Prognostic Significance of *TOP2A* Gene Dosage in HER-2-Negative Breast Cancer

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LEARNING OBJECTIVES

After completing this course, the reader will be able to:

1. Describe the prognostic role of *TOP2A* gene dosage determined by quantitative PCR in HER-2-negative breast cancer.
2. Describe the relationship between HER-2 status and *TOP2A* status in breast cancer tumors.
3. Gain greater understanding of methods used for *TOP2A* status determination, including the advantages of quantitative PCR.

CME

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ABSTRACT

Background. Previous studies showed the prognostic and predictive impact of human epidermal growth factor receptor 2 (*HER-2*) gene alterations analyzed separately and

jointly with topoisomerase II α (*TOP2A*) gene alterations; however, the role of *TOP2A* gene abnormalities alone has not been thoroughly investigated. Additionally, *TOP2A* ab-

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errations were typically studied in HER-2-positive (HER-2⁺) tumors because these genes are frequently coamplified. Therefore, the knowledge concerning the impact of *TOP2A* abnormalities in HER-2-negative (HER-2⁻) patients is scarce. This study aimed to investigate the clinical significance of *TOP2A* anomalies in breast cancer patients with HER-2⁻ and HER-2⁺ tumors.

Materials and Methods. Snap-frozen tumor samples from 322 consecutive stage I–III breast cancer patients were analyzed for *TOP2A* gene dosage using quantitative real-time PCR (qPCR).

Results. A high *TOP2A* gene dosage was found in 94 tumors (29%)—32% and 27% of HER-2⁺ and HER-2⁻ tumors, respectively. The mean *TOP2A* gene dosages in the

HER-2⁺ and HER-2⁻ groups were 1.49 ± 1.03 and 1.09 ± 0.35 , respectively. High *TOP2A* gene dosage had an inverse prognostic impact in terms of shorter disease-free survival (DFS) and overall survival (OS) times in the entire group and in both the HER-2⁻ and HER-2⁺ subgroups. The unfavorable prognostic impact of *TOP2A* gene dosage was maintained in the multivariate Cox regression analysis in the entire group and in HER-2⁻ patients.

Conclusions. A high gene dosage of *TOP2A* determined using qPCR occurs frequently both in HER-2⁺ and HER-2⁻ tumors and has a strong adverse prognostic impact. *The Oncologist* 2012;17:1246–1255

INTRODUCTION

Topoisomerase II α (TOP2A) is an essential nuclear enzyme that changes DNA topology, and thus is required in almost any process involving movement and untangling of DNA [1]. This enzyme is encoded by the *TOP2A* gene located in the 17q12–21 region that also contains the gene encoding human epidermal growth factor receptor (*HER-2*), one of the most frequently amplified genes in breast cancer [2, 3]. The exact mechanism of rearrangement of this region is not yet understood; however, data suggest that even though high copy numbers of *TOP2A* and *HER-2* are common findings, these genes belong to separate amplicons [4–6]. Nevertheless, *HER-2* status has been used to select patients for analysis of *TOP2A* alterations, and their clinical relevance has usually been investigated in the context of *HER-2* overexpression or *HER-2* amplification [7–9].

Both *TOP2A* amplifications and deletions were shown to confer an inferior prognosis but also a greater benefit from anthracycline-containing therapy [10, 11]. A large body of literature has suggested the possibility of guiding therapy based on *TOP2A* status [8, 11, 12]; however, a recent meta-analysis did not confirm the predictive value of *TOP2A* alterations [9]. We recently found a relatively high frequency of *TOP2A* amplifications in HER-2⁻ breast cancers [13], which challenges the common opinion that *TOP2A* alteration is mostly restricted to HER-2⁺ tumors and may be considered as a prognostic and predictive marker only in this subgroup [7, 12, 14]. Thus, the primary aim of this study was to investigate the clinical significance of *TOP2A* anomalies measured using quantitative real-time polymerase chain reaction (qPCR) in a large group of consecutive breast cancer patients with both HER-2⁻ and HER-2⁺ tumors. We also aimed to compare our qPCR method with commonly used fluorescence in situ hybridization (FISH) and immunohistochemistry (IHC) assays and to check the clinical utility of our method by assessing the performance of a *TOP2A* gene dosage analysis in formalin-fixed, paraffin-embedded (FFPE) tissues.

PATIENTS AND METHODS

The study group included 322 consecutive stage I–III breast cancer patients treated in 1999–2009 in three Polish institutions (Table 1). Primary tumor samples were obtained using surgical excision or excisional biopsy prior to any systemic treatment, snap-frozen in liquid nitrogen, and stored at -80°C for further analyses. Additionally, from eight patients, healthy

breast tissue samples were obtained during surgery from a site distant from the primary tumor location and subjected to the preservation procedure. No systemic therapy was administered prior to tumor excision. The majority of patients (59%) underwent primary surgery followed by systemic treatment, radiotherapy, or both, and 22% of patients received induction chemotherapy. The mean age of the patients was 58 years (range, 27–86 years). Follow-up data were available for 319 patients; the median follow-up time was 54 months (range, 0.2–137 months) and the last record was taken in August 2011. Eighty-five patients (26.6%) developed tumor recurrence, 49 of whom (15.4%) died. The study was accepted by the Ethics Committee of the Medical University of Gdańsk (a coordinating center), and all patients signed informed consent forms.

From five healthy donors who signed informed consent forms, full blood samples were drawn in order to isolate leukocyte DNA that served as a calibrator in a real-time PCR experiment. Moreover, in order to compare the performance of the *TOP2A* gene dosage analysis protocol in frozen and FFPE samples, 37 matched FFPE samples were collected.

HER-2 Status Determination

HER-2 protein status was analyzed by IHC using the HercepTestTM (Dako, Glostrup, Denmark) according to the manufacturer's instructions. A 3+ score was considered as a positive result [15, 16]. A 2+ was considered score as equivocal and was tested for *HER-2* gene amplification by FISH with the PathVysion[®] HER- DNA probe kit (Abbott Molecular, Abbott Park, IL) in accordance with the manufacturer's instructions. The mean numbers of HER-2 and centromere 17 (CEP17) signals were estimated for each tumor sample. A HER-2/CEP-17 ratio ≥ 2 was considered as *HER-2* amplification. Cases that were positive using FISH were considered HER-2⁺. Cases with a 2+ IHC score for which FISH analysis was impossible or unsuccessful (as a result of technical reasons and poor quality of the 7- to 10-year-old paraffin-embedded tissue blocks) were excluded from the statistical analysis in which HER-2 status was taken into account.

TOP2A Gene Dosage Evaluation Using qPCR

DNA was isolated from up to 20 mg of macrodissected tumor and healthy breast samples and from 200- μL full blood samples

Table 1. Patient characteristics (*n* = 322)

Variable	<i>n</i> of cases (%)
Age	
>50 yrs	83 (25.8)
≤50 yrs	239 (74.2)
Tumor stage	
T1	100 (31)
T2	147 (45.7)
T3	35 (10.9)
T4	37 (11.5)
Missing data	3 (0.9)
Node stage	
N0	164 (51)
N1	88 (27.3)
N2	59 (18.3)
Missing data	11 (3.4)
Grade	
1	19 (5.9)
2	118 (36.7)
3	100 (31)
Missing data	85 (26.4)
Human epidermal growth factor receptor 2 protein expression	
0	114 (35.4)
1+	59 (18.3)
2+	33 (10.3)
3+	47 (14.6)
Missing data	69 (21.4)
Estrogen receptor status	
Negative	155 (48.1)
Positive	163 (50.7)
Missing data	4 (1.2)
Progesterone receptor status	
Negative	149 (46.3)
Positive	169 (52.5)
Missing data	4 (1.2)
Histological type	
Ductal	222 (69)
Lobular	57 (17.6)
Other	37 (11.5)
Missing data	6 (1.9)

according to the manufacturer's instructions in the tissue and blood and body fluids protocols of the QIAamp® DNA Mini Kit (Qiagen GmbH, Hilden, Germany). DNA was isolated from FFPE samples using the AllPrep® DNA/RNA FFPE Kit (Qiagen) by taking up to four 10-μm thick tissue sections and

using the xylene and ethanol deparaffinization procedure according to the manufacturer's instructions. The quantity and quality of the isolated DNA were measured on a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE). Good-quality DNA was defined by an $A_{260\text{nm}}/A_{280\text{nm}}$ ratio of 1.70–1.90. Isolated DNA samples were stored at –20°C.

Conditions of the qPCR analysis, including the mastermix composition, primer and probe sequences, and temperature parameters are described elsewhere [13]. Briefly, *TOP2A* gene copy number was analyzed in a relative manner using the Pfaffl quantification method. *APP* was the chosen reference gene, and DNA from leukocytes and FFPE sections of healthy breast tissue served as calibrators in the analysis of frozen and FFPE material, respectively. Reactions were performed using a CFX cycler (Bio-Rad, Hercules, CA) on 96-well plates in duplicate, with two negative controls and two calibrators on each plate.

TOP2A gene dosage was dichotomized using the cutoff value below which 99.7% of the *TOP2A* normal population should fall, namely, below three standard deviations from the mean *TOP2A* gene dosage measured in leukocyte DNA from five healthy donors (for frozen samples) and from FFPE sections of healthy breast tissue (for FFPE samples) [17]. Interassay variation was calculated as a coefficient of variance of *TOP2A* gene dosage measured in leukocyte DNA in 17 independent runs or in healthy FFPE breast tissue in six independent runs. To analyze what fraction of cells with *TOP2A* amplification could be detected with the qPCR assay, DNA obtained from the SKBR3 line with *TOP2A* amplification was serially diluted with genomic DNA (Roche Diagnostics, Indianapolis, IN) in proportions corresponding to 100% to 0% in 10% increments. One hundred nanograms of DNA was analyzed in each reaction.

TOP2A Copy Number Evaluation Using FISH

FISH analysis was carried out on tissue microarrays (TMAs). TMAs were constructed from FFPE surgical resection tumor specimens as described previously [18]. Briefly, two 1.5-mm diameter cores from the most representative areas of each tumor were obtained (MTA-I tissue arrayer; Beecher Instruments, Inc., Sun Prairie, WI) and placed in microarray blocks. Punches of tonsils and noncancerous breast tissue samples added to the microarray served as built-in internal controls. Consecutive 4-μm thick TMA sections were cut and placed on charged polylysine-coated slides (Superfrost Plus, BDH Laboratory Supplies, Menzel, Braunschweig, Germany) for subsequent FISH and IHC analyses. *TOP2A* dosages were determined with the U.S. Food and Drug Administration (FDA) approved *TOP2A* FISH pharmDx™ Kit (Dako, Glostrup, Denmark) as described previously [18].

TOP2A Protein Status Determination Using IHC

TOP2A protein expression was examined using TMA IHC staining with Ki-S1 monoclonal antibody (Dako, Glostrup, Denmark) at a dilution of 1:100 for 1.5 hours. Microwave antigen retrieval in citrate buffer and detection with the

NovoLink™ Max-Polymer detection system (Novocastra Laboratories, Newcastle upon Tyne, U.K.) were used. Samples of tonsil were used as positive controls. Negative controls were obtained by omission of the primary antibody. All slides were read independently by two pathologists in a blinded manner without any knowledge of the patient clinical data. At least 500 cancer cells for each core were scored and only nuclear staining was considered. IHC expression was scored according to the percentage of tumor cells with positive staining, because staining intensity was found to be redundant to the frequency. Because no cutoff for positivity has been validated to define TOP2A overexpression, we analyzed this variable based on the obtained median value, similarly to others [19–21]. Thus, a distributional cutoff of $\geq 45\%$ stained cells was used to classify samples as TOP2A⁺.

Statistical Analysis

Correlation between TOP2A status (as a continuous variable) and clinicopathological characteristics was tested using the Mann-Whitney test, correlations between continuous variables were tested using Pearson's correlation test, and correlations between dichotomized variables were tested using the χ^2 test. The endpoints for the study were the disease-free survival (DFS) and overall survival (OS) probabilities. The DFS interval was defined as the time from sample collection to the first of the following events: relapse (local or distant), second malignancy, death, or censoring. Censoring was defined as lost to follow-up or being alive without relapse at the end of follow-up. The OS time was defined as the time from sample collection to death or censoring. Kaplan-Meier estimation was employed for the survival analysis, and the curves were compared using the log-rank test. The multivariate analysis was carried out using the Cox proportional hazard model. Univariate predictors with a p -value $\leq .10$ were entered into a stepwise multivariate model to identify factors that independently predicted DFS and OS probabilities. Statistical significance was assumed when $p \leq .05$. Calculations were performed using Statistica software, version 10 (StatSoft Inc., Tulsa, OK), licensed to the Medical University of Gdańsk. Agreement between different methods corrected for chance was determined by estimating Cohen's κ coefficient using MedCalc software, version 12.2.1.0 (MedCalc Software, Mariakerke, Belgium).

RESULTS

TOP2A Status by qPCR

In the control DNA from the SKBR3 cell line, the median TOP2A gene dosage equaled 3.99 (average of three measurements, 4.01 ± 0.15 ; range, 3.85–4.16). Using the calculated cutoff point for frozen samples, TOP2A amplification was still detected when DNA from the SKBR3 cell line constituted 10% of the total DNA added. The mean TOP2A gene dosages in leukocyte DNA and in frozen and FFPE healthy breast cancer samples equaled 1 ± 0.05 (range, 0.9–1.1), 0.95 ± 0.03 (range, 0.90–0.99), and 0.98 ± 0.07 (range, 0.85–1.04), respectively. In the entire series of frozen tumor samples, the average TOP2A gene dosage equaled 1.14. Samples were

classified as having high TOP2A when the TOP2A gene dosage was ≥ 1.15 for frozen tumors and ≥ 1.19 for FFPE samples. Calculated interassay variations were 5% for frozen tissue and 7% for FFPE tissue.

Comparison of qPCR Results for Frozen and FFPE Breast Cancer Samples

The TOP2A gene dosage measured in FFPE samples was strongly correlated with the results obtained from matching frozen samples ($r = 0.851$; $p < .00001$) (supplemental online Fig. 1S). Concordance between the results was 92% (supplemental online Table 1S). κ equaled 0.83, which indicates high diagnostic agreement.

Comparison of TOP2A Status by qPCR, FISH, and IHC

TOP2A amplification as measured using FISH was found in 24% (34 of 139) of cases. The rates of TOP2A amplification in the HER-2⁺ and HER-2[−] subgroups were 45% (nine of 20) and 20% (20 of 99), respectively ($p = .02$). The results obtained using qPCR and FISH expressed as a continuous scale were correlated with each other ($r = 0.34$; $p = .00002$) (supplemental online Fig. 2S), although the concordance of binary values (positive/negative) was relatively weak (65%; $p = .09$) (supplemental online Table 2S). TOP2A protein measured by IHC was not correlated with either qPCR or FISH ($r = -0.01$; $p = .89$ and $r = .03$; $p = .77$, respectively). TOP2A status was concordant with TOP2A status as assessed using qPCR and FISH in only 49% ($p = .81$) and 47% ($p = .54$) of cases, respectively (supplemental online Tables 2S, 3S).

TOP2A Status in Relation to Clinicopathological Data and Patient Outcome

Of the 322 tumor samples analyzed using qPCR, a high TOP2A gene dosage was found in 94 (29%). Of the 33 cases with a HER-2 2+ score, paraffin-embedded tissue blocks were available for 24 patients. Among these, amplification of HER-2 was found in two cases and a normal HER-2 status was found in 16 cases. For six cases, the FISH analysis was not successful. The occurrences of a high TOP2A gene dosage in HER-2⁺ and HER-2[−] tumors did not differ significantly—32% (16 of 49) and 27% (51 of 189) of cases, respectively ($p = .43$). The mean TOP2A gene dosage was highly correlated with HER-2 status: in the HER-2⁺ and HER-2[−] groups—the mean ($\pm$ standard deviation) values were $1.49 (\pm 1.03)$ and $1.09 (\pm 0.35)$, respectively ($p = .0002$) (Table 2, Fig. 1). No other clinicopathological variables correlated with TOP2A gene dosage (Table 2).

In the group of 319 patients with full follow-up data, a high TOP2A gene dosage was associated with significantly worse DFS ($p = .03$) and OS ($p = .01$) outcomes (Fig. 2). The adverse impact of TOP2A status was also sustained in the subgroup of 189 patients with HER-2[−] tumors— $p = .002$ and $p = .0002$ for the DFS and OS probabilities, respectively (Fig. 2)—and for the subgroup of 49 HER-2⁺ cases— $p = .007$ and $p = .04$ for the DFS and OS probabilities, respectively (Fig. 3). No correlation with clinicopathological data or DFS and OS out-

Table 2. TOP2A gene dosage according to clinicopathological parameters

Variable	n of cases	TOP2A average gene dosage ($\pm$ standard deviation)	p-value
Tumor stage			
T1–2	247	1.17 $\pm$ 0.58	.41
T3–4	72	1.15 $\pm$ 0.33	
Node stage			
N0	164	1.16 $\pm$ 0.65	.15
N1–2	153	1.17 $\pm$ 0.37	
Grade			
1–2	135	1.11 $\pm$ 0.37	.43
3	100	1.17 $\pm$ 0.46	
Human epidermal growth factor receptor 2 status			
Negative	189	1.09 $\pm$ 0.35	.0002
Positive	49	1.49 $\pm$ 1.03	
Hormone receptor status			
ER ⁻	155	1.15 $\pm$ 0.44	.81
ER ⁺	163	1.18 $\pm$ 0.62	
PgR ⁻	149	1.13 $\pm$ 0.42	.10
PgR ⁺	169	1.20 $\pm$ 0.62	
Histological type			
Ductal	222	1.13 $\pm$ 0.39	.12
Lobular	57	1.30 $\pm$ 0.93	

Abbreviations: ER, estrogen receptor; PgR, progesterone receptor; TOP2A, topoisomerase II α .

comes was observed for TOP2A status determined using FISH or IHC.

In the univariate analysis, factors predicting a shorter DFS interval in the entire group of patients included older age, higher tumor (T) stage (T3–4), lymph node involvement, higher grade (3), negative progesterone receptor (PgR) status, HER-2 positivity, and high TOP2A gene dosage. Factors predictive for a shorter OS time were older age, higher T stage (T3–4), lymph node involvement, higher grade (3), and high TOP2A gene dosage (Table 3). In the HER-2⁻ subgroup, higher T stage (T3–4), lymph node involvement, negative estrogen receptor (ER) and PgR status, and high TOP2A gene dosage were associated with a worse DFS outcome and higher T stage (T3–4), lymph node involvement, and high TOP2A gene dosage were associated with a worse OS outcome. In the HER-2⁺ subgroup, none of the clinical variables correlated with the TOP2A gene dosage.

In the stepwise multivariate regression analysis including all patients, older age, higher T stage (T3–4), lymph node involvement, and high TOP2A gene dosage were associated with a worse DFS outcome. A lower OS probability was associated with older age, lymph node involvement, higher tumor grade (3), and high TOP2A gene dosage (Table 4). Multivariate analysis performed on the HER-2⁻ subgroup showed that higher T

stage, lymph node involvement, and high TOP2A gene dosage were associated with a shorter DFS interval, whereas a shorter OS time was correlated with a higher T stage and high TOP2A gene dosage.

Although the study was not designed to examine the predictive value of TOP2A, we performed a statistical analysis in a subgroup of 132 patients administered chemotherapy: either an anthracycline-based ($n = 62$) or a nonanthracycline-based schema (cyclophosphamide, methotrexate, and 5-fluorouracil or taxane based, $n = 70$). In the univariate analysis, anthracycline use was associated with a higher hazard ratio for the OS outcome, compared with non-anthracycline schemas (supplemental online Table 4S). However, the correlation was at a borderline level ($p = .04$) and might merely reflect the fact that more aggressive cancers had been selected for anthracycline-based schemas. The impact of treatment was not maintained in the multivariate analysis (supplemental online Table 5S).

DISCUSSION

TOP2A copy number changes often have been associated with rearrangements of the neighboring HER-2 gene; therefore, studies analyzing TOP2A gene aberrations were typically limited to the HER-2–amplified or HER-2–overexpressing tumors [7, 12]. In the present study, using a qPCR method developed by our group, TOP2A gene dosage was measured in a large consecutive series of primary breast cancer samples, most of which were HER-2⁻. In this material, the frequency of a high TOP2A gene dosage did not differ significantly between tumors with and without HER-2 overexpression ($p = .43$). Notably, for the first time, we present here the prognostic significance of a high TOP2A gene dosage in HER-2⁻ patients.

Our results show that a high TOP2A gene dosage measured using qPCR and FISH occurred in 29% and 24% of all breast cancers, respectively. TOP2A amplifications detected using FISH have been reported in 7%–24% of breast cancers [10, 11, 22–24]. The higher rate of TOP2A abnormalities detected using qPCR could be a result of not only the different method used and the cutoff level applied but also the greater resolution of the PCR method than the commonly used FISH technique. Indeed, FISH uses long oligonucleotide probes and detects regional rather than gene-specific rearrangements. Therefore, PCR might detect smaller rearrangements that can be missed by FISH [25]. It is of note that elevated levels of TOP2A gene expression determined using reverse transcription qPCR have also been observed in HER-2⁻ tumors [26–28], whereas the TOP2A gene was earlier found to be amplified when assessed using FISH in only 0%–6.4% of HER-2⁻ cases [10, 11, 22, 23, 29]. In our material, a high TOP2A gene dosage was detected using qPCR in 32% of HER-2⁺ tumors, which is within the previously reported range of values of 24.3%–50% [4, 23, 24]. The percentage of tumors with a high TOP2A gene dosage in HER-2⁻ tumors was relatively high (27%), but the corresponding average TOP2A gene dosage was significantly lower than in HER-2⁺ tumors ($p = .0002$), a finding that might mirror the link between the two amplicons. Using FISH, the TOP2A amplification rate was more than two times higher in the HER-2⁺ than in the HER-2⁻ group ($p = .02$), which is in

Figure 1. Correlation between the dosage of the gene encoding topoisomerase II α (*TOP2A*) and human epidermal growth factor receptor 2 (HER-2) status ($p = .0002$).

agreement with the literature [30] and again indicates that FISH and qPCR might differ in their resolutions and detected targets.

In the current study, we compared our qPCR-based method [13] with other standard techniques: FISH using the FDA-approved *TOP2A* FISH pharmDx™ Kit (Dako, Glostrup, Denmark) and IHC using the commonly used Ki-S1 antibody [19, 20, 31–33]. Results of the *TOP2A* gene status examined using FISH and qPCR were slightly concordant. No correlation was found between the results using IHC and those using either FISH or qPCR. The lack of correlation of *TOP2A* on the gene and protein levels remains in agreement with data in the literature [16, 21, 33]. It has clearly been demonstrated that *TOP2A* protein expression is strongly influenced by the cellular proliferative rate [34]. Moreover, alternative mRNA splicing generates diverse *TOP2A* protein isomers with different activity levels and different subcellular localizations. Thus, the *TOP2A* gene level alone cannot predict the protein level.

To check the clinical utility of our assay, we performed a comparative analysis of matched frozen and FFPE samples. Comparison of the *TOP2A* gene dosages in the two types of material showed the robustness of the method because the results were highly correlated. Concordance of the *TOP2A* gene dosage status reached 92% ($\kappa = 0.83$). A few discordant cases might not result from the inability of the method to reliably quantify *TOP2A* gene dosage but rather from sampling bias, that is, the frozen and FFPE sections of the tumors taken for DNA isolation might have come from different parts of the tumor.

The frequency analysis of *TOP2A* alterations is clouded by technical issues and the variety of methods, scoring systems, and cutoffs used by particular groups [7, 10, 12]. High inter-laboratory variations for *TOP2A* quantification using FISH were shown recently [9], indicating the need for validated

methods, standardized signal scoring systems, and consistent cut-off levels. The methodologies applied in *TOP2A* copy number change analyses have different sensitivities and, as described by Moelans et al. [35], low-level *TOP2A* amplifications can be detected using multiple-ligation probe amplification but not using chromogenic in situ hybridization. The real-time PCR technique used in our study has several advantages, including speed, low cost, reproducibility, possibility of quantitative analysis, high resolution of analysis, and high specificity of target-sequence detection by hydrolysis probes. This technique has recently been used more frequently for examination of *TOP2A* gene dosage [36] and mRNA level [21, 26, 28].

The tumor samples in our analyses were not microdissected and therefore were, to some degree, contaminated with stromal cells. However, the results of our admixing experiment (in which DNA from the SKBR3 cancer cell line bearing *TOP2A* amplification was serially diluted in genomic DNA) show that this method could detect a high *TOP2A* gene dosage when the percentage of admixed DNA from the SKBR3 cell line was as low as 10%. The analysis of Lehmann et al. [37] showed that low-level amplification of *HER-2* cannot be detected when the cells with the amplification constitute <30% of all tumor cells.

In this series, the prognostic impact of the *TOP2A* gene dosage was found both in the entire group and in the subsets of *HER-2*[−] and *HER-2*⁺ patients. Similarly to other groups [10–12], patients with *TOP2A*-amplified tumors had a shorter DFS interval ($p = .03$, $p = .002$, and $p = .007$, respectively, in all, *HER-2*⁺, and *HER-2*[−] patients) and OS time ($p = .01$, $p = .0002$, and $p = .04$, respectively). In the multivariate analysis including all patients, high *TOP2A* gene dosage was associated

Figure 2. Kaplan-Meier curves according to TOP2A status. (A): Disease-free survival probability according to TOP2A dosage in the entire population ($p = .03$). (B): Overall survival probability according to TOP2A dosage in the entire population ($p = .01$). (C): Disease-free survival probability according to TOP2A dosage in the HER-2⁺ population ($p = .002$). (D): Overall survival probability according to TOP2A dosage in the HER-2⁺ population ($p = .0002$).

Abbreviations: HER-2, human epidermal growth factor receptor 2; TOP2A, topoisomerase II α .

Figure 3. Kaplan-Meier curves according to TOP2A status in HER-2⁺ population. (A): Disease-free survival probability according to TOP2A dosage in the HER-2⁺ population ($p = .007$). (B): Overall survival probability according to TOP2A dosage in the HER-2⁺ population ($p = .04$).

Abbreviations: HER-2, human epidermal growth factor receptor 2; TOP2A, topoisomerase II α .

Table 3. DFS and OS outcomes of all patients and of the HER-2[−] and HER-2⁺ subgroups (univariate analysis)

Variable	<i>n</i>	DFS		OS	
		HR (95% CI)	<i>p</i> -value	HR (95% CI)	<i>p</i> -value
All patients					
Age (≤50 yrs versus >50 yrs)	319	2.06 (1.17–3.60)	.01	2.57 (1.15–5.73)	.02
Tumor stage (T3–4 versus T1–2)	316	3.77 (2.40–5.90)	<.000001	3.48 (1.94–6.27)	.00003
Lymph nodes (positive versus negative)	314	2.98 (1.89–4.70)	.000003	2.96 (1.61–5.41)	.0005
ER (positive versus negative)	315	0.67 (0.43–1.03)	.07	0.67 (0.37–1.18)	.16
PgR (positive versus negative)	315	0.49 (0.32–0.77)	.002	0.60 (0.34–1.07)	.08
Histological type (lobular versus ductal)	276	0.57 (0.31–1.06)	.08	0.40 (0.16–1.02)	.06
Grade (3 versus 1–2)	233	1.99 (1.19–3.32)	.009	2.54 (1.28–5.01)	.008
HER-2 status (positive versus negative)	238	1.84 (1.04–3.27)	.03	1.53 (0.71–3.3)	.27
<i>TOP2A</i> (high versus normal)	319	1.61 (1.04–2.50)	.03	2.05 (1.17–3.60)	.01
HER-2 [−] patients					
Age (≤50 yrs versus >50 yrs)	189	1.7 (0.82–3.65)	.15	1.86 (0.69–5.02)	.22
Tumor size (T3–4 versus T1–2)	188	3.2 (1.5–6.69)	.002	3.92 (1.63–9.5)	.002
Lymph nodes (positive versus negative)	185	3.2 (1.63–6.24)	.0007	3.41 (1.41–8.27)	.006
ER (positive versus negative)	189	0.5 (0.26–0.94)	.03	0.56 (0.25–1.25)	.16
PgR (positive versus negative)	189	0.5 (0.26–0.93)	.03	0.69 (0.3–1.53)	.36
Histological type (lobular versus ductal)	159	1.19 (0.58–2.47)	.63	0.82 (0.29–2.27)	.7
Grade (3 versus 1–2)	134	1.47 (0.62–3.5)	.38	1.82 (0.64–5.22)	.26
<i>TOP2A</i> (high versus normal)	189	2.6 (1.39–4.87)	.003	4.18 (1.85–9.42)	.0006
HER-2 ⁺ patients					
Age (≤50 yrs versus >50 yrs)	49	1.35 (0.44–4.2)	.6	1.34 (0.27–6.53)	.72
Tumor stage (T3–4 versus T1–2)	47	2 (0.62–6.44)	.24	1.99 (0.39–10.1)	.39
Lymph nodes (positive versus negative)	49	1.74 (0.64–4.73)	.28	1.82 (0.48–6.97)	.38
ER status (positive versus negative)	49	0.76 (0.26–2.17)	.6	0.58 (0.12–2.85)	.51
PgR status (positive versus negative)	49	0.39 (0.13–1.23)	.19	0.18 (0.02–1.44)	.1
Histological type (lobular versus ductal) ^a	47	0.59 (0.08–4.49)	.61	X	
Grade (3 versus 1–2) ^a		X		X	
<i>TOP2A</i> (high versus normal)	49	1.47 (0.56–3.83)	.43	1.36 (0.36–5.08)	.64

^aStatistical analysis not possible because of a lack of events in one of the subgroups (marked as X).

Abbreviations: CI, confidence interval; DFS, disease-free survival; ER, estrogen receptor; HER-2, human epidermal growth factor receptor 2; HR, hazard ratio; OS, overall survival; PgR, progesterone receptor; TOP2A, topoisomerase 2α.

with worse DFS ($p = .02$) and OS ($p = .03$) probabilities. In the HER-2[−] subgroup, it was also an independent predictor of poor DFS ($p = .004$) and OS ($p = .0001$) outcomes. Results similar to those of our multivariate analysis were reported by Nielsen et al. [38], wherein *TOP2A* amplification was associated with worse DFS and OS outcomes in the prospectively designed, biological substudy of the Danish Breast Cancer Cooperative Group Trial 89D. However, to the best of our knowledge, we are the first to report the independent adverse prognostic impact of a high *TOP2A* gene dosage in HER-2[−] patients.

Our results show that a high *TOP2A* gene dosage occurs in both HER-2[−] and HER-2⁺ tumors, and this also holds true with higher cutoff values. With an arbitrary selected cutoff level of 2, used by Lamy et al. [36], we found a high *TOP2A*

gene dosage in five of 49 (10%) HER-2⁺ and five of 189 (3%) HER-2[−] tumors. The percentages of positive cases were obviously lower than with the lower cutoff level used in our study, but the general tendency was virtually the same—a high *TOP2A* gene dosage was found in tumors irrespective of their HER-2 status. These data suggest that excluding HER-2[−] samples from analyses of *TOP2A* gene alterations may underestimate the true number of *TOP2A* copy number changes in the entire population of breast cancer patients. In the majority of studies, the frequency of *TOP2A* alterations in HER-2[−] tumors was low or absent [4, 8, 14, 39]; however, this subgroup of tumors should not be omitted in *TOP2A* aberration analyses. Indeed, 20%–38% of the *TOP2A* alterations in some studies were found in HER-2[−] samples [6, 10, 11, 15].

Our finding suggests that assessment of *TOP2A* gene dos-

Table 4. DFS and OS outcomes of all studied patients and of the HER-2⁻ subgroup (multivariate analysis)

Variable	DFS		OS	
	HR (95% CI)	p-value	HR (95% CI)	p-value
All patients				
Age (≤50 yrs versus >50 yrs)	1.84 (1.04–3.25)	.04	5.26 (1.59–17.4)	.007
Tumor stage (T3–4 versus T1–2)	2.75 (1.69–4.46)	.00005		NS
Lymph nodes (positive versus negative)	2.61 (1.59–4.29)	.0001	2.54 (1.23–5.26)	.01
Grade (3 versus 1–2)		NS	2.32 (1.14–4.71)	.02
TOP2A (high versus normal)	1.71 (1.09–2.69)	.02	2.11 (1.08–4.12)	.03
HER-2 ⁻ patients				
Tumor stage (T3–4 versus T1–2)	3.19 (1.45–6.99)	.004	5.33 (2.15–13.22)	.0003
Lymph nodes (positive versus negative)	2.42 (1.2–4.87)	.01		NS
TOP2A (high versus normal)	2.58 (1.36–4.9)	.004	5.05 (2.19–11.6)	.0001

Abbreviations: CI, confidence interval; DFS, disease-free survival; ER, estrogen receptor; HER-2, human epidermal growth factor receptor 2; HR, hazard ratio; NS, not significant in the multivariate Cox regression model; OS, overall survival; PgR, progesterone receptor; TOP2A, topoisomerase 2α.

age using qPCR may provide more valuable clinical information than just determining TOP2A amplification using the most commonly used in situ hybridization techniques.

qPCR recently applied in TOP2A gene expression examinations also showed elevated levels of TOP2A in HER-2⁻ tumors [26–28]. Interestingly, in the study of Rody et al. [27], elevated TOP2A gene expression occurred in 48% and 71% of HER-2⁻ and HER-2⁺ tumors, respectively, but TOP2A status was only of prognostic value in the HER-2⁻ subgroup. In the same study, multivariate Cox regression analysis showed that elevated expression of TOP2A is an independent predictor of a poor survival outcome in ER⁺ patients. The prognostic value of TOP2A RNA expression in the HER-2⁻ subgroup has been shown by others [16, 28]. Additionally, elevated TOP2A RNA expression has been associated with cell proliferation and with highly proliferative intrinsic breast cancer subtypes—both HER-2⁺ (luminal B, HER-2-enriched) and HER-2⁻ (basal-like) cases [21].

The presence of TOP2A amplification in the subset of HER-2⁻ patients challenges the theory that TOP2A complex genetic aberrations are secondary to HER-2 amplification [5]. According to our results, TOP2A alterations could be independent of HER-2 alterations, which was also shown by Nielsen et al. [6]. Comparing TOP2A and HER-2 copy numbers using FISH in breast cancer cell lines, they found that the simultaneous amplification of both genes is not a simple coamplification of a whole amplicon containing both genes. HER-2, but not TOP2A, was present in tandem amplicons, leading to a higher level of HER-2 amplification than TOP2A amplification [6]. A similar observation of a higher level of HER-2 than TOP2A amplification was found in clinical samples of invasive breast cancer [4, 6, 15]. Moreover, there are other data showing that TOP2A is not a part of the HER-2 smallest region of amplification, and its copy number differs from that of HER-2 [15, 36]. Gene dosage analysis using real-time PCR revealed that the frequency of gene copy number alterations decreases with

distance from the HER-2 gene [36]. That study also pinpointed the possible location of a recombinant hotspot between the GRB7 and THRA genes, which lies between HER-2 and TOP2A [36]. Nevertheless, a high gene dosage of TOP2A may occur without HER-2 amplification, which suggests the involvement of alternative breaking points on the long arm of chromosome 17.

In conclusion, we showed that a high TOP2A gene dosage measured using qPCR is a strong negative prognostic factor and could refine risk assessment in HER-2⁻ patients as well; however, further research is needed to confirm this finding.

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