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Expression of Topoisomerase IIα Is Associated with Rapid Cell Proliferation, Aneuploidy, and c-*erb*B2 Overexpression in Breast Cancer

Tero A. H. Järvinen,* Juha Kononen,* Markku Pelto-Huikko,† and Jorma Isola*

From the Laboratory of Cancer Genetics, Tampere University Hospital and Institute of Medical Technology, and the Medical School,† University of Tampere, Tampere, Finland*

The role of molecular markers predicting the response to cytotoxic chemotherapy is not established. A potential predictive factor, topoisomerase IIα (topo IIα), is a target for certain cytotoxic drugs, and its concentration has been shown to correlate with chemosensitivity* in vitro. *We evaluated expression of topo IIα immunohistochemically in 230 breast cancer samples and studied its association with known clinicopathological factors and factors previously shown to predict response to cytotoxic drugs. Topo IIα protein expression was found in 0.6 to 39.4% (10.6 ± 7.9%, mean ± SD) of breast carcinoma cells, whereas expression was undetectable in nonmalignant breast epithelium. Topo IIα protein expression correlated well with semiquantitative mRNA* in situ *hybridization (P = 0.007). A significant association was found between the proportion of topo-IIα-positive cells and low estrogen and progesterone receptor content (P < 0.0001), high grade (P < 0.0001), DNA aneuploidy (P = 0.003), and c-erbB-2 oncoprotein overexpression (P < 0.0001). Topo IIα expression was not associated with clinical variables, such as age of the patient, primary tumor size, or axillary nodal status. A highly significant linear correlation was found between topo IIα and tumor proliferation rate (S-phase fraction, r = 0.46; P < 0.0001). Because hormone receptors, grade, and ploidy are associated with tumor proliferation rate, topo IIα expression was adjusted for S-phase fraction to reveal the proliferation-independent clinopathological associations. According to the analysis of co-variance, only aneuploidy (P = 0.0003) and c-erbB-2 overexpression (P = 0.01) were associated with proliferation-adjusted expression of topo IIα. In conclusion, the close association of Topo IIα with other potential predictive factors (tumor proliferation rate, c-erbB-2 oncoprotein) suggests that topo IIα, having a defined role as a target for cytotoxic drugs, may be a valuable predictor of response to chemotherapy. (Am J Pathol 1996, 148:2073–2082)

There are no commonly accepted markers that could be used to predict response to cytotoxic chemotherapy in breast cancer patients. Response to endocrine therapy can be predicted by hormone receptor analyses,[1] but so far, prediction of response to cytotoxic chemotherapy lacks reliable predictive markers. These factors would be of utmost utility in the clinical management of breast cancer patients. Predictive factors should not only predict response to chemotherapy in general but should also be able to aid in the selection between different forms of chemotherapy. With the increased use of doxorubicin-containing regimens instead of conventional cyclophosphamide-methotrexane-fluorouracil, the selection between different regimens has become increasingly important. Topoisomerase (topo) IIα is one of the enzymes suggested as a clinically relevant predictive factor.[2]

DNA topo II is a eukaryotic homodimeric enzyme that exists in two isoforms in human cells; the 170-kd topo IIα and the 180-kd topo IIβ.[3–8] These two isoforms of topo II share considerable homology but are products of different genes located on chromosomes 17q and 3p, respectively. Topo IIα is a key

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Address reprint requests to Dr. Jorma Isola, University of Tampere, Institute of Medical Technology, Laboratory of Cancer Genetics, PO Box 607, FIN-33101, Tampere, Finland.

enzyme in DNA metabolism, having a central key role in DNA replication.[3–8] Topo IIα acts first by generating and then resealing double-stranded DNA breaks, which are necessary for segregation of chromosomes at the end of mitosis.[6,7] Although recent reports indicate that topo IIβ may have a physiologically relevant function as an integral component of nuclear matrix and nucleoli[9,10] and that it may play a more significant role in mediating drug resistance than has been previously appreciated,[9–12] the function of topo IIβ is still less clearly defined than that of topo IIα.

Topo IIα is a target for a wide variety of structurally diverse cytotoxic drugs, including some of the most important anticancer drugs such as doxorubicin, m-AMSA, mitoxantrone, and etoposide.[4,8] Drugs targeted against topo II trap the enzyme in a so-called cleavable complex, thereby inhibiting its proper function.[4] This results in the accumulation of double-stranded DNA breaks, which are lethal to the cell.[4] It has been shown that sensitivity to topo II-inhibiting drugs is correlated with expression to topo IIα *in vitro*.[13,14] This forms a hypothesis to test whether assays of topo IIα could be used in a clinical setting to predict a patient's response to chemotherapy.

Studies published so far suggest that a favorable response to cytotoxic chemotherapy is associated with a high tumor proliferation rate.[15–18] A recent study indicated that overexpression of c-*erb*B-2 oncoprotein may be an even stronger predictor of response to chemotherapy containing the topo II inhibitor doxorubicin.[2] The biological background of this finding is unknown, because the c-*erb*B-2 proto-oncogene encodes for a transmembrane growth factor receptor, with no reported interaction with cytotoxic drugs. Because topo IIα is located very close to the c-*erb*B-2 oncogene at chromosome 17q12, it has been speculated whether there is a mechanism causing aberrant co-expression of these two proteins.[2,19–21] At the genomic DNA level, both amplification[19,20] and deletion[21] of the topo IIα gene have been described in association with c-*erb*B-2 amplification. Correlation between topo IIα gene copy number aberrations and topo IIα protein expression are not known.[19–21]

So far, only rather limited data are available on the activity and expression of topo IIα in human neoplasms.[22–26] Previous studies using antibodies to topo II have confirmed that immunohistochemically detectable levels of topo II are expressed in virtually all breast cancer samples.[25,26] In a pilot study of 63 invasive ductal breast cancers, Hellemans et al[26] found a relationship between high expression of topo IIα and high tumor grade, large tumor size, nodal status, and the distant metastases at the time of diagnosis.

In this study we optimized an immunohistochemical method for analysis of topo IIα expression from frozen tissue sections and analyzed topo IIα expression in 230 primary invasive breast cancers. The topo IIα expression was correlated with known clinicopathological indicators of malignant potential, among which were tumor proliferation rate and c-*erb*B-2, the potential predictors of chemosensitivity.

Materials and Methods

Patients and Tumors

We studied surgical biopsy specimens from a set of 230 randomly selected female breast cancer patients, whose tumor samples were sent for steroid hormone receptor analysis to the Laboratory of Cancer Genetics, Tampere University Hospital. Entered in this study were tumor samples from invasive, primary breast cancers. The median age of the patients was 61 years (range, 28 to 89). Patients did not receive any preoperative chemotherapy or endocrine therapy.

Tumor samples were snap-frozen in OCT tissue-embedding medium (Tissue-Tek, Miles Laboratories, Naperville, IL) within 15 minutes of removal. Cryostat sections were cut and stained for rapid intra-operative histopathological diagnosis with routine methods. Extra sections (5 to 7 μm) were cut and stored air-tight at −70°C until used in immunocytochemistry and mRNA *in situ* hybridization. Histopathological grading was performed according to the Bloom and Richardson system using routine formalin-fixed, paraffin-embedded sections, respectively.[27]

Immunohistochemistry of Topo IIα

Frozen sections were fixed with Zamboni's fluid for 15 minutes, washed with phosphate-buffered saline (PBS), and incubated with 1.0% bovine serum albumin (BSA)/PBS for blocking nonspecific antibody binding. A rabbit polyclonal antibody (TopoGEN, Columbus, OH) raised against a synthetic peptide of the carboxyl-terminal region of human topo II was incubated overnight (4°C) at a dilution of 1:1000 (from manufacturer's suggested stock solution). A standard avidin-biotin-peroxidase complex (ABC) technique (Vectastain Elite, Vector Laboratories, Burlingame, CA) was used for visualization with diaminobenzidine as a chromogen. The diaminobenzidine reaction was intensified with the methenamine silver method as described elsewhere.[28] Sections

were then counterstained with hematoxylin and mounted. Placenta was used as a positive control. Conventional immunohistochemical controls (dilution series of primary antibody and omission of primary or secondary antibody or chromogenic substrate) indicated that immunostaining was specific for topo IIα. The percentage of positively stained carcinoma cells (topo IIα score) was evaluated using a 20× objective in a blind fashion, ie, unaware of estrogen receptor (ER), progesterone receptor (PR), or c-*erb*B-2 content or other data. In the beginning, the whole histological section was examined under the light microscope, and from morphologically well preserved areas the highest topo IIα staining region was selected for analysis. From 400 to 1000 (average, 600) carcinoma cells were counted to obtain the topo IIα score. Correlation of topo IIα scores between two observers was 0.960 (n = 10). Intra-observer reproducibility of topo IIα scores was 0.867 (n = 15).

ERs and PRs were immunostained using Abbott's ER-ICA and PR-ICA kits (Abbott Laboratories, Chicago, IL). Overexpression of the c-*erb*B-2 protein was detected immunohistochemically using a monoclonal antibody (NCL-BII, Novocastra Labs, Newcastle, UK).[20,21] The c-*erb*B-2, ER-ICA, and PR-ICA antibodies were visualized as explained above for topo IIα. Only intense membranous immunostaining present in a majority of cells was taken to represent overexpression of the c-*erb*B-2 protein, as it has been shown to yield the best prognostic associations.[29,30]

Western Blot

Exponentially growing, BT 474 human breast cancer cells were used for Western blots. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli[31] with some modifications. Total cell lysates were prepared by placing cultured cells directly into SDS sample buffer (10% SDS, 5% 2-mercaptoethanol, 62.5 mmol/L Tris-HCl, pH 6.8), subjected to 8.5% SDS-PAGE gels, and finally transferred to nitrocellulose membranes. Immunostaining was carried out using topo IIα as a primary antibody overnight at 4°C (concentration 1:10,000). To detect topo IIα, peroxidase-conjugated swine anti-rabbit IgG antibody (concentration 1:1000) (Dakopatts, Glostrup, Denmark) was incubated with blotting membranes for 60 minutes at room temperature. The peroxidase-catalyzed reaction was visualized with enhanced chemiluminescence (Amersham Life Sciences, Arlington Heights, IL). Molecular weight marker proteins contained myosin (220 kd), phosphorylase b (97.4 kd), and BSA (66 kd; Amersham Life Sciences, Arlington Heights, IL).

Double Immunofluorescence Staining with Topo IIα and Ki-S1

Ki-S1 is an IgG mouse monoclonal antibody generated by immunizing BALB/c mice with crude nuclear extracts from the human lymphoma cell line U937.[32] As Ki-S1 has recently been shown to recognize human topo IIα,[33] we double immunostained breast cancer samples with Ki-S1 (generous gift from Prof. H. Kreipe, University of Wurzburg, Wurzburg, Germany) and anti-topo-IIα to further demonstrate the specificity of the immunohistochemical staining. Briefly, frozen sections were fixed with Zamboni's fluid for 15 minutes, washed with PBS, and incubated with 1.0% BSA-PBS for blocking nonspecific antibody binding. Topo IIα (1:1000) and Ki-S1 (1:10,000) were incubated overnight in a cocktail (4°C). Topo IIα polyclonal antibody was detected by fluorescein-isothiocyanate-conjugated goat anti-rabbit IgG antibody (1:50; Jackson ImmunoResearch Laboratories, West Grove, PA), whereas Ki-S1 was visualized by biotinylated anti-mouse IgG antibody (1:400; Vector Laboratories) followed by rhodamine-avidin (1:200; Vector Laboratories). Sections were then mounted in Vectashield A (Vector Laboratories) and were analyzed with an Olympus B × 50 epifluorescence microscope equipped with a 10× UPlanFI objective (Olympus Optical, Tokyo, Japan). Two separate images (matching green fluorescein isothiocyanate fluorescence and red rhodamine fluorescence) were captured with a scientific cooled CCD videocamera (Photometrics Image Point, Photometrics, Tuscon, AZ).

In Situ *Hybridization*

Frozen sections were cut at −20°C and thawed onto Superfrost Plus (Menzel, Germany) slides. Two synthetic oligonucleotide probes directed against topo IIα mRNA (nucleotides 2382 to 2427 and 3656 to 3701, GenBank accession number J04088[34]) were labeled at the 3′ end with [^{33}P]dATP (DuPont-NEN Research Products, Boston, MA) using terminal deoxynucleotidyltransferase (Amersham International, Buckinghamshire, UK). A detailed description of the hybridization method has been published elsewhere.[35] Briefly, unfixed, frozen, and air-dried sections were hybridized at 42°C for 18 hours with 1 × 10^7 cpm/ml of the probe, washed four times (15 minutes each) in 1× standard saline citrate at 55°C,

and, while in the final rinse, left to cool at room temperature (approximately 1 hour). Autoradiograph films (Amersham β-max; Amersham International) were overlaid on slides, exposed for 2 weeks, and developed using LX24 developer and AL4 fixative (Kodak, Rochester, NY). Histology was controlled afterwards by staining the hybridized tissue sections with hematoxylin.

Autoradiograph films were analyzed as described previously elsewhere.[36] Briefly, the measurements were done by an image analysis system, and the optical densities of radiographic films (representing mRNA expression) were than converted and expressed as + for low expression, ++ for moderate expression, and +++ for high expression of topo IIα mRNA by one investigator (J. Kononen) without prior knowledge of the immunohistochemical topo IIα data. Evaluation was done only on the histologically most representative areas. The quantitation between different hybridizations was based on the topo IIα mRNA levels of the breast cancer cell lines BT 474, MCF-7, and UACC 812, which were included in each hybridization experiment and formed an internal standard for semi-quantitative analysis. The borderline cases were scored as the lower of the two expression categories in question.

DNA Flow Cytometry

DNA flow cytometry was performed using 200-μm-thick frozen sections as starting material.[37] An EPICS C flow cytometer (Coulter Electronics, Hialeah, FL) was used for data acquisition, and a multicycle software program (Phoenix Flow Systems, San Diego, CA) was used for DNA histogram analysis. A DNA index over 1.07 (in more than 20% of cells) was used as a criterion for DNA aneuploidy. In DNA-aneuploid cases, only the S phase of the aneuploid clone was registered. Cell cycle evaluation was successful in 77% (177/230) of the tumors.

Statistical Methods

Statistical analyses were carried out with an IBM-compatible personal computer and the Biomedical Data Processing software (BMDP Statistical Software, Los Angeles, CA). Association of topo IIα and individual clinicopathological variables was done using one-way analysis of variance (ANOVA with Welch correction for unequal variances). Adjustment of topo IIα for the S-phase fraction was done by analyzing the ratio of topo IIα to the S-phase fraction. Analysis of co-variance was used to evaluate the proliferation-independent associations of topo IIα.

Results

Immunohistochemical Staining for Topo IIα.

Immunohistochemical stainings using different concentrations of topo IIα antibody showed that 1:1000 dilution of topo IIα polyclonal antibody yielded the best result, a strong nuclear immunoreaction without significant background staining (Figure 1, A and B). Positive immunostaining was restricted to the nuclei of carcinoma cells in all 230 invasive breast cancer samples whereas stroma and nonmalignant breast epithelium remained always negative. The proportion of topo-IIα-immunopositive cells in breast carcinomas varied from 0.6 to 37.4% (10.6 ± 7.9%, mean ± SD). The specificity of topo IIα was confirmed with Western blots, which showed a single immunoreactive band at 170 kd, which is in good agreement with the molecular mass of human topo IIα[32,33] (Figure 2). The specificity of topo IIα immunohistochemistry was demonstrated by conventional immunohistochemical controls (not shown) and by a double-immunofluorescence experiment in which two different topo IIα-specific antibodies labeled exactly the same cells (Figure 3, A and B).

In Situ *Hybridization of Topo IIα.*

Topo IIα gene expression was studied with mRNA *in situ* hybridization using synthetic oligonucleotide probes. Close association was obtained between the topo IIα score by immunohistochemistry and for topo IIα mRNA expression from adjacent tissue sections ($P = 0.007$, n = 22, ANOVA; Figures 4 and 5). As a control for specificity, the two distinct topo IIα oligonucleotide probes gave identical hybridization signals when hybridizated separately on adjacent tissue sections (data not shown). Addition of a 100-fold excess of respective unlabeled probe into the hybridization cocktail abolished the hybridization signal completely (not shown).

Association of Topo IIα Score with Clinicopathological Features of the Tumors

Topo IIα expression was not associated with the age of the patient, primary tumor size, or axillary nodal status ($P > 0.05$; Table 1). Topo IIα scores were significantly higher in ER- and PR-negative tumors compared with positive ones ($P < 0.0001$ for both), and there was a strong association between high topo IIα scores and DNA aneuploidy ($P = 0.0003$; Table 1). Poorly differentiated breast carcinomas (grade III) had significantly higher

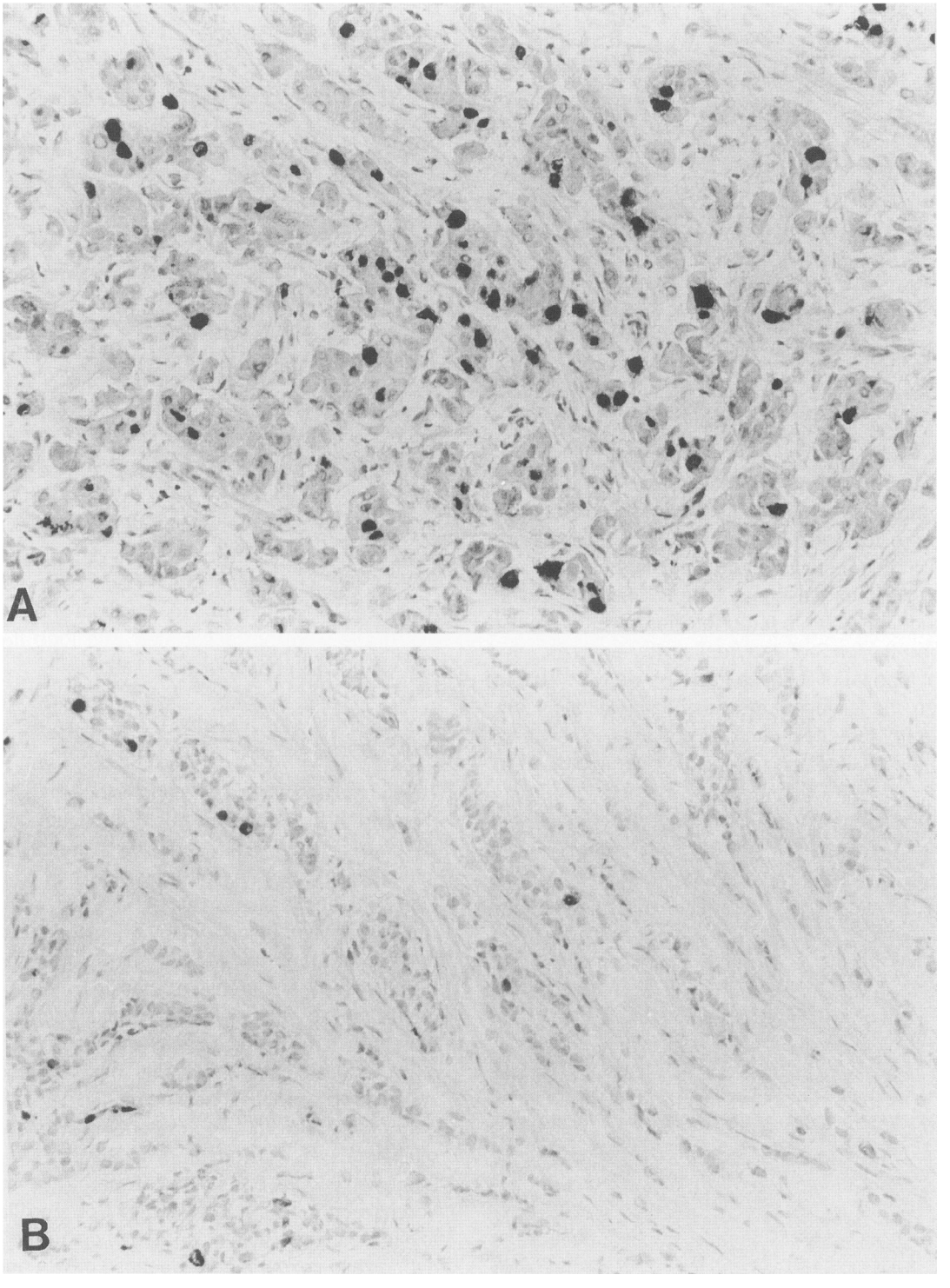

Figure 1. *Immunohistochemical staining of human breast carcinomas with a polyclonal topo IIα antibody. Tumors with a high* (A) *and low* (B) *proportion of topo-IIα-positive breast cancer cells are shown. Hematoxylin counterstain; magnification, ×320.*

topo IIα scores than grade II tumors, which had significantly higher topo IIα scores than grade I tumors ($P < 0.0001$, ANOVA; Table 1). The topo IIα scores were high in tumors with c-*erb*B-2 oncoprotein overexpression ($P < 0.0001$). The proliferative activity of the tumors was determined by flow cytometric S-phase analysis. In the linear regression analysis, topo IIα score correlated highly significantly with S-phase fraction ($r = 0.46$, $P < 0.0001$; Figure 6).

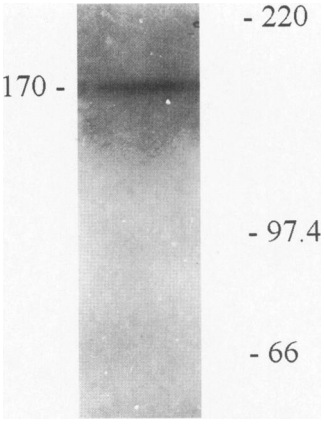

Figure 2. *Western blot of human cancer cell line BT 474 was probed with a polyclonal antibody to topo IIα. The immunoreactive band is found at 170 kd, the reported size of the topo IIα molecule.*[32,33]

Because hormone receptors, tumor grade, and ploidy are known to be significantly associated with tumor proliferation rate (all ANOVA *P* values were <0.01 in this material), it is possible that the clinopathological correlations of topo IIα were due to the confounding effect to tumor proliferation. By adjusting topo IIα for S-phase fraction (by analyzing the ratio of topo IIα of S-phase fraction), we were able to analyze the clinocopathological correlations independent of the effect of tumor proliferation. According to the analysis of co-variance, aneuploidy (*P* = 0.0003) and c-*erb*B-2 overexpression (*P* = 0.01) were independently associated with topo IIα expression in 157 cases from which complete data were available (Table 2). Hormone receptors and histological grade were not associated with topo IIα after adjusting for S-phase fraction (Table 2).

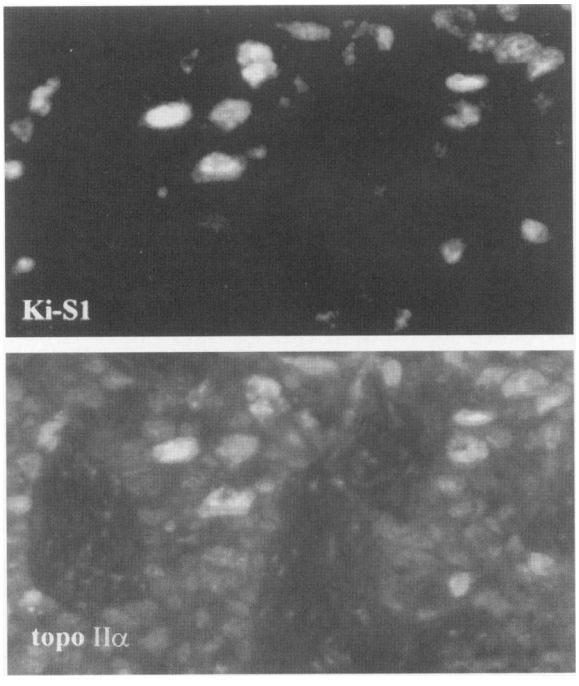

Figure 3. *A double-immunofluorescence experiment confirming the specificity of the topo IIα immunostaining. The two different topo IIα antibodies (Ki-S1 in* upper panel *and polyclonal topo IIα in* lower panel *) localize to exactly the same cells in an invasive ductal breast cancer.*

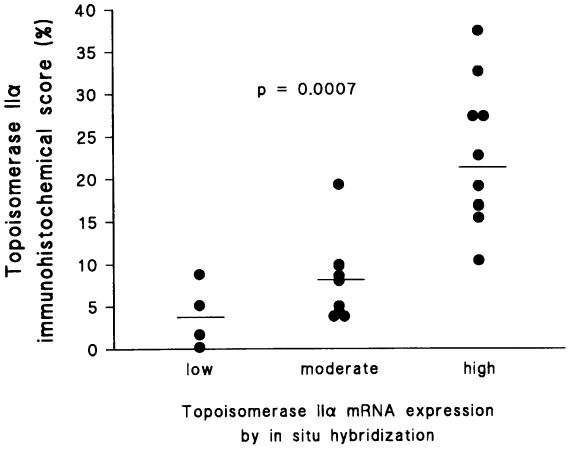

Figure 4. *Association between topo IIα immunohistochemical scores and the semiquantitative mRNA* in situ *hybridization in 22 invasive breast cancers. A highly significant association was found* (P = *0.0007, ANOVA).* Horizontal bars *indicate group means.*

Discussion

These results show that immunohistochemical determination of topo IIα is an easily applicable method for determination of this enzyme in tumor tissues. Immunohistochemistry of topo IIα may therefore have wide applications in tumor pathology not only in breast cancer but also in other cancers that are treated with topo IIα inhibitor chemotherapy. Compared with other currently available detection methods, such as the polymerase-chain-reaction-aided transcript titration assay, enzyme activity assays, and Western blotting,[22,38,39] the immunohistochemical assay of topo IIα is cost competitive and easy to perform. Second, immunohistochemistry enables simultaneous morphological evaluation of the tumor specimen and the distinction between malignant and nonmalignant cells. Measurements of topo IIα protein is meaningful also in the light of our mRNA *in situ* hybridization experiments, because mRNA and protein expressions analyzed on adjacent tissue sections were highly concordant. This finding also indicates that regulation of topo IIα protein expression appears to be a transcriptional event *in vivo*. This is in agreement with previous *in vitro* studies.[40,41]

So far, there is little evidence whether nuclear levels of topo IIα *in vivo* correlate with clinical response to chemotherapy.[24] In the preliminary report

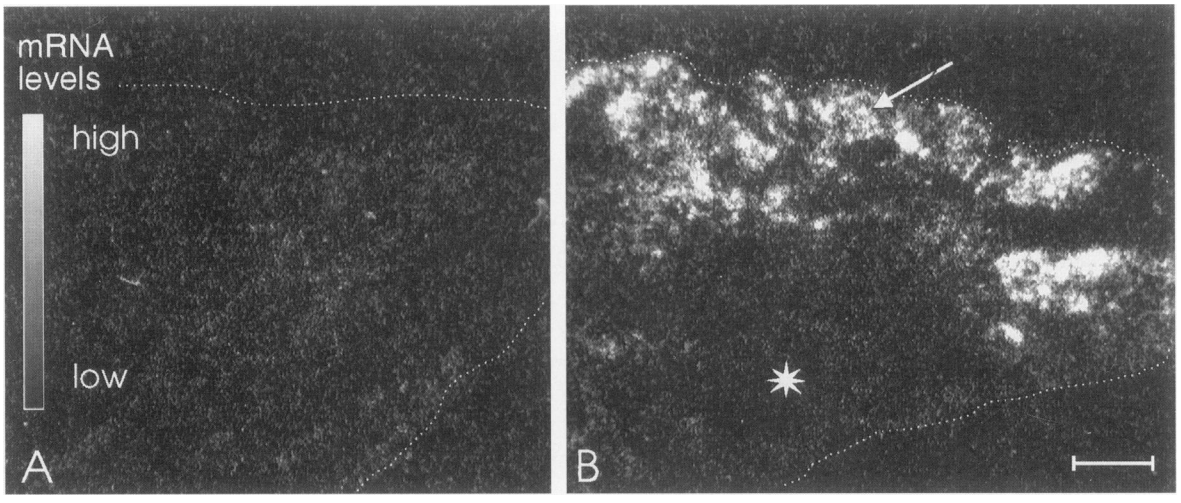

Figure 5. *Messenger RNA* in situ *hybridization of topo IIα. A low magnification autoradiogram of a tumor with low* (A) *and high* (B) *topo IIα mRNA expression level. The immunohistochemical topo IIα scores for these tumors were 1.7 and 16.9%, respectively.* Arrow *indicates the highly cellular tumor margin.* Dotted lines *show margins of the tissue section, and the* asterisk *shows a poorly cellular necrotic tumor area. Scale bar, 0.1 cm. Magnification, ×10.*

of 41 patients with acute myelogenous leukemia, no correlation between either isoform of topo II and clinical response to topo II inhibitors was identified.[24] However, the application of Western blotting as a quantitation method could not distinguish between normal bone marrow and leukemia cells, which may have made the detection less specific.[24] Therefore, the evaluation of topo IIα as a predictive factor for chemotherapy awaits to be studied from morphologically well defined malignant lesions. The current data (good correlation of topo IIα with previously described predictive factors, S-phase fraction, and c-*erb*B-2) suggests indirectly that topo IIα may be a strong predictor of response to chemotherapy. These indirect findings are supported by *in vitro* studies, in which the cellular concentration of topo IIα has been shown to correlate with chemosensitivity.[13,14]

Our present study is the first one in which the expression of topo IIα was correlated with the S-phase fraction in a large number of cancer samples. Although

Table 1. *Association of Topo IIα Expression with Clinicopathological Variables in 230 Primary Breast Cancers*

Variable	n	Topo IIα score (mean ± SD)	*P* value
All tumors	230	10.6 ± 7.9%	
Age			
<50 years	55	12.1 ± 8.1%	
>50 years	175	10.1 ± 7.8%	NS
Histological grade			
I	71	6.3 ± 5.1%	
II	106	11.3 ± 7.6%	
III	47	15.3 ± 8.4%	<0.0001
Tumor size			
<2 cm	127	9.7 ± 7.5%	
>2 cm	85	11.8 ± 8.1%	NS
Axillary nodal status			
Negative	117	9.8 ± 7.6%	
Positive	75	11.5 ± 7.7%	NS
DNA ploidy			
Diploid	94	8.1 ± 6.4%	
Aneuploid	101	12.0 ± 8.1%	0.0003
ER*			
Negative	59	16.8 ± 9.4%	
Positive	171	8.5 ± 6.0%	<0.0001
PR*			
Negative	102	13.4 ± 8.9%	
Positive	128	8.3 ± 6.2%	<0.0001
c-*erb*B-2 overexpression			
Negative	172	9.0 ± 6.7%	
Positive	41	15.8 ± 8.7%	<0.0001

NS, not significant.
*Negative, <20% immunopositive cells; positive, ≥20% immunopositive cells.

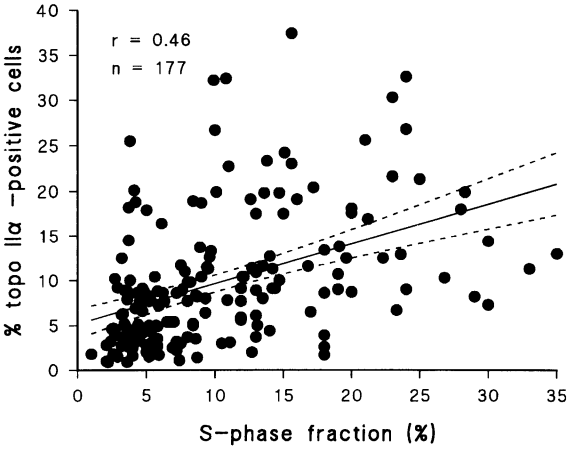

Figure 6. *Correlation between topo IIα score and flow cytometric S-phase fraction. The correlation coefficient* (r = 0.46) *is highly significant* (P < 0.0001). Dashed lines *show 95% confidence intervals for the regression line* (solid line).

Table 2. *Analysis of Co-Variance of Clinopathological Factors Associated with Expression of Topo IIα after Adjusting for Tumor Proliferation Rate (S-Phase Fraction)*

Variable	*F* value	*P* value
Aneuploidy	13.6	0.0003
c-*erb*B2 overexpression	6.8	0.01

The ratio of Topo IIα to S-phase fraction was used for analysis. Other co-variates studied were age of patient, tumor size, nodal status, ER and PR content, and histological grade. A total of 157 cases with complete data were analyzed.

a highly significant correlation ($r = 0.46$, $P < 0.0001$) was found between topo IIα and S-phase fraction, two additional clinopathological factors (aneuploidy and c-*erb*B-2 overexpression) showed proliferation-independent association with topo IIα. Among these, the association between topo IIα and c-*erb*B-2 is particularly interesting, because of simultaneous aberrations in topo IIα and c-*erb*B-2 genes[19,20] and the association of c-*erb*B-2 with resistance to cytotoxic chemotherapy.[42–44] Recently Muss et al[2] showed that patients whose tumors overexpress c-*erb*B-2 were relatively resistant to chemotherapy at conventional doses of the topo II inhibitor doxorubicin. The relative resistance could be overcome by increasing dosage of doxorubicin, the use of which improved survival.[2] The association of c-*erb*B-2 with chemosensitivity was obtained with a statistical multivariate analysis after adjustment for S-phase fraction and p53.[2] The high correlation of c-*erb*B-2 and topo IIα in our study brings up the need to evaluate the predictive value of c-*erb*B-2 together with topo IIα, the molecular target of many cytotoxic drugs (eg, doxorubicin).

Even though a close association between topo IIα, c-*erb*B-2, proliferation rate, and other indicators of high malignant potential was identified, only approximately one-half of the variability of topo IIα expression could be explained by these factors. Thus, these results suggest that topo IIα may have a unique, yet unidentified role as a predictive factor in breast cancer. Therefore, additional studies are needed to clarify whether topo IIα has value was a clinically relevant, predictive factor of response to topoisomerase inhibitor chemotherapy.

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