Alteration of Topoisomerase II-alpha Gene in Human Breast Cancer: Association with Responsiveness to Anthracycline-Based Chemotherapy.

Michael F. Press, Guido Sauter, Marc Buyse, Leslie Bernstein, Roberta Guzman, Angela Santiago, Ivonne Villalobos, Wolfgang Eiermann, Tadeusz Pienkowski, Miguel Martin, Nicholas Robert, John Crown, Valerie Bee, Henri Taupin , Kerry Flom, Isabelle Tabah-Fisch, Giovanni Pauletti , Mary-Ann Lindsay, Alessandro Riva, and Dennis J. Slamon for the Cancer International Research Group

Norris Comprehensive Cancer Center, University of Southern California, Los Angeles, CA; Dept of Pathology, University Medical Center Hamburg-Eppendorf, University of Hamburg, Hamburg, Germany; International Drug Development Institute (IDDI), Louvai-la-Neuve, Belgium; City of Hope National Medical Center, Duarte, CA; Frauenklinik vom Roten Kreuz, Munich, Germany; Maria Sklodowska-Curie Centre, Warsaw, Poland; Hospital Universitario San Carlos, Madrid, Spain; Inova Fairfax Hospital Institute of Research, Falls Church, VA, USA; ICORG, St Vincent's University Hospital, Dublin, Ireland; Cancer International Research Group, Paris, France; Cepheid, Sunnyvale, CA; Sanofi-Aventis, Paris, France; and Jonsson Comprehensive Cancer Center, UCLA, LosAngeles, CA

Abstract

Purpose. Approximately 35% of *HER2*-amplified breast cancers have co-amplification of the topoisomerase II-alpha (*TOP2A*) gene encoding an enzyme that is a major target of anthracyclines. This study was designed to evaluate whether *TOP2A* gene alterations may predict incremental responsiveness to anthracyclines in some breast cancers.

Methods. A total of 4,943 breast cancers were analyzed for alterations in *TOP2A* and *HER2*. Primary tumor tissues from patients with metastatic breast cancer treated in a trial of chemotherapy plus/minus trastuzumab were studied for amplification/deletion of *TOP2A* and *HER2* as a "test set" followed by evaluation of malignancies from two separate, large trials for changes in these same genes as a "validation set". Association between the alterations and clinical outcomes was determined.

Results. "Test set" cases containing *HER2* amplification treated with doxorubicin, cyclophosphamide (AC) plus trastuzumab, demonstrated longer progression-free survival compared to those treated with AC alone (p=0.03). However, patients treated with AC alone whose tumors contain *HER2*/*TOP2A* co-amplification experienced a similar improvement in survival (p=0.004). Conversely, in patients treated with paclitaxel, *HER2*/*TOP2A* co-amplification was not associated with improved outcomes. The same observations were then confirmed in a larger "validation set", where *HER2*/*TOP2A* co-amplification was again associated with longer survival when anthracycline-containing chemotherapy alone was used for treatment compared to outcome in HER2-positive cancers lacking *TOP2A* co-amplification.

Conclusion. In a study involving nearly 5,000 breast malignancies, both "test set" and "validation set" demonstrate that *TOP2A* co-amplification, not *HER2* amplification, is the clinically useful predictive marker of an incremental response to anthracycline-based chemotherapy.

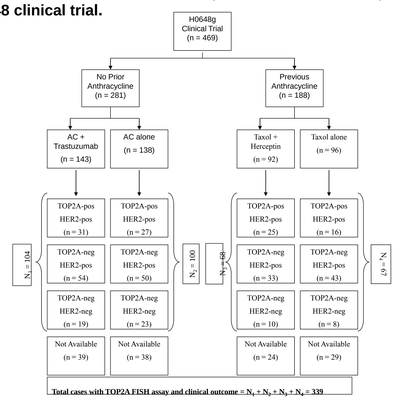
Background

Anthracycline-based chemotherapy is the mainstay of current adjuvant treatments for early stage breast cancer. This is supported by a meta-analysis of several randomized studies showing slightly higher (~4%) disease-free and overall survival rates achieved with anthracycline-based versus non-anthracycline chemotherapies. However, anthracyclines have significant long-term toxicities including cardiac dysfunction and/or induction of myelodysplasia and acute leukemias. Several studies have reported an association between *HER2* amplification/overexpression and increased responsiveness to anthracycline-based chemotherapy; however, underlying biological mechanism(s) are unclear. Indeed, *in vitro* and *in vivo* studies indicate that *HER2* overexpression alone does not alter anthracycline sensitivity. *HER2* is located on the long arm of chromosome 17(17q11.2-12) in close proximity to topoisomerase II-alpha (*TOP2A*) at 17q21-22. Although *HER2* is considered the "target" of the amplification event, *HER2*-amplicon size is variable and contains other genes occasionally including *TOP2A*. Because TOP2A is a target of anthracyclines it is possible that this gene, not *HER2*, is the link between HER2-positive disease and anthracycline responsiveness. We addressed these questions using a retrospective evaluation of 4,943 breast cancers. The first group was a hypothesis generating "test set" of 339 cancers from women enrolled in a trial of HER2-positive metastatic disease in which patients were treated with anthracycline-based or non-anthracycline chemotherapy plus/minus trastuzumab. Clinical response data was then correlated with the presence or absence of *HER2* and *TOP2A* alterations. To validate any observed associations from the "test set", we next evaluated 4,604 samples from two larger studies, BCIRG-006 (2,990 patients) and BCIRG-005 (1,614 patients). This "validation set" was used to define the frequency of *TOP2A* copy-number changes in *HER2*-amplifed and *HER2*-normal cases and determine whether *TOP2A* or *HER2* alterations were correlated with anthracycline response.

Patients

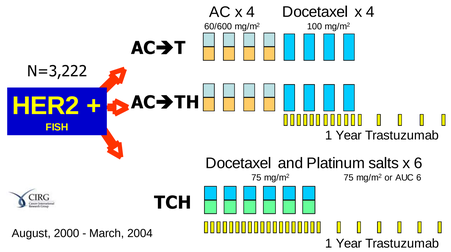
Test Set. Patients evaluated in the "test set" consisted of women with immunohistochemically HER2-positive breast cancer who were enrolled on the H0648g trastuzumab (Herceptin®) study. This study was a randomized phase III multinational trial evaluating trastuzumab (Herceptin®) combined with chemotherapy versus chemotherapy alone as first-line therapy for patients with HER2 overexpressing metastatic breast cancer . A total of 469 patients were enrolled in the study between June 1995 and March 1997; 235 patients received trastuzumab plus chemotherapy and 234 patients received chemotherapy alone. Women with no prior adjuvant treatment with anthracyclines (n = 281) were randomized to receive anthracycline and cyclophosphamide (AC) either with (n = 143) or without (n = 138) trastuzumab. Women previously exposed to anthracyclines (n = 188) were randomized to receive paclitaxel (Taxol®) either with (n = 92) or without (n = 96) trastuzumab (Figure 1).

Figure 1. Test set: Patient and Specimen Accountability in the H0648 clinical trial.



Validation Set. The cases comprising the "validation set" were women participating in the BCIRG 005 and BCIRG 006 (Figure 2) clinical trials which accrued 3,298 and 3,222 patients, respectively, between August 2000 and March 2004. All women entered in the BCIRG006 trial had HER2-amplified primary breast cancers determined by centralized fluorescence *in situ* hybridization (FISH). The BCIRG 006 study compared two trastuzumab–containing adjuvant treatments to a standard chemotherapy alone arm (doxorubicin/ cyclophosphamide followed by docetaxel) in women with resected node-positive or high-risk, node-negative primary breast cancers.

Figure 2. Validation Set: BCIRG 006 clinical trial.



Objectives

1. **To determine the nature and frequency of TOP2A alterations in clinically annotated breast cancers using molecularly-validated cutoffs**
2. **To determine how often such alterations are found in HER2-positive and HER2-negative breast cancers**
3. **To determine the association (if any) between such alterations and response to anthracycline-based chemotherapy regimens**

Materials and Methods

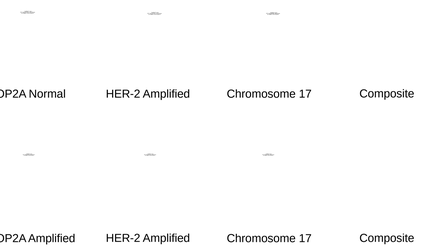
Tissue Specimens. Archived tissue from the H0648g trial consisted of 4 to 6 mM sections previously cut from fixed, paraffin-embedded breast cancer tissue. The tissue sections from 451 of these cases, used as control sections for immunohistochemistry, were processed by FISH to evaluate HER2 gene amplification status and, subsequently, for TOP2A using FISH. The tissue sections were stripped of probe under high stringency conditions and re-processed for TOP2A and HER2 gene amplification by FISH.

The BCIRG studies were prospectively designed to address molecular determinants of treatment response, including analysis of TOP2A, and required centralized tissue assessment for study entry. Fixed, paraffin-embedded tissue specimens from the BCIRG 005 and 006 studies were available as tissue microarrays, prepared as described elsewhere, for 2531 of the 3298 women accrued to the BCIRG 005 clinical trial and 2120 of the 3222 women accrued to the BCIRG 006 clinical trial. Additional cases were available as unstained tissue sections for the BCIRG006 clinical trial and were processed as individual sections. Breast cancers from these two trials were categorized as either amplified (FISH positive) or non-amplified (FISH negative) for both TOP2A and HER2, as described below.

TOP2A and HER2 Gene Amplification by Fluorescence *In Situ* Hybridization. Topoisomerase II-alpha and HER2 gene amplification were determined by fluorescence *in situ* hybridization (FISH) using commercially available probes. We used the same FISH assay method as described elsewhere in detail for HER2 gene amplification (Mass et al., 2005). By using an LSI HER2 probe labeled with SpectrumGreen (Vysis, Inc.) and an LSI TOP2A probe labeled with SpectrumOrange (Vysis, Inc.) simultaneous analysis of HER2 and TOP2A were performed with the FISH assay. The number of chromosome 17 centromeres was determined by FISH using a CEP 17 probe labeled with SpectrumAqua (Vysis, Inc.).

*Mass RD, Press MF, Anderson S, Cobleigh MA, Vogel CL, Dybdal N, Leiberman G, Slamon DJ. Evaluation of clinical outcomes according to HER2 detection by fluorescence *in situ* hybridization in women with metastatic breast cancer treated with trastuzumab. *Clinical Breast Cancer* 6: 240-246, 2005.

Figure 3. Gene amplification assessed by FISH.



Statistics

We tested for differences in clinical outcomes (overall response rates {ORR} and progression-free survival {PFS} in trial H0648, disease-free survival {DFS} in trials BCIRG 005 and BCIRG 006 and overall survival {OS} in all trials) between TOP2A gene-amplified and non-amplified subgroups; data from patients whose tumors had TOP2A gene deletions were grouped with data from patients who had a "normal" TOP2A gene ratio to form the non-amplified subgroup as well as analyzed separately as "deleted". Differences in response rates were compared using a chi-square test; to control for trastuzumab therapy the Mantel-Haenszel test was used for overall comparison. Differences in PFS, DFS and OS were compared using the log-rank test .

Results

Validation of FISH Ratios for TOP2A Amplification and Deletion. To insure that the probes and cutoffs used to generate the amplification/deletion status of *TOP2A* and *HER2* were correct, the status of these two genes was first determined in a molecularly characterized panel of 38 cell lines with known *HER2* and *TOP2A* status. All 38 lines had *HER2* and *TOP2A* gene copy numbers determined by physical mapping of the 17q12-q21 amplicon using overlapping BAC probes in both metaphase spreads and intact interphase nuclei (Pauletti et. al., unpublished). This approach provided the actual size and boundaries of each 17q12-q21 amplicon as well as the correct amplification/deletion status of all genes centromeric and telomeric to *HER2*. This analysis yielded the exact copy number of each normal, co-amplified or deleted gene including *TOP2A* for all 38 cell lines. To replicate the conditions and procedures used for the "test" and "validation" specimens, pellets of each of these fully-characterized cell lines were then prepared and processed as formalin-fixed, paraffin-embedded (FFPE) specimens and sections were cut for FISH analyses as described for clinical samples. Scoring these FFPE sections was performed blinded to their individual identity to ensure an unbiased result. These *HER2* and *TOP2A* results were then compared to the results determined by direct chromosome 17q12-q21 mapping. These analyses yielded complete concordance between FISH copy numbers for both *HER2* and *TOP2A* as determined in FFPE sections and using overlapping BAC array analyses or hybridization of metaphase spreads for each of the 38 cell lines. We subsequently utilized these validated probes and cutoff ratios for analysis of all clinical specimens.

FISH Analysis for *TOP2A* and *HER2* Alterations in the "Test Set" Study. In the H0648 study the per case average *TOP2A* gene copy number per tumor cell nucleus ranged from 1.25 to 30.60; the average chromosome 17 centromere number per tumor cell nucleus varied from 1.20 to 6.40; and the average *TOP2A*-to-chromosome 17 centromere ratio varied from 0.43 to 10.85 (Figure 3). Ninety-nine (29.2%) of the 339 breast cancers successfully tested showed *TOP2A* gene amplification (*TOP2A*-to-chromosome 17 centromere ratio ≥ 2.0), while 44 / 339 (13.0%) showed TOP2A gene deletion (*TOP2A*-to-chromosome 17 centromere ratio ≤ 0.82) (Table 1). The remaining 196 cases (57.8%) showed no alteration in the *TOP2A* gene copy number (Table 1).

The average *HER2* gene copy number per tumor cell varied from 1.85 to 52.80; and the ratio of average HER2 gene copy number-to-chromosome 17 copy number varied from 0.99 to 16.50 (Figure 3). Since all women entering the H0648g clinical trial had HER2 overexpressing breast cancers by Clinical Trial Assay immunohistochemistry (CTA-IHC) (2+ or 3+)(Table 1), it was anticipated that a high proportion would have HER2 gene amplification. The FISH analyses showed 279 (82.3%) of these 339 breast cancers had HER2 gene amplification, while 60 (17.7%) did not show HER2 gene amplification (Table 1).

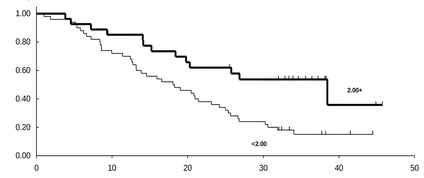
Table 1. HER2 and TOP2A Gene Amplification in the Genentech H0648g Pivotal Clinical Trial by FISH Assay.

	HER2 Gene		
	Not Amplified	Amplified	Totals
TOP2A Del	2 (3%)	42 (15%)	44
TOP2A Normal	58 (97%)	138 (49%)	196
TOP2A Ampl	0 (0%)	99 (36%)	99
Totals:	60 (100%)	279 (100%)	339

Del = deleted, Ampl = amplified.

Overall survival of women having metastatic breast cancer treated with adriamycin-containing chemotherapy. Among women treated with AC-containing chemotherapy (AC+trastuzumab and AC alone) (n=162), women whose breast cancers had co-amplification of *HER2* and *TOP2A* (n = 58) had a longer overall survival than women whose breast cancers had amplification of *HER2* but not *TOP2A* (n = 104). Approximately half of these women were treated with trastuzumab (Herceptin®) and the other half were not. Analysis of the clinical outcome data by treatment arm (AC alone versus AC+trastuzumab) and by *TOP2A* amplification status demonstrated a significant (p = 0.004) survival advantage among those women who were treated with AC alone and had *TOP2A*coamplification (Figure 4).

Figure 4. Women treated with AC alone (n=77) in the "test set" H0648 trial. The overall survival of women whose breast cancers had co-amplification of *HER2* and *TOP2A* (upper thick line, 2.00+) (n = 27) was significantly longer than women whose breast cancers had amplification of *HER2* but not *TOP2A* (thin line, <2.00) (n = 50) (p= 0.004).



In contrast, among those women who were treated with AC and trastuzumab there was no difference in survival for those women whose breast cancers had *TOP2A* gene amplification compared to those women whose breast cancers lacked *TOP2A* gene amplification (p = 0.662). However, it should be noted that this survival comparison is confounded by patient cross-over from the AC alone to the AC-trastuzumab treatment arm.

FISH Analysis for *TOP2A* and *HER2* Alterations in the "Validation Set" Study. *TOP2A* gene status was determined by FISH (Figure 3) for 3001 of the 3222 (93.1%). Eleven cases were excluded from further analysis for various reasons leaving 2,990 cases. All were *HER2* amplified. One thousand fifty-seven of these 2990 (35.4%) showed *HER2*/*TOP2A* co-amplification, while 1788 (59.8%) were *TOP2A* normal and 145 (4.8%) cases were *TOP2A* deleted. There were no significant differences in the distribution of *TOP2A* gene amplified, normal or deleted breast cancer cases by age, Karnofsky performance status, axillary lymph node status, treatment distribution, or stage distribution (data not shown).

In an attempt to determine the frequency of gross genomic alterations of the TOP2A gene in human breast cancers that do not contain the HER2 amplicon, we analyzed a cohort of samples from the BCIRG 005 study. None of 1610 breast cancers lacking *HER2* amplification had *TOP2A* gene amplification while 42 (2.6%) had *TOP2A* gene deletions. There was no significant association between *TOP2A* gene deletion and clinical outcome in this trial.

Analysis of the disease-free survival (DFS) and recurrence-free survival (RFS) in the BCIRG 006 study by *TOP2A* status showed a statistically significantly longer DFS (p < 0.001) and RFS (p < 0.001) among all women whose breast cancers had *TOP2A* gene co-amplification compared to those whose breast cancers did NOT.

Among women randomized to standard chemotherapy alone, i.e. no trastuzumab (arm 1, AC->T), those women whose breast cancers had co-amplification of *HER2* and *TOP2A* genes also showed a significantly longer DFS (p < 0.001) (Figure 5) and RFS (p < 0.001) compared to women whose breast cancers lacked *TOP2A* gene co-amplification.

Figure 5. Disease-free survival (DFS) of women enrolled in control treatment arm (AC→T) by TOP2A gene amplification.

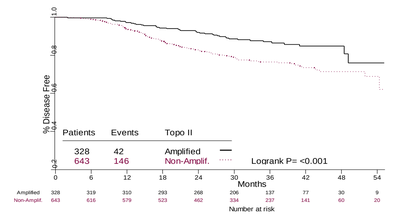


Figure 6. Disease-free survival of women whose breast cancers lacked TOP2A gene amplification by treatment arm (AC→T, AC→TH and TCH).

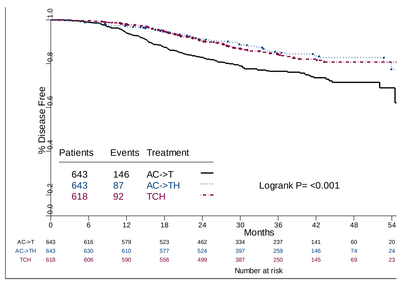


Figure 7. Disease-free survival of women whose breast cancers had *TOP2A* gene co-amplification by treatment arm (AC→T, AC→TH and TCH).

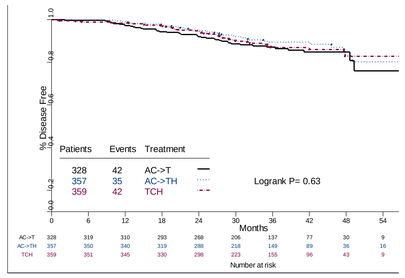


Figure 8. Comparison of disease-free survival (DFS) of women in the BCIRG 006 clinical trial by TOP2A gene co-amplification status and by treatment arm.

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Conclusions

- For women treated with anthracycline-containing chemotherapy alone whose tumors had co-amplification of *HER2* and *TOP2A*, there was a significant clinical outcome advantage (OS or DFS) compared to women whose tumors had *HER2* amplification and lacked *TOP2A* amplification.
- Although disease recurrence rates are low, among women whose breast cancers had co-amplification of *HER2* and *TOP2A*, the disease-free survival was not significantly different among women who were treated with either anthracycline-containing chemotherapy alone or chemotherapy + trastuzumab.

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