[17,18] whereas others have evaluated *TOP2A* copy number.[19,20] In any case, the results of these studies are contradictory and it is still unclear whether investigating *TOP2A* provides useful clinical information concerning anthracycline benefit. Current techniques for measuring *TOP2A* are not standardized, which may account, at least partly, for these apparently contradictory results. In addition, the agreement among techniques and the correlation between *TOP2A* expression values measured by different methods is also not well established. On the other hand, *TOP2A* is considered a surrogate marker of cell proliferation and its expression has been associated with high tumor grade[21,22] and the absence of hormone receptors,[23] suggesting that overexpression of *TOP2A* could be characteristic of a specific type of breast tumor. Investigation of the predictive significance of *TOP2A* for anthracycline-containing regimens requires knowing the correlation between different methods and the characterization of *TOP2A*-expressing tumors to control potential confounding factors.

The aim of this study was to evaluate the status of *TOP2A* in breast cancer tumors by four different methods [ie, fluorescence *in situ* hybridization (FISH), gene expression via DNA microarrays, gene expression via real-time PCR (Q-PCR), and immunohistochemistry (IHC)]. We also sought to assess the correlation and agreement among these techniques when classifying the tumors as *TOP2A* positive or negative. In addition, *TOP2A* status was correlated to clinical features, such as cell proliferation, hormone receptor status, and breast cancer intrinsic subtypes, that identified many significant correlations.

Material and Methods

Study Population

Tumor biopsy specimens were obtained from a set of 61 pretreated patients diagnosed as having locally advanced breast cancer; these patients participated in a neoadjuvant clinical trial (registered at the following Web site: *http://www. clinicaltrials.gov*; identifier NCT00123929). The clinical trial was approved by the Hospital Clínico San Carlos Ethics Committee, Madrid, Spain. Briefly, eligibility criteria included the following: women aged between 18 and 78 years; clinical stage IIB, IIIA, or IIIB breast cancer; and palpable breast tumors not amenable to breast-preserving surgery. Before the start of the trial, an informed

consent was obtained from every patient. A total of 226 registered patients were enrolled in the trial; 204 underwent a complete evaluation. No significant differences were observed between the subset of 61 patients and the whole cohort of 226 patients. Clinical and pathological characteristics of each of the 61 patients are available in Supplemental Table S1 (at *http://ajp. amjpathol.org*).

RNA Isolation and Microarray Expression Profiling

Total RNA was extracted from tumor biopsy specimens using a kit (Qiagen RNeasy Mini Kit; Qiagen Inc., Valencia, CA), following the instructions of the manufacturer. The biopsy specimens were obtained before neoadjuvant chemotherapy. To check cellularity, an H&E image was obtained from all tumors; only samples with more than 80% tumor cells were used. The amount of RNA was assessed with a device (Nanodrop ND-1000 UV Spectrophotometer; Thermo Fisher Scientific, Wilmington, DE). RNA integrity was assessed using a kit (RNA 6000 Nano Chip kit), followed by analysis (Bioanalyzer 2100; Agilent Technologies, Santa Clara, CA). Whole human genome oligo 4 $\times$ 44 microarrays (Agilent Technologies) were hybridized after low RNA input fluorescence amplification of tumor total RNA, according to the manufacturer's protocol. Arrays were scanned (GenePix 4000B scanner; Molecular Devices Corporation, Sunnyvale, CA), analyzed using software (GenePix 5.1), and uploaded into a University of North Carolina microarray database, in which a Lowess normalization of the log2 ratio (Cyanine 5/Cyanine 3) intensity values is automatically performed. For all analyses, genes were filtered by requiring the Lowess-normalized intensity values in both channels to be greater than 10; only genes that reported values in 70% or more of the samples were included. The genes were median centered across all samples. Breast cancer subtypes were assigned as described by Parker et al.[24] In addition, the recently identified subtype named Claudin-low was assigned using a separate centroid-based predictor[25]; therefore, tumors were categorized into Luminal A, Luminal B, Basal-like, Her2-enriched, Claudin-low, and Normal-like. The primary microarray data presented in this study are available in the Gene Expression Omnibus database (accession No. GSE21997).

Q-PCR Analysis

Total RNA, 200 ng, was used as a template to obtain first-strand cDNA using a system (SuperScript First-Strand Synthesis System for RT-PCR; Invitrogen, Parsley, UK), following the manufacturer's instructions. To determine that cDNA was synthesized, the first-strand cDNA was further amplified by conventional PCR using primers for β-actin, as previously described.[26] Real-time PCR was performed using gene expression assays (TaqMan; Applied Biosystems, Foster City, CA) and a PCR system (ABI PRISM 7900HT Fast Real Time PCR System; Applied Biosystems). Briefly, 4 μL of

cDNA template was mixed with 1 μL of ×20 *TOP2A* primers, probes labeled with 5,6 carboxifluoresceina reporter dye (assay Hs00172214_m1), 1 μL of ×20 human β-actin primers, and probes labeled with VIC-reported dye (TaqMan endogenous control, part 4326315E; Applied Biosystems), 10 μL of mix (TaqMan Universal PCR Mastermix), and 4 μL of RNase-free water. Human β-actin served as an endogenous control to normalize the *TOP2A* mRNA levels in the subsequent quantitative analysis. The PCR conditions were those recommended by the manufacturer. A pool of RNA from 11 breast tissue samples of healthy individuals who underwent cosmetic or plastic surgery was used as a control. Each sample was measured in triplicate. The data were analyzed by the comparative Ct method, in which the amount of *TOP2A*, normalized to β-actin and relative to control, is given as follows: $2^{-(\Delta Ct\ sample\ -\ \Delta Ct\ control)}$. Tumors that yielded values of two or greater were considered as *TOP2A* positive.

IHC and Tumor Grading

Paraffin-embedded tumor samples from core biopsy specimens were evaluated by IHC analysis for *TOP2A* (*TOP2A*-mouse monoclonal antibody Novocastra Leica-TopoIIA, clone 3F6, 1:40; Leica Microsystems, Wetzlar, Germany); estrogen receptor (ER) (clone 1D5, 1:35; Dako Cytomation, Glostrup, Denmark), progesterone re-

ceptor (clone PgR 636, 1:50; Dako Cytomation), and Ki-67 (clone MIB-1, 1:75; Dako Cytomation). After incubation with the primary antibodies, immunohistochemical studies were performed using a Bond-Max immunostainer (Vision BioSystems, Hingham, MA) with polymer-defined peroxidase detection.

Because no cutoff for positivity has been validated to define *TOP2A* overexpression, we analyzed this variable based on a cutoff of 20% or greater (mean value in this study) and median values (10% of stained cells). The Ki-67 positivity was defined as 20% or greater of stained cells because the staining in our normal control breast tissue (from the tissue bank of Hospital Clínico San Carlos) was always lower than this value. The cut points for ER and progesterone receptor positivity were established at 10% or greater of stained cells. All tumors were graded by the study pathologist (JALGA) by using Elston-Ellis histological grading.[27]

Measurement of TOP2A and ERBB2 Amplification

The amplification of *TOP2A* and *ERBB2* was measured by FISH. The probes used were as follows: locus-specific identifier *TOP2A*, labeled in orange; centromere enumeration probe 17, labeled in green; and locus-specific identifier *ERBB2* probe, labeled in orange (Vysis-Abbott, Downers Grove, IL). Slides were prepared according to

Table 1. Characteristics of Study Participants and Distributions of Characteristics by *TOP2A* Status

	FISH data			Q-PCR data		Microarray data		IHC data	
Characteristics	No. of participants (*n* = 61)	No. of tumors with *TOP2A* CNAs (*n* = 15)	*P* value	*TOP2A* expression*	*P* value	*TOP2A* expression*	*P* value	No. of *TOP2A*-positive tumors (*n* = 25)	*P* value
Tumor size (cm)									
≤5	24	2	0.03[†]	0.92 (1.8)	0.44	0.08 (1.0)	0.45	9	0.79
>5	37	13		0.57 (1.7)		−0.09 (0.7)		16	
Histological feature									
Ductal	48	13	0.87	0.93 (1.8)	0.12	0.051 (0.9)	0.38	21	0.78
Lobular	10	2		−0.35 (1.5)		−0.37 (0.8)		3	
Other	3	0		0.72 (1.5)		−0.11 (1.0)		1	
Stage at diagnosis									
II	21	2	0.03[†]	0.58 (2.0)	0.91	0.07 (1.0)	0.84	8	0.28
IIIA	20	4		0.73 (1.6)		−0.06 (0.8)		6	
IIIB	20	9		0.83 (1.8)		−0.08 (0.8)		11	
Histological grade									
II	40	9	0.76	0.56 (1.8)	0.38	−0.11 (0.8)	0.26	10	<0.01[†]
III	21	6		0.99 (1.8)		0.15 (0.9)		15	
Ki-67									
Low	18	3	0.52	−0.21 (1.4)	<0.01[†]	−0.48 (0.7)	<0.01[†]	4	0.086
High	43	12		1.10 (1.8)		0.17 (0.8)		21	
Estrogen receptor									
Positive	36	12	0.07	0.55 (1.8)	0.39	−0.07 (0.9)	0.59	13	0.43
Negative	25	3		0.95 (1.7)		0.05 (0.8)		12	
Progesterone receptor									
Positive	37	10	0.76	0.67 (1.9)	0.86	0.01 (0.9)	0.76	12	0.11
Negative	24	5		0.76 (1.6)		−0.06 (0.7)		13	
ERBB2 status									
Positive	19	8	0.05[†]	0.58 (1.9)	0.71	−0.11 (0.9)	0.60	8	1.0
Negative	42	7		0.76 (1.7)		0.02 (0.9)		17	

CNA, copy number alteration.
*Data are given as the mean (SD).
[†]Significant difference.

the manufacturer's instructions for paraffin sections. A positive result was defined as an *ERBB2* and *TOP2A* genes/chromosome 17 ratio of 2.2 or greater (for *ERBB2*) or greater than 2 (for *TOP2A*). The cutoff for the deletion was established as a *TOP2A* gene/chromosome 17 ratio of 0.5 or lower. Images were visualized on a fluorescence microscope and captured on a workstation (MetaSystems, Altlussheim, Germany). A minimum of 100 nuclei were counted per case. All cut points were predefined before the correlations were performed; in all of the cases, the pathologist (JALGA) was blinded from the patient's identity and outcome.

Statistical Analysis

Qualitative variables were summarized by their frequency distribution, and quantitative variables were summarized by their mean ± SD. The continuous values of Q-PCR were log transformed. For qualitative variables, comparison was evaluated by the χ^2 or Fisher exact test in cases in which more than 25% of the expected values were less than five. The association between the expression of *TOP2A*, measured by gene expression microarrays or Q-PCR, and other qualitative variables was assessed by *t*-test. To compare between more than two groups, a one-way analysis of variance for quantitative variables was used. The correlation among the quantitative values of *TOP2A* expression, measured by Q-PCR and gene expression microarrays, was evaluated with simple linear regression analysis. The comparison between *TOP2A* expression data, assessed by IHC, and gene expression microarrays or Q-PCR was performed using receiver operating characteristic curve analysis. The area under the curve, sensitivity, and specificity were also determined. The adjusted false-positive, false-negative, and error rates of the determined cutoffs were estimated using a bootstrapping method. Simple Cohen's κ coefficients and percentage agreement with its 95% confidence interval were used to assess the agreement when classifying the tumors as *TOP2A* positive or *TOP2A* negative. The strength of agreement is considered to be slight when κ values are between 0.00 and 0.20; fair, 0.21 and 0.40; moderate, 0.41 and 0.60; good, 0.61 and 0.80; and almost perfect, 0.81 and 1.00. All statistical tests were two sided, and $P < 0.05$ was considered significant. The statistical analysis was performed using software (SPSS 17.0 and R 2.10.1).

Results

Clinicopathological Characteristics of the Study Population

Table 1 summarizes the pathological characteristics of the 61 tumor samples/patients and the distribution of characteristics by *TOP2A* status. As shown, *TOP2A* copy number alterations were significantly associated with tumor size, stage, and *ERBB2* amplification status. Interestingly, *TOP2A* expression, as assessed by Q-PCR or microarray, was not significantly associated with any of

these characteristics. On the other hand, *TOP2A* status, by Q-PCR and gene expression microarrays, was significantly associated with Ki-67 positivity ($P = 0.008$ and $P = 0.005$, respectively). Similarly, according to IHC data, *TOP2A* protein overexpression was more frequent in Ki-67–positive tumors, although Ki-67 positivity was not significantly associated with *TOP2A* IHC positivity ($P = 0.08$).

In addition, we found that *TOP2A* mRNA expression, as assessed by Q-PCR or gene expression microarrays, varied significantly with intrinsic subtype ($P < 0.001$ in both cases). High-proliferative subtypes, such as Basal-like, Luminal B, and Her2-enriched, expressed higher levels of *TOP2A* than Luminal A, Claudin-low, or Normal-like tumors (Figure 1, A and B). In the same way, according to IHC data, the proportion of *TOP2A*-positive tumors was statistically different depending on tumor subtype ($P = 0.002$). As shown in Figure 1C, Basal-like, Her2-enriched, and Luminal B tumors had the most *TOP2A*-positive tumors, whereas Luminal A tumors were mostly *TOP2A* negative and all Normal-like tumors were *TOP2A* negative. Therefore, *TOP2A* expression can be considered a proliferative marker that is highly characteristic of the rapidly growing tumor subtypes. Finally, we observed that *TOP2A* copy number alterations occurred in Luminal A, Luminal B, Her2-enriched, and Claudin-low subtypes; no alterations were found in Basal-like and Normal-like tumors (Figure 1D).
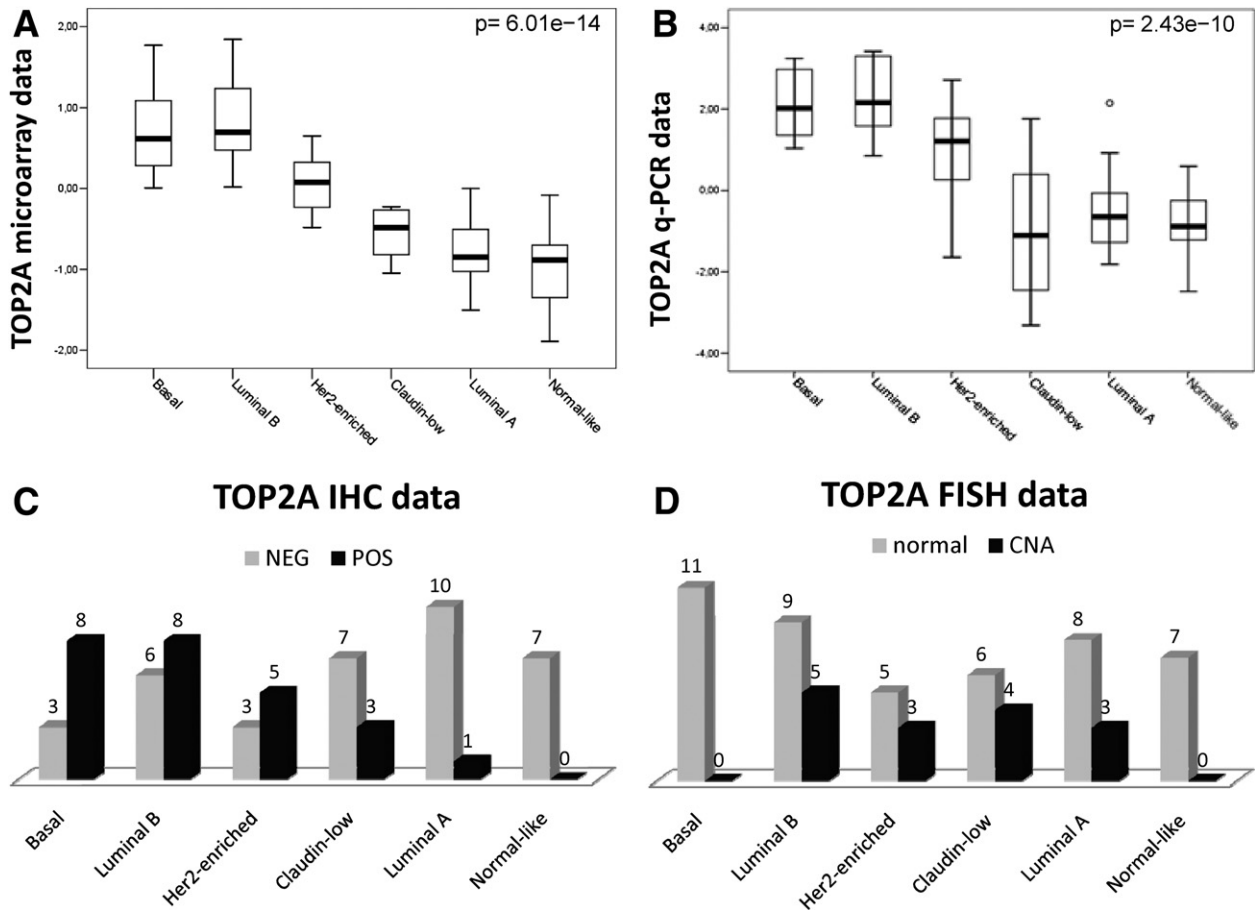
Correlation Between TOP2A Status Assessed by Q-PCR, Gene Expression Array, and IHC

The *TOP2A* expression values measured by Q-PCR and gene expression microarrays significantly correlated with each other (Pearson correlation coefficient, 0.816; 95% confidence interval, 0.71 to 0.89; $P = 1.2 \times 10^{-15}$) (Figure 2). In addition, tumors with positive *TOP2A* expression by IHC had relatively higher mean levels of *TOP2A* expression, as measured by Q-PCR ($P = 0.002$) or gene expression microarrays ($P < 0.001$).

To demonstrate the concordance of *TOP2A* expression status, determined by these techniques, we performed receiver operating characteristic curve analysis to assess how *TOP2A* gene expression by Q-PCR or microarrays can predict *TOP2A*-positive status by IHC. For Q-PCR, the area under the curve was 0.741 (sensitivity, 80%; specificity, 72%; best cutoff value, 1.04; adjusted error rate, 27%; adjusted false-positive rate, 23%; adjusted false-negative rate, 30%); for gene expression microarrays, the area under the curve was 0.791 (sensitivity, 100%; specificity, 53%; best cutoff value, −0.48; adjusted error rate, 30%; adjusted false-positive rate, 4%; adjusted false-negative rate, 49%) (Figure 3).

TOP2A Copy Number Alteration and TOP2A Expression

The *TOP2A* was amplified in 13 cases (21.3%) and deleted in 2 cases (3.3%). Unlike RNA and protein levels, no significant association was observed between *TOP2A*
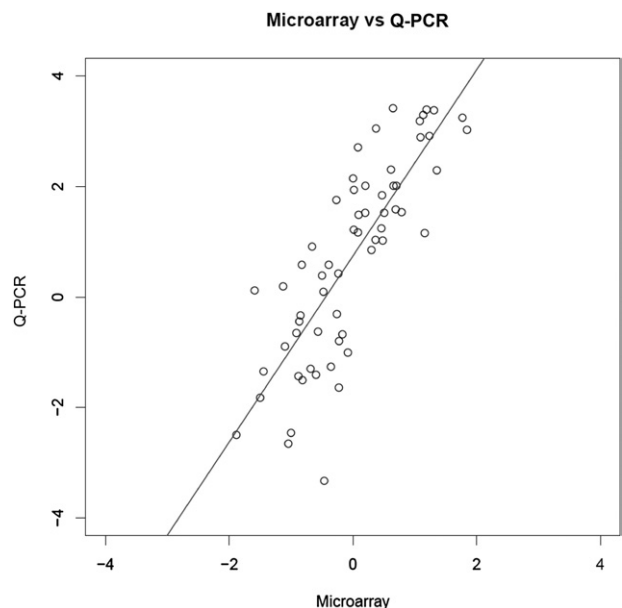
Figure 1. A: Box plot. The *TOP2A* mRNA expression values, measured by gene expression microarrays, according to genomic subtype. **B:** Box plot. The *TOP2A* mRNA expression values, measured by Q-PCR, according to genomic subtype. **C:** Bar chart. The number of *TOP2A*-positive (POS) and *TOP2A*-negative (NEG) tumors, according to IHC, for each subtype. **D:** Bar chart. The number of *TOP2A*-positive and *TOP2A*-negative tumors, according to FISH, for each subtype. CNA indicates copy number alteration.
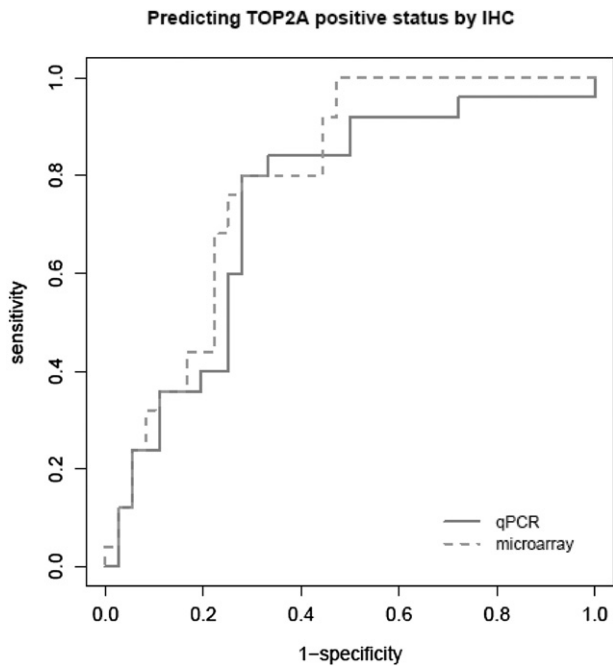
copy number alterations and the expression of the gene measured by Q-PCR or gene expression microarrays. In addition, we did not find agreement between IHC and FISH when assessing *TOP2A* status in tumor samples ($\kappa = 0.134$, $P = 0.26$) (Table 2).

Discussion

In this study, *TOP2A* gene expression, as assessed by DNA microarray or Q-PCR, was significantly associated with Ki-67 IHC positivity. When *TOP2A* was assessed by IHC, a similar tendency was observed. Taken together, these data suggest that *TOP2A* expression across all breast cancers is likely a proliferation marker, which is concordant with previous reports about breast cancer[28–30] and tumor types.[31,32] Consistent with these data, we found higher *TOP2A* gene expression in highly proliferative subtypes, such as Basal-like, Luminal B, and Her2-enriched tumors, when compared with Luminal A, Normal-like, and Claudin-low tumors. Therefore, tumor proliferation may have a confounding effect on the predictive value of *TOP2A* and, therefore, *TOP2A* expression may possibly need to be adjusted for Ki-67 or other proliferative markers.

Figure 2. Correlation between *TOP2A* expression values, assessed by gene expression microarrays and Q-PCR.

Figure 3. Receiver operating characteristic curves. Prediction of *TOP2A* IHC positivity according to gene expression microarrays and Q-PCR data.

There is growing evidence supporting that breast cancer is a heterogeneous disease, rather than a single disease; and breast cancer subtypes have specific clinicopathological characteristics with different prognoses.[24,25,33,34] The relative benefit of anthracyclines has not been tested for each specific breast cancer molecular subtype, although therapeutic outcome might be affected by molecular subtype.[35] Different results among researchers who have assessed the predictive value of *TOP2A* expression might be, in part, because of different proportions of breast cancer subtypes between study populations; we demonstrated that *TOP2A* expression is substantially different across the known subtypes.

In our study, *TOP2A* amplification was associated with tumor size, stage, and *ERBB2* positivity. Interestingly, none of these characteristics was associated with *TOP2A* expression. This might suggest that TOP2A protein–overexpressing tumors and tumors with *TOP2A* copy number alterations might be biologically different. Indeed, we found that the distribution of *TOP2A*-amplified tumors among subtypes is different and that the protein expression is also uniquely different; thus, it is not surprising to find that the FISH and IHC data are not that highly cor-

Table 2. The *TOP2A*-Positive and *TOP2A*-Negative Tumors According to FISH and IHC Results

	IHC results		
FISH results	Positive	Negative	κ (*P*) value*
Positive	8 (13)	7 (11)	0.134 (0.26)
Negative	17 (28)	29 (48)	

Data are given as number (percentage) of 61 tumors unless otherwise indicated.
*Cohen's κ coefficient for agreement between FISH and IHC results.

related. The expression of *TOP2A* has been extensively studied in human breast cancers by different methods. However, the correlation between techniques has been scarcely reported. As expected, our results showed a good correlation among Q-PCR and gene expression microarrays (Pearson correlation coefficient, 0.816).

A threshold value for *TOP2A* expression that could define subgroups associated with treatment outcome remains to be established. Some researchers[17,36–38] have used an arbitrary IHC cutoff of greater than 10%, whereas others[39] have used a 25% cutoff. We used a cutoff of 20% because staining in our control healthy breast tissue was always lower than this value; however, similar results were obtained when considering the cutoff of 10% (data not shown). We found that both microarrays and Q-PCR predicted fairly well the *TOP2A* expression values measured by IHC. In addition, our results showed a significant association between *TOP2A* mRNA and protein levels. However, IHC is potentially a more subjective technique and may have a lower precision when assessing the status of *TOP2A* relative to Q-PCR. Interestingly, no association between *TOP2A* copy number alterations and the expression of the gene was observed, which is in agreement with previous reports.[17,40]

Because *TOP2A* is considered the molecular target of anthracyclines,[3] the predictive value of this gene in patients with breast cancer has been widely studied. Several retrospective studies[12–16] have shown that *ERBB2*-positive tumors are rather sensitive to anthracyclines. In addition, amplification of *ERBB2* is variable in size. Some flanking genes in the 17q12-q21 region, such as *TOP2A*, are frequently either coamplified or deleted in breast cancers with *ERBB2* amplification.[41–43] Because of this coincidence, many researchers have hypothesized that *TOP2A* amplification might become the gold standard predictive factor for anthracyclines, rather than *ERBB2*; and many retrospective studies have been conducted to assess its predictive value. However, although several studies[19,39,42,44] have shown that *TOP2A* alterations are associated with an increased responsiveness to anthracycline-containing regimens, others[20,45] have been inconclusive.

Our data suggest that *TOP2A* amplification and *TOP2A* overexpression are indicative of different biological processes because these two events are not associated and are characteristic of different tumor subtypes. For example, Basal-like tumors typically overexpressed *TOP2A* protein and/or mRNA, but no Basal-like tumor had *TOP2A* DNA copy number changes. This might suggest that the predictive value of *TOP2A* copy number may not be because of a *TOP2A* protein function. Rather, it may be indicative of some other biological process or genomic aberration that could be characteristic of *ERBB2*-amplified tumors because *TOP2A* amplification occurs almost exclusively within *ERBB2*-amplified tumors. In support of this hypothesis, a previous study,[39] performed in a cohort of 245 patients, found that *TOP2A* amplification predicted benefit from adjuvant anthracyclines only in the subset of *ERBB2*-positive breast cancer but failed to predict response when the entire population of the study was considered. Similarly, the TOP trial showed that *TOP2A* am-

plification, which was always associated with *ERBB2* amplification, predicted benefit from regimens containing anthracyclines in ER-negative tumors.[46] In both studies, *TOP2A* expression did not correlate with treatment outcome. Contrary to these results, we recently observed that *TOP2A* expression is predictive of response to a single agent, doxorubicin, but not *TOP2A* amplification.[47] Because the target of anthracyclines is the TOP2A protein, not the gene, it seems that the study of the expression (RNA and protein) might predict more accurately the treatment outcome with anthracyclines than TOP2A copy number alterations (amplification or deletion); further studies on this point are clearly warranted.

In summary, *TOP2A* copy number alterations do not correlate with gene expression in breast cancers, and the expression of the gene/protein varies significantly depending on the tumor subtype and proliferation status. These facts could explain, in part, the previously reported discrepancies regarding the predictive value of *TOP2A* for anthracycline-based chemotherapy regimens.

References

1. Ryberg M, Nielsen D, Cortese G, Nielsen G, Skovsgaard T, Andersen P: New insight into epirubicin cardiac toxicity: competing risks analysis of 1097 breast cancer patients. J Natl Cancer Inst 2008, 100:1058–1067
2. Diamandidou E, Buzdar AU, Smith TL, Frye D, Witjaksono M, Hortobagyi GN: Treatment-related leukemia in breast cancer patients treated with fluorouracil-doxorubicin-cyclophosphamide combination adjuvant chemotherapy: the University of Texas M.D. Anderson Cancer Center experience. J Clin Oncol 1996, 14:2722–2723
3. Tewey KM, Rowe TC, Yang L, Halligan BD, Liu LF: Adriamycin-induced DNA damage mediated by mammalian DNA topoisomerase II. Science 1984, 226:466–468
4. Minotti G, Menna P, Salvatorelli E, Cairo G, Gianni L: Anthracyclines: molecular advances and pharmacologic developments in antitumor activity and cardiotoxicity. Pharmacol Rev 2004, 56:185–229
5. Osheroff N: Biochemical basis for the interaction of type I and type II topoisomerases with DNA. Pharmacol Ther 1989, 41:233–241
6. Wang JC: DNA topoisomerase. Annu Rev Biochem 1996, 58:635–692
7. Davies SM, Robson CN, Davies SL, Hickson ID: Nuclear topoisomerase II levels correlate with the sensitivity of mammalian cells to intercalating agents and epipodophyllotoxins. J Biol Chem 1988, 263:17724–17729
8. Asano T, An T, Mayes J, Zwelling LA, Kleinerman ES: Transfection of human topoisomerase IIa into etoposide-resistant cells: transient increase in sensitivity followed by down-regulation of the endogenous gene. Biochem J 1996, 319:307–313
9. Withoff S, Keith WN, Knol AJ, Coutts JC, Hoare SF, Mulder NH, de Vries EG: Selection of a subpopulation with fewer DNA topoisomerase II alpha gene copies in a doxorubicin-resistant cell line panel. Br J Cancer 1996, 74:502–507
10. Withoff S, de Vries EGE, Keith WN, Nienhuis EF, van der Graaf WT, Uges DR, Mulder NH: Differential expression of DNA topoisomerase II alpha and -beta in P-gp and MRP-negative VM26, mAMSA and mitoxantrone-resistant sublines of the human SCLC cell line GLC4. Br J Cancer 1996, 74:1869–1876
11. Järvinen TA, Tanner M, Rantanen V, Bärlund M, Borg A, Grénman S, Isola J: Amplification and deletion of topoisomerase II associate with HER-2 amplification and affect sensitivity to topoisomerase II inhibitor doxorubicin in breast cancer. Am J Pathol 2000, 156:839–847
12. Thor AD, Berry DA, Budman DR, Muss HB, Kute T, Henderson IC, Barcos M, Cirrincione C, Edgerton S, Allred C, Norton L, Liu ET: ErbB-2, p53, and efficacy of adjuvant therapy in lymph node-positive breast cancer. J Natl Cancer Inst 1998, 90:1346–1360
13. Paik S, Bryant J, Park C, Fisher B, Tan-Chiu E, Hyams D, Fisher ER, Lippman ME, Wickerham DL, Wolmark N: erbB-2 and response to doxorubicin in patients with axillary lymph node-positive, hormone receptor-negative breast cancer. J Natl Cancer Inst 1998, 90:1361–1370
14. Paik S, Bryant J, Tan-Chiu E, Yothers G, Park C, Wickerham DL, Wolmark N: ERBB2 and choice of adjuvant chemotherapy for invasive breast cancer: National Surgical Adjuvant Breast and Bowel Project Protocol B-15. J Natl Cancer Inst 2000, 92:1991–1998
15. Pritchard KI, Shepherd LE, O'Malley FP, Andrulis IL, Tu D, Bramwell VH, Levine MN: ERBB2 and responsiveness of breast cancer to adjuvant chemotherapy. N Engl J Med 2006, 354:2103–2111
16. Gennari A, Sormani MP, Pronzato P, Puntoni M, Colozza M, Pfeffer U, Bruzzi P: ERBB2 status and efficacy of adjuvant anthracyclines in early breast cancer: a pooled analysis of randomized trials. J Natl Cancer Inst 2008, 100:14–20
17. Petit T, Wilt M, Velten M, Millond R, Rodier JF, Borel C, Mors R, Haegelé P, Eber M, Ghnassia JP: Comparative value of tumour grade, hormonal receptors, Ki-67, HER-2 and topoisomerase II alpha status as predictive markers in breast cancer patients treated with neoadjuvant anthracycline-based chemotherapy. Eur J Cancer 2004, 40:205–211
18. Sparano JA, Goldstein L, Childs B, Shak S, Brassard D, Badve S, Baehner FL, Bugarini R, Rowley S, Perez E, Shulman LN, Martino S, Davidson NE, Sledge GW, Gray R: Relationship between topoisomerase 2A RNA expression and recurrence after adjuvant chemotherapy for breast cancer. Clin Cancer Res 2009, 15:7693–7700
19. O'Malley FP, Chia S, Tu D, Shepherd LE, Levine MN, Bramwell VH, Andrulis IL, Pritchard KI: Topoisomerase II alpha and responsiveness of breast cancer to adjuvant chemotherapy. J Natl Cancer Inst 2009, 101:644–650
20. Harris LN, Broadwater G, Abu-Khalaf M, Cowan D, Thor AD, Budman D, Cirrincione CT, Berry DA, Winer EP, Hudis CA, Hayes DF, Friedman P, Ellis M, Dressler L: Topoisomerase II{alpha} amplification does not predict benefit from dose-intense cyclophosphamide, doxorubicin, and fluorouracil therapy in ERBB2-amplified early breast cancer: results of CALGB 8541/150013. J Clin Oncol 2009, 27:3430–3436
21. Tuccari G, Rizzo A, Giuffrè G, Barresi G: Immunocytochemical detection of DNA topoisomerase type II in primary breast carcinomas: correlation with clinico-pathological features. Virchows Arch A Pathol Anat Histopathol 1993, 423:51–55
22. Lynch BJ, Guinee DG Jr, Holden JA: Human DNA topoisomerase II-alpha: a new marker of cell proliferation in invasive breast cancer. Hum Pathol 1997, 28:1180–1188
23. Nakopoulou L, Lazaris AC, Kavantzas N: DNA topoisomerase II alfa inmunoreactivity as a marker of aggressiveness in invasive breast cancer. Pathobiology 2000, 68:137–143
24. Parker JS, Mullins M, Cheang MC, Leung S, Voduc D, Vickery T, Davies S, Fauron C, He X, Hu Z, Quackenbush JF, Stijleman IJ, Palazzo J, Marron JS, Nobel AB, Mardis E, Nielsen TO, Ellis MJ, Perou CM, Bernard PS: Supervised risk predictor of breast cancer based on intrinsic subtypes. J Clin Oncol 2009, 27:1160–1167
25. Prat A, Parker JS, Karginova O, Fan C, Livasy C, Herschkowitz JI, He X, Perou CM: Phenotypic and molecular characterization of the claudin-low intrinsic subtype of breast cancer. Breast Cancer Res 2010, 12:R68
26. Kreuzer KA, Lass U, Landt O, Nitsche A, Laser J, Ellerbrok H, Pauli G, Huhn D, Schmidt CA: Highly sensitive and specific fluorescence reverse transcription-PCR assay for pseudogene-free detection of beta-actin transcripts as quantitative reference. Clin Chem 1999, 45:297–300
27. Elston CW, Ellis IO: Pathological prognostic factors in breast cancer, I: the value of histological grade in breast cancer: experience from a large study with long-term follow-up. Histopathology 1991, 19:403–410
28. Hellemans P, van Dan PA, Geysken M, Oosterom AT, Buytaert PH, van Marck E: Immunohistochemical study of topoisomerase II α expression in primary ductal carcinoma of the breast. J Clin Pathol 1995, 48:147–150
29. Järvinen TA, Kononen J, Pelto-Huikko M, Isola J: Expression of topoisomerase II alpha is associated with rapid cell proliferation, aneuploidy, and c-ERBB2 overexpression in breast cancer. Am J Pathol 1996, 148:2073–2082
30. Boege F, Andersen A, Jensen S, Zeidler R, Kreipe H: Proliferation-associated nuclear antigen ki-64 is identical with topoisomerase IIα: delineation of a carboxyl-terminal epitope with peptide antibodies. Am J Pathol 1996, 146:1302–1308

31. Shvero J, Koren R, Shvili I, Yaniv E, Sadov R, Hadar T: Expression of human DNA topoisomerase II-alpha in squamous cell carcinoma of the larynx and its correlation with clinicopathologic variables. Am J Clin Pathol 2008, 130:934–939

32. Wong N, Yeo W, Wong WL, Wong NL, Chan KY, Mo FK, Koh J, Chan SL, Chan AT, Lai PB, Ching AK, Tong JH, Ng HK, Johnson PJ, To KF: TOP2A overexpression in hepatocellular carcinoma correlates with early age onset, shorter patients survival and chemoresistance. Int J Cancer 2009, 124:644–652

33. Perou CM, Sørlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, Pollack JR, Ross DT, Johnsen H, Akslen LA, Fluge O, Pergamen-schikov A, Williams C, Zhu SX, Lønning PE, Børresen-Dale AL, Brown PO, Botstein D: Molecular portraits of human breast tumors. Nature 2000, 406:747–752

34. Carey LA, Perou CM, Livasy CA, Dressler LG, Cowan D, Conway K, Karaca G, Troester MA, Tse CK, Edmiston S, Deming SL, Geradts J, Cheang MC, Nielsen TO, Moorman PG, Earp HS, Millikan RC: Race, breast cancer subtypes, and survival in the Carolina Breast Cancer Study. JAMA 2006, 295:2492–2502

35. Rouzier R, Perou CM, Symmans WF, Ibrahim N, Cristofanilli M, An-derson K, Hess KR, Stec J, Ayers M, Wagner P, Morandi P, Fan C, Rabiul I, Ross JS, Hortobagyi GN, Pusztai L: Breast cancer molecular subtypes respond differently to preoperative chemotherapy. Clin Cancer Res 2005, 11:5678–5685

36. Durbecq V, Paesmans M, Cardoso F, Desmedt C, Di Leo A, Chan S, Friedrichs K, Pinter T, Van Belle S, Murray E, Bodrogi I, Walpole E, Lesperance B, Korec S, Crown J, Simmonds P, Perren JT, Leroy J, Rouas G, Sotiriou C, Piccart M, Larsimon D: Topoisomerase-II alpha expression as a predictive marker in a population of advanced breast cancer patients randomly treated either with single-agent doxorubicin or single-agent docetaxel. Mol Cancer Ther 2004, 3:1207–1214

37. Di Leo A, Larsimont D, Gancberg D, Jarvinen T, Beauduin M, Vinde-voghel A, Michel J, Focan CH, Ries F, Gobert PH, Closon-Dejardin MT, Dolci S, Rouas G, Paesmans M, Lobelle JP, Isola J, Piccart MJ: HER-2 and topo-isomerase IIα as predictive markers in a population of node-positive breast cancer patients randomly treated with adjuvant CMF or epirubicin plus cyclophosphamide. Ann Oncol 2001, 12:1081–1089

38. Cardoso F, Di Leo A, Larsimont D, Gancberg D, Rouas G, Dolci S, Ferreira F, Paesmans M, Piccart M: Evaluation of HER-2, p53, bcl-2, topoisomerase II-α, heat shock proteins 27 and 70 in primary breast cancer and metastatic ipsilateral axillary lymph nodes. Ann Oncol 2001, 12:615–620

39. Arriola E, Rodriguez-Pinilla SM, Lambros MB, Jones RL, James M, Savage K, Smith IE, Dowsett M, Reis-Filho JS: Topoisomerase II alpha amplification may predict benefit from adjuvant anthracyclines in HER2 positive early breast cancer. Breast Cancer Res Treat 2007, 106:181–189

40. Muller RE, Parkers RK, Andrulis I, O'Malley FP: Amplification of the TOP2A gene does not predict high levels of topoisomerase II alpha protein in human breast tumor samples. Genes Chromosomes Can-cer 2004, 39:288–297

41. Olsen KE, Knudsen H, Rasmussen BB, Balslev E, Knoop A, Ejlertsen B, Nielsen KV, Schönau A, Overgaard J; Danish Breast Cancer Co-operative Group: Amplification of ERBB2 and TOP2A and deletion of TOP2A genes in breast cancer investigated by new FISH probes. Acta Oncol 2004, 43:35–42

42. Knoop AS, Knudsen H, Balslev E, Rasmussen BB, Overgaard J, Nielsen KV, Schonau A, Gunnarsdóttir K, Olsen KE, Mouridsen H, Ejlertsen B; Danish Breast Cancer Cooperative Group: Retrospective analysis of topoisomerase IIa amplifications and deletions as predic-tive markers in primary breast cancer patients randomly assigned to cyclophosphamide, methotrexate, and fluorouracil or cyclophospha-mide, epirubicin, and fluorouracil: Danish Breast Cancer Cooperative Group. J Clin Oncol 2005, 23:7483–7490

43. Park K, Han S, Gwak GH, Kim HJ, Kim J, Kim KM: Topoisomerase II-alpha gene deletion is not frequent as its amplification in breast cancer. Breast Cancer Res Treat 2006, 98:337–342

44. Scandinavian Breast Group Trial 9401, Tanner M, Isola J, Wiklund T, Erikstein B, Kellokumpu-Lehtinen P, Malmström P, Wilking N, Nilsson J, Bergh J: Topoisomerase IIalpha gene amplification predicts favor-able treatment response to tailored and dose-escalated anthracy-cline-based adjuvant chemotherapy in HER-2/neu-amplified breast cancer: Scandinavian Breast Group Trial 9401. J Clin Oncol 2006, 24:2428–2436

45. Hannemann J, Kristel P, van Tinteren H, Bontenbal M, van Hoesel QG, Smit WM, Nooij MA, Voest EE, van der Wall E, Hupperets P, de Vries EG, Rodenhuis S, van de Vijver MJ: Molecular subtypes of breast cancer and amplification of topoisomerase IIa: predictive role in dose intensive ad-juvant chemotherapy. Br J Cancer 2006, 95:1334–1341

46. Desmedt C, Azambuja E, Larsimont D, Haibe-Kains FL, Rouas G, Selleslags J, Delaloge S, Duhem C, Kains J-P, Carly B, Maerevoet M, Vindevoghel A, Cardoso F, Durbecq V, Nogaret J-M, Veys I, Scob-bens J-C, Noterman D, Salgado R, Di Leo, A, D'Hondt V, Piccart M, Sotiriou C: Predicting the efficacy of anthracyclines in breast cancer (BC): The results of the TOP trial and their validation in the BIG 00-01 trial. Abstract 1893. Presented at the AACR Annual Meeting, April 18–22, 2009, Denver, CO

47. Chia SK, Ung K, Bramwekk VH, Tu D, Perou CM, Ellis MJ, Pernard PS, Vickery T, Shepherd LE, Nielsen TO: Prognostic and Predictive im-pact of biologic classification by qRT-PCR with a 50-gene subtype predictor (PAM50) for adjuvant tamoxifen in premenopausal breast cancer: Results from the NCIC CTG MA.12 randomized trial. Abstract 508. Presented at the ASCO Annual Meeting, June 4–8, 2010, Chi-cago, IL