Topoisomerase II-α Expression in Different Cell Cycle Phases in Fresh Human Breast Carcinomas

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Topoisomerase II-α (topo IIα) is the key target enzyme for the topoisomerase inhibitor class of anticancer drugs. In normal cells, topo IIα is expressed predominantly in the S/G2/M phase of the cell cycle. In malignant cells, *in vitro* studies have indicated that the expression of topo IIα is both higher and less dependent on proliferation state in the cell. We studied fresh specimens from 50 cases of primary breast cancer. The expression of topo IIα in different cell cycle phases was analyzed with two-parameter flow cytometry using the monoclonal antibody SWT3D1 and propidium iodide staining. The expression of topo IIα was significantly higher in the S/G2/M phase of the cell cycle than in the G0/G1 phase in both DNA diploid and DNA nondiploid tumors. In 18 of 21 diploid tumors, and in 25 of 29 nondiploid tumors, >50% of the topo IIα–positive cells were in the G0/G1 phase. This significant expression of topo IIα in the G0/G1 phase of the cell cycle may have clinically important implications for treatment efficacy of topoisomerase II inhibitors.

KEY WORDS: Breast cancer, Cell cycle, DNA flow cytometry, Topoisomerase IIα.

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Breast cancer is the most common cancer among women in Sweden and in the United States, and the disease is fatal to every fourth woman despite wide use of adjuvant chemotherapy (1–3). The limited efficacy of chemotherapy is due to intrinsic or acquired resistance to the drugs in use. Anthracyclines are the most active chemotherapeutic agents in advanced breast cancer (4) and are today included in most adjuvant chemotherapy regimens for breast cancer. Anthracyclines belong to the anticancer agents called topoisomerase (topo) II inhibitors.

Topoisomerases are enzymes, present in the nuclei in mammalian cells, that regulate topological changes in DNA that are vital for many cellular processes such as replication and transcription (5). They perform their function by introducing transient protein-bridged DNA breaks on one (topoisomerase I) or both DNA strands (topoisomerase II; 6). There are two isoenzymes of topoisomerase II, with genetically and biochemical distinct features. Topoisomerase II-α (topo IIα), with a molecular weight of 170 kd, is located on chromosome 17 (7), and topoisomerase II-β (topo IIβ), with a molecular weight of 180 kd, is located on chromosome 3 (8). Topo IIα is the primary drug target for anthracyclines (6, 9).

Topoisomerase II inhibitors inhibit the rejoining step in the breakage-rejoining cycle of topo IIα and thereby shift the equilibrium toward a key covalent reaction intermediate termed the cleavable complex (10). This leads to double-stranded DNA breaks, which can, by a partly unknown mechanism, lead to cell death (11). *In vitro* studies on cell lines from different human malignancies has shown that sensitivity to topoisomerase II inhibitors is dependent on the cellular level of topo IIα (12–14).

Topo IIβ is expressed relatively constantly throughout the cell cycle in both normal and transformed cells (15, 16). In cell lines, it has been shown that in normal cells, topo IIα is a marker of proliferation and that expression is restricted to S and G2/M phases of the cell cycle, whereas in transformed cells, the topo IIα expression is both higher and less dependent on proliferation state in the cell (15, 17–21). The altered topo IIα distribution may be caused by prolonged intracellular half-life of topo IIα associated with the malignant transformation (17). This dysregulation of the expression of topo IIα in the cell cycle in malignant cells may have implications for the efficacy of treatment with topoisomerase II inhibitors.

There is today little information regarding topo IIα expression related to different cell cycle phases in human neoplasms. A study on ovarian cancer with two-parameter flow cytometric analysis has detected topo IIα–positive cells in the G1 phase in 14 of 29 cases (22).

The aim of the present study was to examine the distribution of topo IIα in the different cell cycle phases with two-parameter flow cytometry (FCM) in fresh human breast carcinomas.

MATERIALS AND METHODS

Patients

Material from 50 tumors from 49 women operated on at Örebro University Hospital was used in this study. All breast cancer samples where sent fresh from the operation theater to the Department of Pathology. Only patients with tumor size big enough to allow adequate material for routine histology, DNA analysis, estrogen receptor and progesterone receptor determinations, and FCM for topo IIα were included.

Patient age at operation ranged from 33 to 94 years, with a median age of 61 years. All tumors were invasive breast carcinomas. Tumor characteristics are shown in Table 1. No patient had received preoperative chemotherapy, hormonal therapy, or radiotherapy. Patients were given oral and written information regarding the study, and informed consent was obtained.

Preparation of Fresh Breast Tumor Tissue

The breast tumor samples were stored at 4° C in 0.05 M phosphate buffered saline (PBS) and pre-

TABLE 1. Characteristics of the 50 Breast Carcinomas

	Number	%
Tumor size		
pT1	16	32
pT2	32	64
pT3	2	4
Histologic type		
Ductal	43	86
Lobular	3	6
Other	4	8
Tumor grade[a]		
1	9	18
2	25	50
3	16	32
Estrogen receptor status		
Positive	39	78
Negative	11	22
Ploidy status		
Diploid	21	42
Nondiploid	29	58
Axillary nodal status		
pN0	26	52
pN1	22	44
ND[b]	2	4

[a] Using the Elston modification of the Bloom and Richardson system.
[b] ND, axillary node dissection not done.

pared within 18 hours of excision. Fresh breast tissue was mechanically disaggregated with Medimachine (ConsulT.S., Rivalta di Torino, Italy) in 0.05 M PBS for 1–2 minutes. The cell suspension was filtered through a 40-μm filter and centrifuged at 300 × g for 5 minutes. After one wash in PBS the cells were diluted to a concentration of 10×10^6/mL in 0.05 M PBS.

Determination of S-Phase Fraction and Ploidy

A regular DNA staining with DNAcon 3 tubes (ConsulT.S.) was performed. The tubes contained a dehydrated buffer (lysing agent, RNAse and cromatin stabiliser). Propidium iodide solution (PI) was added and the tube left at room temperature for 5 minutes in the dark. Two different control cells, chicken and salmon, were added to 1×10^6 sample cells and then incubated for 1 hour in an ice bath in the dark.

The stained nuclei were analyzed in a FACScan equipped with a 488-nm argon laser (Becton Dickinson, Immunocytometry Systems, CA) with CellQuest (Becton Dickinson) software. The DNA analysis was based on 15 000 events. To calculate DNA ploidy and S-phase fraction (SPF), Modfit (Becton Dickinson) software was used. Tumors were defined as either diploid (one stemline) or nondiploid (two or more cell populations; 23). The SPF was compared with that of the total number of cells expressing topo IIα. SPF was technically feasible in 82% (41/50) of the tumors.

Flow Cytometry TopoIIα Analysis

Simultaneous measurement of topo IIα and DNA content was done by two-parameter FCM. After 1×10^6 cells was added to each of two tubes, the tubes were incubated with 600-μL of lysing solution (0.5% Triton X-100 [Sigma Chemical Co, St. Louis, MO], 1% BSA [Life Technologies, Paisley, Scotland], and 0.2 μg/mL EDTA [Sigma] in 0.05 M PBS), pH 8.6. The cells were incubated for 10 minutes in an ice bath in the dark. The nuclei were fixed in 2 mL of $-20°$ C 99.8% methanol pa (J.T. Baker. Deventer, Holland) for 10 minutes in an ice-bath in the dark. After one wash with 0.05 M PBS, the tubes were centrifuged for 5 minutes at 300 × g. Topo IIα clone SWT3D1 IgG1κ (DAKO, Glostrup, Denmark; 1:100) was added to one of the tubes. After incubation, a FITC-conjugated rat anti-mouse (RAM) antibody (DAKO; 1:50) was added to both tubes and incubated, followed by another incubation with 400 μL of PI (10 μg/mL; Sigma), with RNAse (200 μg/mL; Sigma). The last three incubations were performed for 30 minutes in an ice bath in the dark.

The stained nuclei were analyzed in a FACScan equipped with a 488-nm argon laser (Becton Dickinson) and CellQuest (Becton Dickinson) software.
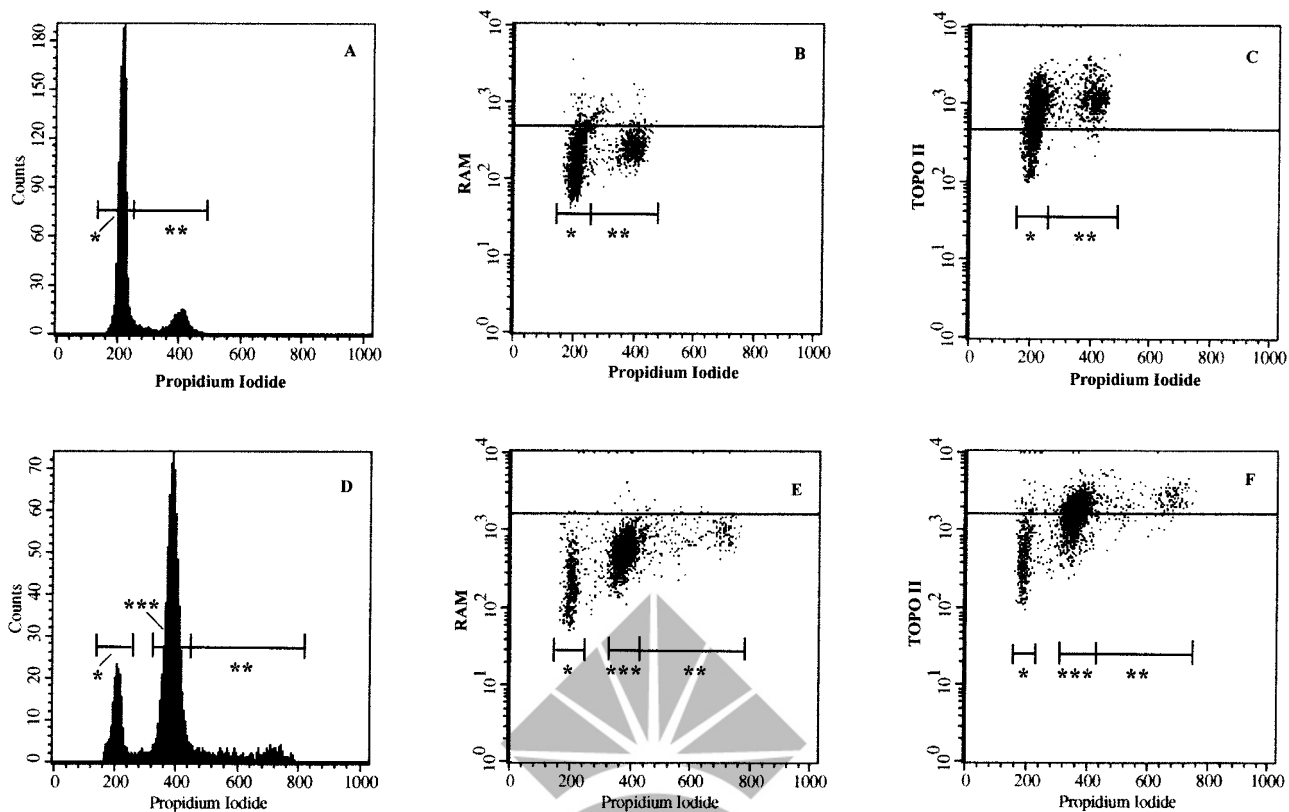
FIGURE 1. Flow cytometric analysis of topo IIα and DNA contents in fresh breast carcinomas. The DNA content of the investigated nuclei, reflecting the cell cycle phase, was determined after incubation with propidium iodide and RNAse. (**A** and **D**) show DNA histograms used for separation of the different cell cycle phases. **A–C,** tumor with diploid DNA content showing cells in G0/G1 phase (*) and S/G2/M phase (**). **D–F,** tumor with nondiploid DNA content showing diploid cells in G0/G1 phase (*), nondiploid cells in G0/G1 phase (***), and cells in S/G2/M phase (**). (**B** and **E**) represent negative controls with FITC-labeled rat anti-mouse antibody (RAM) only. The cutoff limit for topo IIα positivity was set for each population based on the negative control.

The two-parametric analysis was based on 5000 events. The green fluorescence of FITC was recorded as the total amount of detected topo IIα, and the red fluorescence of PI was recorded as the total amount of DNA.

In both the analysis of the total topo IIα expression and of the expression in the separate cell cycle phases, the cutoff limits were set individually on the negative control samples according to the appearance of the scatterplot. The percentage of background events in the negative control was subtracted from the percentage of true-positive cells. The separation of the different cell cycle phases was
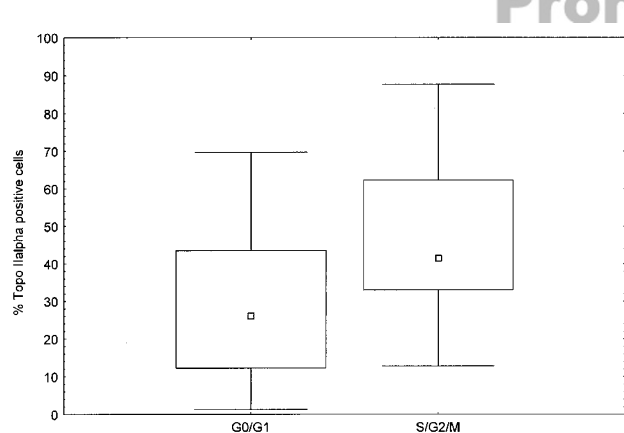

FIGURE 2. Percentage of topo IIα–positive cells in G0/G1 phase and S/G2/M phase in 21diploid breast tumors. The upper and lower quartiles and the median values are depicted as box plots. Whiskers indicate maximum and minimum values.

FIGURE 3. Percentage of topo IIα–positive cells in diploid cells in G0/G1 phase, nondiploid cells in G0/G1 phase, and nondiploid cells in S/G2/M phase in 29 nondiploid breast tumors. The upper and lower quartiles and the median values are depicted as box plots. Whiskers indicate maximum and minimum values.
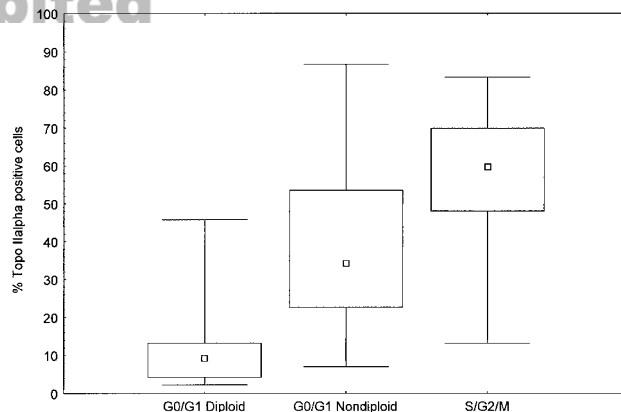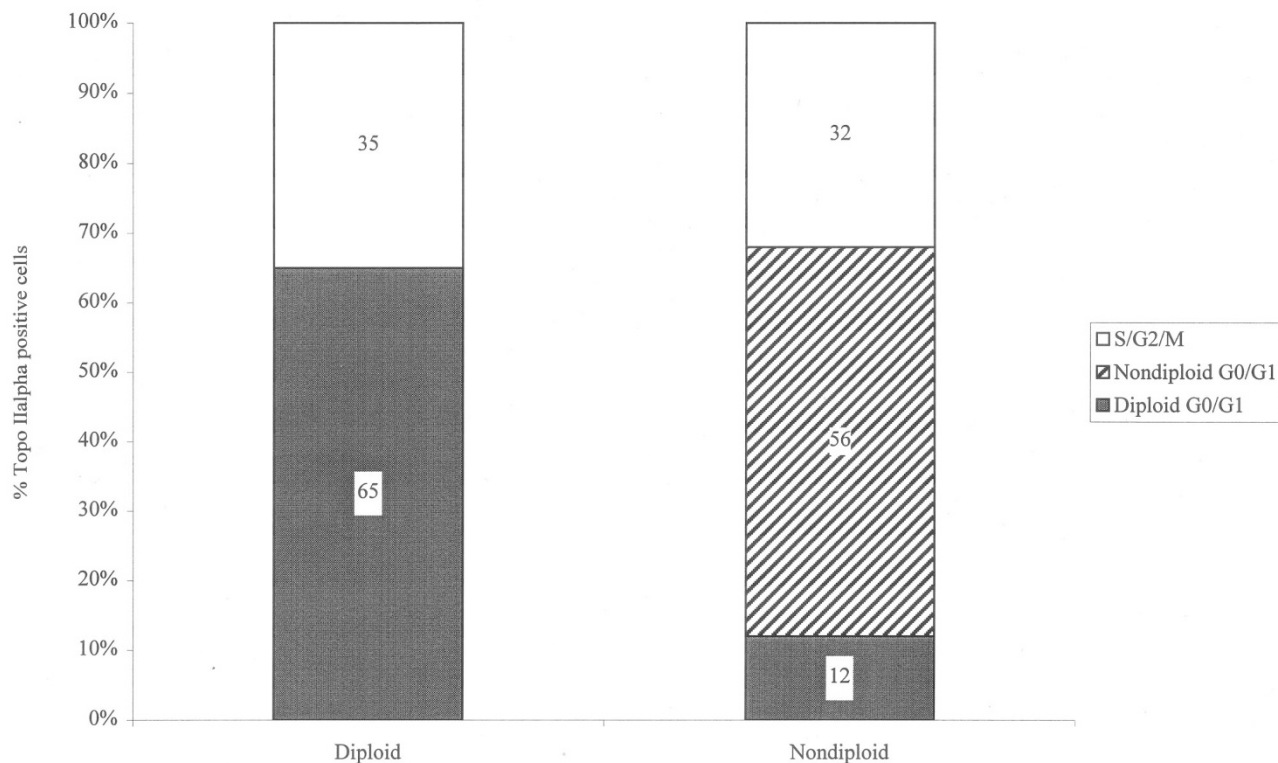
FIGURE 4. Median distribution of the topo IIα–positive cells in different cell cycle phases in 21 diploid and 29 nondiploid breast tumors.

done from the DNA histogram, as illustrated in Figure 1.

Statistical Methods

Statistica software (StatSoft, OK) was used for all calculations. Values are presented as the median and difference between upper and lower quartiles (interquartile range, IQR), or as the mean and standard deviation (SD). Difference in proportion of topo IIα–positive cells between diploid and nondiploid tumors was tested by the Mann-Whitney test. Correlation between the proportion of topo IIα–positive cells and SPF was determined by the Spearman's rank correlation. Difference in expression of topo IIα between cells in G0/G1 phase and S/G2/M phase in diploid tumors was tested by the Wilcoxon matched-pairs test. The three group analysis in nondiploid tumors was done with Friedman ANOVA test and posthoc test with Wilcoxon signed rank sum test with Bonferroni correction. A level of $P < .05$ was accepted as statistically significant.

RESULTS

Expression of Topo IIα in the Total Cell Population

When analyzed on the total cell population, the median expression of topo IIα in all 50 cases was 25% (IQR 23%) positive cells. There was no statistically significant difference between the proportion of topo IIα–positive cells in 21 diploid and 29 nondiploid tumors ($P = .8$). No statistically significant correlation between the proportion of topo IIα–positive cells and SPF was reached ($P = .1$).

Topo IIα in Relation to Cell Cycle Phase in Diploid Tumors

The expression of topo IIα in G0/G1 phase and S/G2/M phase from 21 diploid tumors is shown in Figure 2. The median expression of positive cells in G0/G1 phase was 26% (IQR 31%), compared with 41% (IQR 29%) in S/G2/M phase ($P = .002$). Of all topo IIα–positive cells, a mean of 65% (SD 18%) were in G0/G1 phase (Figure 4). In 18 cases, >50% of the positive cells were in G0/G1 phase.

Topo IIα in Relation to Cell Cycle Phase in Nondiploid Tumors

The expression of topo IIα in G0/G1 phase for the diploid cells and G0/G1 phase and S/G2/M phase for the nondiploid cells from 29 nondiploid tumors is shown in Figure 3. The median expression of positive cells in G0/G1 phase for the diploid cells was 9% (IQR 9%), compared with 34% (IQR 30%) in G0/G1 phase and 60% (IQR 22%) in S/G2/M phase

for the nondiploid cells. These differences were statistically significant ($P < .001$).

In these nondiploid tumors, the expression in diploid cells in G0/G1 phase was significantly lower than the expression in cells in G0/G1 phase in the diploid tumors ($P = .003$). Of all topo IIα–positive cells, a mean of 56% (SD 15%) were in nondiploid cells in G0/G1 phase, 12% (SD 11%) in nondiploid cells in S/G2/M phase, and 32% (SD 13%) in diploid cells in G0/G1 phase (Fig. 4). In 25 cases, >50% of the positive cells were in cells in G0/G1 phase.

DISCUSSION

There is today no established factor for clinical use in prediction of chemotherapy efficacy in breast cancer. SPF and erbB-2, a tyrosine kinase membrane receptor, are two factors that have shown predictive potential. High SPF, a marker of high tumor cell proliferation, has shown positive correlation with response to chemotherapy (24–26). Topo IIα is also a marker of proliferation, and in a previous study using immunohistochemistry, a good correlation with SPF has been shown (27). In our study, we found no clear correlation between the proportion of topo IIα–positive cells determined by FCM and SPF.

Patients with tumors expressing high levels of erbB-2 have been shown to benefit from addition of doxorubicin to L-phenylalanine mustard and 5-FU (28) and from dose-escalated compared with a low-dose combination of cyclophosphamide, doxorubicin, and 5-FU (29). There is however no molecular rationale for a relationship between erbB-2 level and anthracycline efficacy. In a study by Pegram *et al.* (30), it has been shown that there is no difference in chemosensitivity *in vitro* or *in vivo* between erbB-2–negative and transfected breast cancer cells and breast cancer xenografts. Both the erbB-2 gene and the topo IIα gene are located close to each other on chromosome 17q. *In vitro* studies on breast cancer cell lines have shown increased gene copy number for topo IIα in two of five erbB-2-amplified cell lines. This topo IIα amplification was associated with increased topo IIα protein expression and increased sensitivity to the topo II inhibitor doxorubicin (31). Co-amplification of these two genes has also been shown in breast cancer samples (31–33). This co-amplification may explain the observed relationship between erbB-2 overexpression and anthracycline efficacy.

In normal cells, topo IIα is a marker of proliferation, and expression is restricted to S/G2/M phases of the cell cycle. In accordance with this, we also found in this study a significantly higher expression of topo IIα in S/G2/M phase than in G0/G1 phase, both in diploid and in nondiploid tumors.

Our results show that in clinical breast cancer samples topo IIα is expressed also in the G0/G1 phase. Although the expression is lower than in S/G2/M phase, it might be high enough to have implications for the efficacy of topoisomerase II inhibitors. In fact, in 18 of 21 diploid tumors and in 25 of 29 nondiploid tumors, >50% of the topo IIα–positive cells were in G0/G1 phase. These results on fresh breast cancer tissue are in accordance with the findings from *in vitro* studies in which malignant transformation was accompanied by qualitative changes in the expression of topo IIα (17). However, the level of topo IIα in individual tumor cells in G0/G1 phase may be too low to be detected with immunohistochemistry. This may explain why a study using immunohistochemical analysis of topo IIα has failed to predict response to epirubicin in metastatic breast cancer (34).

With a significant expression of topo IIα in G0/G1 phase, it can be postulated that topoisomerase II inhibitors exert their action by the same mechanism in nonproliferating as in proliferating tumor cells. These findings may explain why some human neoplasms can be cured with chemotherapy despite the fact that the majority of the tumor cells are in a non-proliferative cell cycle phase. It may be that tumor cells with none or low expression of topo IIα in G0/G1 phase, like normal cells, can escape from the topoisomerase II inhibitors and repair the DNA damage, whereas tumor cells with persistent expression of topo IIα throughout the cell cycle can not.

In summary, our findings show that topo IIα, the key target enzyme of topoisomerase II inhibitors, is significantly expressed in G0/G1 phase of breast cancer cells. This finding may have clinically important implications for treatment efficacy of topoisomerase II inhibitors.

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