Amplification and Deletion of Topoisomerase II α Associate with ErbB-2 Amplification and Affect Sensitivity to Topoisomerase II Inhibitor Doxorubicin in Breast Cancer

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Topoisomerase II α (topoII α) is a key enzyme in DNA replication and a molecular target for many anti-cancer drugs called topoII inhibitors. The topoII α gene is located at chromosome band 17q12-q21, close to the ErbB-2 oncogene (HER-2/*neu*), which is the most commonly amplified oncogene in breast cancer. Because of the physical proximity to ErbB-2, copy number aberrations may also occur in the topoII α gene. These topoII α gene copy number aberrations may be related to the altered chemosensitivity to topoII inhibitors that breast cancers with ErbB-2 amplification are known to have. We used fluorescence *in situ* hybridization to study copy number aberrations of both topoII α and ErbB-2 in nine breast cancer cell lines and in 97 clinical breast tumors, which were selected for the study according to their ErbB-2 status by Southern blotting. TopoII α -protein expression was studied with Western blot and sensitivity to doxorubicin (a topoII inhibitor) with a 96-well clonogenic *in vitro* assay. Two of the five cell lines with ErbB-2 gene amplification (SK-BR-3 and UACC-812) showed amplification of topoII α . In MDA-361 cells, ErbB-2 amplification (14 copies/cell) was associated with a physical deletion of topoII α (four copies of chromosome 17 centromere and two copies of topoII α). The topoII α amplification in UACC-812 cells was associated with 5.9-fold-increased topoII α protein expression and 2.5-fold-increased sensitivity to the topoII inhibitor, doxorubicin, whereas the deletion in MDA-361 leads to decreased protein expression (45% of control) and a 2.4-fold-increased chemoresistance *in vitro*. Of 57 ErbB-2-amplified primary breast carcinomas, 25

(44%) showed ErbB-2-topoII α coamplification and 24 (42%) showed a physical deletion of the topoII α gene. No topoII α copy number aberrations were found in 40 primary tumors without ErbB-2 amplification. TopoII α gene amplification and deletion are common in ErbB-2-amplified breast cancer and are associated with increased or decreased sensitivity to topoII inhibitors *in vitro*, respectively. These findings may explain the altered chemosensitivity to topoII inhibitors reported in ErbB-2-amplified breast cancers. (Am J Pathol 2000, 156:839–847)

The ErbB-2 oncogene (also termed HER-2/*neu*) is the most frequently amplified oncogene in breast cancer. A number of studies have shown that the ErbB-2 oncogene is amplified in 20 to 35% of breast and ovarian cancers, and the amplification is known to be associated with shortened disease-free and overall survival.^{1–3} ErbB-2 amplification has also been linked with altered sensitivity to cytotoxic drugs, especially to those targeting topoisomerase II α (topoII inhibitors), in many clinical trials.^{4–13} Most studies have linked ErbB-2 to chemoresistance to topoII inhibitors,^{4–9} but there are also clinical trials reporting either no association^{10–12} or even a higher likelihood for a response in ErbB-2-amplified tumors.^{13,14} According to *in vitro* studies, ErbB-2 amplification and overexpression associate exclusively with resistance to cytotoxic drugs.^{15–17}

Biological mechanisms that explain the association between ErbB-2 amplification and altered sensitivity to to-

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poll inhibitors are not known. Experiments with mouse and human cells have indicated that the *in vitro*-induced ErbB-2 protein overexpression does not alter the chemosensitivity of cancer cells to the topoll inhibitor.^{16,17} Neither is the ErbB-2 protein as a transmembrane growth factor receptor known to interact physically with topoll inhibitors. Although the ErbB-2 oncogene is considered to be the target gene for 17q12-q21 amplification, the amplicon harbors other closely located genes, such as *v-erbA*/thyroid hormone receptor α (THRA)¹⁸; retinoic acid receptor α ¹⁹; MLNs 50, 51, 62, and 64^{20,21}; and topoll α .^{19,22–24} Among the coamplified genes, topoll α is particularly interesting in breast cancer, because it is the molecular target for topoll inhibitors.^{25,26} *In vitro* studies with different experimental designs have established that sensitivity to topoll inhibitors is dependent on the expression level of topoll α in target cancer cells.^{27–33} The cells with a low concentration of topoll α protein are less sensitive to topoll-inhibiting drugs than cells containing a high concentration of topoll α because they contain less of the molecular target enzyme of topoll inhibitors, topoll α , than cells with a high concentration of topoll α .^{27–33}

Studies with only a few primary breast carcinomas indicate that ErbB-2 and topoll α can both be amplified simultaneously in breast cancer.^{19,22,23} In accordance with coamplification, immunohistochemically detectable topoll α expression is significantly correlated with ErbB-2 overexpression in breast cancer.³⁴ We studied ErbB-2 and topoll α gene copy number aberrations in breast cancer cell lines and primary breast carcinomas and determined the association between topoll α copy number aberrations, protein expression, and sensitivity to doxorubicin, a widely used topoll inhibitor.

Materials and Methods

Preparation of Cells for Fluorescence in Situ Hybridization (FISH)

Breast cancer cell lines BT-474, DU-4475, MCF-7, MDA-157, MDA-361, SK-BR-3, UACC-812, UACC-893, and ZR-75-1 were obtained from the American Type Culture collection (ATCC, Rockville, MD) and were cultured in recommended conditions. The confluent cultures were harvested to obtain interphase nuclei from the cells that were predominantly in the G₁ phase of the cell cycle. The cells were fixed in Carnoy's fluid (75% methanol, 25% acetic acid) and dropped on microscope slides.³⁵ Primary breast tumors (97) were derived from the tumor bank of the University of Lund, Sweden. The primary tumors were selected from the set of tumors that had been studied previously for ErbB-2 amplification by Southern blotting.³⁶ The primary tumors were freshly frozen and stored at -70°C . Imprint touch preparations were prepared for FISH by lightly pressing a semithawed frozen tumor piece onto Superfrost Plus microscope slides (Menzel, Germany).³⁷

Probes for FISH

A PAC clone for ErbB-2 (RMC17P077) was obtained from Resource for Molecular Genetics (Berkeley, CA), and a P1 probe for topoll α was obtained by a polymerase chain reaction (PCR)-based screening of a P1 library (Genome Systems Inc., St. Louis, MO).^{38,39} A chromosome 17 pericentromeric probe (p17H8) was used as a reference probe to determine the overall copy number of chromosome 17.^{38,39}

The specificity of the large-insert-size genomic DNA probes was confirmed by PCR with primers amplifying the sequences for ErbB-2 and topoll α and with a probe DNA as a template. The PCR conditions were optimized for each primer pair, using PTC-100 thermocycler (MJ Research Inc., Watertown, MA). Approximately 100 ng of each template probe and 25 pmol/L of corresponding primers were used in a 25- μL reaction volume in a standard reaction mixture recommended with Dynazyme II thermostable DNA polymerase (Finnzymes Oy, Espoo, Finland). The PCR analysis showed that the ErbB-2 probe did not recognize sequences from topoll α and vice versa (data not shown).

FISH

Two-color FISH was done as previously described.^{34,36,37} Before hybridization, imprint touch specimens were fixed at room temperature with 50%, 70%, and 100% Carnoy's fluid (10 minutes each). The probes were labeled with biotin-14-deoxyadenosine triphosphate and digoxigenin-11-deoxyuridine triphosphate by standard nick translation. The hybridization was carried out overnight at 42°C in a hybridization mixture^{35,37} containing 5 ng of pericentromeric probe, 20 ng of gene-specific probe, and 10 μg of human placental DNA. After hybridization, the slides were washed with 0.4 $\times$ standard saline citrate (2 minutes at 74°C) and 2 $\times$ standard saline citrate (1 minute at room temperature). Hybridization was detected immunohistochemically with avidin-fluorescein isothiocyanate (for biotin-labeled probe) and anti-digoxigenin rhodamine. The slides were counterstained with 0.2 mM 4,6-diamidino-2-phenylindole (DAPI) in an antifade solution (Vectashield, Vector Laboratories, Burlingame, CA).^{35,37}

Hybridizations were evaluated using an Olympus BX50 epifluorescence microscope equipped with a 63 $\times$ oil-immersion objective (numeric aperture 1.4). A dual band-pass fluorescence filter (Chromotechnology, Brattleboro, VT) was used to visualize the fluorescein isothiocyanate and rhodamine signals simultaneously. At least 80 non-overlapping nuclei with intact morphology (based on DAPI counterstaining) were scored to determine the number of hybridization signals for each topoll α , ErbB-2, and 17 centromere probe. Control hybridizations to normal lymphocytes were done to ascertain that the probes recognized a single-copy target and that the hybridization efficiencies were sufficient. Both absolute copy numbers and the relative copy number ratio (ratio between mean number of ErbB-2 or topoll α and mean number of chromosome 17 centromere signals) were determined. The

amplification of ErbB-2 and topoll α was defined if the copy number ratio was 1.5 or more. Topoll α was defined as deleted if the ratio was 0.7 or less.

Assays for Sensitivity to Doxorubicin

The chemosensitivity of cancer cell lines to doxorubicin was determined by the 96-well clonogenic assay and growth rate experiments, essentially as described previously.^{40,41} A 96-well clonogenic assay was selected, because it is considered the most reliable method for assessing cell killing after genotoxic stress.⁴² Briefly, cells in the midlogarithmic growth were used for the experiments. Variable concentrations (0–32 ng/ml) of doxorubicin (Adriamycin, Farmitalia Carlo Erba AB, Nerviano, Italy) were added to the plates for the incubation period of 4 weeks. Plating efficiency (PE) was calculated using the formula $PE = -\ln(\text{number of negative wells}/\text{total number of wells})/\text{number of cells plated per well}$. The fraction of surviving cells as a function of the doxorubicin dose was fitted with a linear quadratic equation. The comparison of drug sensitivity was made using 50% inhibitory concentration (IC₅₀) values, corresponding to 50% inhibition of the surviving fraction, which were obtained from the fitted dose-response curves.^{40,41}

Owing to the slow growth rate of the UACC-812 cell line, its sensitivity was determined by growth rate experiments (using MDA-361 cell line as an internal control to standardize the two assays).^{40,41} The cell density of the suspension used for plating was adjusted to 15,000 cells/ml for the MDA-361 cell line and to 25,000 cells/ml for the UACC-812 cells. Multiple 24-well plates were prepared to establish replicates for each drug concentration and time point studied. The cell number was presented as a function of time on the semilogarithmic scale.^{40,41} Values obtained from the clonogenic assay and growth rate experiments are expressed as a mean $\pm$ SD of six ($n = 6$) separate experiments.

Western Blotting

Nuclear extracts were prepared from cancer cell lines as described previously.⁴³ Whole cell extracts of primary breast tumors were prepared for hormone receptor analysis and frozen at -70°C until used in Western blots.³⁶ Protein concentrations were determined by the Bio-Rad protein assay (Bio-Rad, Richmond, CA). Nuclear extracts and cytosols with equal protein concentrations were electrophoretically separated on 8.0% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel and subsequently transferred to nitrocellulose membranes (Hybond-C Extra, Amersham, Arlington Heights, IL). Immunostaining was carried out using either topoll α -specific monoclonal antibody Ki-S1 (Boehringer Mannheim, Mannheim, Germany; dilution 1:1000),^{34,44–46} or a monoclonal antibody to cyclin B1 (Neomarkers, Union City, CA; dilution 1:300).⁴⁷ A topoll α -specific monoclonal antibody, Ki-S1, is specific for a COOH-terminal epitope of human topoll α and does not cross-react with topoll β .^{34,44–46} The specificity of the antibody used for the detection of

topoll α (Ki-S1) was also confirmed by doing a Western blot with another topoll α -specific monoclonal antibody, Ki-S4 (a kind gift from Dr. Udo Kellner, University of Kiel, Kiel, Germany).⁴⁶ The two topoll α antibodies gave identical bands from the same control samples (data not shown). Both topoll α and cyclin B1 antibodies were detected with the rabbit anti-mouse IgG secondary antibody conjugated to horseradish peroxidase (dilution 1:1000) (Dakopatts, Glostrup, Denmark). The peroxidase-catalyzed reaction was visualized with enhanced chemiluminescence (Amersham Life Sciences, Arlington Heights, IL).

For quantitative determination of topoll α protein expression, the immunoreactive bands were quantitated with a densitometer. Because the expression of topoll α is proliferation-dependent^{25,26,34,44–46} and the proliferation rates of the cell lines are not equal, the topoll α band intensities were adjusted with band intensities of cyclin B1, which has a cell cycle-specific (G₂/M-phase) expression pattern identical to that of topoll α .⁴⁷

Results

Topoll α and ErbB-2 Gene Copy Numbers in Breast Cancer Cell Lines

Five of nine studied breast cancer cell lines (MDA-361, SK-BR-3, UACC-812, BT474, UACC-893) showed high-level amplifications of the ErbB-2 oncogene (Figure 1). Two of these ErbB-2-amplified cell lines, UACC-812 and SK-BR-3, showed increased gene copy numbers for topoll α . SK-BR-3 was defined to have a low-level topoll α amplification (9 copies of topoll α and 6 copies of chromosome 17 centromere), whereas a high-level topoll α amplification was found in UACC-812 cells (27 copies of topoll α gene and 4 copies of chromosome 17 centromere; Figure 1). The MDA-361 cells showed ErbB-2 amplification (mean, 14 copies/cell) and, surprisingly, a physical deletion of the other allele of topoll α . On average, four copies of chromosome 17 centromere were found, compared with only two copies of topoll α (mean relative gene copy number 0.46; Figure 1).

The Relationship between Topoll α Gene Copy Number and Protein Expression

Topoll α protein expression of UACC-812, SK-BR-3, and MDA-361 cell lines was compared with that of BT-474 (ErbB-2-amplified) and MCF-7 (no ErbB-2 gene amplification) cell lines, which do not show copy number alterations of topoll α (normal copy number of topoll α gene). Only one immunoreactive band was seen in Western blots at 170 kd, which is the known molecular mass of topoll α . Strong bands were identified for UACC-812 and SK-BR-3 cells and only the weak band in MDA-361 cells (Figure 2). To rule out the possibility that the differences in the topoll α protein expression resulted from differences in the proliferation rates of the breast cancer cell lines, the bands were quantitated after they were ad-

Figure 1. Copy number aberrations of ErbB-2 and topoisomerase II α in breast cancer. ErbB-2 amplification in cell lines BT-474, MDA-361, and UACC-812 (**A–C**) was associated with no change, deletion or amplification of topoiII α gene (relative to 17 centromere, **D–F**, respectively). **G** and **H**: Clinical breast tumors with ErbB-2 and topoiII α amplification. **I**: Physical deletion of topoisomerase II α gene in primary breast tumor. The fluorochrome colors used for each probe are indicated in each panel. DAPI (blue) was used as counterstain. Note that not all signals demonstrating amplified gene copies are in the same focal plane.

justed for the variation in cell proliferation (amount of the cell population in the G₂/M phase of the cell cycle, where topoiII α is expressed). This was determined by a parallel Western blot of the cyclin B1 gene (Figure 2). After the adjustment, the UACC-812 and SK-BR-3 cells showed 5.9- and 2.0-fold-increased topoiII α protein expression when compared with that of control cell lines (mean of BT-474 and MCF-7; Table 1). The MDA361 cell line, with a physical deletion of the topoiII α gene, showed decreased topoiII α expression (45% of controls; Table 1).

TopoiII α Aberrations and Sensitivity to TopoiII Inhibitor Doxorubicin

Among the cell lines studied, MDA-361 (with topoiII α deletion and the lowest protein expression) was the most

resistant to doxorubicin (IC₅₀ 24 $\pm$ 2 ng/ml, six different experiments). The IC₅₀ values for the control cell lines BT-474 and MCF-7 were 8 and 12 ng/ml, respectively. The UACC-812 cell line grew too slowly to be analyzed by the 96-well clonogenic assay. Therefore, its IC₅₀ value was determined from growth inhibition experiments with various concentrations of doxorubicin (1–32 ng/ml). MDA-361 cells served as an internal control to confirm the similarity of the IC₅₀ values by the two methods. UACC-812 cells were hypersensitive to doxorubicin. The use of 5, 10, or 30 ng/ml of doxorubicin caused almost complete inhibition of growth. The concentration of 1 ng/ml doxorubicin suppressed the growth only slightly, whereas the dose of 3 ng/ml doxorubicin caused a growth inhibition of 36% in the UACC-812 cells. Thus, it can be estimated from these growth inhibition curves that

Figure 2. Protein expression of topoisomerase II α and cyclin B1 in five breast cancer cell lines by Western blotting. The quantitated band intensities of topo II α were adjusted by those of cyclin B1 to eliminate the differences in proliferation rates of the breast cancer cell lines (see Table 1). Even without any adjustments, strong immunoreactive bands can be seen in cell lines UACC-812 and SK-BR-3 (topoII α amplification in both cell lines), and the weakest bands in MDA-361 (with topoII α deletion). High-level topoisomerase II α expression in five primary breast carcinomas with amplification of topoisomerase II α (lanes 1–5) compared with two primary tumors with normal copy number of topoisomerase II α (lanes 6 and 7).

the IC₅₀ value of UACC-812 for doxorubicin is slightly less than 4 ng/ml (Table 1).

TopoII α Gene Copy Number and Protein Expression in Clinical Breast Tumors

Because topoII α aberrations were found only in cell lines with ErbB-2 amplification, we studied topoII α copy num-

bers in 57 breast tumors whose ErbB-2 amplification had previously been determined by Southern blot³⁶ and was now confirmed by FISH (Figure 1). In this set of 57 ErbB-2-amplified primary breast tumors, ErbB-2-topoII α coamplification was seen in 25 tumors (44%) and topoII α deletion in 24 tumors (42%; Figure 1; Table 3). The relative topoII α copy number was unaltered only in eight tumors with ErbB-2 amplification (14%; Table 3). The average number of topoII α gene copies in amplified tumors ($n = 25$) was 12.9 ± 6.4 . Seventeen of the topoII α amplifications (68%) were classified as high-level amplifications (more than 10 gene copies/cell, over threefold relative copy number increase, or both; Table 4). The average gene copy numbers of amplified ErbB-2 (by FISH) were not associated with topoII α copy number. The ErbB-2 gene copy number was 19.3 ± 10.3 in topoII α amplified tumors ($n = 25$), 18.9 ± 10.4 in topoII α deleted tumors ($n = 24$), and 21.9 ± 12.2 in tumors with a normal topoII α copy number ($n = 8$; Table 4).

To confirm that topoII α gene aberrations associate with ErbB-2 amplification also *in vivo*, we studied the topoII α gene aberrations in 40 primary tumors with normal ErbB-2 gene status (no amplification by FISH and/or Southern blot). No topoII α gene copy number aberrations were found in these 40 primary tumors (Table 3).

Whole-tissue extracts, which were left over from hormone receptor analysis,³⁶ were available only from nine ErbB-2 amplified primary breast cancers. Five of these had topoII α amplification, two had deletion, and two had normal copy numbers of topoII α . All five tumors with topoII α amplification showed a strong immunoreactive band for topoII α in Western blot (Figure 2). The sensitivity

Table 1. Absolute and Relative Copy Numbers of Topoisomerase II α and ErbB-2 in Nine Breast Cancer Cell Lines by FISH

Cell line	ErbB-2 copy number		Topoisomerase II α copy number	
	Absolute (mean $\pm$ SD)	Relative to 17 centromere	Absolute (mean $\pm$ SD)	Relative to 17 centromere
Lymphocytes	2.0 $\pm$ 0.4	1.0	2.1 $\pm$ 0.4	1.1
BT-474	53.0 $\pm$ 6.2	8.0*	4.2 $\pm$ 0.6	1.0
DU-4475	4.3 $\pm$ 0.9	1.1	4.0 $\pm$ 0.4	1.0
MCF-7	2.7 $\pm$ 0.8	0.7	3.9 $\pm$ 0.9	1.0
MDA-157	3.4 $\pm$ 1.1	0.9	4.0 $\pm$ 0.8	1.0
MDA-361	14.0 $\pm$ 2.3	3.5*	1.9 $\pm$ 0.7	0.5†
SK-BR-3	44.0 $\pm$ 6.1	7.1*	9.2 $\pm$ 4.8	1.5*
UACC-812	41.0 $\pm$ 7.5	10.0*	27.0 $\pm$ 5.6	6.7*
UACC-893	66.0 $\pm$ 12.0	32.0*	2.3 $\pm$ 0.7	1.1
ZR-75-1	3.3 $\pm$ 1.0	1.2	3.6 $\pm$ 0.8	1.3

*, Gene amplification; †, physical deletion.

Table 2. Topoisomerase II α Copy Number Aberrations, Protein Expression, and Sensitivity to Doxorubicin in Four Breast Cancer Cell Lines

Cell line	ErbB-2 status	Topoisomerase II α copy number status	Topoisomerase II α protein expression*	Sensitivity to doxorubicin (IC ₅₀ , ng/ml)
MCF-7	No amplified	Not altered	1.0 $\times$ †	12 $\pm$ 2
BT-474	Amplified	Not altered	1.0 $\times$ †	8 $\pm$ 1
UACC-812	Amplified	6.7 $\times$ amplification	5.9 $\times$	<4
MDA-361	Amplified	4:2 deletion	0.45 $\times$	24 $\pm$ 2

*Data from Western blots after adjustment with cell proliferation (Cyclin B1).

†Protein expression of MCF-7 and BT-474 combined and set as a control value.

Table 3. Prevalence of Topoisomerase II α Amplification and Deletion in 97 Breast Cancers According to ErbB-2 Oncogene Amplification

ErbB-2 amplification*	No Topoll α copy number aberrations*	Topoll α amplification*	Topoll α deletion*
No amplification (N = 40)	40 (100%)	0	0
Amplification (N = 57)	8 (14%)	25 (44%)	24 (42%)

*Determined by FISH.

of Western blot using whole-tissue cytosol samples was not sufficient to detect the topoll α protein in tumors with the normal or deleted topoll α gene copy number.

Discussion

Our data from breast cancer cell lines and primary tumors indicate that amplification or deletion of the topoll α gene is present in almost 90% of ErbB-2-amplified breast cancers. Together with our *in vitro* chemosensitivity experiments, these findings may explain at least partly the reported associations between ErbB-2 amplification and the altered sensitivity of these tumors to topoll inhibitors.⁴⁻¹³ The topoll α aberrations are most likely driven by ErbB-2 oncogene amplification, because none of the cell lines or primary tumors without ErbB-2 amplification showed gene copy number aberrations of topoll α .

ErbB-2 amplification was most frequently associated with topoll α amplification. Topoll α amplification has previously been described in the SK-BR-3 cell line^{19,24} and in a few clinical tumor samples studied.^{19,22,23} We confirmed topoll α amplification in the SK-BR-3 cells and defined it as a low-level amplification (nine copies of topoll α and six copies of chromosome 17). The UACC-812 cells, in turn, showed a mean of 27 copies of the topoll α /cell, which is comparable to that in many clinical tumor samples. Thus, the UACC-812 cell line may provide a clinically relevant model to study the effects of topoll α gene amplification. In clinical tumors, topoll α was amplified in 25 of 57 tumors (44%) with ErbB-2 amplification. Thus, calculating from the 20 to 30% prevalence of ErbB-2 amplification, one can estimate that topoll α is amplified in as many as 5 to 15% of all breast cancers.

The amplification of the target genes of the cytotoxic drugs is a common and a well-known mechanism by which cancer cell lines confer resistance to cytotoxic drugs *in vitro*.⁴⁸ For example, the cytotoxic actions of such agents as coformycin and methotrexate can be overcome by the amplification of the genes (adenylate-deaminase 2 and dihydrofolate-reductase), which me-

tabolize these drugs, in cancer cell lines.⁴⁸ However, the target genes of the cytotoxic drugs have not been implicated to induce resistance to chemotherapy in human tumors until recently.⁴⁹⁻⁵² We showed several years ago that hormone refractory prostate cancer cells circumvent the androgen blockade therapy by amplification of the androgen receptor gene, and thereby adapt to the lower levels of circulating androgens.^{49,50} After our novel finding,⁴⁹ the gene copy number aberrations of other target genes have been implicated in causing resistance to different forms of chemotherapy.^{51,52} In this study, we present completely opposite evidence, the amplification of the target gene induced hypersensitivity to the certain class of chemotherapy. We identified that the amplification of topoll α , a target enzyme for topoll inhibitors, causes an increased sensitivity to topoll inhibitors by inducing the overexpression of the topoll α protein. This may be a very important finding, because topoll α amplifications were seen in clinical tumors with the amplification of the ErbB-2 oncogene. The amplification of the ErbB-2 oncogene, in turn, is known to be associated with shortened disease-free and overall survival.¹⁻³ In addition, in two large trials it was reported that patients with ErbB-2 amplification derived the greatest benefit from topoll inhibitor chemotherapy^{13,14} and it was speculated in these studies that the effect was due to the simultaneous overexpression of topoll α .^{3,14} Because we found topoll α amplification in almost 50% of the ErbB-2-amplified breast tumors, the preliminary speculations may indeed be true. We may have identified a mechanism (gene amplification of topoll α) by which these patients with poor prognosis may obtain a favorable response to conventional chemotherapy with topoll inhibitors.

Physical deletion of the other allele of the topoll α gene was unexpected and has not been described previously in breast cancer. We found topoll α deletions in one cell line and in more than 40% (23/57) of primary tumors with an ErbB-2 amplification. The type of topoll α deletion in MDA-361 cells (four copies of chromosome 17 and two copies of topoll α) was the same as in most of the clinical

Table 4. Characterization of 57 Primary Breast Carcinomas with ErbB-2 Oncogene Amplification According to Topoisomerase II α Gene Status

Parameter	No topoll α copy number aberrations	Topoll α amplification	Topoll α deletion
Number of tumors	8 (14%)	25 (44%)	24 (42%)
ERBB2 copy number*	21.9 $\pm$ 12.2	19.3 $\pm$ 10.3	18.9 $\pm$ 10.4
Topoll α copy number*	2.5 $\pm$ 0.8	12.9 $\pm$ 6.4	2.3 $\pm$ 0.6
Chromosome 17 copy number*	2.5 $\pm$ 0.9	3.3 $\pm$ 1.2	5.2 $\pm$ 1.6

*Determined by FISH. The results are expressed as mean $\pm$ SD, counted from at least 50 cells.

tumors that showed topoll α deletion. The multiplied gene copy numbers for both topoll α and 17 centromere indicate that the deletion had occurred before the tumor became aneuploid (tetraploid for chromosome 17). The evidence from tumor suppressor genes indicates that a deletion of the other allele of the gene is a typical finding in breast cancer and is sufficient to cause a biologically relevant reduction in the function of the deleted gene (eg, p53; reviewed in Reference 53). The molecular mechanisms underlying the adjacent ErbB-2 amplification and topoll α gene deletion are unknown, although a pathway for identical chromosomal rearrangement has recently been presented *in vitro*.⁵⁴ Like topoll α deletion, telomeric deletion has also been documented to follow cyclin D1 amplification at 11q13 in breast cancer.^{37,55} The chromosomal breakpoints and the size of the deleted chromosomal segment (including topoll α) are currently not known.

Although topoll α amplification and deletion appear to be consequences of ErbB-2 amplification, topoll α aberrations may clinically be highly relevant, because topoll α is the molecular target for several important anti-cancer drugs, termed topoll inhibitors. Numerous *in vitro* studies have established that chemosensitivity to topoll inhibitors is dependent on the cellular concentration of topoll α in cancer cells.^{25,27-33,56} High concentrations of the topoll α protein are associated with increased sensitivity, whereas low levels lead to decreased drug sensitivity.²⁷⁻³³ Unfortunately, the regulation of the topoll α expression in cancer cells is largely unknown.^{34,56} Mutations of topoll α are known to alter the activity of the topoll α protein, but they are rare and, thus, not considered to have any diagnostic value (reviewed in Reference 56).

Our *in vitro* results showed that the topoll α gene amplification and deletion have opposite effects on both topoll α protein expression and on sensitivity to the topoll inhibitor, doxorubicin. The UACC-812 cells with topoll α amplification were hypersensitive to doxorubicin, whereas the MDA-361 cells with the topoll α deletion were the most resistant. These results are unlikely to be biased by differences in the proliferation rates, because the most resistant cell line (MDA-361) was the fastest growing, which is commonly considered a sign of increased chemosensitivity. In Western blots, the confounding effect of cell proliferation on topoll α protein expression measurements was eliminated, because topoll α expression was adjusted by that of cyclin B1, which has an identical (G₂/M phase) cell cycle-specific expression pattern as topoll α .⁴⁷

Although we do not have direct evidence to link topoll α gene copy number aberrations to drug sensitivity to topoll inhibitors in a clinical material, topoll α amplification was associated with protein overexpression also in clinical tumors. This suggests that topoll α -amplified tumors might be more sensitive to topoll inhibitors also *in vivo*. The topoll α deletion, in turn, may be a marker of tumors that are resistant to doxorubicin. This finding is supported by data reported from an *in vitro* model of lung cancer^{30,31} in which topoll α deletions that were acquired during exposure to doxorubicin led to decreased topoll α messenger RNA expression and, ultimately, to increased chemoresistance to the topoll inhibitor in lung cancer cell

lines.^{30,31} The association of the topoll α deletion with decreased protein expression needs to be established in a clinical material with a more sensitive method, because our old whole-tissue extracts did not allow sufficient sensitivity to detect low amounts of topoll α . However, the topoll α deletion has to be considered as a very interesting resistance mechanism to topoll inhibitors, especially at the moment, when P-glycoprotein-mediated resistance to anthracyclines has been seriously questioned.⁵⁷ P-glycoprotein is a transmembrane transporter protein that has been implicated in inducing chemoresistance to a wide variety of cytotoxic drugs, including topoll inhibitors, by transporting these drugs out of the cancer cells.⁵⁷ However, a recent study showed conclusively that neither P-glycoprotein nor its messenger RNA is expressed in untreated or anthracycline-treated breast cancer samples.⁵⁷ Therefore, P-glycoprotein probably does not determine chemosensitivity to anthracycline chemotherapy in breast cancer nor contribute to the development of resistance to topoll inhibitors in breast cancer.⁵⁷

So far, clinical studies have highlighted the role of ErbB-2 as a predictive factor for topoll inhibitor chemotherapy. Most studies have linked ErbB-2 to chemoresistance to topoll inhibitors,⁴⁻⁹ but there are also clinical trials reporting either no association¹⁰⁻¹² or a tendency for higher response rates among ErbB-2-amplified breast tumors.^{13,14} *In vitro* studies, in turn, have correlated ErbB-2 amplification and overexpression exclusively to chemoresistance to topoll inhibitors.¹⁶ Despite an increased number of clinical findings linking ErbB-2 to altered drug sensitivity, the molecular mechanisms behind these associations have not been defined. Sophisticated experiments in mouse and human *in vitro* models transfecting the ErbB-2 complementary DNA have shown no direct effects of ErbB-2 protein overexpression on chemosensitivity to topoll inhibitors.^{16,17} Neither is the ErbB-2 protein as a growth factor receptor known to interact with topoll inhibitors physically.⁵⁸ Thus, some authors have recently suggested that ErbB-2 overexpression may be just a surrogate for altered topoll α activity.^{3,14} Our present findings support this hypothesis. However, the relationship between ErbB-2 amplification and chemosensitivity is probably more complex than previously thought, because ErbB-2 amplification was associated equally often with topoll α amplification and deletion, which may have opposite effects on chemosensitivity *in vivo*. Thus, our results encourage us to correlate the response to topoll inhibitors directly with topoll α gene amplification and deletion.

Note Added in Proof

We have recently confirmed the high prevalence of topoll α gene amplification and deletion in primary breast cancers with ErbB-2 oncogene amplification with larger patient material.⁵⁹ In addition, we have shown that topoll α and ErbB-2 genes are separately amplified in breast cancer.⁵⁹

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