NEW POSSIBILITIES FOR THE TAILORED THERAPY OF BREAST CANCER PATIENTS

Ph.D. Thesis

Dr Nikolényi Aliz

Supervisor:

Prof. Zsuzsanna Kahán M.D., Ph.D.

Department of Oncotherapy

Faculty of Medicine, University of Szeged

Szeged, Hungary

Szeged

2011

List of full papers that served as the basis of the Ph.D. thesis

I.   **Nikolényi A.**, Sükösd F., Kaizer L., Csörgő E., Vörös A., Uhercsák G., Ormándi K., Lázár G., Thurzó L., Brodowicz T., Kahán Z. Tumor topoisomerase II alpha status and response to anthracycline-based neoadjuvant chemotherapy in breast cancer. *Oncology – accepted for publication*

IF: 2.390

II.   **Nikolényi A.**, Uhercsák G., Csenki M., Hamar S., Csörgő E., Tánczos E., Thurzó L., Brodowicz T., Wagnerova M., Kahán Z. Tumor topoisomerase II alpha protein expression and outcome after adjuvant dose-dense anthracycline-based chemotherapy. *Pathology and Oncology Research – under review*

IF:1.152

III.   Varga Z., Hideghéty K., Mező T., **Nikolényi A**., Thurzó L., Kahán Zs. Individual positioning: a comparative study of adjuvant breast radiotherapy in the prone vs. the supine position. *Int. J. Radiation Oncology Biol. Phys.* 2009; 75:94-100.

IF: 4.639

IV.   Kahán Z, **Nikolényi A**, Uhercsák G, Thurzó L. Neoadjuvant systemic therapy in breast cancer. *Orv Hetil.* 2009; 150:65-71.

Related article

I.   Varga L, Baradnay G, Hohn J, Simonka Z, Hideghéthy K, Maráz A, **Nikolényi A**, Veréb B, Tiszlavicz L, Németh I, Mán E, Lázár G. Klinikai és hisztopatológiai eredmények előrehaladott rectumtumorok neoadjuváns kezelése után. *Magy Onkol*. 2010; 54:129-135.

Table of contents

List of abbreviation

2D	two-dimensional
3D	three-dimensional
A	adriamycin
ADC	adriamycin (A)-docetaxel (D)-cyclophosphamide (C)
ANOVA	analysis of variance
ATC	adriamycin (A)-paclitaxel (T)-cyclophosphamide (C)
BMI	body mass index
C	cyclophosphamide
CECOG	Central European Cooperative Oncology Group
CEP17	centromere of the chromosome 17
CI	confidence interval
CLD	central lung distance
CR	complete regression
CT	computed tomography
D	docetaxel
DCIS	ductal cancer *in situ*
DRR	digitally reconstructed radiograph
DVH	dose-volume histogram
E	epirubicin
ED	epirubicin-docetaxel
EDC	epirubicin-docetaxel-capecitabine
ER	estrogen receptor
FEC	5-fluorouracil-epirubicin-cyclophosphamide chemotherapy
FISH	fluorescence in situ hybridisation
GCSF	granulocyte colony stimulating factor
H_2O_2	hydrogen peroxide
IHC	immunohistochemistry
LVI	lymphovascular invasion
MLD	mean lung dose
MRI	magnetic resonance imaging
OAR	organ at risk
OS	overall survival

pCR	patologically complete regression
PTV	planning target volume
PgR	progesterone receptor
PR	partial regression
RFS	recurrence-free survival
RNA	ribonucleic acid
SD	stable disease
T	paclitaxel
TMA	tissue micro array
TOP2A	topoisomerase II alpha
TRG	tumor regression grade
V_{5Gy}	volume receiving more than 5 Gy
V_{20Gy}	volume receiving more than 20 Gy
V_{25Gy}	volume receiving more than 25 Gy
V_{30Gy}	volume receiving more than 30 Gy
$V_{95\%-107\%}$	volume receiving at least 47.5 Gy, but less than 53.5 Gy

1.   Introduction

Breast cancer mortality shows a decline which is attributed in part to the wide-spread use of adjuvant treatments including systemic therapy and postoperative irradiation (1). The selection of the most appropriate individualized therapy is extremely important considering the efficiency and the long-term side-effects of the adjuvant and neoadjuvant treatments. The adjuvant systemic therapy reduces the risk of systemic relapse with 20-40%. (2). The long-term effect of the neoadjuvant systemic therapy are equivalent to that of adjuvant therapy (3). The anthracyclines have been widely used during the past 30 years for the adjuvant therapy of breast cancer, and have proved superior efficacy to non-anthracycline-containing regimens (4). The use of anthracyclines, however, involves a higher risk of long-term toxicity such as cardiac failure and myeloproliferative disease, and restriction of their use was suggested in view of the results of the adjuvant BCIRG006 Trial (5). There is clearly a need for the identification of predictive factors and the selection of cancers likely to benefit most from the use of anthracyclines. Many experimental and clinical data support the possible role of the topoisomerase II alpha (TOP2A) status of the tumor in the prediction of anthracycline sensitivity. TOP2A is an enzyme that plays a pivotal role in DNA replication and cell proliferation (6-8). Targeted inhibition of this enzyme at a molecular level is responsible for the cytotoxic effect of the TOP2A inhibitor anthracyclines. TOP2A is located on chromosome 17 q12-q21, next to the HER2 gene, and its aberrations (amplification or deletion) have been demonstrated mostly (6,7), but not exclusively (9), in HER2-positive breast cancers. Around one-third of all HER2-positive breast tumours, and at least one-tenth of all breast cancers, present with TOP2A gene amplifications, and 4-13% with deletion of the gene (5,7,9-16). The protein expression of TOP2A does not depend on the presence of gene aberrations (14,15,17-20), and is highly regulated at the RNA level (21). Both TOP2A gene abnormalities (5,9-14,17,22) and high TOP2A expression (15,22,23) have been related to the greater efficiency of anthracycline-based chemotherapy.

Adjuvant radiotherapy is the standard treatment after breast-conserving surgery and in well-determinated cases after mastectomy, too. Modern CT-based 3D conformal radiotherapy is able to reduce the radiation dose to the organs at risk, and to improve dose homogenity within the target volume. The simplest possibility to

protect the OARs is individual patient positioning during breast radiotherapy. Prone positioning has been shown to reduce the dose to the ipsilateral lung and the heart during breast radiotherapy.

2. Aims

2.1.   We aimed at performing a retrospective study of the presence of gene abnormalities and the expression of TOP2A in a cohort of breast cancers treated with neoadjuvant anthracycline-based chemotherapy.

2.2.   We set out to perform a retrospective study of the expression of TOP2A in 3 cohorts of breast cancers treated with adjuvant dose-dense anthracycline-based chemotherapy, with the aim of an analysis of the TOP2A status in relation to other tumour features and the outcome.

2.3.   We initiated a prospective study to compare radiotherapy in the prone position with our usual technique in the supine position with excellent repositioning accuracy. The identification of those patients who benefit from prone positioning by means of dosimetry (dose homogeneity and protection of the OARs) and feasibility.

3. Patients and methods

All the clinical studies had been approved by the Institutional Review Board of the University of Szeged, and all the enrolled patients gave their written informed consent before being registered as participating in the study.

3.1. *Tumor topoisomerase II alpha status and response to anthracycline-based neoadjuvant chemotherapy in breast cancer*

3.1.1. *Patients*

Patients with operable T2≥3 cm or T3-4 and/or N1-2 and M0 breast cancer were eligible (if clinically node-positive, T1 tumor size was permitted). Through physical examination, mammography, ultrasonography and breast MRI, the initial local/regional tumor status and that after six cycles of chemotherapy were evaluated. Via core needle biopsy, 3 tissue cylinders were taken in each case preoperatively with a 16 G core needle for histopathological examinations.

3.1.2. *Methods*

Chemotherapy

All patients received docetaxel 75 mg/m^2 and epirubicin 75 mg/m^2 on day 1 (ED regimen), which in the case of tumor stage T1-T3 was supplemented with capecitebine 2x1000 mg/m^2 daily on days 1-14 (EDC regimen), irrespective of the nodal status.

Tissue micro array (TMA) construction

From the biopsied tissues or postsurgical specimens, an experienced pathologist selected the most cellular region. A tissue core 2 mm in diameter was punched for the TMA and embedded in an acceptor block. Slides for TOP2A FISH and IHC examinations were made from every block.

Fluorescent in situ hybridization (FISH)

The TMA slides were subjected to triplet color FISH assay (LSI TOP2A spectrum Green /HER2 spectrum Orange/ CEP 17 Spectrum Aqua, Vysis, Downers Grove, IL, USA) for simultaneous evaluation of TOP2A and HER2 genes and chromosome 17-copy number according to the manufacturer's instructions. A ZEISS Axioimager Z2 fluorescence microscope and the Mark and Find System (Carl Zeiss, AxioVision 4.8) were used to identify every spot, in each of which 20 cells were counted and the number of gene copies was assessed. The numbers of the green signals of

TOP2A and the orange signals of the HER2 gene and centromere 17 (CEP17) were recorded for each nucleus, and the ratios of the numbers of signals for the gene probes TOP2A and HER2 divided by the number of signals for CEP17 were calculated. TOP2A/CEP17 and HER2/CEP17 ratios >2.2 were defined as gene amplification, and those of <0.8 as deletion. Polysomy was taken as 5 or more copy numbers of centromeres for chromosome 17 per cell. (Fig.1.)
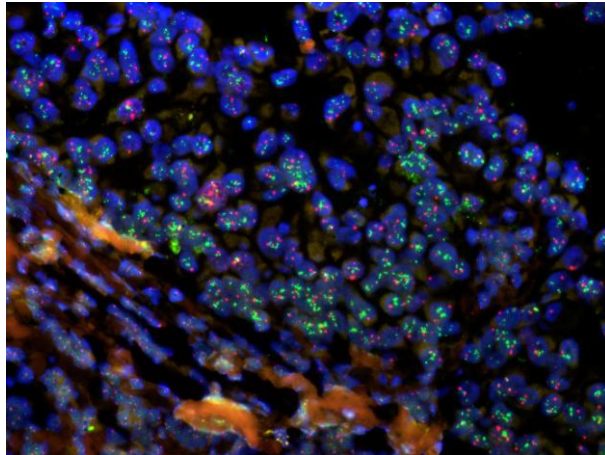

Figure 1. Simultaneous evaluation of TOP2A and HER2 genes and chromosome 17-copy number with triplet color FISH assay (TOP2A: green, HER2: orange, CEP 17: aqua). A case of TOP2A amplification.

Immunohistochemistry (IHC)

IHC was done on paired tumor samples taken from the pretreatment biopsies and surgical specimens. All samples were formalin-fixed and paraffin-embedded. If the complete disappearance of the cancer was obtained, only the pre-chemotherapy value could be determined.

ER, PgR, HER2 and Ki67 IHC was carried out with an automatic staining system applying the peroxidase-streptavidin-biotin technique (Dako Autostainer). A peroxydase-based detection system was used according to the manufacturer's instructions. For HER2 IHC, the HercepTest (Dako Glostrup, Denmark) was used. The Ki67 labeling index was assessed with the MIB1 monoclonal antibody. The threshold for ER or PgR positivity was 10%. HER2 expression was scored semiquantitavely according to the ASCO/CAP guidelines.

TOP2A IHC involved use of the primary specific monoclonal antibody Topoisomerase II alpha Ki-s1 (Lab Vision, Fremont, CA, USA). Antigen retrieval

was achieved by autoclaving in citrate buffer, pH 6.0, for 10 min at 121°C, and an EnVision + System (Dako) was applied as the detection system. Immunostained sections were evaluated by two independent pathologists who had no prior knowledge of the clinicopathologic variables. Each pathologist counted at least 50 cells within randomly selected and outlined areas on each slide, and the percentage of immunostained cells was determined. Disagreement between the pathologists prompted reassesment of the results and a consensus was reached by a joint re-evaluation of the slide.

A cut-off value of 15% separated negative (≤15%) and positive cases (>15%). For HER2 IHC, the standard method was used. HER2 expression was scored semiquantitatively with scores 0-3+, following the accepted criteria; HER2 2+ was regarded as indeterminate and required HER2 FISH examination. We used Ki67 labeling indices as continuous variables, thus no threshold was used in the analyses. (Fig.2.).

Figure 2. TOP2A protein expression in the nuclei of the tumor cell (20 x magnification)

Evaluation of the tumor response, the relapse free survival and the overall survival
The tumor characteristics were determined with standardized methods. Tumor regression was graded via the semiquantitative scoring system developed by Sinn et al. A pCR was taken as the absence of any invasive or *in situ* tumor in the breast or the axilla. Analyses were carried out on the associations of the tumor response, the RFS and the OS with the tumor characteristics, such as the histological type, the pathological stage, the grade, the ER, PgR and HER2 status, the Ki67 and TOP2A

protein expressions and the amplification of the TOP2A gene, and the relation between the tumor characteristics before and after chemotherapy.

Statistical analysis

The associations between the binary or multiple versus the continuous variables were analyzed by the independent sample t-test or one-way ANOVA, respectively. The relationships of the qualitative data were tested by chi-square tests. To examine the changes in the tumor markers after chemotherapy, the paired sample t-test and McNemar test were used for the continuous and categorical variables, respectively. The relationship between the continuous variables was examined by correlational analysis. The effects of the tumor markers on RFS and OS were analyzed with the linear regression model. SPSS version 17.0 for Windows (SPSS Inc., Chicago, IL, USA) was applied for statistical analysis.

3.2. *Tumor topoisomerase II alpha protein expression and outcome after adjuvant dose-dense anthracycline-based chemotherapy*

3.2.1. *Patients*

Data from 3 phase II clinical studies with adjuvant dose-dense anthracycline-based chemotherapy were collected. In the dose-dense sequential adriamycin (A)-paclitaxel (T)-cyclophosphamide (C) chemotherapy study (ATC group), 55 high-risk breast cancer patients were treated. In the very similar dose-dense sequential adriamycin (A)-docetaxel (D)-cyclophosphamide (C) chemotherapy study (ADC group), 34 breast cancer patients were treated. Of the 34 patients enrolled, 33 (97%) completed all 12 cycles, whereas one was excluded after the first 7 cycles because of disease progression. In the dose-dense FEC study (CECOG group), most of the enrolled 51 patients completed the study, but the clinical data and the tumour samples were accessable in only 43 cases treated at the Hungarian and the Slovakian centres.

3.2.2. *Methods*

Chemotherapy

Patients received 60 mg/m^2 A for 4 cycles, 200 mg/m^2 T for 4 cycles, and 800 mg/m^2 C for 4 cycles, all chemotherapy cycles 2 weeks apart with GCSF support in the A-T-C group (24). Patients received 60 mg/m^2 A for 4 cycles, 75 mg/m^2 D for 4 cycles, and 800 mg/m^2 C for 4 cycles, all chemotherapy cycles 2 weeks apart, with GCSF support In the A-D-C group. Patients were randomized to 6 cycles of FEC$_{75}$

or FEC_{90} (fluorouracil 500 mg/m^2, epirubicin 75 or 90 mg/m^2, respectively and cyclophosphamide 500 mg/m^2) with pegfilgasrtim support in the CECOG group (25)

Evaluation of the tumor features, the relapse free survival and the overall survival

Analyses were carried out on the associations of the RFS and the OS with the tumour characteristics.

3.3.   *The effect of individual postioning on the radiation exposure of the risk organs*

3.3.1.   *Patients*

Early breast cancer patients after surgery requiring only radiotherapy of the operated breast were included in the study. In the first phase of the study (n=20), although radiotherapy planning was performed in both positions, all patients received radiotherapy in the supine position. The 41 patients enrolled in the second phase were randomized to radiotherapy in the prone *vs.* the supine position, but the position for radiotherapy randomized to the patient was blinded to the physician who performed the contouring.

3.3.2.   *Methods*

Radiotherapy

The patients were positioned on the supine thorax and the prone breast modules of the AIO (All In One) Solution^{TM} (ORFIT, Belgium) system, which contains special cushion sets fixed to a universal baseplate. In the supine position, the patient was laid on a 15° thorax wedge cushion with both arms elevated, resting on an arm support, and held on an adjustable grip pole. The head was placed in the head support secured to a supplementary baseplate attached to the thorax cushion. In the prone position, the head was resting on a pillow, both arms were placed superolaterally, supported by the cranial part of the prone breast cushion, and the target breast was hanging across the semicircular aperture of the platform. The patient was rotated slightly so as to allow the ipsilateral chest wall to extend into the aperture. A thermoplastic mask (5-point fixation, breast precut; ORFIT, Belgium) was applied in the supine position, moulded around the chin, the neck, the thorax (excluding the target breast) and the abdomen. The opposite breast was covered with the mask and carefully positioned away from the radiation fields. Mask fixation was not used in the prone position, but a polyfoam wedge was placed under

the contralateral breast in order to displace it. Based on the experience gained during the first phase of the study, in the second 41 patients, a different polyfoam wedge was applied as a new development of the AIO system, for better protection of the opposite breast (Fig. 3).
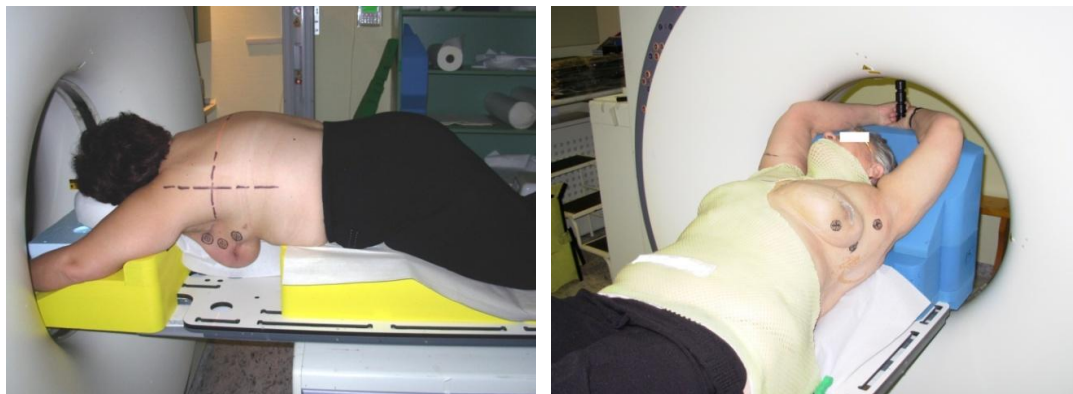

Figure 3. Typical prone and supine positioning during breast radiotherapy

Positioning landmarks were drawn on the skin or the mask, using two lateral lasers and one overhead laser. All patients were scanned on a Somatom Emotion 6 CT simulator (Siemens, Germany) in both positions. The planning target volume (PTV) and OARs were contoured on the CT slices throughout the entire planning volume in the XIO^{TM} (CMS) treatment planning system, according to the local protocol (26) The PTV was defined as the entire breast delineated on the CT data set, extending to within 4 mm of the skin surface. Individual conformal radiotherapy plans were generated. A mean dose to the PTV of 50 Gy, and a uniform distribution (±10%) of the prescribed dose to 95% of the PTV, were aimed at. Dose homogeneity within the PTV was characterized by the volume of the breast receiving at least 47.5 Gy, but less than 53.5 Gy ($V_{95-107\%}$). The radiation exposure of the OARs (the volume of the ipsilateral lung receiving more than 20 Gy [V_{20Gy}], the mean lung dose [MLD], the mean dose to the heart [MHD], the volume of the heart receiving more than 25 or 30 Gy [V_{25Gy} and V_{30Gy}], the volume of the contralateral breast receiving more than 5 Gy [V_{5Gy}] and the mean dose to the contralateral breast) was registered in both positions. The central lung distance (CLD) and breast separation were determined in the supine position as measures of the patient anatomy.

Evaluation of repositioning accuracy

The objectives in the second phase of the study were patient adherence to the protocol and repositioning accuracy and toxicity during radiotherapy. Prior to the commencement of radiotherapy, the position of the isocenter in the patient was checked under the CT simulator. The necessary displacement in 3D was registered as the first datum of the repositioning accuracy. The radiotherapy was delivered with a linear accelerator (Primus, Siemens) in 5 fractions per week. The accuracy of patient repositioning during radiotherapy was checked 3 times per week with an electronic portal imaging device (Beamview[TM] vs. 2.2, Siemens), with the help of radiopaque markers placed on the skin/mask as reference markers. One portal image for one of the tangentional beams was recorded, and compared with the corresponding beam's eye view digitally reconstructed radiograph generated from the planning system. The need to correct the position of the table in 2D was established and recorded. Analysis of each port image involved determination of the distances between the radiopaque skin markers, and measurements of the CLD, the lung area included in the field, the central flash distance and the inferior central margin (27,28). The action level was set at 3 mm. Systematic and random errors generated from the 3D vector of displacement during the CT simulation and the 2D vector of displacement during the radiotherapy were calculated according to conventional definitions (29,30). Acute skin reactions (graded by the CTC AE vs. 3.0) were compared in 41 patients randomized to radiotherapy the prone *vs.* the supine position, at the end of the whole breast irradiation.

Statistical analysis

The relations between the data obtained by analysis of the radiotherapy plans and repositioning accuracy *vs.* the patient characteristics were analyzed with the aid of the Student t-test, the chi-square test, regression analysis, ANOVA and logistic regression. Statistical analysis was performed with SPSS 11.0 for Windows (SPSS Inc., Chicago, IL).

4.   Results

4.1.   *Tumor topoisomerase II alpha status and response to anthracycline-based neoadjuvant chemotherapy in breast cancer*

Between 12/2003 and 08/2010, 43 patients (with 45 tumors) received neoadjuvant anthracycline-based chemotherapy: 12 patients with the ED regimen (one with bilateral tumor) and 31 patients with the EDC regimen (one with bilateral tumor). The mean age (±SD) of the patients was 47.2 (±12.8) years. Forty-two patients (97.7%) completed 6 cycles of chemotherapy, while one patient received only 5 cycles of ED because of disease progression. Complete regression (CR) was revealed by the imaging methods in 15 cases (33.3%), and partial regression (PR) in 26 cases (57.8%); 3 cases (6.7%) did not indicate any significant change (stable disease, SD), while 1 case (2.2%) progressed. Most patients participated in mastectomy (62.2%) and axillary block dissection (97.8%) after the chemotherapy, but 1 patient did not undertake surgery. The initial and post-chemotherapy clinical tumor stages are included in Table 1.

	T					N				Stage			
	T0	T1	T2	T3	T4	N0	N1	N2	N3	0	I	II	III
Pre-chemotherapy (n=45)	0	3	17	18	7	12	9	21	3	0	0	14	31
Post-chemotherapy (n=44)	12	17	13	2	0	25	12	5	2	10	11	15	9

Table 1 Pre-chemotherapy (clinical) and post-chemotherapy (pathological) tumor and lymph node status before and after neoadjuvant chemotherapy according to the UICC/AJCC TNM classification. Note that one patient did not undergo surgery after neoadjuvant chemotherapy.

About half of the tumors were ER-positive, and one-third of them PgR-positive. HER2 positivity was demonstrated by HER2 IHC and/or FISH in 18% of all samples (Table 2).

Tumor feature		N	%
Histological type	IDC	38	84.4
	ILC	3	6.7
	other	4	8.9
Histologic grade	1	0	0
	2	11	24.4
	3	33	73.3
	unknown	1	2.2
ER	negative	25	55.6
	positive	20	44.4
PR	negative	28	62.2
	positive	17	37.8
HER2	negative	37	82.2
	positive	8	17.8
Proportion (±SD) of Ki67-positive cells (%)	56.1±23.6		
TOP2A	Negative (≤15%)	6	15.8
	Positive (>15%)	32	84.2
	unknown	7	
Proportion (±SD) of TOP2A-positive cells (%)	41.0±27.9		

Table 2 Pathological features of breast cancers before neoadjuvant chemotherapy

No significant change was observed in the ER, PgR or HER2 status of the tumors after chemotherapy. The proportion of Ki67-positive tumor cells was significantly reduced by the chemotherapy (56.1±23.6 vs. 19.0±27.7%, p=0.004).

The pathological tumor responses to chemotherapy are listed in Table 3.

Histological tumor regression grade (TRG)	Overall n=44 (%)	EDC n=31 (%)	EC n=13 (%)
TRG0	1 (2.3)	0 (0.0)	1 (7.7)
TRG1	20 (45.5)	16 (51.6)	4 (30.8)
TRG2	10 (22.6)	6 (19.4)	4 (30.8)
TRG3	1 (2.3)	1 (3.2)	0 (0.0)
TRG4	12 (27.3)	8 (25.8)	4 (30.7)

Table 3 Pathological tumor response after neoadjuvant ED or EDC chemotherapy (p=0.50)

Although complete disappearance of the primary tumor (TRG 4) was detected in 12 cases, the axillary lymph nodes were still involved in 3 of these cases, and 9 (20%) cases were therefore classified as pCR. No significant difference existed between tumor response according to the chemotherapy regimen (p=0.50): the proportions of major tumor responses (TRG3-4) were 29% (n=9) and 30.7% (n=4) among the patients treated with the EDC or the ED regimens, respectively (Table 3), while the respective rates of pCR were 22.6% (n=7) and 15.4% (n=2). In an additional lymph node-negative case, only a small DCIS focus remained. The association between the clinical and pathological tumor responses proved to be statistically significant (p<0.001).

TOP2A FISH/IHC

For technical reasons, the TOP2A FISH and TOP2A IHC results were assessable in only 25 and 38 cases, respectively. With FISH, 23 tumors (92%) exhibited a normal TOP2A gene copy number, while in 2 (8%), the TOP2A gene was amplified; both were HER2-positive by means of IHC and FISH. Despite the fact that the median proportion of IHC-stained cells was 50%, in view of the reference data in the literature (31-33), we used >15% as a cut-off value for the definition of TOP2A positivity (Table 2). Thirty-two (84.2%) tumors were classified as TOP2A-positive

and 6 (15.8%) as TOP2A-negative from the core biopsy. No significant correlation was found between the TOP2A status as determined by FISH and IHC (p=0.52). The average (±SD) proportion of TOP2A-positive cells in the evaluable samples was 41.0±27.9% before, and 12.7±24.8% after the chemotherapy (p<0.001). The expression of TOP2A showed a strong correlation with that of Ki67 (R=0.743, p<0.001), and was negatively correlated with ER (R=0.404, p=0.012) and PgR (R=0.430, p=0.007) (Fig. 4), irrespective of the HER2 status (data not shown).
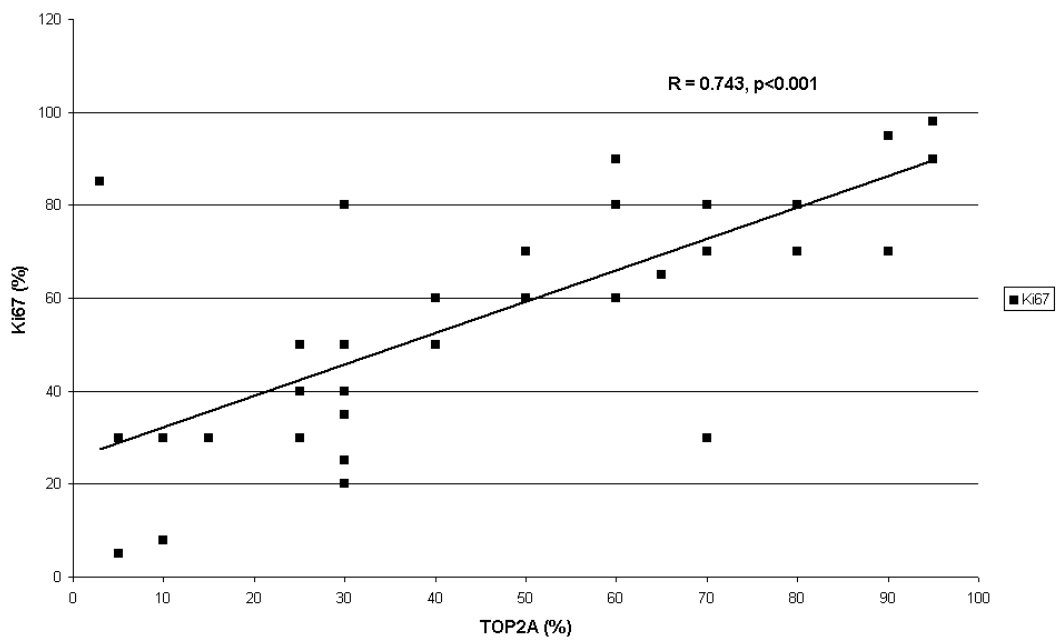

Figure 4. Correlation between the expression of TOP2A and Ki67

The expression of TOP2A was not related to the HER2 status of the tumor (Table 4).

	HER2-positive	HER2-negative	P
TOP2A-positive	6 (100.0 %)	26 (81.2%)	0.328
TOP2A-negative	0 (0.0 %)	6 (18.8%)	
TOP2A mean (±SD)	37.5±16.7	50.9±29.5	0.291

Table 4 TOP2A protein expression according to the HER2 status of the tumor (n=38)

Grade 3 cancers displayed higher TOP2A and Ki67 expressions than those of grade 2 cancers (Table 5).

	Grade 2 (%)	Grade3 (%)	p
Ki67 mean (±SD)	42.3±26.6	62.3±24.8	0.036
TOP2A mean (±SD)	35.5 ±26.4	53.5±27.6	0.082

Table 5 Association between tumor grade and initial Ki67 and TOP2A expression

Association between tumor response and tumor characteristics

A major tumor response was seen mostly for grade 3 and ER-negative cancers (Fig. 5.).
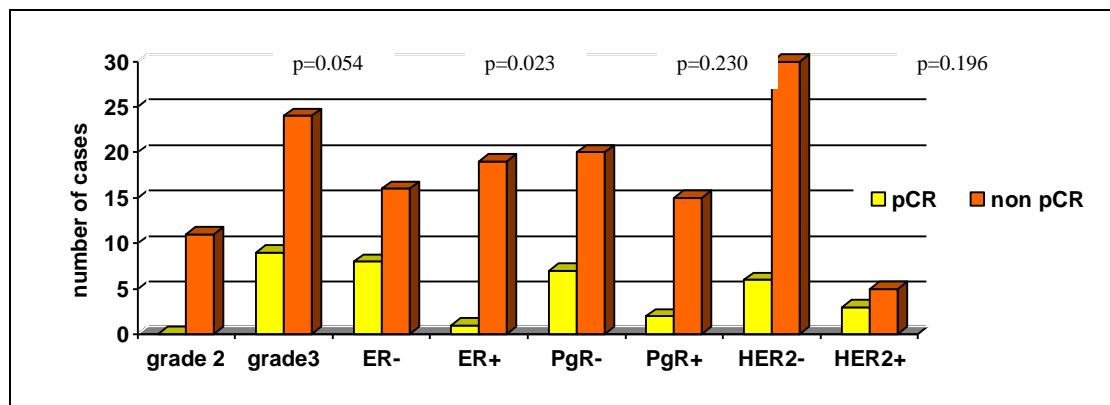

Figure 5. Tumor characteristics in relation with tumor response to neoadjuvant anthracycline-based chemotherapy (n=44)

The development of pCR was related to high grade (grade 3) (p=0.054) and ER negativity (p=0.027). While the mean (±SD) pre-chemotherapy TOP2A expression was 66.9±26.3% in cases with pCR, it was 41.8±26.6% in cases without pCR (p=0.037). Eight pCRs (21%) occurred among those cases that were assessed for TOP2A IHC, and all the pCRs occurred in TOP2A-positive cancers. Although no association was found with TOP2A amplification, both TOP2A-amplified tumors gave a major response: pCR in one, and a reduction in tumor size from 70 to 15 mm in the other. Ki67 was not predictive of the tumor response in univariate analysis (OR=1.027, 95% CI: 0.992-1.062, p=0.167). In the logistic regression model including the grade, ER, the expression of TOP2A was an independent predictor of pCR (OR=1.460, for every 10% increase, 95% CI: 1.016-2.096, p=0.041).

Survival

The median follow-up time was 31.0 months. Fourteen patients developed local or distant recurrence, and 3 died. The median RFS and OS were 23.7 and 31.0 months, respectively (Fig. 6).
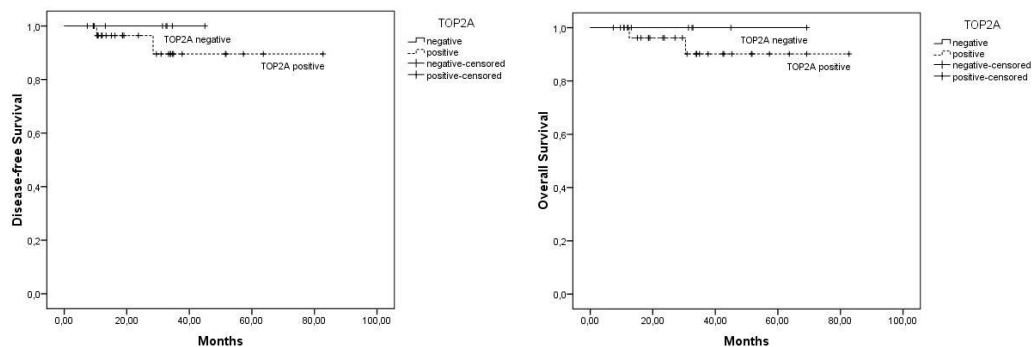

Figure 6. Survival (DFS and OS) according to the TOP2A status of the breast cancer

RFS was shorter in cases with PgR-negative than in those with PgR-positive cancers (23.0 vs. 32.6 months, p=0.07, linear regression: R=0.350, p=0.018), but OS did not depend on any of the tumor features. The RFS and OS were not related to the tumor response or the decrease of TOP2A protein expression.

4.2.   *Tumor topoisomerase II alpha protein expression and outcome after adjuvant dose-dense anthracycline-based chemotherapy*

The patient- and tumour-related characteristics within the 3 study cohorts and in the overall population are included in Table 6.

	ATC (n=55)	ADC (n=34)	CECOG (n=43)	Overall (n=132)
Age (mean±SE)	59.8±1.2	54.9±1.6	54.9±1.6	57.0±0.8
pT (mean±SE, mm)	35.6±2.8	16.7±2.7	22.5±2.3	26.3±1.6
pN+ (median)	6	0.5	2	3
Histological type (%) IDC ILC Medullary Other	43 (78.2) 6 (10.9) 1 (1.8) 5 (9.1)	30 (88.3) 1 (2.9) 0 (0) 3 (8.8)	35 (81.4) 4 (9.3) 3 (7.0) 1 (2.3)	108 (81.8) 11 (8.3) 4 (3.0) 9 (6.9)
LVI present (%)	38 (69.1)	9 (26.5)	28 (65.1)	75 (56.8)
Grade 1 **Grade 2** **Grade 3** **Grade unknown**	3 (5.4) 10 (18.2) 27 (49.1) 15 (27.3)	0 (0) 12 (35.3) 17 (50) 5 (14.7)	2 (4.7) 18 (41.9) 22 (51.2) 1 (2.3)	5 (3.8) 40 (30.3) 66 (50.0) 21 (15.9)
ER positive (%)	25 (45.5)	17 (50)	16 (37.2)	33 (25)
PgR positive (%)	23 (41.8)	15 (44.1)	15 (34.9)	30 (22.7)
HER2 positive(%)	13 (23.6)	9 (26.5)	7 (16.3)	29 (22.0)
Ki67 (mean±SE, %)	29.3±4.2	25.0±4.5	42.3±5.5	32.3±2.7
Ki67 (median, %)	20	20	30	25

Table 6 Patient- and tumour-related characteristics within the study groups and the overall population

The median follow-up time for the entire population was 64.5 months, and for the ATC, ADC and CECOG cohorts was 103, 44.5 and 60 months, respectively. Altogether 31 relapses (23.5%) and 23 deaths (17.4%) occurred. The OS differed significantly in the 3 cohorts: the ATC cohort exhibited the worst, and the ADC cohort the best survival (p<0.01). Among the standard prognostic factors, the pathological tumor size (pT) and the number of positive lymph nodes were associated with the RFS in the overall study population (p<0.05), while the presence of LVI was related to the RFS in the ADC cohort.

TOP2A IHC

For technical reasons, the TOP2A IHC results were assessable in only 106 cases. In the overall population, the average and median proportions of the TOP2A-positive cells were 21% and 10%, respectively. With a cut-off value of 15%, 48% of the tumours were classified as TOP2A-positive (Table 7).

	ATC	ADC	CECOG	Overall
TOP2 A IHC (n)	40	27	39	106
TOP2A (mean±SE, %)	18.3±3.4	17.33±5.0	24.5±5.0	21.02±2.3
TOP2A (median, %)	10	15	10	10
TOP2A+ (n)(%)	16 (40)	14 (51.9)	21 (53.8)	51 (48.1)

Table 7 TOP2A IHC status in the study groups and the overall population

Most of the TOP2A-positive tumours were of grade 3 (p=0.004). The expression of TOP2A correleted significantly with that of Ki67 (R=0.532, p<0.001), but not with ER or PgR. Among the ER- and/or PgR-positive cancers, more were TOP2A-negative than among the ER- and PgR-negative cancers (p=0.021 and p=0.002, respectively) (Table 8).

	ATC (n=40)		**ADC** (n=27)		**CECOG** (n=39)		**Overall** (n=106)	
	TOP2 A-	TOP2A +	TOP2 A-	TOP2A +	TOP2 A-	TOP2A +	TOP2 A-	TOP2A +
ER-	11	11	6	8	5	13	22	32
ER+	13	5	7	6	13	8	33	19
p	0.203		0.706		0.054		0.021	
PgR -	10	12	7	10	6	15	23	37
PgR +	14	4	6	4	12	6	32	14
p	0.054		0.440		0.026		0.002	

Table 8 TOP2A IHC status according to the ER/PR status of the tumour

All hormone receptor-negative cancers were of grade 2 or 3, and TOP2A-positive cases were more frequently of grade 3 (p=0.066 and p=0.040 in the ER-negative and the PgR-negative groups, respectively). No association was detected between the TOP2A status and the grade of the tumour in the hormone receptor-positive group. The expression of TOP2A was not related to the tumour size, the number of positive nodes or the HER2 status of the tumour. The protein expressions of TOP2A and Ki67 increased with the grade (p=0.162 and p=0.005, respectively).

Association between outcome and tumour TOP2A status

In the overall population, more relapses and more deaths occurred among the TOP2A-negative cases than among the TOP2A-positive cases, and the RFS and OS were longer accordingly (Table 9, Fig. 7.).

TOP2A IHC	number of deaths (%)	OS (mean±SE) (months)	number of relapses (%)	RFS (mean±SE) (months)
Negative	14/55 (25.5)	93.3±6.0	14/55 (25.5)	93.7±6.1
Positive	6/51 (11.8)	103.8±4.3	8/51 (15.7)	96.8±5.9
p (Mantel-Cox)		0.081		0.229

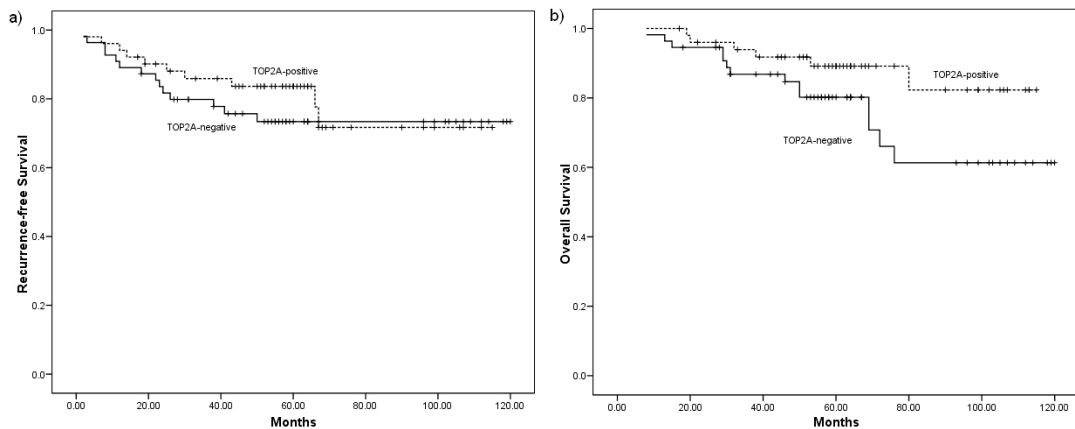**Table 9** Survival (OS and RFS) according to the TOP2A status of the tumour

Figure 7. Survival (OS and RFS) according to the TOP2A status of the tumour

The outcome in the hormone receptor-positive and hormone receptor-negative subgroups was analysed separately (Table 10, Fig. 8).

	ER-negative		ER-positive		PgR-negative		PgR-positive	
	TOP2 A-	TOP2 A+	TOP2 A-	TOP2 A+	TOP2 A-	TOP2 A+	TOP2 A-	TOP2 A+
number of deaths	6/22	2/32	8/33	4/19	7/23	2/37	7/32	4/14
OS (mean±SE) (months)	93.1± 9.3	109.3± 3.9	92.9± 7.6	82.7± 6.8	89.0± 9.6	110.5± 3.1	95.7± 7.4	81.3± 10.2
p (Mantel-Cox)	0.035		0.916		0.005		0.494	
number of relapses	7/22	5/32	7/33	5/19	7/23	6/37	7/32	4/14
RFS(mean ±SE) (months)	87.2± 10.3	97.1± 7.5	97.2± 7.3	77.6± 8.1	87.3± 10.3	97.2± 6.7	97.7± 7.1	79.5± 11.2
p (Mantel-Cox)	0.176		0.774		0.169		0.639	

Table 10 Survival (OS and RFS) according to the TOP2A and ER/PR status of the tumor
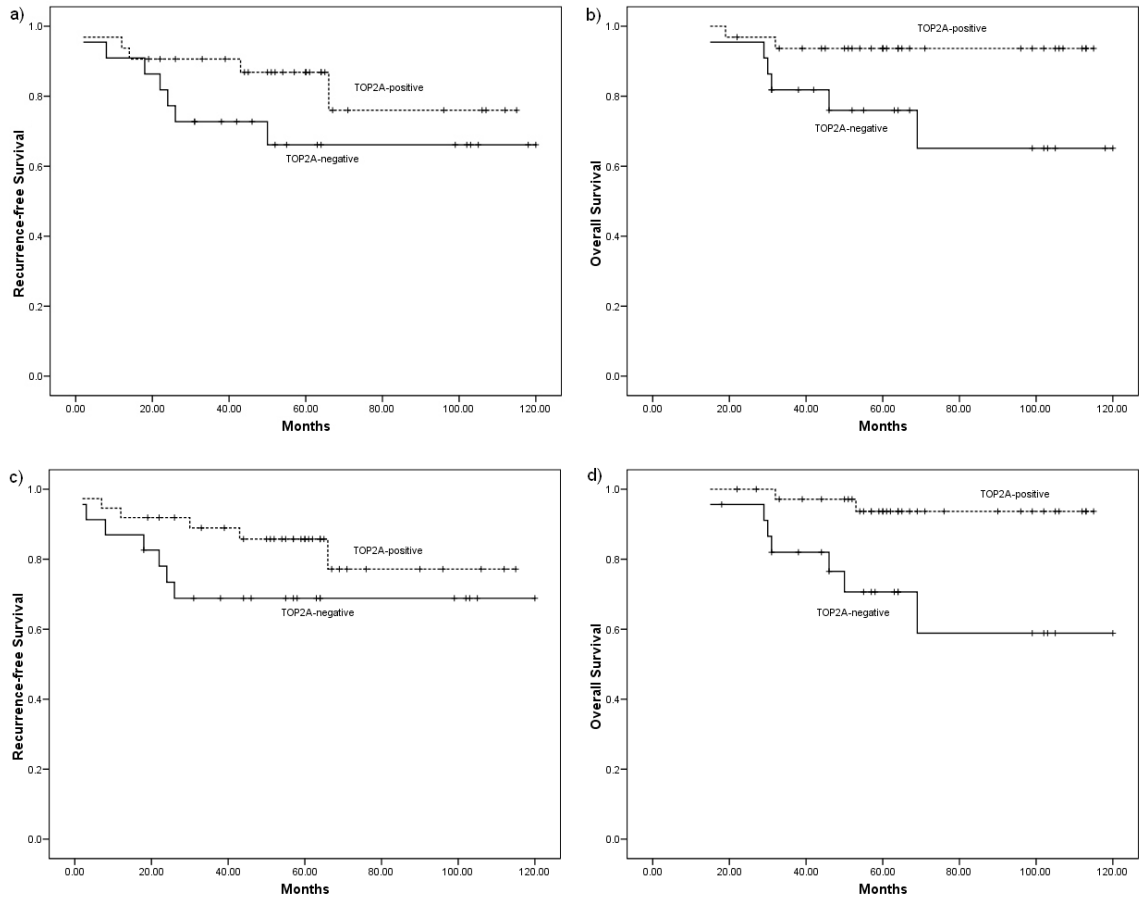
Figure 8. **a,c** Survival (OS and RFS) according to the tumor TOP2A IHC status in the ER and/or PR positive tumors

Figure 8 b,d Survival (OS and RFS) according to the tumor TOP2A IHC status in the ER and/or PR negative tumors

While there was no difference in the number of events, or in the OS and the RFS in the ER- and the PgR-positive subgroups according to the TOP2A status, the OS and RFS were significantly improved in the ER- or PgR-negative and TOP2A-positive cases as compared with the TOP2A-negative cases (Table 10, Fig. 8). Figure 7 presents the RFS and OS as functions of the TOP2A expression status in ER/PR-negative cases.

In order to estimate the dependence of the OS and the RFS on the tumour TOP2A and Ki67 status, the tumour grade and the nodal status in ER- and/or PgR-negative cancer, these variables were studied in a Cox proportional hazards model. In grade 3 cases, the risk of death was decreased, with HR= 0.216 (95% CI: 0.047-0.990, p=0.048) as compared with grade 2 cases. In the TOP2A-positive cases, the risk of death was decreased, with HR=0.211 (95% CI: 0.042-1.05, p=0.056). In multivariate analysis, no interaction was detected between these variables.

4.3.   *The effect of individual postioning on the radiation exposure of the risk organs*

4.3.1.   *General statistics*

The first phase of the study and the second, feasibility phase involved 20 and 41 patients, respectively. The mean (±SD) age of the overall study population was 56.0±9.6 (29.3-73.9), and that in the second phase was 56.6±9.9 (29.3-73.6) years. Twenty-seven patients needed right-sided, and 34 underwent left-sided breast irradiation. The age, weight, waist, hip size and breast separation did not differ significantly between the patients randomized to radiotherapy in the prone or the supine position (Table 11).

	Age (years)	Weight (kg)	Height (cm)	BMI (kg/cm^2)	Waist size (cm)	Hip size (cm)	Breast separation (cm)
Supine n=21	59.1±9.3 (42.1-75.0)	71.6±12.4 (52.0-96.0)	162.1±7.7 (150-175)	27.2±3.9 (20.9-33.2)	93.3±14.4 (78-145)	107.4±12.1 (95-150)	21.1±2.7 (16.4-26.9)
Prone n=20	56.9±10.7 (30.7-72.4)	69.9±12.4 (50.0-102.0)	161.0±4.3 (152-168)	27.1±5.3 (17.7-38.9)	89.3±10.6 (69-108)	104.4±9.9 (87-124)	20.7±3.1 (14.2-26.9)
p	0.49	0.66	0.56	0.94	0.32	0.40	0.64

Table 11 Patient characteristics (mean±SD) among patients randomized to radiotherapy in the prone *vs.* the supine position

Tumor bed boost irradiation and systemic treatments did not differ significantly between the two groups.

4.3.2. *Radiation plans for the prone vs. the supine position*

The radiotherapy plans were first analyzed in the overall population. The mean (±SD) percentage PTV covered by 47.5-53.5 Gy ($V_{95-107\%}$) in the prone *vs.* the supine position was 85.1±4.2% and 89.2±2.2%, respectively (p<0.0001). The dose homogeneity did not depend on the PTV or the breast separation. The irradiated volume of and the dose to the ipsilateral lung determined in terms of the MLD and the V_{20Gy} were dramatically lower in the prone position than in the supine position (Table 12).

	Lung (n=61)		Heart (n=34)		
	MLD (Gy)	V_{20Gy} (%)	Mean dose (Gy)	V_{25Gy} (%)	V_{30Gy} (%)
Supine	7.45±2.62	14.3±5.4	3.51±2.33	4.7±4.6	4.1±4.3
Prone	2.02±1,23	3.3±2.5	3.18±1.31	3.6±2.5	3.0±2.2
p	<0.0001	<0.0001	0.413	0.171	0.152

Table 12 Radiation doses to the ipsilateral lung and the heart in the overall study population. The mean values±SD are shown.

No significant difference was detected in the mean dose to the heart and the volumes of the heart receiving at least 25 Gy or 30 Gy in 34 left-sided breast cancer patients according to their position during radiotherapy (Table 12). The first 20 pairs of treatment plans revealed significantly higher doses to the contralateral breast in the prone position than in the supine position. In the second phase of the study (n=41), as a consequence of the more complete displacement of the opposite breast due to the use of a new polyfoam wedge, there was no longer any significant difference (Table 13).

	First phase n=20		Second phase n=41		p for first *vs.* second phase	
	Mean dose (Gy)	V_{5Gy} (%)	Mean dose (Gy)	V_{5Gy} (%)	Mean dose	V_{5Gy}
Supine	0.85±0.47	2.7±2.0	0.61±0.73	1.7±2.8	0.096	0.073
Prone	1.26±0.78	4.5±3.4	0.74±0.44	2.2±2.0	0.00092	0.001
p for supine *vs.* prone	0.0038	0.0057	0.162	0.159		

Table 13 Radiation dose to the opposite breast in the 2 consecutive cohorts of the study

We hoped to identify those parameters related to the patient anatomy which indicate high lung doses if radiotherapy is given in the supine position, in order to select those patients who would benefit most from radiotherapy in the prone position. As regards the volume of the target breast, the breast separation and the CLD, only the CLD was significantly associated with the MLD (r=0.843, p<0.0001) and the V_{20Gy} (r=0.733, p<0.0001).

### 4.3.3 *Implementation of breast radiotherapy in the prone position*

In the second phase of the study, the adherence to the study protocol, the repositioning accuracy and the early skin reactions were analyzed. The protocol was tolerated well by all the patients; only one patient treated in the prone position needed a 1-week break because of radiodermatitis. It was necessary to correct the location of the isocenter in the simulator or the position of the table during radiotherapy in 20.3% (61/301) and 20.3% (62/306) of all the checks in the prone

and the supine position, respectively (p=0.999). The mean length of the displacement vector was 8.06±4.66 (3.00–22.56) mm and 6.60±3.05 (3.00–21.19) mm in the prone and supine position, respectively (p=0.021). The population random errors were 17.39 mm and 13.63 mm, while the population systematic errors were 0.86 mm and 0.82 mm, for the prone and the supine position, respectively. The random errors in the two groups are shown in Table 14.

	Mean ± SE (mm)	Median (mm)
Supine	2.75 ± 0.27	2.58
Prone	3.46 ± 0.37	3.48
P	0.061	

Table 14 Random errors for repositioning in the prone and supine positions

A trend was detected for better overall repositioning accuracy in the supine position (p=0.061). We analyzed whether the repositioning accuracy changed from patient to patient during the study period. The individual random errors for repositioning in the prone position decreased with time, while no change was detected in the group randomized to radiotherapy in the supine position (Fig. 9).
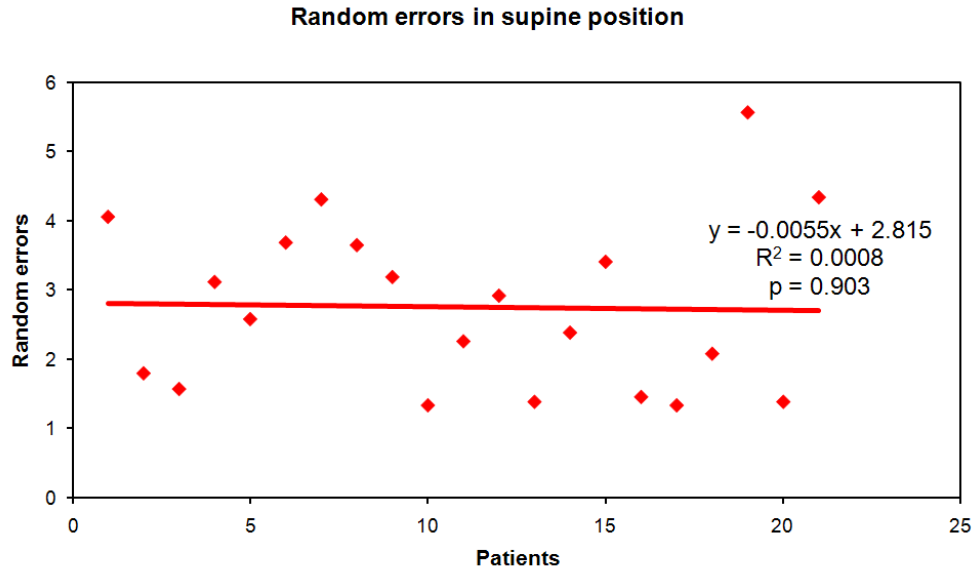
Random errors in prone position

$$y = -0.1161x + 4.6829$$
$$R^2 = 0.1761$$
$$p = 0.065$$

Random errors in supine position
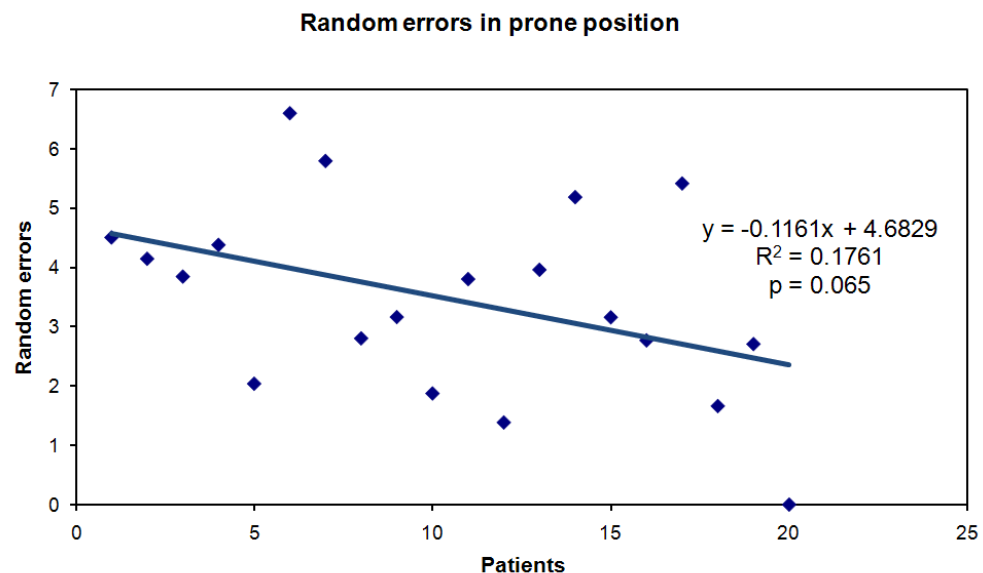
Figure 9 Random errors for repositioning among the patients who received radiotherapy prone and those received radiotherapy supine by sequence of enrolment in the study

The repositioning accuracy in the prone position, did not depend on any of the patient-related parameters. In the supine position, however, the repositioning accuracy was significantly related to lower weight (p=0.01), the BMI (p=0.011), the waist size (p=0.039), the volume of the ipsilateral breast (p=0.007) and the breast separation (p=0.001). Radiodermatitis of grade 1 developed in 55% and 38.1%, and radiodermatitis of grade 2 in 35% and 19.5% of the patients receiving radiotherapy in the prone or the supine position, respectively (p= 0.025). Acute skin reactions were not related to dose homogeneity in the PTV or the random errors for repositioning, regarded as measures of systematic and random overdosage, respectively.

5.   Discussion

5.1   *Tumor topoisomerase II alpha status and response to anthracycline-based neoadjuvant chemotherapy in breast cancer*

The amplification of the TOP2A gene is a rare abnormality, and is restricted to HER2 positive tumors (5,10-12,15,22,23,34). The deletion of the gene is less frequent, and its role in anthracycline-sensitivity is controversial (5,10,12,16,34-37). In our cohort, 2 cases with TOP2A gene amplification exhibited HER2 amplification and high TOP2A expression (data not shown), and showed excellent response to the therapy, however, the small number of cases limits the interpretation of the findings.

The cut-off point for defining TOP2A-positivity by IHC varied in different studies. The most often used threshold value was 10-15% (range 5-25%) (11, 23,38, 39-42), and the rate of TOP2A-positive tumors varied between 5-45% (15, 23, 42). In a series of 245 tumors, the median proportion of TOP2A-positive cells was 27%, and about half of the tumors rated positive (11). For the TOP2A-positive category, we used the cut-off value of >15%. In our cohort, the median proportion of TOP2A-stained cells was relatively high (50%), and the majority of tumors classified as TOP2A-positive. The first reason for this finding is the selected nature of our study population: only patients with rapidly proliferating tumors (based on the knowledge of standard tumor characteristics), likely chemosensitive were chosen for neoadjuvant chemotherapy. Second, the methods applied (the tumor regions with the highest cellularity were used; TOP2A-positive cells were counted regardless of the intensity of the staining) also favored high TOP2A values. Of note is that using the same methods, the median TOP2A IHC value in another cohort of our patients selected for adjuvant chemotherapy and in the whole population irrespective of tumor characteristics was 20 (range: 0-90) and 5 (range: 0-80), respectively (data not shown).

In our study, TOP2A protein expression was found an independent predictor of pCR after neoadjuvant docetaxel-epirubicin chemotherapy, and the probability of pCR increased by almost 50% with every 10% increase of the TOP2A positive tumor cells. The predictive role of the tumor TOP2A status for anthracycline-based chemotherapy has been investigated with different methods, in different settings. The correlation between the efficiency of adjuvant anthracycline-based

chemotherapy and the presence of TOP2A gene amplification (5, 16, 43) or the amplification and deletion of the gene (9,10,12,35) in the breast tumor is well demonstrated. In those randomized trials which compared an anthracycline-containing chemotherapy with a non-anthracycline containing regimen, the benefit of the former was limited to tumors with the presence of the amplification (5, 43), or the amplification or the deletion of the TOP2A gene (9,12,35). Some studies demonstrated that the presence of TOP2A gene amplification is predictive for the benefit of the dose elevation of the anthracylines (16, 44). In contrast, a retrospective analysis of the CALGB 8541 study did not support a difference of benefit if doxorubicin was administered at different doses in tumors with TOP2A gene aberrations, but in that study, standard dose was compared with suboptimal doses (34). In the neoadjuvant setting, the retrospective study of 350 cases showed that the amplification of the TOP2A gene involves a 3 times higher probability of pCR in patients treated with neoadjuvant anthracycline-based chemotherapy (22). Likewise, the amplification of the TOP2A gene or the polysomy of chromosome 17 indicated a more than 4-times increased probability to obtain pCR in HER2-positive and ER-, PgR-negative tumors (36). Park et al. found that the chance of obtaining a response including pCR after neoadjuvant doxorubicin was associated with TOP2A and HER2 coamplification (34). In a relatively small neoadjuvant study, in consistence with our findings, serial TOP2A IHC determination showed a trend for better response to anthracyclines in tumors with higher TOP2A expression, and a significant decrease after therapy, in responders (38). Mukherjee et al. found the extent of TOP2A protein expression predictive of the pCR in 91 patients treated with preoperative FEC chemotherapy (45). The robust study of Press et al. provides evidence that the coamplification of the TOP2A and HER2 genes is a clinically useful predictive marker of an enhanced response to anthracycline-based chemotherapy in metastatic breast cancer (46). In a phase III study in advanced breast cancer, the TOP2A IHC status was predictive for response to doxorubicin monotherapy, and every 10% increase in TOP2A expression was associated with a 9% increase in the probability of response, while no such effect was demonstrated for the treatment with docetaxel monotherapy (23).

The main outcome and novelty of our study is the demonstration that a simple tool such as TOP2A IHC is a useful, semiquantitative predictive marker of the benefit of neoadjuvant anthracycline-based chemotherapy in breast cancer. The design of our

study is not appropriate to answer whether TOP2A IHC staining is a specific marker of anthracycline sensitivity or that of chemosensitivity only. Other studies, however, support the hypothesis that high TOP2A protein expression is predictive of anthracycline sensitivity. In an early study of Di Leo et al., it seemed that the >10% expression of TOP2A protein favors the benefit of both the choice and the higher dose of an adjuvant EC regimen (42). Likewise, Durbecque et al. in a retrospective analysis of the TAX 303 randomized study, demonstrated that although docetaxel is more efficient than doxorubicin in the population of advanced breast cancer patients overall, increasing TOP2A expression is associated with a higher chance to obtain a response in the doxorubicin arm, but not in the docetaxel arm (23).

Many studies examined the correlation between the TOP2A gene status and the TOP2A protein expression (11, 15, 39, 41, 47, 48). Although gene amplification favored high protein expression, the presence of the enzyme was not dependent on the gene abnormality. Jarvinen et al. found that TOP2A protein expression correlated well with TOP2A mRNA (48). Brase et al., analyzed TOP2A at the levels of gene amplification, RNA expression and protein expression, and studied their correlations. No correlation was found between gene amplification and RNA or protein expression, but a strong correlation existed between TOP2A RNA and protein levels (21). The conclusion was drawn that unlike in the case of the HER2 status, TOP2A protein expression is highly regulated at the RNA level. In our cohort, most tumors exhibited high TOP2A expression without the presence of TOP2A gene alteration, as a function of high proliferative activity. Schindlbeck et al. based on their study on patients treated with adjuvant anthracycline-based chemotherapy, concluded that it is the TOP2A IHC and not the gene status that predicts benefit of the treatment (15). The relevance of TOP2A expression in the prediction of anthracycline-sensitivity merits further studies, however, the reconsideration of the optimal method is needed.

We did not find association between outcome and TOP2A expression or the fall of TOP2A expression after neoadjuvant chemotherapy. This result is limited by the relatively short follow-up time. Survival was neither different in other studies by the TOP2A status among patients treated with anthracyclines (11,37,40). Of note is, however, that the administration of anthracyclines did improve outcome in HER2-

and TOP2A-coamplified tumors to a level that was obtained without such chemotherapy, but with the addition of Herceptin (5).

In conclusion, our findings suggest that the IHC determination of the TOP2A protein is a useful tool for the estimation of the sensitivity of breast cancer to anthracycline-based chemotherapy.

5.2 *Tumor topoisomerase II alpha protein expression and outcome after adjuvant dose-dense anthracycline-based chemotherapy*

The TOP2A status in breast cancer has been studied as a prognostic and predictive factor by different methods in multiple studies. Most investigators agree that the amplification or the deletion of the TOP2A gene is restricted to HER2-positive cancers (5,6,14,22). Co-amplification of the HER2 and TOP2A genes indicated an increased anthracycline sensitivity in most (5,6,14,22,43), but not all studies (49,13). The design of these retrospective studies, however, was not always appropriate for detection of the benefit of anthracycline therapy according to the presence of TOP2A gene abnormality (13,37). In those randomized trials which compared anthracycline-containing chemotherapy with a non-anthracycline-containing regimen, the benefit of the former was limited to tumours with an abnormal TOP2A gene status (5,9,11,12,43). Some studies have demonstrated that the presence of a TOP2A gene alteration is predictive of the benefit of an elevation of the anthracyline dose (17,44). Deletion of the gene is less frequent, and its role in anthracycline sensitivity seems rather controversial (5,10-12,16,22,37). In line with the contradictory results, it is noteworthy that, although TOP2A gene abnormalities have been observed exclusively in HER2-positive breast cancers, high anthracycline sensitivity is not limited to this special group (50).

Investigations of whether the expression of TOP2A is a specific marker of anthracycline sensitivity gave more concordant results. The early study by Di Leo et al. led to the conclusion that a finding of TOP2A positivity by means of IHC determination favoured the benefit of both the choice and a higher dose of an adjuvant EC regimen (42). Likewise, in a retrospective analysis of the TAX 303 randomized study, Durbecque et al. demonstrated that, although docetaxel is more efficient than doxorubicin in the population of advanced breast cancer patients overall, increase of the TOP2A protein expression is associated with a higher chance of obtaining a response in the doxorubicin arm, but not in the docetaxel arm

(23). The greater sensitivity to anthracycline-based adjuvant chemotherapy of ER/PgR-negative breast cancers as compared with ER- or PgR-positive tumours has been well demonstrated (50). Our own study suggests that one of the related key factors is the more frequent TOP2A positivity among the ER/PgR-negative tumours, and we advocate TOP2A IHC as a tool to select those hormone receptor-negative cases which would benefit from adjuvant anthracyclines. In a patient population treated with adjuvant anthracycline-containing chemotherapy, Schindlbeck et al. retrospectively examined the TOP2A status. About 50% of the cases proved to be TOP2A-positive, and after a median survival time of 42 months, the survival was significantly poorer among the TOP2A-negative cases (15). Brase et al. demonstrated the strong negative prognostic power of an elevated TOP2A RNA level in 782 untreated breast cancer patients, which remained significant after further analyses in the ER-positive and the HER2-negative and triple-negative subgroups. In the same paper, complete tumour regression to chemotherapy with EC was reported to be related to the high TOP2A and low ER RNA levels, results which support our finding that anthracyclines result in a favourable outcome in ER-negative and TOP2A-positive cancers (21). Rody et al.followed up more than 1300 patients, and found that the TOP2A expression was the strongest indicator of a poor prognosis among hormone receptor-positive cases, while no such effect was detected among the ER-negative cases (51). Although the prognostic effect of TOP2A positivity was found to be independent of the systemic therapy, the nature of the chemotherapy given in about half of the patients, was not reported. It may be speculated that the similar outcome in the TOP2A-positive and -negative cases in the ER-negative group may be due to the higher chemosensitivity of the TOP2A-positive cases.

The expression of TOP2A seems to be regulated most strongly at the RNA level, and its gene status is probably less determinative of its functional capacity. Jarvinen et al. and Brase et al. found no correlation between gene amplification and protein expression, but there was a strong correlation between the TOP2A RNA and protein levels (21,48). Accordingly, although gene amplification favoured a high protein expression in those studies that examined the correlation between the TOP2A gene status and the TOP2A protein expression, the presence of the enzyme was not dependent on the gene abnormality (14,15,18-20). Their findings led Brase et al. to recommend determination of the RNA expression, while Schindlbeck et al.

suggested determination of the protein expression of TOP2A for patient selection, rather than examination of the gene status (15,21).

5.3   *The effect of individual postioning on the radiation exposure of the risk organs*

We evaluated our initial experience regarding the dosimetry and feasibility of conformal breast radiotherapy in the prone position, and identified its place in everyday practice. Our results indicate that its primary advantage is the significantly reduced radiation exposure of the ipsilateral lung. The doses to the heart and the contralateral breast are similar in the prone and supine positions. Special practice in and attention to accurate repositioning are needed if the prone position is applied, and the dose inhomogeneity and acute skin reactions may be slightly increased. There have been few studies on prone breast radiotherapy. Some of them focused on the dose distribution (52-55), and others on clinical implementation (56-60), and only one study dealt with both dosimetric aspects and feasibility (61). The present study is the first randomized clinical trial to compare breast radiotherapy in the prone *vs.* the supine position.

Utilization of the prone position during breast radiotherapy raises special considerations because of the altered shape, motion and position of the organs present in the region. The altered shape of the target breast hanging down across the aperture of the positioning device results in a different dose distribution relative to that in the supine position. Improved dose uniformity, and especially the avoidance of an overdosage within the PTV, have been associated with a better cosmetic outcome (62, 63). A higher dose inhomogeneity is related to larger breasts if conventional tangent beams are used (62). Buijsen et al. (54) compared prone and supine breast irradiation in 10 patients with pendulous breasts, and concluded that the dose homogeneity was better in the prone than in the supine position. In fact, this was based on a comparison of the PTV overdosed ($V_{105\%}$ and $V_{107\%}$) in the supine *vs.* the prone position, while the significantly lower mean dose and PTV coverage representing an underdosage were neglected. Similarly, larger volumes receiving >52.5 Gy within the PTV were found in the supine than in the prone position, but no other information on dose distribution was reported in another study (6). We examined $V_{95-107\%}$ as a measure of dose homogeneity within the PTV, according to ICRU Report 62 (64), and found that the dose distribution was

significantly more uniform in the supine position, regardless of the size or shape of the target breast. None of the radiotherapy plans indicated measurable volumes receiving >53.5 Gy.

Because of the different shape of the chest wall when the patient is positioned prone, the lung volume included in the tangent fields is considerably less. All authors agree that the lung doses are dramatically reduced if breast radiotherapy is performed with the patient prone (52-54,65,66). The beneficial effect of prone positioning on the protection of the ipsilateral lung is further enhanced if the almost absent intrafractional motion of the chest wall is taken into account for the calculation of safety margins around the CTV (60, 67, 68).

When left-sided irradiation is performed, the irradiated volume of the heart is not reduced, despite the fact that less intrathoracic volume is exposed to radiation in the prone than in the supine position. Reports on heart doses, however, are not concordant. Some studies suggest a reduction in heart doses as a result of prone positioning, but do not provide direct comparisons with supine positioning (65, 66). Others are consistent with our results in showing no significant difference in the doses to the heart as a function of the treatment position (52-54). This finding may be accepted if the change in position of the heart by treatment position is taken into consideration. In fact, the prone position causes an anterior displacement of the heart within the thorax by 19 mm on average, as demonstrated by CT and MRI measurements in breast cancer patients receiving radiotherapy (69).

Since breast radiotherapy increases the risk of the late development of contralateral breast cancer by 18-34%, special attention is needed for the protection of the opposite breast during radiotherapy (70,71). Although some studies allude to the radiation dose to the opposite breast in the prone position, detailed dose volume histogram data have not been provided (52,65). No widely accepted dose constraints exist for the contralateral breast. We registered V_{5Gy} and the mean dose to the healthy breast. In the first phase of the study, in consequence of the suboptimal positioning of the patient in the prone position, we detected higher doses to the opposite breast in the prone than in the supine position. Following revision of the positioning method, in the second phase of the study, no difference was observed. We consider careful application of the polyfoam wedge in the prone position, and of mask fixation in the supine position to be very important, in order to remove the opposite breast from the radiation fields.

The largest prospective phase I-II study on prone breast irradiation is that of Formenti et al. (66). Accelerated whole breast radiotherapy was feasible in 90 patients, with high set-up reproducibility, although numerical data were not provided. In another feasibility study (61), prolonged adequate immobilization could not be achieved in 3 of 35 patients with large pendulous breasts in the prone position. In one retrospective study (56), 5% of the patients during prone breast radiotherapy complained of chest wall or rib pain, and 2 of 248 patients suffered a rib fracture (56), as did 1 of 35 in the previous study (61). All our patients considered the prone radiotherapy convenient, and completed the course of radiotherapy. We believe, that the comfortable positioning system in use, was essential to achieve such good adherence to the protocol. It is our view that repositioning accuracy is a key condition for radiotherapy, especially if inverse or forward intensity modulation is applied (67,68). During simulation in 308 patients with various cancer sites, Schüller et al. (72) found that the repositioning accuracy was better in the entire patient population if positioning aids or mask fixation were used, but did not differ by prone or supine positioning. Breast irradiation was performed in the supine position for 64 patients, without mask fixation. Of the various tumor sites, the breast exhibited the poorest repositioning accuracy. Displacement was carried out in 27 patients (42.2%), and in many cases exceeded 1 cm. In another study of 25 breast cancer patients irradiated in the supine position (73), the isocenter displacement on simulation was on average 5.7 mm. Morrow et al. (60) studied the interfractional error in repositioning in 15 patients, and recommended image guidance during prone breast radiotherapy because of the need for frequent and large displacements. In accord with our results, they observed no relation between the breast size and the repositioning accuracy. Interestingly, however, we found that the repositioning accuracy in the supine position is significantly worse in obese patients. To the best of our knowledge, no such data have been published previously. If confirmed, they indicate that increased attention must be payed to the position of overweight patients during breast radiotherapy. We believe that the relatively good repositioning accuracy in our study, was related to the comfortable positioning device used for both the prone and the supine position, and to the mask fixation used in the supine position. The repositioning accuracy in the prone position improved over time, indicating the need for experience and expertise if the method is newly introduced. Furthermore, our study warrants the

development of mask fixation in the prone position, which would reduce the set-up uncertainity.

In other publications (56,61), acute skin reactions after breast radiotherapy in the prone position were reported in similar incidences as among our patients. Mahe et al. (61) found that acute skin reactions were most frequent at the top and the bottom of the fields, in accordance with the high dose regions. In our study, radiodermatitis in the prone position was not related to the size of the breast or the dose-inhomogeneity in it.

Merchant and McCormick (65) recommend breast radiotherapy in the prone position if that in the supine position is likely to result in unacceptable dose inhomogeneity or significant doses to normal tissues. We hoped to identify those patients who would benefit most from the prone position during breast radiotherapy. Since we could not detect any advantage of prone radiotherapy other than the absence of radiation exposure of the lung, we set out to identify those patient-related parameters that are associated with a higher lung dose if the patient is irradiated in a supine position. Consideration of the volume of the breast, the breast separation and the CLD as measures of the shape of the PTV indicated that only the CLD was related to the dose to the ipsilateral lung. Thus, we recommend monitoring of the CLD as a primary measure for an indication for prone radiotherapy. Moreover, since the risk of early and late radiation lung sequelae is strongly related to the age of the patient (26) and the presence of lung diseases, and possibly also to certain systemic therapies, these factors should be taken into account when a decision is made concerning the position during breast radiotherapy.

6. Summary, conclusions

6.1.   We found that despite that the amplification of the TOP2A gene was rare, and restricted to HER2-positivity, the protein expression was usually elevated in tumors with high proliferation rate; anthracycline-based neoadjuvant chemotherapy resulted in the reduction of the expression of TOP2A. TOP2A positivity was an independent predictor of pCR, and a 10% increase of TOP2A IHC staining resulted in a 46% increase of the likelihood of obtaining a pCR.

6.2.   We found TOP2A positivity in about half of the cancers treated with adjuvant dose-dense anthracycline-based chemotherapy. TOP2A positivity was more frequent among the ER- and/or PR-negative cancers. Among the hormone receptor-negative cases, TOP2A positivity and grade 3 indicated improved OS and RFS as a possible consequence of the higher sensitivity to the applied regimen. Our data indicate that a simple tool such as TOP2A IHC (together with the grade) is an useful predictive marker, at least in the hormone receptor-negative cases, and should be implemented in routine practice for the selection of those who can be expected to benefit from adjuvant anthracycline-based chemotherapy. The usually poor outcome in the group of hormone receptor-negative and TOP2A-positive cases may be reversed by the application of anthracycline-containing chemotherapy.

6.3.   Conformal breast radiotherapy is feasible in the prone position. Its primary advantage is the substantially lower radiation dose to the ipsilateral lung. The higher dose inhomogeneity and the enhanced rate of the grade 1-2 skin toxicity, however, may be concerns. We recommend monitoring of the CLD as a primary measure for an indication for prone radiotherapy. Special practice in and attention to accurate repositioning are needed if the prone position is applied.

7. Acknowledgements

8.   References

1. Nowak AK, Wilcken NR, Stockler MR, Hamilton A, Ghersi D. Systematic review of taxane-containing versus non-taxane-containing regimens for adjuvant and neoadjuvant treatment of early breast cancer. *Lancet Oncol*. 2004; 5:372-380.

2. Early Breast Cancer Trialists' Collaborative Group. Favourable and unfavourable effects on long-term survival of radiotherapy for early breast cancer: an overview of the randomised trials. *Lancet* 2000; 355:1739-1740.

3. Kaufmann M, von Minckwitz G, Bear HD, Buzdar A, McGale P, Bonnefoi H, Colleoni M, Denkert C, Eiermann W, Jackesz R, Makris A, Miller W, Pierga JY, Semiglazov V, Schneeweiss A, Souchon R, Stearns V, Untch M, Loibl S. Recommendations from an international expert panel on the use of neoadjuvant (primary) systemic treatment of operable breast cancer: new perspectives 2006. *Ann Oncol*. 2007; 18:1927-1934.

4. Early Breast Cancer Trialists' Collaborative Group (EBCTCG): Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. *Lancet.* 2005; 365:1687-1717.

5. Slamon D, EiermannW, Robert N et al. BCIRG 006: 2nd interim analysis phase II randomized trial comparing doxorubicin and cyclophosphamide followed by docetaxel positive early (AC®TH) with docetaxel, carboplatin and trastuzumab (TCH) in HER2 positive early breast cancer patients: BCIRG 006 study. *Breast Cancer Res Treat* 2005; 94:S5 [abstr. 1]

6. Di Leo A, Biganzoli L, Claudion W, Licitra S, Pestrin M, Larsimont D: Topoisomerase II alpha as a marker predicting anthracyclines' activity in early breast cancer patients: ready for the primetime? *Eur J Cancer*, 44: 2791-2798, 2008

7. Järvinen TA, Liu ET. HER-2/neu and topoisomerase IIalpha in breast cancer. *Breast Cancer Res Treat*. 2003; 78:299-311.

8. Isaacs RJ, Davies SL, Sandri MI, Redwood C, Wells NJ, Hickson ID. Physiological regulation of eukaryotic topoisomerase II. *Biochim Biophys Acta*. 1998;1400:121-137.

9. Nielsen KV, Ejlertsen B, Moller S, Jorgensen JT, Knoop A, Knudsen H, Mouridsen HT: The value of TOP2A gene copy number variation as a biomarker in breast cancer: Update of DBCG trial 89D. *Acta Oncol* 2008; 47: 725-734.

10. Di Leo A, Gancberg D, Larsimont D, Tanner M, Jarvinen T, Rouas G, Dolci S, Leroy JY, Paesmans M, Isola J, Piccart MJ. HER-2 amplification and topoisomerase IIalpha gene aberrations as predictive markers in node-positive breast cancer patients randomly treated either with an anthracycline-based therapy or with cyclophosphamide, methotrexate, and 5-fluorouracil. *Clin Cancer Res* 2002; 8:1107-1116.

11. Knoop AS, Knudsen H, Balslev E, Rasmussen BB, Overgaard J, Nielsen KV, Schonau A, Gunnarsdóttir K, Olsen KE, Mouridsen H, Ejlertsen B. Danish Breast Cancer Cooperative Group. Retrospective analysis of topoisomerase IIa amplifications and deletions as predictive markers in primary breast cancer patients randomly assigned to cyclophosphamide, methotrexate, and fluorouracil or cyclophosphamide, epirubicin, and fluorouracil: Danish Breast Cancer Cooperative Group. *J Clin Oncol* 2005; 23:7483-7490.

12. O'Malley FP, Chia S, Tu D, Shepherd LE, Levine MN, Bramwell VH, Andrulis IL, Pritchard KI. Topoisomerase II alpha and responsiveness of breast cancer to adjuvant chemotherapy. *J Natl Cancer Inst*. 2009; 101:644-650.

13. Harris LN, Broadwater G, Abu-Khalaf M, Cowan D, Thor AD, Budman D, Cirrincione CT, Berry DA, Winer EP, Hudis CA, Hayes DF, Friedman P, Ellis M, Dressler L. Topoisomerase II{alpha} amplification does not predict benefit from dose-intense cyclophosphamide, doxorubicin, and fluorouracil therapy in HER2-amplified early breast cancer: results of CALGB 8541/150013. *J Clin Oncol*. 2009; 27:3430-3436.

14. Arriola E, Rodriguez-Pinilla SM, Lambros MBK, Jones RL, James M, Savage K, Smith IE, Dowsett M, Reis-Filho JS: Topoisomerase II alpha amplification may predict benefit from adjuvant anthracyclines in HER2 positive early breast cancer. *Breast Cancer Res Treat* 2007; 106:181-189.

15. Schindlbeck C, Mayr D, Olivier C, Rack B, Engelstaedter V, Jueckstock J, Jenderek C, Andergassen U, Jeschke U, Friese K. Topoisomerase IIalpha expression rather than gene amplification predicts responsiveness of adjuvant anthracycline-based chemotherapy in women with primary breast cancer. *J Cancer Res Clin Oncol*. 2010;136:1029-1037.

16. Tanner M, Isola J, Wiklund T, Erikstein B, Kellokumpu-Lehtinen P, Malmström P, Wilking N, Nilsson J, Bergh J: Topoisomerase IIalpha gene amplification predicts favorable treatment response to tailored and dose-escalated

anthracycline-based adjuvant chemotherapy in HER-2/neu-amplified breast cancer: Scandinavian Breast Group Trial 9401. *J Clin Oncol* 2006; 24: 2428-2436

17. Fritz P, Cabrera CM, Dippon J, Gerteis A, Simon W, Aulitzky WE, van der Kuip H. c-erbB2 and topoisomerase IIalpha protein expression independently predict poor survival in primary human breast cancer: a retrospective study. *Breast Cancer Res.* 2005; 7:R374-384.

18. Durbecq V, Desmed C, Paesmans M, Cardoso F, Di Leo A, Mano M, Rouas G, Leroy JY,
Sotiriou C, Piccart M, Larsimont D: Correlation between topoisomerase-IIalpha gene amplification and protein expression in HER-2 amplified breast cancer. *Int J Oncol* 2004; 25:1473-1479.

19. Mueller RE, Parkes RK, Andrulis I, O'Malley FP: Amplification of the TOP2A gene does not predict high levels of topoisomerase II alpha protein in human breast tumor samples. *Genes Chromosomes Cancer* 2004; 39:288-297.

20. Callagy G, Pharoah P, Chin SF, Sangan T, Daigo Y, Jackson L, Caldas C: Identification and validation of prognostic markers in breast cancer with the complementary use of array-CGH and tissue microarrays. *J Pathol* 2005; 205:388-396.

21. Brase JC, Schmidt M, Fischbach T, Sültmann H, Bojar H, Koelbl H, Hellwig B, Rahnenführer J, Hengstler JG, Gehrmann MC. ERBB2 and TOP2A in breast cancer: a comprehensive analysis of gene amplification, RNA levels, and protein expression and their influence on prognosis and prediction. *Clin Cancer Res*. 2010;16:2391-2401.

22. Konecny GE, Pauletti G, Untch M, Wang HJ, Möbus V, Kuhn W, Thomssen C, Harbeck N, Wang L, Apple S, Jänicke F, Slamon DJ. Association between HER2, TOP2A, and response to anthracycline-based preoperative chemotherapy in high-risk primary breast cancer. *Breast Cancer Res Treat.* 2010; 120:481-489.

23. Durbecq V, Paesmans M, Cardoso F, Desmedt C, Di Leo A, Chan S, Friedrichs K, Pinter T, Van Belle S, Murray E, Bodrogi I, Walpole E, Lesperance B, Korec S, Crown J, Simmonds P, Perren TJ, Leroy JY, Rouas G, Sotiriou C, Piccart M, Larsimont D: Topoisomerase-II alpha expression as a predictive marker in a population of advanced breast cancer patients randomly treated either with single-agent doxorubicin or single-agent docetaxel. *Mol Cancer Ther* 2004; 3:1207-1214.

24. Kahan Z, Uhercsak G, Hajnal-Papp R, Boda K, Thurzo L. Dose-dense sequential adriamycin-Paclitaxel-cyclophosphamide chemotherapy is well tolerated and safe in high-risk early breast cancer. *Oncology.* 2005;68:446-453.

25. Kahán Z, Spanik S, Wagnerova M, Skacel T, Planko B, Fitzthum E, Lindner E, Soldatenkova V, Zielinski CC, Brodowicz T.: Feasibility of two dose-dense FEC regimens with growth factor support for adjuvant therapy in patients with early breast cancer: results from a randomised study of the Central European Cooperative Oncology Group (CECOG). *Breast Cancer Res Treat*. 2008; 112:557-563.

26. Kahán Z., Csenki M., Varga Z., *et al.* The risk of early and late lung sequelae after conformal radiotherapy in breast cancer patients. *Int J Radiat Oncol Biol Phys* 2007; 68:673-681.

27. Fein DA, McGee KP, Schultheiss TE, Fowble B, Hanks GE. Intra- and interfractional reproducibility of tangential breast fields: A prospective on-line portal imaging study. *Int J Radiation Oncology Biol Phys* 1996; 34:733-740.

28. Lirette A, Pouliot J, Aubin M, Larochelle M. The role of electronic portal imaging in tangential breast irradiation: a prospective study. *Radiother Oncol* 1995; 37:241-245.

29. Hurkmans CW, Remeijer P, Lebesque JV, Mijnheer B. Set-up verification using portal imaging; review of current clinical practice. *Radiother Oncol* 2001; 58:105-120.

30. Stroom JC, Heijmen BJM. Geometrical uncertainties, radiotherapy planning margins, and the ICRU-62 report. *Radiother Oncol* 2002; 64:75-83.

31. Arriola E, Rodriguez-Pinilla SM, Lambros MBK, Jones RL, James M, Savage K, Smith IE, Dowsett M, Reis-Filho JS: Topoisomerase II alpha amplification may predict benefit from adjuvant anthracyclines in HER2 positive early breast cancer. *Breast Cancer Res Treat* 2007; 106:181-189.

32. Arpino G, Ciocca DR, Weiss H, Allred DC, Daguerre P, Vargas-Roig L, Leuzzi M, Gago F, Elledge R, Mohsin SK. Predictive value of apoptosis, proliferation, HER-2, and topoisomerase IIalpha for anthracycline chemotherapy in locally advanced breast cancer. *Breast Cancer Res Treat*. 2005;92:69-75.

33. Nocito A, Kononen J, Kallioniemi OP, Sauter G. Tissue microarrays (TMAs) for high-throughput molecular pathology research. *Int J Cancer* 2001;94:1–5.

34. Park K, Kim J, Lim S, Han S. Topoisomerase II-alpha (topoII) and HER2 amplification in breast cancers and response to preoperative doxorubicin chemotherapy. *Eur J Cancer.* 2003;395:631-634.

35. Harris LN, Broadwater G, Abu-Khalaf M, Cowan D, Thor AD, Budman D, Cirrincione CT, Berry DA, Winer EP, Hudis CA, Hayes DF, Friedman P, Ellis M, Dressler L. Topoisomerase II{alpha} amplification does not predict benefit from dose-intense cyclophosphamide, doxorubicin, and fluorouracil therapy in HER2-amplified early breast cancer: results of CALGB 8541/150013. *J Clin Oncol.* 2009; 27:3430-3436.

36. Orlando L, Del Curto B, Gandini S, Ghisini R, Pietri E, Torrisi R, Balduzzi A, Cardillo A, Dellapasqua S, Veronesi P, Viale G, Goldhirsch A, Colleoni M: Topoisomerase IIalpha gene status and prediction of pathological complete remission after anthracycline-based neoadjuvant chemotherapy in endocrine non-responsive Her2/neu-positive breast cancer. *Breast* 2008; 17: 506-511.

37. Tubbs R, Barlow WE, Budd GT, Swain E, Porter P, Gown A, Yeh IT, Sledge G, Shapiro C, Ingle J, Haskell C, Albain KS, Livingston R, Hayes DF. Outcome of patients with early-stage breast cancer treated with doxorubicin-based adjuvant chemotherapy as a function of HER2 and TOP2A status. *J Clin Oncol.* 2009;27:3881-3886.

38. Arpino G, Ciocca DR, Weiss H, Allred DC, Daguerre P, Vargas-Roig L, Leuzzi M, Gago F, Elledge R, Mohsin SK. Predictive value of apoptosis, proliferation, HER-2, and topoisomerase IIalpha for anthracycline chemotherapy in locally advanced breast cancer. *Breast Cancer Res Treat.* 2005;92:69-75.

39. Bhargava R, Lal P, Chen B: HER-2/neu and topoisomerase IIa gene amplification and protein expression in invasive breast carcinomas: chromogenic in situ hybridization and immunohistochemical analyses. *Am J Clin Pathol* 2005; 123:889-895

40. Gómez HL, Pinto JA, Olivera M, Vidaurre T, Doimi FD, Vigil CE, Velarde RG, Abugattas JE, Alarcón E, Vallejos CS. Topoisomerase II-alpha as a predictive factor of response to therapy with anthracyclines in locally advanced breast cancer. *Breast.* 2010 Aug 10. [Epub ahead of print]

41. Cardoso F, Durbecq V, Larsimont D, Paesmans M, Leroy JY, Rouas G, Sotiriou C, Renard N, Richard V, Piccart MJ, Di Leo A. Correlation between complete response to anthracycline-based chemotherapy and topoisomerase II-alpha gene

amplification and protein overexpression in locally advanced/metastatic breast cancer. *Int J Oncol* 2004; 24:201-209.

42. Di Leo A, Larsimont D, Gancberg D, Jarvinen T, Beauduin M, Vindevoghel A, Michel J, Focan CH, Ries F, Gobert PH, Closon-Dejardin MT, Dolci S, Rouas G, Paesmans M, Lobelle JP, Isola J, Piccart MJ: HER-2 and topo-isomerase IIalpha as predictive markers in a population of node-positive breast cancer patients randomly treated with adjuvant CMF or epirubicin plus cyclophosphamide. *Ann Oncol* 2001; 12:1081-1089.

43. Gunnarsdóttir KA, Jensen MB, Zahrieh D, Gelber RD, Knoop A, Bonetti M, Mouridsen H, Ejlertsen B. CEF is superior to CMF for tumours with TOP2A aberrations: a Subpopulation Treatment Effect Pattern Plot (STEPP) analysis on Danish Breast Cancer Cooperative Group Study 89D. *Breast Cancer Res Treat* 2010; 123:163-169.

44. Hannemann J, Kristel P, van Tinteren H, Bontenbal M, van Hoesel QG, Smit WM, Nooij MA, Voest EE, van der Wall E, Hupperets P, de Vries EG, Rodenhuis S, van de Vijver MJ. Molecular subtypes of breast cancer and amplification of topoisomerase II alpha: predictive role in dose intensive adjuvant chemotherapy. *Br J Cancer* 2006; 95:1334-1341.

45. Mukherjee A, Shehata M, Moseley P, Rakha E, Ellis I, Chan S. Topo2α protein expression predicts response to anthracycline combination neo-adjuvant chemotherapy in locally advanced primary breast cancer. *Br J Cancer*. 2010;103:1794-1800.

46. Press MF, Sauter G, Buyse M, Bernstein L, Guzman R, Santiago A, Villalobos IE, Eiermann W, Pienkowski T, Martin M, Robert N, Crown J, Bee V, Taupin H, Flom KJ, Tabah-Fisch I, Pauletti G, Lindsay MA, Riva A, Slamon DJ. Alteration of Topoisomerase II-Alpha Gene in Human Breast Cancer: Association With Responsiveness to Anthracycline-Based Chemotherapy. *J Clin Oncol*. 2010 Dec 28. [Epub ahead of print]

47. Mueller RE, Parkes RK, Andrulis I, O'Malley FP: Amplification of the TOP2A gene does not predict high levels of topoisomerase II alpha protein in human breast tumor samples. *Genes Chromosomes Cancer* 2004; 39:288-297.

48. Järvinen TA, Kononen J, Pelto-Huikko M, Isola J. Expression of topoisomerase IIalpha is associated with rapid cell proliferation, aneuploidy, and c-erbB2 overexpression in breast cancer. *Am J Pathol*. 1996;148:2073-2082.

49. Bartlett JM, Munro A, Cameron DA, Thomas J, Prescott R, Twelves CJ. Type 1 receptor tyrosine kinase profiles identify patients with enhanced benefit from anthracyclines in the BR9601 adjuvant breast cancer chemotherapy trial. *J Clin Oncol.* 2008; 26: 5027-5035.

50. Rouzier R, Perou CM, Symmans WF, Ibrahim N, Cristofanilli M, Anderson K, Hess KR, Stec J, Ayers M, Wagner P, Morandi P, Fan C, Rabiul I, Ross JS, Hortobagyi GN, Pusztai L. Breast cancer subtypes respond differently to preoperative chemotherapy. *Clin Cancer Res* 2005; 11:5678-5685.

51. Rody A, Karn T, Ruckhäberle E, Müller V, Gehrmann M, Solbach C, Ahr A, Gätje R, Holtrich U, Kaufmann M: Gene expression of topoisomerase II alpha (TOP2A) by microarray analysis is highly prognostic in estrogen receptor (ER) positive breast cancer. *Breast Cancer Res Treat*, 2009;113:4574-66

52. Griem KL, Fetherston P, Kuznetsova M, *et al*. Three-dimensional photon dosimetry: A comparison of treatment of the intact breast in the supine and prone position. *Int J Radiation Oncology Biol Phys* 2003; 57:891-899.

53. Kurtman C, Andrieu MN, Hicsönmez A, Celebioglu B. Three-dimensional conformal breast irradiation in the prone position. *Brazilian Journal of Medical and Biological Research* 2003; 36:1441-1446.

54. Buijsen J, Jager JJ, Bovendeerd J, *et al*. Prone breast irradiation for pendulous breasts. *Radiotherapy and Oncology* 2007; 82:337-340.

55. Goodman KA, Hong L, Wagman R, Hunt MA, McCormick B. Dosimetric analysis of a simplified intensity modulation technique for prone breast radiotherapy. *Int J Radiation Oncology Biol Phys* 2004; 60:95-102.

56. Stegman LD, Beal KP, Hunt MA, Fornier MN, McCormick B. Long-term clinical outcomes of whole-breast irradiation delivered in the prone position. *Int J Radiation Oncology Biol Phys* 2007; 68:73-81.

57. Grann A, McCormick B, Chabner ES, *et al.* Prone breast radiotherapy in early-stage breast cancer: A preliminary analysis. *Int J Radiation Oncology Biol Phys* 2000; 47:319-325.

58. Fein DA, McGee KP, Schultheiss TE, Fowble B, Hanks GE. Intra- and interfractional reproducibility of tangential breast fields: A prospective on-line portal imaging study. *Int J Radiation Oncology Biol Phys* 1996; 34:733-740.

59. Algan Ö, Fowble B, McNeeley S, Fein D. Use of the prone position in radiation treatment for women with early stage breast cancer. *Int J Radiation Oncology Biol Phys* 1998; 40:1137-1140.

60. Morrow NV, Stepaniak C, White J, Wilson JF, Li AX. Intra- and interfractional variations for prone breast irradiation: An indication for image-guided radiotherapy. *Int J Radiation Oncology Biol Phys* 2007; 69:910-917.

61. Mahe M-A, Classe J-M, Dravet F, Cussac A, Cuilliere J-C. Preliminary results for prone-position breast irradiation. *Int J Radiation Oncology Biol Phys* 2002; 52:156-160.

62. Moody AM, Mayles WP, Bliss JM, *et al.* The influence of breast size on late radiation effects and association with radiotherapy dose inhomogeneity. *Int J Radiation Oncology Biol Phys* 1994; 33:106-112.

63. Johansen J, Overgaard J, Rose C, *et al*. Cosmetic outcome and breast morbidity in breast-conserving treatment. Results from the Danish DBCG-82TM national randomized trial in breast cancer. *Acta Oncol* 2002; 41:369-380.

64. International Commission on Radiation Units and Measurements. Prescribing, recording and reporting photon beam therapy (supplement to ICRU Report 50) ICRU Report 62. Bethesda, MD, USA: ICRU Publications, 1999.

65. Merchant TE, McCormick B. Prone position breast irradiation. *Int J Radiat Oncol Biol Phys* 1994; 30:197-203.

66. Formenti SC, Gidea-Addeo D, Goldberg JD, *et al.* Phase I-II trial of prone accelerated intensity modulated radiation therapy to the breast to optimally spare normal tissue. *J Clin Oncol* 2007; 25:2236-2242.

67. van Herk M. Errors and margins in radiotherapy. *Semin Radiat Oncol* 2004; 14:52-64.

68. Saliou MG, Giraud P, Simon L, *et al*. Radiotherapy for breast cancer: Respiratory and set-up uncertainties. *Cancer Radiother* 2005; 9:414-421.

69. Chino JP, Marks LB. Prone positioning causes the heart to be displaced anteriorly within the thorax: Implications for breast cancer treatment. *Int J Radiation Oncology Biol Phys* 2008; 70:916-920.

70. Early Breast Cancer Trialists' Collaborative Group (EBCTCG). Effects of radiotherapy and of differences in the extent of surgery for early breast cancer on local recurrence and 15-year survival: an overview of the randomised trials. *Lancet* 2005; 366:2087-2106.

71. Levi F, Randimbison L, Te VC, La Vecchia C. Cancer risk after radiotherapy for breast cancer. *Br J Cancer* 2006; 95:390-392.

72. Schüller P, Bruns F, Hesselmann S, *et al*. Simulator verification of the accuracy of patient repositioning after virtual simulation. *Strahlenther Onkol* 2002; 178:715-721.

73. Horst E, Schuck A, Moustakis C*, et al*. CT simulation in nodal positive breast cancer. *Strahlenther Onkol* 2001; 177:511-516.

APPENDIX

I

Tumor topoisomerase II alpha status and response to anthracycline-based neoadjuvant chemotherapy in breast cancer

Alíz Nikolényi[1], Farkas Sükösd[2], László Kaizer[2], Erika Csörgő[2], András Vörös[2], Gabriella Uhercsák[1], Katalin Ormándi[3, 5], György Lázár[4], László Thurzó[1], Thomas Brodowicz[6], Zsuzsanna Kahán[1]

[1]Department of Oncotherapy, [2]Department of Pathology, [3]Department of Radiology, [4]Department of Surgery, University of Szeged, [5]Euromedic Diagnostics Szeged Ltd, Szeged, Hungary; [6]Central European Cooperative Oncology Group, Vienna, Austria

Corresponding author: Zsuzsanna Kahán, Department of Oncotherapy, University of Szeged, Korányi fasor 12, H-6720 Szeged, Hungary; Phone: 36-62-545406; Fax: 36-62-545922; E-mail: kahan@onko.szote.u-szeged.hu

Running title: Topoisomerase II alpha in breast cancer

Key-words: anthracyclines, breast cancer, chemosensitivity, primary neoadjuvant chemotherapy, topoisomerase II alpha

Abstract

Objectives: The individualized chemotherapy of breast cancer improves the outcome. Anthracyclines target the enzyme topoisomerase II alpha (TOP2A).

Methods: Forty-three patients with 45 breast cancers were treated with neoadjuvant Taxotere-Epirubicin±Xeloda chemotherapy. The TOP2A status of the cancers, determined retrospectively by FISH and IHC, was analyzed in relation to the standard clinical and pathological data.

Results: Clinically and pathologically complete remission (pCR) was achieved in 15 (33.3%) and 9 (20%) cases, respectively. The TOP2A gene was amplified in 2 HER2-positive cancers (8%), and 32 (84.2%) overall exhibited TOP2A expression in >15% of the cells. The expression of TOP2A exhibited a strong correlation with the expression of Ki67 (R=0.743, p<0.001), and was negatively correlated with ER (R=0.404, p=0.012) and PgR (R=0.430, p=0.007). The expression of TOP2A was not related to the amplification of the TOP2A gene or the HER2 status of the tumor. The proportions of Ki67- and TOP2A-positive tumor cells were significantly reduced after chemotherapy (56.1±23.6 vs. 19.0±27.7%, p=0.004, and 41.0±27.9% vs. 12.7±24.8%, p<0.001, respectively). The development of pCR was related to a high grade (p=0.054), ER negativity (p=0.027) and high TOP2A expression (p=0.037). The expression of TOP2A was an independent predictor of pCR (OR=1.460, for every 10% increase, 95% CI: 1.016-2.096, p=0.041). After a median follow-up time of 31.0 months, neither RFS nor OS was related to the tumor response.

Conclusions: TOP2A expression is a marker of the tumor's proliferation rate and sensitivity to anthracycline-based chemotherapy, and does not depend on the amplification of its gene.

Introduction

The delivery of chemotherapy preoperatively in the neoadjuvant setting is an option in all cases that would require adjuvant chemotherapy after surgery [1]. The response to neoadjuvant chemotherapy has been demonstrated by multiple studies to correlate with the prognosis [1]. The achievement of pathologically complete regression (pCR), i.e. the absence of any remaining cancerous tissue after primary systemic therapy, indicates an excellent outcome, and is commonly applied as a surrogate end-point in clinical studies on neoadjuvant systemic therapy [1, 2]. Since breast cancer is a heterogeneous disease entity at all stages, the identification of predictive factors with the aim of facilitating the choice of systemic therapy is a relevant approach to improvement of the long-term outcome.

Topoisomerase II alpha (TOP2A) is an enzyme that plays a key role in DNA replication and cell proliferation [3-5]. Targeted inhibition of this enzyme at a molecular level is responsible for the cytotoxic effect of the TOP2A inhibitors, including the anthracycline class. TOP2A is located on chromosome 17 q12-q21, next to the HER2 gene, and its aberrations (amplification or deletion) have been demonstrated mostly in HER2-positive breast cancers [3, 4]. Around one-third of all HER2-positive breast tumors, and at least one-tenth of all breast cancers present with TOP2A gene amplifications, and 4-13% with deletion of the gene [4, 6-14]. TOP2A gene abnormalities of such as amplification or deletion have been related to chemosensitivity in many studies [6-11, 13-16]. The expression of TOP2A has been less extensively studied than its gene abnormalities, but tumors with high TOP2A

expression have been found to be more responsive to anthracycline-based chemotherapy in the adjuvant [12], neoadjuvant [16, 17] and metastatic setting [18]. Amplification [19, 20] or increased expression [21] of the TOP2A gene have been shown to be a predictor of a poor prognosis among patients with ER-positive breast cancer, and of a good prognosis in HER2-positive cases [11]. Despite these achievements, at present the data are inconclusive concerning the predictive role of the TOP2A gene or protein status due to the different methods and study designs used, and the retrospective nature of most analyses.

We set out to perform a retrospective study of the presence of gene abnormalities and the expression of TOP2A in a cohort of breast cancers treated with neoadjuvant anthracycline-based chemotherapy.

Patients and Methods

Patients with operable T2≥3 cm or T3-4 and/or N1-2 and M0 breast cancer were eligible (if clinically node-positive, T1 tumor size was permitted). Full blood count, standard serum biochemistry and imaging examinations including chest X-ray, abdominal ultrasonography and bone scan, were carried out to rule out distant metastases. Through physical examination, mammography, ultrasonography and breast MRI, the initial local/regional tumor status and that after six cycles of chemotherapy were evaluated. Via core needle biopsy, 3 tissue cylinders were taken in each case preoperatively with a 16 G core needle for histopathological examinations. Before or during the chemotherapy, a clip (O-Twist-Marker, BIP Biomed. Instrumente & Produkte GmbH, Germany) was inserted into the tumor for visualization purposes.

All patients received docetaxel 75 mg/m^2 and epirubicin 75 mg/m^2 on day 1 (TE regimen), which in the case of tumor stage T1-T3 was supplemented with capecitebine 2x1000 mg/m^2 daily on days 1-14 (EDC regimen), irrespective of the nodal status. Six cycles of neoadjuvant chemotherapy were delivered every 3 weeks with filgrastim or pegfilgrastim supportation. The clinical response was classified according to the WHO criteria [22]. The tumor characteristics were determined with standardized methods. Tumor regression was graded via the semiquantitative scoring system developed by Sinn et al., as follows: 0 = no effect, 1 = resorption and tumor sclerosis, 2 = minimal residual invasive tumor [< 0.5 cm], 3 = residual noninvasive tumor only, 4 = no tumor detectable [23]. A pCR was taken as the absence of any invasive or *in situ* tumor in the breast or the axilla.

Relapse-free survival (RFS) and overall survival (OS) were calculated from day 1 of chemotherapy to the date of appearance of local/distant metastasis, or the date of death for any reason (or the date of the last follow-up), respectively. Analyses were carried out on the associations of the tumor response, the RFS and the OS with the tumor characteristics, such as the histological type, the pathological stage, the grade, the ER, PgR and HER2 status, the Ki67 and TOP2A protein expressions and the amplification of the TOP2A gene, and the relation between the tumor characteristics before and after chemotherapy.

Tissue micro array (TMA) construction

From the postsurgical specimens or biopsied tissues, an experienced pathologist (LK) selected the most cellular region. The TMA was constructed as described previously [24]. A tissue core 2 mm in diameter was punched for the TMA and

embedded in an acceptor block.   Slides for TOP2A FISH and IHC examinations were made from every block.

Fluorescent in situ hybridization (FISH)

The TMA slides were subjected to triplet color FISH assay (LSI TOP2A spectrum Green /HER2 spectrum Orange/ CEP 17 Spectrum Aqua, Vysis, Downers Grove, IL, USA) for simultaneous evaluation of TOP2A and HER2 genes and chromosome 17-copy number according to the manufacturer's instructions. A ZEISS Axioimager Z2 fluorescence microscope and the Mark and Find System (Carl Zeiss, AxioVision 4.8) were used to identify every spot, in each of which 20 cells were counted and the number of gene copies was assessed. The numbers of the green signals of TOP2A and the orange signals of the HER2 gene and centromere 17 (CEP17) were recorded for each nucleus, and the ratios of the numbers of signals for the gene probes TOP2A and HER2 divided by the number of signals for CEP17 were calculated. TOP2A/CEP17 and HER2/CEP17 ratios >2.2 were defined as gene amplification, and those of <0.8 as deletion. Polysomy was taken as 5 or more copy numbers of centromeres for chromosome 17 per cell.

Immunohistochemistry (IHC)

IHC was done on paired tumor samples taken from the pretreatment biopsies and surgical specimens. All samples were formalin-fixed and paraffin-embedded. If the complete disappearance of the cancer was obtained, only the pre-chemotherapy value could be determined.

ER, PgR, HER2 and Ki67 IHC was carried out with an automatic staining system applying the peroxidase-streptavidin-biotin technique (Dako Autostainer). Slides

were deparaffinized, rehydrated and treated with 3% H_2O_2 in methanol for 10 minutes to block endogenous peroxidase activity. Sections were immersed in 10 mmol/L sodium citrate buffer (pH 6.0), subjected to heat-induced antigen retrieval in a microwave oven for 15 minutes, and then cooled for 20 minutes. A peroxydase-based detection system was used according to the manufacturer's instructions. For HER2 IHC, the HercepTest (Dako Glostrup, Denmark) was used. The Ki67 labeling index was assessed with the MIB1 monoclonal antibody.

The threshold for ER or PgR positivity was 10%. HER2 expression was scored semiquantitavely according to the ASCO/CAP guidelines.

TOP2A IHC involved use of the primary specific monoclonal antibody Topoisomerase II alpha Ki-s1 (Lab Vision, Fremont, CA, USA). Antigen retrieval was achieved by autoclaving in citrate buffer, pH 6.0, for 10 min at 121°C, and an EnVision + System (Dako) was applied as the detection system. Immunostained sections were evaluated by two independent pathologists who had no prior knowledge of the clinicopathologic variables. Each pathologist counted at least 50 cells within randomly selected and outlined areas on each slide, and the percentage of immunostained cells was determined. Disagreement between the pathologists prompted reassesment of the results and a consensus was reached by a joint re-evaluation of the slide.

A cut-off value of 15% separated negative ($\leq$15%) and positive cases (>15%). For HER2 IHC, the standard method was used. HER2 expression was scored semiquantitatively with scores 0-3+, following the accepted criteria; HER2 2+ was regarded as indeterminate, and required HER2 FISH examination. We used Ki67 labeling indices as continuous variables, thus no threshold was used in the analyses.

Statistical analysis

The associations between the binary or multiple versus the continuous variables were analyzed by the independent sample t-test or one-way ANOVA, respectively. The relationships of the qualitative data were tested by chi-square tests. To examine the changes in the tumor markers after chemotherapy, the paired sample t-test and McNemar test were used for the continuous and categorical variables, respectively. The relationship between the continuous variables was examined by correlational analysis. The effects of the tumor markers on RFS and OS were analyzed with the linear regression model. SPSS version 17.0 for Windows (SPSS Inc., Chicago, IL, USA) was applied for statistical analysis.

Results

Between 12/2003 and 08/2010, 43 patients (with 45 tumors) received neoadjuvant anthracycline-based chemotherapy: 12 patients with the ED regimen (one with bilateral tumor) and 31 patients with the EDC regimen (one with bilateral tumor). The mean age (±SD) of the patients was 47.2 (±12.8) years. Forty-two patients (97.7%) completed 6 cycles of chemotherapy, while one patient received only 5 cycles of ED because of disease progression. Complete regression (CR) was revealed by the imaging methods in 15 cases (33.3%), and partial regression (PR) in 26 cases (57.8%); 3 cases (6.7%) did not indicate any significant change (stable disease, SD), while 1 case (2.2%) progressed. Most patients participated in mastectomy (62.2%) and axillary block dissection (97.8%) after the chemotherapy, but 1 patient did not undertake surgery. The initial and post-chemotherapy clinical tumor stages are included in Table 1.

	T					N				Stage			
	T0	T1	T2	T3	T4	N0	N1	N2	N3	0	I	II	III
Pre-chemotherapy (n=45)	0	3	17	18	7	12	9	21	3	0	0	14	31
Post-chemotherapy (n=44)	12	17	13	2	0	25	12	5	2	10	11	15	9

Table 1 Pre-chemotherapy (clinical) and post-chemotherapy (pathological) tumor and lymph node status before and after neoadjuvant chemotherapy according to the

UICC/AJCC TNM classification. Note that one patient did not undergo surgery after neoadjuvant chemotherapy.

About half of the tumors were ER-positive, and one-third of them PgR-positive. HER2 positivity was demonstrated by HER2 IHC and/or FISH in 18% of all samples (Table 2).

Tumor feature		N	%
Histological type	IDC	38	84.4
	ILC	3	6.7
	other	4	8.9
Histologic grade	1	0	0
	2	11	24.4
	3	33	73.3
	unknown	1	2.2
ER	negative	25	55.6
	positive	20	44.4
PR	negative	28	62.2
	positive	17	37.8
HER2	negative	37	82.2
	positive	8	17.8
Proportion (±SD) of Ki67-positive cells (%)	56.1±23.6		
TOP2A	Negative (≤15%)	6	15.8
	Positive (>15%)	32	84.2
	unknown	7	
Proportion (±SD) of TOP2A-positive cells (%)	41.0±27.9		

Table 2 Pathological features of breast cancers before neoadjuvant chemotherapy

No significant change was observed in the ER, PgR or HER2 status of the tumors after chemotherapy. The proportion of Ki67-positive tumor cells was significantly reduced by the chemotherapy (56.1±23.6 vs. 19.0±27.7%, p=0.004). The pathological tumor responses to chemotherapy are listed in Table 3.

Histological tumor regression grade (TRG)	Overall n=44 (%)	EDC n=31 (%)	EC n=13 (%)
TRG0	1 (2.3)	0 (0.0)	1 (7.7)
TRG1	20 (45.5)	16 (51.6)	4 (30.8)
TRG2	10 (22.6)	6 (19.4)	4 (30.8)
TRG3	1 (2.3)	1 (3.2)	0 (0.0)
TRG4	12 (27.3)	8 (25.8)	4 (30.7)

Table 3 Pathological tumor response after neoadjuvant ED or EDC chemotherapy (p=0.50)

Although complete disappearance of the primary tumor (TRG 4) was detected in 12 cases, the axillary lymph nodes were still involved in 3 of these cases, and 9 (20%) cases were therefore classified as pCR. No significant difference existed between tumor response according to the chemotherapy regimen (p=0.50): the proportions of major tumor responses (TRG3-4) were 29% (n=9) and 30.7% (n=4) among the patients treated with the TEX or the TE regimens, respectively (Table 3), while the respective rates of pCR were 22.6% (n=7) and 15.4% (n=2). In an additional lymph node-negative case, only a small DCIS focus remained. The association between the

clinical and pathological tumor responses proved to be statistically significant (p<0.001).

TOP2A FISH/IHC

For technical reasons, the TOP2A FISH and TOP2A IHC results were assessable in only 25 and 38 cases, respectively. With FISH, 23 tumors (92%) exhibited a normal TOP2A gene copy number, while in 2 (8%), the TOP2A gene was amplified; both were HER2-positive by means of IHC and FISH. Despite the fact that the median proportion of IHC-stained cells was 50%, in view of the reference data in the literature [11, 17, 18], we used >15% as a cut-off value for the definition of TOP2A positivity (Table 2). Thirty-two (84.2%) tumors were classified as TOP2A-positive and 6 (15.8%) as TOP2A-negative from the core biopsy. No significant correlation was found between the TOP2A status as determined by FISH and IHC (p=0.52). The average (±SD) proportion of TOP2A-positive cells in the evaluable samples was 41.0±27.9% before, and 12.7±24.8% after the chemotherapy (p<0.001). The expression of TOP2A showed a strong correlation with that of Ki67 (R=0.743, p<0.001), and was negatively correlated with ER (R=0.404, p=0.012) and PgR (R=0.430, p=0.007) (Figure 1), irrespective of the HER2 status (data not shown).

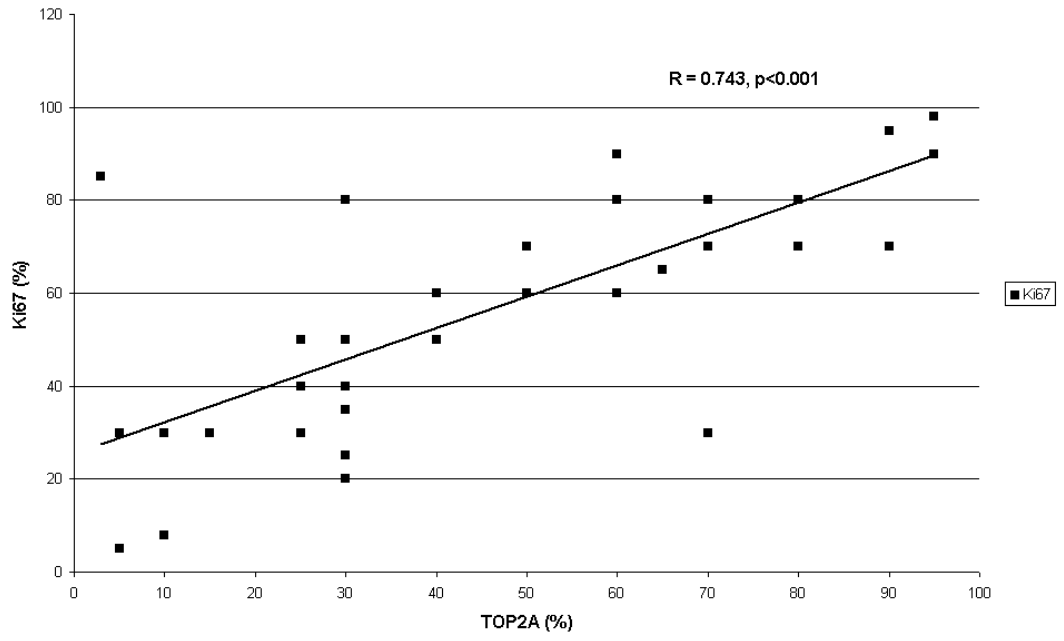**Figure 1.** Correlation between the expression of TOP2A and Ki67

The expression of TOP2A was not related to the HER2 status of the tumor (Table 4)

	HER2-positive	HER2-negative	P
TOP2A-positive	6 (100.0 %)	26 (81.2%)	0.328
TOP2A-negative	0 (0.0 %)	6 (18.8%)	
TOP2A mean (±SD)	37.5±16.7	50.9±29.5	0.291

Table 4 TOP2A protein expression according to the HER2 status of the tumor (n=38)

. Grade 3 cancers displayed higher TOP2A and Ki67 expressions than those of grade 2 cancers (Table 5).

	Grade 2 (%)	Grade3 (%)	p
Ki67 mean (±SD)	42.3±26.6	62.3±24.8	0.036
TOP2A mean (±SD)	35.5 ±26.4	53.5±27.6	0.082

Table 5 Association between tumor grade and initial Ki67 and TOP2A expression

Association between tumor response and tumor characteristics

A major tumor response was seen mostly for grade 3 and ER-negative cancers

(Table 6).

	pCR		
	yes	**no**	p
Grade2	0	11	p=0.054
Grade3	9	24	
ER-negative	8	16	p=0.023
ER-positive	1	19	
PR-negative	7	20	p=0.230
PR-positive	2	15	
HER2-negative	6	30	p=0.196
HER2-positive	3	5	

Table 6 Tumor characteristics in relation with tumor response to neoadjuvant
anthracycline-based chemotherapy (n=44)

The development of pCR was related to high grade (grade 3) (p=0.054) and ER

negativity (p=0.027). While the mean (±SD) pre-chemotherapy TOP2A expression

was 66.9±26.3% in cases with pCR, it was 41.8±26.6% in cases without pCR

(p=0.037). Eight pCRs (21%) occurred among those cases that were assessed for

TOP2A IHC, and all the pCRs occurred in TOP2A-positive cancers. Although no

association was found with TOP2A amplification, both TOP2A-amplified tumors

gave a major response: pCR in one, and a reduction in tumor size from 70 to 15 mm

in the other. Ki67 was not predictive of the tumor response in univariate analysis

(OR=1.027, 95% CI: 0.992-1.062, p=0.167). In the logistic regression model

including the grade, ER, the expression of TOP2A was an independent predictor of

pCR (OR=1.460, for every 10% increase, 95% CI: 1.016-2.096, p=0.041).

Survival

The median follow-up time was 31.0 months. Fourteen patients developed local or

distant recurrence, and 3 died. The median RFS and OS were 23.7 and 31.0

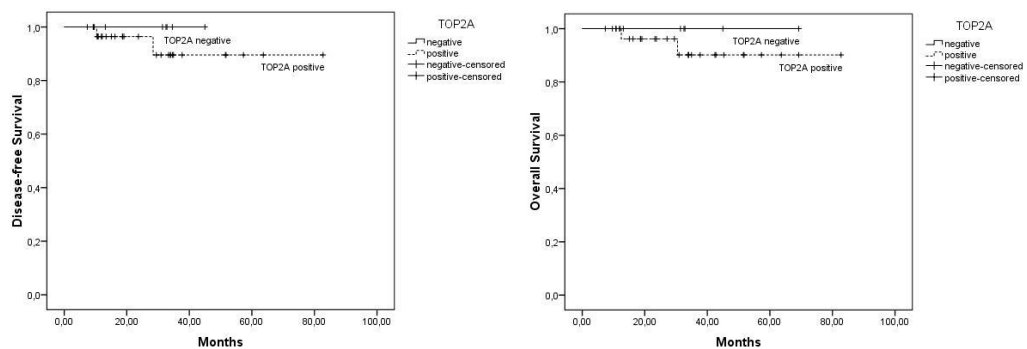months, respectively (Fig. 2).

Figure 2. Survival (DFS and OS) according to the TOP2A status of the breast
cancer

RFS was shorter in cases with PgR-negative than in those with PgR-positive

cancers (23.0 vs. 32.6 months, p=0.07, linear regression: R=0.350, p=0.018), but

OS did not depend on any of the tumor features. The RFS and OS were not related

to the tumor response or the decrease of TOP2A protein expression.

Discussion

We found that despite that the amplification of the TOP2A gene was rare, and

restricted to HER2-positivity, the protein expression was usually elevated in tumors

with high proliferation rate; anthracycline-based chemotherapy resulted in the reduction of the expression of TOP2A. TOP2A positivity was an independent predictor of pCR, and a 10% increase of TOP2A IHC staining resulted in a 46% increase of the likelihood of obtaining a pCR.

The amplification of the TOP2A gene is a rare abnormality, and is restricted to HER2 positive tumors [6, 7, 10-13, 16, 24, 25]. The deletion of the gene is less frequent, and its role in anthracycline-sensitivity is controversial [6, 7, 9, 10, 13-16, 26]. In our cohort, 2 cases with TOP2A gene amplification exhibited HER2 amplification and high TOP2A expression (data not shown), and showed excellent response to the therapy, however, the small number of cases limits the interpretation of the findings.

The cut-off point for defining TOP2A-positivity by IHC varied in different studies. The most often used threshold value was 10-15% (range 5-25%) [11, 17, 18, 27-30], and the rate of TOP2A-positive tumors varied between 5-45% [12, 18, 30]. In a series of 245 tumors, the median proportion of TOP2A-positive cells was 27%, and about half of the tumors rated positive [11]. For the TOP2A-positive category, we used the cut-off value of >15%. In our cohort, the median proportion of TOP2A-stained cells was relatively high (50%), and the majority of tumors classified as TOP2A-positive. The first reason for this finding is the selected nature of our study population: only patients with rapidly proliferating tumors (based on the knowledge of standard tumor characteristics), likely chemosensitive were chosen for neoadjuvant chemotherapy. Second, the methods applied (the tumor regions with the highest cellularity were used; TOP2A-positive cells were counted regardless of the intensity of the staining) also favored high TOP2A values. Of note is that using the same methods, the median TOP2A IHC value in another cohort of our patients

selected for adjuvant chemotherapy and in the whole population irrespective of tumor characteristics was 20 (range: 0-90) and 5 (range: 0-80), respectively (data not shown).

In our study, TOP2A protein expression was found an independent predictor of pCR after neoadjuvant docetaxel-epirubicin chemotherapy, and the probability of pCR increased by almost 50% with every 10% increase of the TOP2A positive tumor cells. The predictive role of the tumor TOP2A status for anthracycline-based chemotherapy has been investigated with different methods, in different settings. The correlation between the efficiency of adjuvant anthracycline-based chemotherapy and the presence of TOP2A gene amplification [13, 14, 31] or the amplification and deletion of the gene [6-8, 10] in the breast tumor is well demonstrated. In those randomized trials which compared an anthracycline-containing chemotherapy with a non-anthracycline containing regimen, the benefit of the former was limited to tumors with the presence of the amplification [13, 31], or the amplification or the deletion of the TOP2A gene [7, 8, 10]. Some studies demonstrated that the presence of TOP2A gene amplification is predictive for the benefit of the dose elevation of the anthracylines [14, 32]. In contrast, a retrospective analysis of the CALGB 8541 study did not support a difference of benefit if doxorubicin was administered at different doses in tumors with TOP2A gene aberrations, but in that study, standard dose was compared with suboptimal doses [9]. In the neoadjuvant setting, the retrospective study of 350 cases showed that the amplification of the TOP2A gene involves a 3 times higher probability of pCR in patients treated with neoadjuvant anthracycline-based chemotherapy [16]. Likewise, the amplification of the TOP2A gene or the polysomy of chromosome 17 indicated a more than 4-times increased probability to obtain pCR in HER2-positive

and ER-, PgR-negative tumors [15]. Park et al. found that the chance of obtaining a response including pCR after neoadjuvant doxorubicin was associated with TOP2A and HER2 coamplification [25]. In a relatively small neoadjuvant study, in consistence with our findings, serial TOP2A IHC determination showed a trend for better response to anthracyclines in tumors with higher TOP2A expression, and a significant decrease after therapy, in responders [17]. Mukherjee et al. found the extent of TOP2A protein expression predictive of the pCR in 91 patients treated with preoperative FEC chemotherapy [33]. The robust study of Press et al. provides evidence that the coamplification of the TOP2A and HER2 genes is a clinically useful predictive marker of an enhanced response to anthracycline-based chemotherapy in metastatic breast cancer [34]. In a phase III study in advanced breast cancer, the TOP2A IHC status was predictive for response to doxorubicin monotherapy, and every 10% increase in TOP2A expression was associated with a 9% increase in the probability of response, while no such effect was demonstrated for the treatment with docetaxel monotherapy [18].

The main outcome and novelty of our study is the demonstration that a simple tool such as TOP2A IHC is an useful, semiquantitative predictive marker of the benefit of neoadjuvant anthracycline-based chemotherapy in breast cancer. The design of our study is not appropriate to answer whether TOP2A IHC staining is a specific marker of anthracycline sensitivity or that of chemosensitivity only. Other studies, however, support the hypothesis that high TOP2A protein expression is predictive of anthracycline sensitivity. In an early study of Di Leo et al., it seemed that the >10% expression of TOP2A protein favors the benefit of both the choice and the higher dose of an adjuvant EC regimen [30]. Likewise, Durbecque et al. in a retrospective analysis of the TAX 303 randomized study, demonstrated that

although docetaxel is more efficient than doxorubicin in the population of advanced breast cancer patients overall, increasing TOP2A expression is associated with a higher chance to obtain a response in the doxorubicin arm, but not in the docetaxel arm [18].

Many studies examined the correlation between the TOP2A gene status and the TOP2A protein expression [11, 12, 27, 29, 35, 37]. Although gene amplification favored high protein expression, the presence of the enzyme was not dependent on the gene abnormality. Jarvinen et al. found that TOP2A protein expression correlated well with TOP2A mRNA [37]. Brase et al., analyzed TOP2A at the levels of gene amplification, RNA expression and protein expression, and studied their correlations. No correlation was found between gene amplification and RNA or protein expression, but a strong correlation existed between TOP2A RNA and protein levels [20]. The conclusion was drawn that unlike in the case of the HER2 status, TOP2A protein expression is highly regulated at the RNA level. In our cohort, most tumors exhibited high TOP2A expression without the presence of TOP2A gene alteration, as a function of high proliferative activity. Schindlbeck et al. based on their study on patients treated with adjuvant anthracycline-based chemotherapy, concluded that it is the TOP2A IHC and not the gene status that predicts benefit of the treatment [12]. The relevance of TOP2A expression in the prediction of anthracycline-sensitivity merits further studies, however, the reconsideration of the optimal method is needed.

We did not find association between outcome and TOP2A expression or the fall of TOP2A expression after neoadjuvant chemotherapy. This result is limited by the relatively short follow-up time. Survival was neither different in other studies by the TOP2A status among patients treated with anthracyclines [11, 26, 28]. Of note is,

however, that the administration of anthracyclines did improve outcome in HER2- and TOP2A-coamplified tumors to a level that was obtained without such chemotherapy, but with the addition of Herceptin [13].

In conclusion, our findings suggest that the IHC determination of the TOP2A protein is a useful tool for the estimation of the sensitivity of breast cancer to anthracycline-based chemotherapy.

References

1. Kaufmann M, von Minckwitz G, Bear HD, Buzdar A, McGale P, Bonnefoi H, Colleoni M, Denkert C, Eiermann W, Jackesz R, Makris A, Miller W, Pierga JY, Semiglazov V, Schneeweiss A, Souchon R, Stearns V, Untch M, Loibl S. Recommendations from an international expert panel on the use of neoadjuvant (primary) systemic treatment of operable breast cancer: new perspectives 2006. Ann Oncol. 2007; 18:1927-1934.

2. Steger GG, Greil R, Jakesz R, Lang A, Mlineritsch B, Melbinger-Zeinitzer E, Marth C, Samonigg H, Kubista E, Gnant M. Final results of ABCSG-24, a randomized phase III study comparing epiribicin, docetaxel, and capecitabine (EDC) to epirubicin and docetaxel (ED) as neoadjuvant treatment for early breast cancer and comparing ED/EDC + trastuzumab (T) to ED/EDC as neoadjuvant treatment for early HER-2 positive breast cancer. Cancer Res 2009; 69: S565 [abstr. 1081]

3. Di Leo A, Biganzoli L, Claudion W, Licitra S, Pestrin M, Larsimont D: Topoisomerase II alpha as a marker predicting anthracyclines' activity in early breast cancer patients: ready for the primetime? Eur J Cancer, 44: 2791-2798, 2008

4. Järvinen TA, Liu ET. HER-2/neu and topoisomerase IIalpha in breast cancer. Breast Cancer Res Treat. 2003; 78:299-311.

5. Isaacs RJ, Davies SL, Sandri MI, Redwood C, Wells NJ, Hickson ID. Physiological regulation of eukaryotic topoisomerase II. Biochim Biophys Acta. 1998;1400:121-137.

6. Di Leo A, Gancberg D, Larsimont D, Tanner M, Jarvinen T, Rouas G, Dolci S, Leroy JY, Paesmans M, Isola J, Piccart MJ. HER-2 amplification and topoisomerase IIalpha gene aberrations as predictive markers in node-positive breast cancer patients randomly treated either with an anthracycline-based therapy or with cyclophosphamide, methotrexate, and 5-fluorouracil. Clin Cancer Res 2002; 8:1107-1116.

7. Knoop AS, Knudsen H, Balslev E, Rasmussen BB, Overgaard J, Nielsen KV, Schonau A, Gunnarsdóttir K, Olsen KE, Mouridsen H, Ejlertsen B. Danish Breast Cancer Cooperative Group. Retrospective analysis of topoisomerase IIa amplifications and deletions as predictive markers in primary breast cancer patients randomly assigned to cyclophosphamide, methotrexate, and fluorouracil or cyclophosphamide, epirubicin, and fluorouracil: Danish Breast Cancer Cooperative Group. J Clin Oncol 2005; 23:7483-7490.

8. Nielsen KV, Ejlertsen B, Moller S, Jorgensen JT, Knoop A, Knudsen H, Mouridsen HT: The value of TOP2A gene copy number variation as a biomarker in breast cancer: Update of DBCG trial 89D. Acta Oncol 2008; 47: 725-734.

9. Harris LN, Broadwater G, Abu-Khalaf M, Cowan D, Thor AD, Budman D, Cirrincione CT, Berry DA, Winer EP, Hudis CA, Hayes DF, Friedman P, Ellis M, Dressler L. Topoisomerase II{alpha} amplification does not predict benefit from dose-intense cyclophosphamide, doxorubicin, and fluorouracil therapy in HER2-amplified early breast cancer: results of CALGB 8541/150013. J Clin Oncol. 2009; 27:3430-3436.

10. O'Malley FP, Chia S, Tu D, Shepherd LE, Levine MN, Bramwell VH, Andrulis IL, Pritchard KI. Topoisomerase II alpha and responsiveness of breast cancer to adjuvant chemotherapy. J Natl Cancer Inst. 2009; 101:644-650.

11. Arriola E, Rodriguez-Pinilla SM, Lambros MBK, Jones RL, James M, Savage K, Smith IE, Dowsett M, Reis-Filho JS: Topoisomerase II alpha amplification may predict benefit from adjuvant anthracyclines in HER2 positive early breast cancer. Breast Cancer Res Treat 2007; 106:181-189.

12. Schindlbeck C, Mayr D, Olivier C, Rack B, Engelstaedter V, Jueckstock J, Jenderek C, Andergassen U, Jeschke U, Friese K. Topoisomerase IIalpha expression rather than gene amplification predicts responsiveness of adjuvant anthracycline-based chemotherapy in women with primary breast cancer. J Cancer Res Clin Oncol. 2010;136:1029-1037.

13. Slamon D, EiermannW, Robert N et al. BCIRG 006: 2nd interim analysis phase II randomized trial comparing doxorubicin and cyclophosphamide followed by docetaxel positive early (AC®TH) with docetaxel, carboplatin and trastuzumab

(TCH) in HER2 positive early breast cancer patients: BCIRG 006 study. Breast Cancer Res Treat 2005; 94:S5 [abstr. 1]

14. Tanner M, Isola J, Wiklund T, Erikstein B, Kellokumpu-Lehtinen P, Malmström P, Wilking N, Nilsson J, Bergh J: Topoisomerase IIalpha gene amplification predicts favorable treatment response to tailored and dose-escalated anthracycline-based adjuvant chemotherapy in HER-2/neu-amplified breast cancer: Scandinavian Breast Group Trial 9401. J Clin Oncol 2006; 24: 2428-2436.

15. Orlando L, Del Curto B, Gandini S, Ghisini R, Pietri E, Torrisi R, Balduzzi A, Cardillo A, Dellapasqua S, Veronesi P, Viale G, Goldhirsch A, Colleoni M: Topoisomerase IIalpha gene status and prediction of pathological complete remission after anthracycline-based neoadjuvant chemotherapy in endocrine non-responsive Her2/neu-positive breast cancer. Breast 2008; 17: 506-511.

16. Konecny GE, Pauletti G, Untch M, Wang HJ, Möbus V, Kuhn W, Thomssen C, Harbeck N, Wang L, Apple S, Jänicke F, Slamon DJ. Association between HER2, TOP2A, and response to anthracycline-based preoperative chemotherapy in high-risk primary breast cancer. Breast Cancer Res Treat. 2010; 120:481-489.

17. Arpino G, Ciocca DR, Weiss H, Allred DC, Daguerre P, Vargas-Roig L, Leuzzi M, Gago F, Elledge R, Mohsin SK. Predictive value of apoptosis, proliferation,

HER-2, and topoisomerase IIalpha for anthracycline chemotherapy in locally advanced breast cancer. Breast Cancer Res Treat. 2005;92:69-75.

18. Durbecq V, Paesmans M, Cardoso F, Desmedt C, Di Leo A, Chan S, Friedrichs K, Pinter T, Van Belle S, Murray E, Bodrogi I, Walpole E, Lesperance B, Korec S, Crown J, Simmonds P, Perren TJ, Leroy JY, Rouas G, Sotiriou C, Piccart M, Larsimont D: Topoisomerase-II alpha expression as a predictive marker in a population of advanced breast cancer patients randomly treated either with single-agent doxorubicin or single-agent docetaxel. Mol Cancer Ther 2004; 3:1207-1214.

19. Rody A, Karn T, Ruckhäberle E, Müller V, Gehrmann M, Solbach C, Ahr A, Gätje R, Holtrich U, Kaufmann M: Gene expression of topoisomerase II alpha (TOP2A) by microarray analysis is highly prognostic in estrogen receptor (ER) positive breast cancer. Breast Cancer Res Treat, 2009;113:4574-66

20. Brase JC, Schmidt M, Fischbach T, Sültmann H, Bojar H, Koelbl H, Hellwig B, Rahnenführer J, Hengstler JG, Gehrmann MC. ERBB2 and TOP2A in breast cancer: a comprehensive analysis of gene amplification, RNA levels, and protein expression and their influence on prognosis and prediction. Clin Cancer Res. 2010;16:2391-2401.

21. Sparano JA, Goldstein LJ, Childs BH, Shak S, Brassard D, Badve S, Baehner FL, Bugarini R, Rowley S, Perez E, Shulman LN, Martino S, Davidson NE, Sledge GW Jr, Gray R. Relationship between Topoisomerase 2A RNA Expression and Recurrence after Adjuvant Chemotherapy for Breast Cancer. Clin Cancer Res. 2009;15:7693-7700.

22. Miller AB, Hoogstraten B, Staquet M, Winkler A. Reporting results of cancer treatment. Cancer 1981; 47:207-214.

23. Sinn HP, Schmid H, Junkermann H, Huober J, Leppien G, Kaufmann M, Bastert G, Otto HF: Histologic regression of breast cancer after primary (neoadjuvant) chemotherapy. Geburtshilfe Frauenheilkd 1994; 54:552-558.

24. Nocito A, Kononen J, Kallioniemi OP, Sauter G. Tissue microarrays (TMAs) for high-throughput molecular pathology research. Int J Cancer 2001;94:1–5.

25. Park K, Kim J, Lim S, Han S. Topoisomerase II-alpha (topoII) and HER2 amplification in breast cancers and response to preoperative doxorubicin chemotherapy. Eur J Cancer. 2003;395:631-634.

26. Tubbs R, Barlow WE, Budd GT, Swain E, Porter P, Gown A, Yeh IT, Sledge G, Shapiro C, Ingle J, Haskell C, Albain KS, Livingston R, Hayes DF. Outcome of

patients with early-stage breast cancer treated with doxorubicin-based adjuvant chemotherapy as a function of HER2 and TOP2A status. J Clin Oncol. 2009;27:3881-3886.

27. Bhargava R, Lal P, Chen B: HER-2/neu and topoisomerase IIa gene amplification and protein expression in invasive breast carcinomas: chromogenic in situ hybridization and immunohistochemical analyses. Am J Clin Pathol 2005; 123:889-895

28. Gómez HL, Pinto JA, Olivera M, Vidaurre T, Doimi FD, Vigil CE, Velarde RG, Abugattas JE, Alarcón E, Vallejos CS. Topoisomerase II-alpha as a predictive factor of response to therapy with anthracyclines in locally advanced breast cancer. Breast. 2010 Aug 10. [Epub ahead of print]

29. Cardoso F, Durbecq V, Larsimont D, Paesmans M, Leroy JY, Rouas G, Sotiriou C, Renard N, Richard V, Piccart MJ, Di Leo A. Correlation between complete response to anthracycline-based chemotherapy and topoisomerase II-alpha gene amplification and protein overexpression in locally advanced/metastatic breast cancer. Int J Oncol 2004; 24:201-209.

30. Di Leo A, Larsimont D, Gancberg D, Jarvinen T, Beauduin M, Vindevoghel A, Michel J, Focan CH, Ries F, Gobert PH, Closon-Dejardin MT, Dolci S, Rouas G, Paesmans M, Lobelle JP, Isola J, Piccart MJ: HER-2 and topo-isomerase IIalpha as predictive markers in a population of node-positive breast cancer patients randomly

treated with adjuvant CMF or epirubicin plus cyclophosphamide. Ann Oncol 2001; 12:1081-1089.

31. Gunnarsdóttir KA, Jensen MB, Zahrieh D, Gelber RD, Knoop A, Bonetti M, Mouridsen H, Ejlertsen B. CEF is superior to CMF for tumours with TOP2A aberrations: a Subpopulation Treatment Effect Pattern Plot (STEPP) analysis on Danish Breast Cancer Cooperative Group Study 89D. Breast Cancer Res Treat 2010; 123:163-169.

32. Hannemann J, Kristel P, van Tinteren H, Bontenbal M, van Hoesel QG, Smit WM, Nooij MA, Voest EE, van der Wall E, Hupperets P, de Vries EG, Rodenhuis S, van de Vijver MJ. Molecular subtypes of breast cancer and amplification of topoisomerase II alpha: predictive role in dose intensive adjuvant chemotherapy. Br J Cancer 2006; 95:1334-1341.

33. Mukherjee A, Shehata M, Moseley P, Rakha E, Ellis I, Chan S. Topo2α protein expression predicts response to anthracycline combination neo-adjuvant chemotherapy in locally advanced primary breast cancer. Br J Cancer. 2010;103:1794-1800.

34. Press MF, Sauter G, Buyse M, Bernstein L, Guzman R, Santiago A, Villalobos IE, Eiermann W, Pienkowski T, Martin M, Robert N, Crown J, Bee V, Taupin H, Flom KJ, Tabah-Fisch I, Pauletti G, Lindsay MA, Riva A, Slamon DJ. Alteration of

Topoisomerase II-Alpha Gene in Human Breast Cancer: Association With Responsiveness to Anthracycline-Based Chemotherapy. J Clin Oncol. 2010 Dec 28. [Epub ahead of print]

35. Mueller RE, Parkes RK, Andrulis I, O'Malley FP: Amplification of the TOP2A gene does not predict high levels of topoisomerase II alpha protein in human breast tumor samples. Genes Chromosomes Cancer 2004; 39:288-297.

36. Callagy G, Pharoah P, Chin SF, Sangan T, Daigo Y, Jackson L, Caldas C: Identification and validation of prognostic markers in breast cancer with the complementary use of array-CGH and tissue microarrays. J Pathol 2005; 205:388-396.

37. Järvinen TA, Kononen J, Pelto-Huikko M, Isola J. Expression of topoisomerase IIalpha is associated with rapid cell proliferation, aneuploidy, and c-erbB2 overexpression in breast cancer. Am J Pathol. 1996;148:2073-2082.

II

Running head: Topoisomerase II alpha expression and adjuvant chemotherapy in breast cancer

Manuscript type: original paper

Tumour topoisomerase II alpha protein expression and outcome after adjuvant dose-dense anthracycline-based chemotherapy

Alíz Nikolényi[1], Gabriella Uhercsák[1], Melinda Csenki[1], Sándor Hamar[2], Erika Csörgő[2], Ervin Tánczos[3], László Thurzó[1], Thomas Brodowicz[4], Maria Wagnerova[5], Zsuzsanna Kahán[1]

[1]Department of Oncotherapy, [2]Department of Pathology, [3]Department of Medical Informatics, [4]Department of Surgery, University of Szeged, Szeged, Hungary; [4]Central European Cooperative Oncology Group, Vienna, Austria; [5]Oncology Institute, Department of Radiotherapy and Oncology, Kosice, Slovakia

Corresponding author: Zsuzsanna Kahán, Department of Oncotherapy, University of Szeged, Korányi fasor 12, H-6720 Szeged, Hungary; Phone: 36-62-545406; Fax: 36-62-545922; E-mail: kahan@onko.szote.u-szeged.hu

Key-words: anthracyclines, adjuvant chemotherapy, breast cancer, dose-dense chemotherapy, topoisomerase II alpha

Abbreviations

A   adriamycin

ADC   adriamycin (A)-docetaxel (D)-cyclophosphamide (C) chemotherapy study

ATC   adriamycin (A)-paclitaxel (T)-cyclophosphamide (C) chemotherapy study

C   cyclophosphamide

CECOG   Central European Cooperative Oncology Group

D   docetaxel

ER   estrogen receptor

FEC   fluorouracil-epirubicin-cyclophosphamide chemotherapy

FISH   fluorescence in situ hybridisation

GCSF   granulocyte colony stimulating factor

IHC   immunohistochemistry

LVI   lymphovascular invasion

OS   overall survival

PR   progesterone receptor

RFS   recurrence-free survival

RNA   ribonucleic acid

T   paclitaxel

TOP2A   topoisomerase II alpha

Abstract

There is a need for the selection of those breast cancers where benefit may be attained from the addition of an anthracycline to the adjuvant chemotherapy. The expression of topoisomerase II alpha (TOP2A) protein in 3 cohorts of breast cancers treated with adjuvant dose-dense anthracycline-based chemotherapy was determined retrospectively. The TOP2A status was analysed in relation with the other standard tumour features and the outcome. TOP2A IHC results were assessable in 106 patients: with a cut-off value of 15%, 48% of the tumours were classified as TOP2A-positive. The expression of TOP2A correlated with that of Ki67 (R=0.532, p<0.001) and a high grade (p=0.04) , but did not correlate with the proportion of ER- or PR-positive cells in the tumour. More tumors were TOP2A-negative among the ER- or PR-positive cancers than among the ER/PR-negative cancers (p=0.021 and p=0.002, respectively). After a median follow-up time of 64.5 months, 31 relapses (23.5%) and 23 deaths (17.4%) had occurred in 131 patients. The overall survival was longer in the TOP2A-positive cases than in the TOP2A-negative cases. The recurrence-free survival and the overall survival were significantly more favourable in the ER/PR-negative and TOP2A-positive tumours than in other subgroups. In a Cox proportional hazards model, the grade and TOP2A remained significant determinants in the ER/PR-negative subgroup. TOP2A positivity and grade 3 indicated a decrease in the risk of death with HR=0.211 (95% CI: 0.042-1.05, p=0.056) and HR=0.216 (95% CI: 0.047-0.990, p=0.048), respectively. A higher sensitivity to anthracycline-containing regimens is suggested in ER/PR-negative and TOP2A-positive cancers.

Introduction

The anthracyclines have been widely used during the past 30 years for the adjuvant therapy of breast cancer, and have proved superior efficacy to non-anthracycline-containing regimens [1]. The use of anthracyclines, however, involves a higher risk of long-term toxicity such as cardiac failure and myeloproliferative disease, and restriction of their use was suggested in view of the results of the adjuvant BCIRG006 Trial [2]. Breast cancers display differences in sensitivity to anthracyclines, use of which should be limited to the anthracycline-sensitive cases [3]. There is clearly a need for the identification of predictive factors and the selection of cancers likely to benefit most from the use of anthracyclines.

The most extensively studied such predictive factor has been HER2. From a pooled analysis of 8 randomized studies involving more than 5000 patients, Gennari et al. concluded that the added benefit of anthracycline-containing chemotherapy is confined to HER2-positive cases [4]. In accord with this, in cases without HER2 gene amplification in the MA.5 randomized clinical trial, CEF chemotherapy did not improve the recurrence-free survival (RFS) or the overall survival (OS) [5]. In the NEAT study, however, the opposite effect was found, i.e. the benefit of the addition of doxorubicin to CMF was limited to HER1-3-negative cancers [6]. Few data exist on the increased efficiency of anthracyclines in certain HER2-negative cancers, such as the triple negative or basal and other undifferentiated breast cancers [7].

Many experimental and clinical data support the possible role of the topoisomerase II alpha (TOP2A) status of the tumour in the prediction of anthracycline sensitivity. TOP2A is an enzyme that plays a pivotal role in DNA replication and cell

proliferation [8-10]. Targeted inhibition of this enzyme at a molecular level is responsible for the cytotoxic effect of the TOP2A inhibitor anthracyclines. TOP2A is located on chromosome 17 q12-q21, next to the HER2 gene, and its aberrations (amplification or deletion) have been demonstrated mostly [8,9], but not exclusively [11], in HER2-positive breast cancers. Around one-third of all HER2-positive breast tumours, and at least one-tenth of all breast cancers, present with TOP2A gene amplifications, and 4-13% with deletion of the gene [2,9,11-18]. The protein expression of TOP2A does not depend on the presence of gene aberrations [16,17,19-22], and is highly regulated at the RNA level [23]. Both TOP2A gene abnormalities [2,11-16,19,24] and high TOP2A expression [17,24,25] have been related to the greater efficiency of anthracycline-based chemotherapy.

We set out to perform a retrospective study of the expression of TOP2A in 3 cohorts of breast cancers treated with adjuvant dose-dense anthracycline-based chemotherapy, with the aim of an analysis of the TOP2A status in relation to other tumour features and the outcome.

Materials and Methods

Data from 3 phase II clinical studies with adjuvant dose-dense anthracycline-based chemotherapy were collected. In the dose-dense sequential adriamycin (A)-paclitaxel (T)-cyclophosphamide (C) chemotherapy study (ATC group), 55 high-risk breast cancer patients received 60 mg/m^2 A for 4 cycles, 200 mg/m^2 T for 4 cycles, and 800 mg/m^2 C for 4 cycles, all chemotherapy cycles 2 weeks apart with GCSF support [26]. All the patients completed the 4 A cycles, and 47 (85.5%)

patients completed all 12 cycles. In the very similar dose-dense sequential adriamycin (A)-docetaxel (D)-cyclophosphamide (C) chemotherapy study (ADC group), 34 breast cancer patients received 60 mg/m^2 A for 4 cycles, 75 mg/m^2 D for 4 cycles, and 800 mg/m^2 C for 4 cycles, all chemotherapy cycles 2 weeks apart, with GCSF support. Of the 34 patients enrolled, 33 (97%) completed all 12 cycles, whereas one was excluded after the first 7 cycles because of disease progression. In the dose-dense FEC study (CECOG group), breast cancer patients were randomized to 6 cycles of FEC$_{75}$ or FEC$_{90}$ (fluorouracil 500 mg/m^2, epirubicin 75 or 90 mg/m^2, respectively and cyclophosphamide 500 mg/m^2) with pegfilgasrtim support [27]. Most of the enrolled 51 patients completed the study, but the clinical data and the tumour samples were accessible in only 43 cases treated at the Hungarian and the Slovakian centres. Patient- and tumour-related data, such as the pathological stage, the grade, and the ER, PR, HER2 and Ki67 status, determined by standard methods in the 3 study populations, are included in Table 1.

The RFS and the OS were calculated from day 1 of chemotherapy to the date of appearance of local recurrence/distant metastasis, or the date of death for any reason (or the date of the last follow-up), respectively. Analyses were carried out on the associations of the RFS and the OS with the tumour characteristics.

Tissue microarray (TMA) construction

TMAs were constructed from formalin-fixed and paraffin-embedded tumour blocks as described previously [28]. An experienced pathologist (SH) selected the most cellular region. A tissue core 2 mm in diameter was punched for the TMA and embedded in an acceptor block. Slides for FISH and IHC examinations were made from every block.

Immunohistochemistry (IHC)

TOP2A IHC involved use of the primary specific monoclonal antibody TOP2A Ki-s1 (Lab Vision, Fremont, CA, USA) with an automatic staining machine (Dako Autostainer). Antigen retrieval was achieved by autoclaving in citrate buffer, pH 6.0, for 10 min at 121°C, and an EnVision + System (Dako) was applied as the detection system. The nuclei of 50 tumour cells were counted under the microscope by two independent examiners, and the proportion of stained cells was recorded. A cut-off value of 15% separated negative ($\leq$15%) and positive cases (>15%).

For HER2 IHC, the standard method was used. HER2 expression was scored semiquantitatively with scores in the range 0-3+, following the accepted criteria; HER2 2+ was regarded as indeterminate, and required HER2 FISH examination.

Statistical analysis

Univariate comparisons of groups was performed by one-way ANOVA and chi-square testing in cases of continuous and categorical variables, respectively. Two-by-two frequency tables were evaluated by means of Fisher's exact test. The relationship between the continuous variables was examined by correlational analysis. The dependence of the durations of the RFS and OS on the possible risk factors was analysed by means of the Kaplan-Meier method. To estimate the effects of the TOP2A protein expression and the conventional prognostic factors on the outcome, the Cox proportional hazards model was utilized. A stepwise selection method was performed, using the likelihood-ratio statistics based on the maximum partial likelihood estimates. SPSS version 17.0 for Windows (SPSS Inc., Chicago, IL, USA) was applied for statistical analysis.

Results

The patient- and tumour-related characteristics within the 3 study cohorts and in the
overall population are included in Table 1.

	ATC (n=55)	ADC (n=34)	CECOG (n=43)	Overall (n=132)
Age (mean±SE)	59.8±1.2	54.9±1.6	54.9±1.6	57.0±0.8
pT (mean±SE, mm)	35.6±2.8	16.7±2.7	22.5±2.3	26.3±1.6
pN+ (median)	6	0.5	2	3
Histological type (%) IDC ILC Medullary Other	43 (78.2) 6 (10.9) 1 (1.8) 5 (9.1)	30 (88.3) 1 (2.9) 0 (0) 3 (8.8)	35 (81.4) 4 (9.3) 3 (7.0) 1 (2.3)	108 (81.8) 11 (8.3) 4 (3.0) 9 (6.9)
LVI present (%)	38 (69.1)	9 (26.5)	28 (65.1)	75 (56.8)
Grade 1 **Grade 2** **Grade 3** **Grade unknown**	3 (5.4) 10 (18.2) 27 (49.1) 15 (27.3)	0 (0) 12 (35.3) 17 (50) 5 (14.7)	2 (4.7) 18 (41.9) 22 (51.2) 1 (2.3)	5 (3.8) 40 (30.3) 66 (50.0) 21 (15.9)
ER positive (%)	25 (45.5)	17 (50)	16 (37.2)	33 (25)
PR positive (%)	23 (41.8)	15 (44.1)	15 (34.9)	30 (22.7)
HER2 positive(%)	13 (23.6)	9 (26.5)	7 (16.3)	29 (22.0)
Ki67 (mean±SE, %)	29.3±4.2	25.0±4.5	42.3±5.5	32.3±2.7
Ki67 (median, %)	20	20	30	25

Table 1. Patient- and tumour-related characteristics within the study groups and the overall population

The median follow-up time for the entire population was 64.5 months, and for the ATC, ADC and CECOG cohorts was 103, 44.5 and 60 months, , respectively. Altogether 31 relapses (23.5%) and 23 deaths (17.4%) occurred. The OS differed significantly in the 3 cohorts: the ATC cohort exhibited the worst, and the ADC cohort the best survival ($p<0.01$). Among the standard prognostic factors, the pathological tumour size (pT) and the number of positive lymph nodes were associated with the RFS in the overall study population ($p<0.05$), while the presence of LVI was related to the RFS in the ADC cohort.

TOP2A IHC

For technical reasons, the TOP2A IHC results were assessable in only 106 cases. In the overall population, the average and median proportions of the TOP2A-positive cells were 21% and 10%, respectively. With a cut-off value of 15%, 48% of the tumours were classified as TOP2A-positive (Table 2).

	ATC	ADC	CECOG	Overall
TOP2 A IHC (n)	40	27	39	106
TOP2A (mean±SE, %)	18.3±3.4	17.33±5.0	24.5±5.0	21.02±2.3
TOP2A (median, %)	10	15	10	10
TOP2A+ (n)(%)	16 (40)	14 (51.9)	21 (53.8)	51 (48.1)

Table 2 TOP2A IHC status in the study groups and the overall population

Most of the TOP2A-positive tumours were of grade 3 (p=0.004). The expression of TOP2A correleted significantly with that of Ki67 (R=0.532, p<0.001), but not with ER or PR. Among the ER- and/or PR-positive cancers, more were TOP2A-negative than among the ER- and PR-negative cancers (p=0.021 and p=0.002, respectively) (Table 3).

	ATC (n=40)		ADC (n=27)		CECOG (n=39)		Overall (n=106)	
	TOP2A-	TOP2A+	TOP2A-	TOP2A+	TOP2A-	TOP2A+	TOP2A-	TOP2A+
ER-	11	11	6	8	5	13	22	32
ER+	13	5	7	6	13	8	33	19
p	0.203		0.706		0.054		0.021	
PR-	10	12	7	10	6	15	23	37
PR+	14	4	6	4	12	6	32	14
p	0.054		0.440		0.026		0.002	

Table 3 TOP2A IHC status according to the ER/PR status of the tumour

All hormone receptor-negative cancers were of grade 2 or 3, and TOP2A-positive cases were more frequently of grade 3 (p=0.066 and p=0.040 in the ER-negative and the PR-negative groups, respectively). No association was detected between the TOP2A status and the grade of the tumour in the hormone receptor-positive group. The expression of TOP2A was not related to the tumour size, the number of positive nodes or the HER2 status of the tumour. The protein expressions of TOP2A and Ki67 increased with the grade (p=0.162 and p=0.005, respectively).

Association between outcome and tumour TOP2A status

In the overall population, more relapses and more deaths occurred among the TOP2A-negative cases than among the TOP2A-positive cases, and the RFS and OS were longer accordingly (Table 4, Fig. 1).

TOP2A IHC	number of deaths (%)	OS (mean±SE) (months)	number of relapses (%)	RFS (mean±SE) (months)
Negative	14/55 (25.5)	93.3±6.0	14/55 (25.5)	93.7±6.1
Positive	6/51 (11.8)	103.8±4.3	8/51 (15.7)	96.8±5.9
p (Mantel-Cox)		0.081		0.229

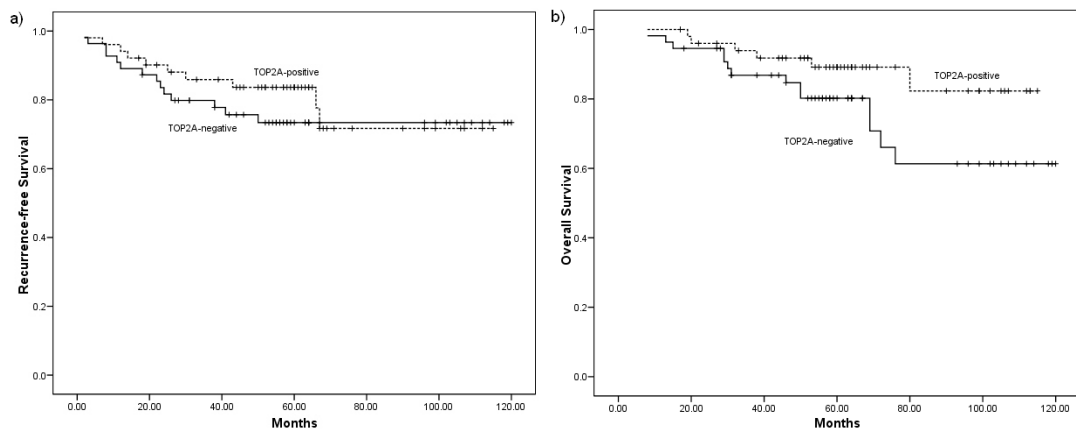**Table 4** Survival (OS and RFS) according to the TOP2A status of the tumour

Figure 1. Survival (OS and RFS) according to the TOP2A status of the tumour

The outcome in the hormone receptor-positive and hormone receptor-negative subgroups was analysed separately (Table 5, Fig. 2).

	ER-negative		ER-positive		PR-negative		PR-positive	
	TOP2 A-	TOP2 A+	TOP2 A-	TOP2 A+	TOP2 A-	TOP2 A+	TOP2 A-	TOP2 A+
number of deaths	6/22	2/32	8/33	4/19	7/23	2/37	7/32	4/14
OS (mean±SE) (months)	93.1± 9.3	109.3± 3.9	92.9± 7.6	82.7± 6.8	89.0± 9.6	110.5± 3.1	95.7± 7.4	81.3± 10.2
p (Mantel-Cox)	0.035		0.916		0.005		0.494	
number of relapses	7/22	5/32	7/33	5/19	7/23	6/37	7/32	4/14
RFS(mean ±SE) (months)	87.2± 10.3	97.1± 7.5	97.2± 7.3	77.6± 8.1	87.3± 10.3	97.2± 6.7	97.7± 7.1	79.5± 11.2
p (Mantel-Cox)	0.176		0.774		0.169		0.639	

Table 5 Survival (OS and RFS) according to the TOP2A and ER/PR status of the tumor
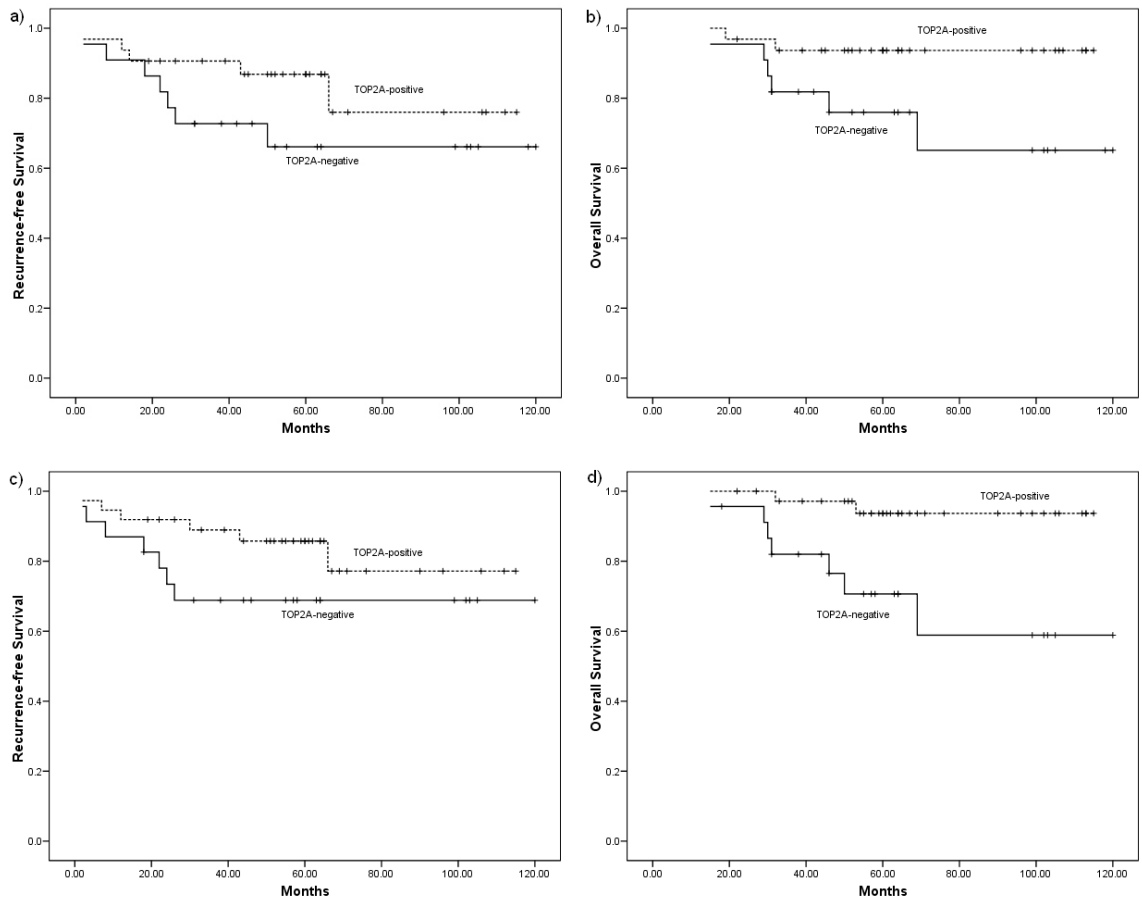
Figure 2. **a,c** Survival (OS and RFS) according to the tumor TOP2A IHC status in the ER and/or PR positive tumors

Figure 2 b,d Survival (OS and RFS) according to the tumor TOP2A IHC status in the ER and/or PR negative tumors

While there was no difference in the number of events, or in the OS and the RFS in the ER- and the PR-positive subgroups according to the TOP2A status, the OS and RFS were significantly improved in the ER- or PR-negative and TOP2A-positive cases as compared with the TOP2A-negative cases (Table 5, Fig. 2). Figure 1 presents the RFS and OS as functions of the TOP2A expression status in ER/PR-negative cases.

In order to estimate the dependence of the OS and the RFS on the tumour TOP2A and Ki67 status, the tumour grade and the nodal status in ER- and/or PR-negative cancer, these variables were studied in a Cox proportional hazards model. In grade

3 cases, the risk of death was decreased, with HR= 0.216 (95% CI: 0.047-0.990, p=0.048) as compared with grade 2 cases. In the TOP2A-positive cases, the risk of death was decreased, with HR=0.211 (95% CI: 0.042-1.05, p=0.056). In multivariate analysis, no interaction was detected between these variables. No other significant effect was emerged.

Discussion

We found TOP2A positivity in about half of the cancers treated with adjuvant dose-dense anthracycline-based chemotherapy. TOP2A positivity was more frequent among the ER- and/or PR-negative cancers. Among the hormone receptor-negative cases, TOP2A positivity and grade 3 indicated improved OS and RFS. Int he light of the findings of a more favourable outcome after adjuvant dose-dense anthracycline-based chemotherapy in the ER/PR-negative and TOP2A-positive and/or grade 3 subgroups, a higher sensitivity to this regimen is suggested in these cases.

The TOP2A status in breast cancer has been studied as a prognostic and predictive factor by different methods in multiple studies. Most investigators agree that the amplification or the deletion of the TOP2A gene is restricted to HER2-positive cancers [2,8,16,24]. Co-amplification of the HER2 and TOP2A genes indicated an increased anthracycline sensitivity in most [2,8,16,24,29], but not all studies [6,15]. The design of these retrospective studies, however, was not always appropriate for detection of the benefit of anthracycline therapy according to the presence of TOP2A gene abnormality [15,30]. In those randomized trials which compared

anthracycline-containing chemotherapy with a non-anthracycline-containing regimen, the benefit of the former was limited to tumours with an abnormal TOP2A gene status [2,11,13,14,31]. Some studies have demonstrated that the presence of a TOP2A gene alteration is predictive of the benefit of an elevation of the anthracyline dose [19,32]. Deletion of the gene is less frequent, and its role in anthracycline sensitivity seems rather controversial [2,12-14,18,24,30]. In line with the contradictory results, it is noteworthy that, although TOP2A gene abnormalities have been observed exclusively in HER2-positive breast cancers, high anthracycline sensitivity is not limited to this special group [7]. Investigations of whether the expression of TOP2A is a specific marker of anthracycline sensitivity gave more concordant results. The early study by Di Leo et al. led to the conclusion that a finding of TOP2A positivity by means of IHC determination favoured the benefit of both the choice and a higher dose of an adjuvant EC regimen [33]. Likewise, in a retrospective analysis of the TAX 303 randomized study, Durbecque et al. demonstrated that, although docetaxel is more efficient than doxorubicin in the population of advanced breast cancer patients overall, increase of the TOP2A protein expression is associated with a higher chance of obtaining a response in the doxorubicin arm, but not in the docetaxel arm [25]. The greater sensitivity to anthracycline-based adjuvant chemotherapy of ER/PR-negative breast cancers as compared with ER- or PR-positive tumours has been well demonstrated [7,34]. Our own study suggests that one of the related key factors is the more frequent TOP2A positivity among the ER/PR-negative tumours, and we advocate TOP2A IHC as a tool to select those hormone receptor-negative cases which would benefit from adjuvant anthracyclines. In a patient population treated with adjuvant anthracycline-containing chemotherapy, Schindlbeck et al.

retrospectively examined the TOP2A status. About 50% of the cases proved to be

TOP2A-positive, and after a median survival time of 42 months, the survival was

significantly poorer among the TOP2A-negative cases [17]. Brase et al.

demonstrated the strong negative prognostic power of an elevated TOP2A RNA

level in 782 untreated breast cancer patients, which remained significant after

further analyses in the ER-positive and the HER2-negative and triple-negative

subgroups. In the same paper, complete tumour regression to chemotherapy with

EC was reported to be related to the high TOP2A and low ER RNA levels, results

which support our finding that anthracyclines result in a favourable outcome in ER-

negative and TOP2A-positive cancers [23]. Rody et al.followed up more than 1300

patients, and found that the TOP2A expression was the strongest indicator of a poor

prognosis among hormone receptor-positive cases, while no such effect was

detected among the ER-negative cases [35]. Although the prognostic effect of

TOP2A positivity was found to be independent of the systemic therapy, the nature

of the chemotherapy given in about half of the patients, was not reported. It may be

speculated that the similar outcome in the TOP2A-positive and -negative cases in

the ER-negative group may be due to the higher chemosensitivity of the TOP2A-

positive cases.

The expression of TOP2A seems to be regulated most strongly at the RNA level,

and its gene status is probably less determinative of its functional capacity. Jarvinen

et al. and Brase et al. found no correlation between gene amplification and protein

expression, but there was a strong correlation between the TOP2A RNA and protein

levels [23,36]. Accordingly, although gene amplification favoured a high protein

expression in those studies that examined the correlation between the TOP2A gene

status and the TOP2A protein expression, the presence of the enzyme was not

dependent on the gene abnormality [16,17,20-22]. Their findings led Brase et al. to recommend determination of the RNA expression, while Schindlbeck et al. suggested determination of the protein expression of TOP2A for patient selection, rather than examination of the gene status [17,23].

Our own data indicate that a simple tool such as TOP2A IHC (together with the grade) is a useful predictive marker, at least in the hormone receptor-negative cases, and should be implemented in routine practice for the selection of those who can be expected to benefit from adjuvant anthracycline-based chemotherapy. The usually poor outcome in the group of hormone receptor-negative and TOP2A-positive cases may be reversed by the application of anthracycline-containing chemotherapy.

References

1. Early Breast Cancer Trialists' Collaborative Group (EBCTCG): Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. Lancet. 2005; 365:1687-1717.

2. Slamon D, EiermannW, Robert N et al. BCIRG 006: 2nd interim analysis phase II randomized trial comparing doxorubicin and cyclophosphamide followed by docetaxel positive early (AC®TH) with docetaxel, carboplatin and trastuzumab (TCH) in HER2 positive early breast cancer patients: BCIRG 006 study. Breast Cancer Res Treat 2005; 94:S5 [abstr. 1]

3. Gianni L, Norton L, Wolmark N, Suter TM, Bonadonna G, Hortobagyi GN. Role of anthracyclines int he treatment of early breast cancer. J Clin Oncol 2009; 27: 4798-4808.

4. Gennari A, Sormani MP, Pronzato P, Puntoni M, Colozza M, Pfeffer U, Bruzzi P. HER2 status and efficacy of adjuvant anthracyclines in early breast cancer: a pooled analysis of randomized trials. J Natl Cancer Inst. 2008; 100:14-20.

5. Pritchard KI, Shepherd LE, O'Malley FP, Andrulis IL, Tu D, Bramwell VH, Levine MN; National Cancer Institute of Canada Clinical Trials Group. HER2 and responsiveness of breast cancer to adjuvant chemotherapy. N Engl J Med. 2006; 354: 2103-2111.

6. Bartlett JM, Munro A, Cameron DA, Thomas J, Prescott R, Twelves CJ. Type 1 receptor tyrosine kinase profiles identify patients with enhanced benefit from anthracyclines in the BR9601 adjuvant breast cancer chemotherapy trial. J Clin Oncol. 2008; 26: 5027-5035.

7. Rouzier R, Perou CM, Symmans WF, Ibrahim N, Cristofanilli M, Anderson K, Hess KR, Stec J, Ayers M, Wagner P, Morandi P, Fan C, Rabiul I, Ross JS,

Hortobagyi GN, Pusztai L. Breast cancer subtypes respond differently to preoperative chemotherapy. Clin Cancer Res 2005; 11:5678-5685.

8. Di Leo A, Biganzoli L, Claudion W, Licitra S, Pestrin M, Larsimont D: Topoisomerase II alpha as a marker predicting anthracyclines' activity in early breast cancer patients: ready for the primetime? Eur J Cancer, 44: 2791-2798, 2008

9. Järvinen TA, Liu ET. HER-2/neu and topoisomerase IIalpha in breast cancer. Breast Cancer Res Treat. 2003; 78:299-311.

10. Isaacs RJ, Davies SL, Sandri MI, Redwood C, Wells NJ, Hickson ID. Physiological regulation of eukaryotic topoisomerase II. Biochim Biophys Acta. 1998;1400:121-137.

11. Nielsen KV, Ejlertsen B, Moller S, Jorgensen JT, Knoop A, Knudsen H, Mouridsen HT: The value of TOP2A gene copy number variation as a biomarker in breast cancer: Update of DBCG trial 89D. Acta Oncol 2008; 47: 725-734.

12. Di Leo A, Gancberg D, Larsimont D, Tanner M, Jarvinen T, Rouas G, Dolci S, Leroy JY, Paesmans M, Isola J, Piccart MJ. HER-2 amplification and topoisomerase IIalpha gene aberrations as predictive markers in node-positive breast cancer patients randomly treated either with an anthracycline-based therapy or with cyclophosphamide, methotrexate, and 5-fluorouracil. Clin Cancer Res 2002; 8:1107-1116.

13. Knoop AS, Knudsen H, Balslev E, Rasmussen BB, Overgaard J, Nielsen KV, Schonau A, Gunnarsdóttir K, Olsen KE, Mouridsen H, Ejlertsen B. Danish Breast Cancer Cooperative Group. Retrospective analysis of topoisomerase IIa

amplifications and deletions as predictive markers in primary breast cancer patients randomly assigned to cyclophosphamide, methotrexate, and fluorouracil or cyclophosphamide, epirubicin, and fluorouracil: Danish Breast Cancer Cooperative Group. J Clin Oncol 2005; 23:7483-7490.

14. O'Malley FP, Chia S, Tu D, Shepherd LE, Levine MN, Bramwell VH, Andrulis IL, Pritchard KI. Topoisomerase II alpha and responsiveness of breast cancer to adjuvant chemotherapy.
J Natl Cancer Inst. 2009; 101:644-650.

15. Harris LN, Broadwater G, Abu-Khalaf M, Cowan D, Thor AD, Budman D, Cirrincione CT, Berry DA, Winer EP, Hudis CA, Hayes DF, Friedman P, Ellis M, Dressler L. Topoisomerase II{alpha} amplification does not predict benefit from dose-intense cyclophosphamide, doxorubicin, and fluorouracil therapy in HER2-amplified early breast cancer: results of CALGB 8541/150013. J Clin Oncol. 2009; 27:3430-3436.

16. Arriola E, Rodriguez-Pinilla SM, Lambros MBK, Jones RL, James M, Savage K, Smith IE, Dowsett M, Reis-Filho JS: Topoisomerase II alpha amplification may predict benefit from adjuvant anthracyclines in HER2 positive early breast cancer. Breast Cancer Res Treat 2007; 106:181-189.

17. Schindlbeck C, Mayr D, Olivier C, Rack B, Engelstaedter V, Jueckstock J, Jenderek C, Andergassen U, Jeschke U, Friese K. Topoisomerase IIalpha expression rather than gene amplification predicts responsiveness of adjuvant anthracycline-based chemotherapy in women with primary breast cancer. J Cancer Res Clin Oncol. 2010;136:1029-1037.

18. Tanner M, Isola J, Wiklund T, Erikstein B, Kellokumpu-Lehtinen P, Malmström P, Wilking N, Nilsson J, Bergh J: Topoisomerase IIalpha gene amplification predicts favorable treatment response to tailored and dose-escalated anthracycline-based adjuvant chemotherapy in HER-2/neu-amplified breast cancer: Scandinavian Breast Group Trial 9401. J Clin Oncol 2006; 24: 2428-2436.

19. Fritz P, Cabrera CM, Dippon J, Gerteis A, Simon W, Aulitzky WE, van der Kuip H. c-erbB2 and topoisomerase IIalpha protein expression independently predict poor survival in primary human breast cancer: a retrospective study. Breast Cancer Res. 2005; 7:R374-384.

20. Durbecq V, Desmed C, Paesmans M, Cardoso F, Di Leo A, Mano M, Rouas G, Leroy JY,
Sotiriou C, Piccart M, Larsimont D: Correlation between topoisomerase-IIalpha gene amplification and protein expression in HER-2 amplified breast cancer. Int J Oncol 2004; 25:1473-1479.

21. . Mueller RE, Parkes RK, Andrulis I, O'Malley FP: Amplification of the TOP2A gene does not predict high levels of topoisomerase II alpha protein in human breast tumor samples. Genes Chromosomes Cancer 2004; 39:288-297.

22. Callagy G, Pharoah P, Chin SF, Sangan T, Daigo Y, Jackson L, Caldas C: Identification and validation of prognostic markers in breast cancer with the complementary use of array-CGH and tissue microarrays. J Pathol 2005; 205:388-396.

23. Brase JC, Schmidt M, Fischbach T, Sültmann H, Bojar H, Koelbl H, Hellwig B, Rahnenführer J, Hengstler JG, Gehrmann MC. ERBB2 and TOP2A in breast cancer: a comprehensive analysis of gene amplification, RNA levels, and protein expression and their influence on prognosis and prediction. Clin Cancer Res. 2010;16:2391-2401.

24. Konecny GE, Pauletti G, Untch M, Wang HJ, Möbus V, Kuhn W, Thomssen C, Harbeck N, Wang L, Apple S, Jänicke F, Slamon DJ. Association between HER2, TOP2A, and response to anthracycline-based preoperative chemotherapy in high-risk primary breast cancer. Breast Cancer Res Treat. 2010; 120:481-489.

25. Durbecq V, Paesmans M, Cardoso F, Desmedt C, Di Leo A, Chan S, Friedrichs K, Pinter T, Van Belle S, Murray E, Bodrogi I, Walpole E, Lesperance B, Korec S, Crown J, Simmonds P, Perren TJ, Leroy JY, Rouas G, Sotiriou C, Piccart M, Larsimont D: Topoisomerase-II alpha expression as a predictive marker in a population of advanced breast cancer patients randomly treated either with single-agent doxorubicin or single-agent docetaxel. Mol Cancer Ther 2004; 3:1207-1214.

26. Kahan Z, Uhercsak G, Hajnal-Papp R, Boda K, Thurzo L. Dose-dense sequential adriamycin-Paclitaxel-cyclophosphamide chemotherapy is well tolerated and safe in high-risk early breast cancer. Oncology. 2005;68:446-453.

27. Kahán Z, Spanik S, Wagnerova M, Skacel T, Planko B, Fitzthum E, Lindner E, Soldatenkova V, Zielinski CC, Brodowicz T.: Feasibility of two dose-dense FEC

regimens with growth factor support for adjuvant therapy in patients with early breast cancer: results from a randomised study of the Central European Cooperative Oncology Group (CECOG). Breast Cancer Res Treat. 2008; 112:557-563.

28. Nocito A, Kononen J, Kallioniemi OP, Sauter G. Tissue microarrays (TMAs) for high-throughput molecular pathology research. Int J Cancer 2001;94:1–5.

29. Park K, Kim J, Lim S, Han S. Topoisomerase II-alpha (topoII) and HER2 amplification in breast cancers and response to preoperative doxorubicin chemotherapy. Eur J Cancer. 2003;395:631-634.

30. Tubbs R, Barlow WE, Budd GT, Swain E, Porter P, Gown A, Yeh IT, Sledge G, Shapiro C, Ingle J, Haskell C, Albain KS, Livingston R, Hayes DF. Outcome of patients with early-stage breast cancer treated with doxorubicin-based adjuvant chemotherapy as a function of HER2 and TOP2A status. J Clin Oncol. 2009;27:3881-3886.

31. Gunnarsdóttir KA, Jensen MB, Zahrieh D, Gelber RD, Knoop A, Bonetti M, Mouridsen H, Ejlertsen B. CEF is superior to CMF for tumours with TOP2A aberrations: a Subpopulation Treatment Effect Pattern Plot (STEPP) analysis on Danish Breast Cancer Cooperative Group Study 89D. Breast Cancer Res Treat 2010; 123:163-169.

32. Hannemann J, Kristel P, van Tinteren H, Bontenbal M, van Hoesel QG, Smit WM, Nooij MA, Voest EE, van der Wall E, Hupperets P, de Vries EG, Rodenhuis S, van de Vijver MJ. Molecular subtypes of breast cancer and amplification of

topoisomerase II alpha: predictive role in dose intensive adjuvant chemotherapy. Br J Cancer 2006; 95:1334-1341.

33. Di Leo A, Larsimont D, Gancberg D, Jarvinen T, Beauduin M, Vindevoghel A, Michel J, Focan CH, Ries F, Gobert PH, Closon-Dejardin MT, Dolci S, Rouas G, Paesmans M, Lobelle JP, Isola J, Piccart MJ: HER-2 and topo-isomerase IIalpha as predictive markers in a population of node-positive breast cancer patients randomly treated with adjuvant CMF or epirubicin plus cyclophosphamide. Ann Oncol 2001; 12:1081-1089.34. Berry DA, Cirrincione C, Henderson IC, Citron ML, Budman DR, Goldstein LJ, Martino S, Perez EA, Muss HB, Norton L, Hudis C, Winer EP. Estrogen-receptor status and outcomes of modern chemotherapy for patients with node-positive breast cancer. JAMA. 2006; 295:1658-1667.

35. Rody A, Karn T, Ruckhäberle E, Müller V, Gehrmann M, Solbach C, Ahr A, Gätje R, Holtrich U, Kaufmann M: Gene expression of topoisomerase II alpha (TOP2A) by microarray analysis is highly prognostic in estrogen receptor (ER) positive breast cancer. Breast Cancer Res Treat, 2009;113:4574-66

36. Järvinen TA, Kononen J, Pelto-Huikko M, Isola J. Expression of topoisomerase IIalpha is associated with rapid cell proliferation, aneuploidy, and c-erbB2 overexpression in breast cancer. Am J Pathol. 1996;148:2073-2082.

III

doi:10.1016/j.ijrobp.2008.10.045

INDIVIDUAL POSITIONING: A COMPARATIVE STUDY OF ADJUVANT BREAST RADIOTHERAPY IN THE PRONE *VERSUS* SUPINE POSITION

Zoltán Varga, Katalin Hidegféty, M.D., Ph.D., Tamás Mező, Alíz Nikolényi, M.D.,
László Thurzó, M.D., Ph.D., and Zsuzsanna Kahán, M.D., Ph.D.

Department of Oncotherapy, University of Szeged, Szeged, Hungary

Purpose: To study breast radiotherapy in the prone vs. supine positions through dosimetry and clinical implementation.

Methods and Materials: Conformal radiotherapy plans in 61 patients requiring only breast irradiation were developed for both the prone and supine positions. After evaluation of the of the first 20 plan pairs, the patients were irradiated in the prone or supine position in a randomized fashion. These cases were analyzed for repositioning accuracy and skin reactions related to treatment position and patient characteristics.

Results: The planning target volume covered with 47.5–53.5 Gy in the prone vs. the supine position was 85.1% ± 4.2% vs. 89.2 ± 2.2%, respectively ($p < 0.0001$). Radiation exposure of the ipsilateral lung, expressed in terms of the mean lung dose and the V_{20Gy}, was dramatically lower in the prone vs. supine position ($p < 0.0001$), but the doses to the heart did not differ. There was no difference in the need to correct positioning during radiotherapy, but the extent of displacement was significantly higher in the prone vs. supine position ($p = 0.021$). The repositioning accuracy in the prone position exhibited an improvement over time and did not depend on any patient-related parameters. Significantly more radiodermatitis of Grade 1–2 developed following prone vs. supine irradiation ($p = 0.025$).

Conclusions: Conformal breast radiotherapy is feasible in the prone position. Its primary advantage is the substantially lower radiation dose to the ipsilateral lung. The higher dose inhomogeneity and increased rate of Grade 1–2 skin toxicity, however, may be of concern.   © 2009 Elsevier Inc.

Breast cancer, Conformal radiotherapy, Prone treatment position, Supine treatment position, Repositioning accuracy.

INTRODUCTION

Postoperative radiotherapy has become an integral part of the complex treatment of breast cancer. The risk of late radiogenic sequelae such as lung fibrosis, cardiovascular events, or secondary cancers increases with radiation exposure of the organs at risk (OARs) (1–4), and selective irradiation of the target organ is therefore mandatory. The simplest way to protect the OARs during breast radiotherapy is individual patient positioning. It has been observed that a prone position during breast radiotherapy results in a substantially lower dose to OARs such as the ipsilateral lung (5–9) and the heart (5, 8), with the additional advantage of improved dose homogeneity (5, 6, 9). This mode of positioning has been shown to be feasible (10, 11), even in obese patients (8), and to provide a similar long-term outcome and toxicity as with standard supine tangents (11, 12). Because we had

earlier found the prone position to be helpful in a few difficult cases, we set out to perform a prospective study to compare radiotherapy in the prone position with our usual technique in the supine position with excellent repositioning accuracy. The study comprised two phases: the first phase served as a setup period for the acquisition of experience with patient positioning and radiotherapy planning in the prone position, but the radiotherapy was in fact delivered in the conventional supine position; in the second phase, radiotherapy administered in the prone vs. the supine position in a randomized fashion was studied. The radiotherapy plans were analyzed for the overall study population, whereas the implementation of breast radiotherapy in the prone position was the subject of only the second phase of the study. We aimed to identify those patients who benefit most from prone positioning by means of dosimetry (dose homogeneity and

---

protection of the OARs) and feasibility (including repositioning accuracy).

METHODS AND MATERIALS

The study was approved by the Institutional Review Board of the University of Szeged, and all enrolled patients gave their written informed consent before being registered in the study.

Early breast cancer patients after surgery requiring only radiotherapy of the operated breast were included in the study. No restriction existed regarding the size of the breast or the patient.

In the first phase of the study ($n = 20$), although radiotherapy planning was performed in both positions, all patients received radiotherapy in the supine position. The 41 patients enrolled in the second phase were randomized to radiotherapy in the prone vs. the supine position, but the position for radiotherapy randomized to the patient was blinded to the physician who performed the contouring.

The patients were positioned on the supine thorax and the prone breast modules of the AIO (All In One) Solution (ORFIT, Wijnegem, Belgium) system, which contains special cushion sets fixed to a universal baseplate. In the supine position, the patient was laid on a 15° thorax wedge cushion with both arms elevated, resting on an arm support, and held on an adjustable grip pole. The head was placed in the head support secured to a supplementary baseplate attached to the thorax cushion. In the prone position, the head was resting on a pillow, both arms were placed superolaterally, supported by the cranial part of the prone breast cushion, and the target breast lay across the semicircular aperture of the platform. The patient was rotated slightly to allow the ipsilateral chest wall to extend into the aperture. A thermoplastic mask (five-point fixation, breast precut; ORFIT) was applied in the supine position, molded around the chin, the neck, the thorax (excluding the target breast), and the abdomen. The opposite breast was covered with the mask and carefully positioned away from the radiation fields. Mask fixation was not used in the prone position, but a polyfoam wedge was placed under the contralateral breast to displace it. On the basis of experience gained during the first phase of the study, in the second 41 patients, a different polyfoam wedge was applied as a new development of the AIO system for better protection of the opposite breast (Fig. 1). Positioning landmarks were drawn on the skin or the mask, using two lateral lasers and one overhead laser. All patients were scanned on a Somatom Emotion 6 CT simulator (Siemens, Erlangen, Germany) in both positions. The planning target volume (PTV) and OARs were contoured on the CT slices throughout the entire planning volume in the XiO (CMS, Maryland Heights, MO) treatment planning system, according to the local protocol (13). The PTV was defined as the entire breast delineated on the CT data set, extending to within 4 mm of the skin surface. Treatment plans were developed by applying conventional 6-MV tangential photon fields set up isocentrically and a median of 2 (range, 1–3) individually weighted 6/15-MV segmental fields superimposed on the tangential fields by using a multileaf collimator. Wedges were used in almost all cases. A mean dose to the PTV of 50 Gy and a uniform distribution ($\pm 10\%$) of the prescribed dose to 95% of the PTV were aimed for. Dose homogeneity within the PTV was characterized by the volume of the breast receiving at least 47.5 Gy but less than 53.5 Gy ($V_{95\%-107\%}$). Radiation exposure of the OARs (the volume of the ipsilateral lung receiving more than 20 Gy [V_{20Gy}], the mean lung dose [MLD], the mean dose to the heart [MHD], the volume of the heart receiving more than 25 or 30 Gy [V_{25Gy} and V_{30Gy}], the volume of the contralateral breast receiving more than 5 Gy [V_{5Gy}], and the mean dose to the contralateral breast) was registered
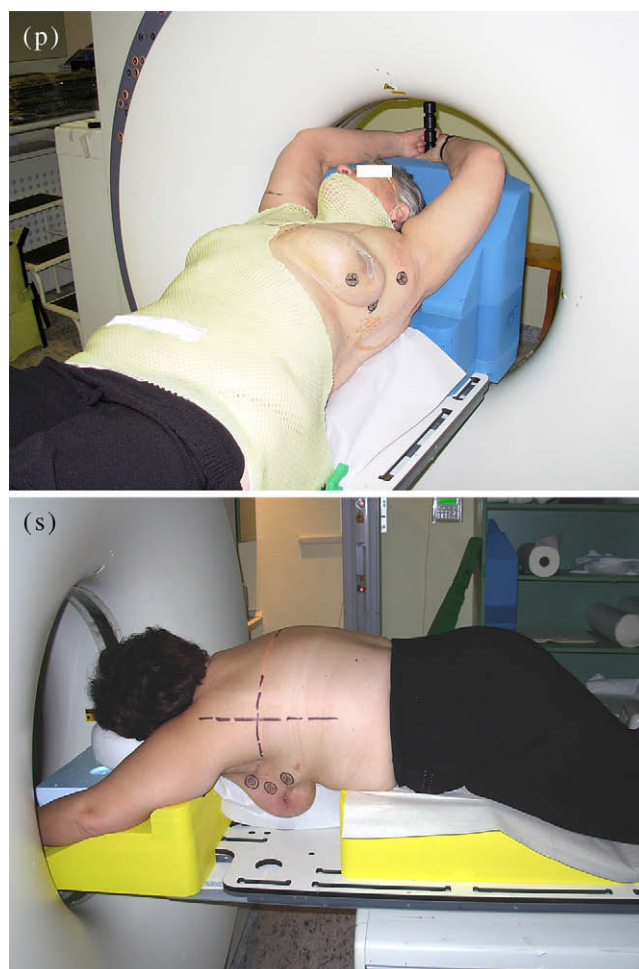

Fig. 1. Typical supine and prone positioning during breast radiotherapy.

in both positions. The central lung distance (CLD) and breast separation were determined in the supine position as measures of the patient anatomy.

The objectives in the second phase of the study were patient adherence to the protocol, repositioning accuracy, and toxicity during radiotherapy. Before the commencement of radiotherapy, the position of the isocenter in the patient was checked under the CT simulator. The necessary displacement in three dimensions was registered as the first datum of the repositioning accuracy. Radiotherapy was delivered with a linear accelerator (Primus, Siemens) in five fractions per week. The accuracy of patient repositioning during radiotherapy was checked three times per week with an electronic portal imaging device (Beamview version 2.2, Siemens), with the help of radio-opaque markers placed on the skin and mask as reference markers. (The dose delivered by portal imaging was taken into consideration in the calculation of the final dose received by the patient.) One portal image for one of the tangentional beams was recorded and compared with the corresponding beam's eye view digitally reconstructed radiograph generated from the planning system. The need to correct the position of the table in two dimensions was established and recorded by one or two physicians (AN or ZK). Analysis of each port image involved determination of the distances between the radio-opaque skin markers, and measurements of the CLD, the lung area included in the field, the central flash distance, and the inferior central margin (14, 15). The action level was set at 3 mm. Systematic and random errors generated from the three-dimensional

vector of displacement during the CT simulation and the two dimensional vector of displacement during the radiotherapy were calculated according to conventional definitions (16, 17). Acute skin reactions (graded by the Common Terminology Criteria for Adverse Events, version 3.0) were compared in 41 patients randomized to radiotherapy in the prone vs. supine position, at the end of the whole breast irradiation. The relations between the data obtained by analysis of the radiotherapy plans and repositioning accuracy vs. the patient characteristics were analyzed with the Student t test, the chi-square test, regression analysis, analysis of variance, and logistic regression. Statistical analysis was performed with SPSS 11.0 for Windows.

RESULTS

The first phase of the study and the second feasibility phase involved 20 and 41 patients, respectively. The mean ($\pm$ SD) age of the overall study population was 56.0 ± 9.6 (range, 29.3–73.9), and that of the second phase was 56.6 ± 9.9 (range, 29.3–73.6) years. Twenty-seven patients underwent right-sided and 34 left-sided breast irradiation. The age, weight, waist, hip size, and breast separation did not differ significantly between patients randomized to radiotherapy in the prone or the supine position (Table 1). Tumor bed boost irradiation and systemic treatments did not differ significantly between the two groups.

Radiotherapy plans for the prone vs. the supine position

The radiotherapy plans were first analyzed in the overall population. Mean ($\pm$ SD) percentage PTV covered by 47.5–53.5 Gy ($V_{95\%-107\%}$) in the prone vs. the supine position was $85.1 \pm 4.2\%$ and $89.2 \pm 2.2\%$, respectively ($p < 0.0001$). Dose homogeneity did not depend on PTV or breast separation. The irradiated volume of and the dose to the ipsilateral lung, determined in terms of MLD and V_{20Gy}, were dramatically lower in the prone position than in the supine position (Table 2). No significant difference was detected in the mean dose to the heart and the volumes of the heart receiving at least 25 Gy or 30 Gy in 34 left-sided breast cancer patients according to their position during radiotherapy (Table 2). The first 20 pairs of treatment plans revealed significantly higher doses to the contralateral breast in the prone position than in the supine position. In the second phase of the study ($n = 41$), as a consequence of the more complete displacement of the opposite breast due to the use of a new polyfoam wedge, there was no longer any significant difference (Table 3).

We hoped to identify parameters related to patient anatomy that indicate high lung doses if radiotherapy is given in the supine position to select those patients who would benefit most from radiotherapy in the prone position. With regard to the volume of the target breast, breast separation, and CLD, only CLD was significantly associated with MLD ($r = 0.843$, $p < 0.0001$) and V_{20Gy} ($r = 0.733$, $p < 0.0001$).

Implementation of breast radiotherapy in the prone position

In the second phase of the study, adherence to the study protocol, repositioning accuracy, and early skin reactions

Table 1. Characteristics (mean $\pm$ SD) of patients randomized to radiotherapy in the prone vs. supine positions

	Age (years)	Weight (kg)	Height (cm)	BMI (kg/cm²)	Waist size (cm)	Hip size (cm)	Breast separation (cm)
Supine (n = 21)	59.1 ± 9.3 (42.1–75.0)	71.6 ± 12.4 (52.0–96.0)	162.1 ± 7.7 (150–175)	27.2 ± 3.9 (20.9–33.2)	93.3 ± 14.4 (78–145)	107.4 ± 12.1 (95–150)	21.1 ± 2.7 (16.4–26.9)
Prone (n = 20)	56.9 ± 10.7 (30.7–72.4)	69.9 ± 12.4 (50.0–102.0)	161.0 ± 4.3 (152–168)	27.1 ± 5.3 (17.7–38.9)	89.3 ± 10.6 (69–108)	104.4 ± 9.9 (87–124)	20.7 ± 3.1 (14.2–26.9)
p value	0.49	0.66	0.56	0.94	0.32	0.40	0.64

Abbreviation: BMI = body mass index.

Table 2. Radiation doses to the ipsilateral lung and the heart in the overall study population (mean $\pm$ SD)

	Lung ($n = 61$)		Heart ($n = 34$)		
	MLD (Gy)	V_{20Gy} (%)	Mean dose (Gy)	V_{25Gy} (%)	V_{30Gy} (%)
Supine	7.45 ± 2.62	14.3 ± 5.4	3.51 ± 2.33	4.7 ± 4.6	4.1 ± 4.3
Prone	2.02 ± 1.23	3.3 ± 2.5	3.18 ± 1.31	3.6 ± 2.5	3.0 ± 2.2
p value	<0.0001	<0.0001	0.413	0.171	0.152

Abbreviation: MLD = mean lung dose.

were analyzed. The protocol was tolerated well by all patients; only one treated in the prone position required a 1-week break because of radiodermatitis. It was necessary to correct the location of the isocenter in the simulator or the position of the table during radiotherapy in 20.3% (61/301) and 20.3% (62/306) of all checks in the prone and the supine position, respectively ($p = 0.999$). The mean length of the displacement vector was 8.06 ± 4.66 mm (range, 3.00–22.56 mm) and 6.60 ± 3.05 mm (range, 3.00–21.19 mm) in the prone and supine positions, respectively ($p = 0.021$). The population random errors were 3.89 mm and 2.97 mm, whereas the population systematic errors were 0.86 mm and 0.82 mm for the prone and the supine position, respectively. The random errors in the two groups are shown in Table 4. A trend was detected for better overall repositioning accuracy in the supine position ($p = 0.061$). We analyzed whether repositioning accuracy changed from patient to patient during the study period. The individual random errors for repositioning in the prone position decreased with time, whereas no change was detected in the group randomized to radiotherapy in the supine position (Fig. 2). Repositioning accuracy in the prone position did not depend on any patient-related parameter. In the supine position, however, it was significantly related to lower weight ($p = 0.01$), body mass index ($p = 0.011$), waist size ($p = 0.039$), volume of the ipsilateral breast ($p = 0.007$), and breast separation ($p = 0.001$). Grade 1 radiodermatitis developed in 55% and 38.1% of patients and Grade 2 radiodermatitis in 35% and 19.5% of the patients receiving radiotherapy in the prone or the supine position, respectively ($p = 0.025$). Acute skin reactions were not related to dose homogeneity in the PTV or the random errors for repositioning, regarded as measures of systematic and random overdosage, respectively.

DISCUSSION

We evaluated our initial experience regarding the dosimetry and feasibility of conformal breast radiotherapy in the prone position and identified its place in everyday practice. Our results indicate that its primary advantage is the significantly reduced radiation exposure of the ipsilateral lung. The doses to the heart and the contralateral breast are similar in the prone and supine positions. Special practice in and attention to accurate repositioning are necessary if the prone position is applied, and dose inhomogeneity and acute skin reactions may increase slightly.

There have been few studies on prone breast radiotherapy. Some focused on dose distribution (6, 7, 9, 18) and others on clinical implementation (11, 12, 14, 19, 20); only one study with both dosimetric aspects and feasibility (10). This study is the first randomized clinical trial to compare breast radiotherapy in the prone vs. the supine position.

Use of the prone position during breast radiotherapy raises special considerations because of the altered shape, motion, and position of the organs present in the region. The altered shape of the target breast hanging down across the aperture of the positioning device results in a different dose distribution relative to that in the supine position. Improved dose uniformity, particularly avoidance of an overdosage within the PTV, have been associated with a better cosmetic outcome (21, 22). A higher dose inhomogeneity is related to larger breasts if conventional tangent beams are used (21). Buijsen *et al.* (9) compared prone and supine breast irradiation in 10 patients with pendulous breasts, and concluded that the dose homogeneity was better in the prone than the supine position. In fact, this was based on a comparison of the PTV overdosed ($V_{105\%}$ and $V_{107\%}$) in the supine vs. prone position, but the significantly lower mean dose and PTV coverage representing an underdosage were neglected. Similarly, in another study (6), larger volumes receiving > 52.5 Gy within the PTV were found in the supine than the prone position, but no other information on dose distribution was reported. We examined $V_{95\%-107\%}$ as a measure of dose homogeneity within the PTV, according to International Commission on Radiation Units and Measurements Report 62 (23), and found that the dose distribution was significantly more

Table 3. Radiation dose to the opposite breast in the two consecutive cohorts of the study

	First phase ($n = 20$)		Second phase ($n = 41$)		*p* for first vs. second phase	
	Mean dose (Gy)	V_{5Gy} (%)	Mean dose (Gy)	V_{5Gy} (%)	Mean dose	V_{5Gy}
Supine	0.85 ± 0.47	2.7 ± 2.0	0.61 ± 0.73	1.7 ± 2.8	0.096	0.073
Prone	1.26 ± 0.78	4.5 ± 3.4	0.74 ± 0.44	2.2 ± 2.0	0.00092	0.001
p for supine vs. prone	0.0038	0.0057	0.162	0.159		

Table 4. Random errors for repositioning in the prone and
supine positions

	Mean ± SE (mm)	Median (mm)
Supine	2.75 ± 0.27	2.58
Prone	3.46 ± 0.37	3.48
p value	0.061	

uniform in the supine position, regardless of the size or shape of the target breast. None of the radiotherapy plans indicated measurable volumes receiving > 53.5 Gy. Our dose prescription strategy was different from those of Buijsen *et al.* (9), and Griem *et al.* (6). A mean dose of 50 Gy was prescribed to the entire PTV, provided that the dose range is between 45 and 55 Gy in at least 95% of the PTV, instead of specifying a dose to a dose prescription point. We believe that our approach reliably represents the dose homogeneity within the PTV. Goodman *et al.* (18) reported a simplified intensity-modulated radiation therapy (IMRT) technique that improved dose homogeneity within the target breast in the prone position compared with the unacceptably high doses generated if conventional tangents were used. The greatest improvement was seen in women with the most pendulous breasts. Although in our study dose uniformity was acceptable in both positions, in certain cases, the IMRT approach could be followed to prevent the early and late consequences of dose inhomogeneity. In accordance with these data, in an

investigation of 35 patients with large pendulous breasts, Mahe *et al.* (10) found that when conventional tangents were used, the dose was 105%–110% in one third of patients. Despite the use of in-field segments, we observed hot spots at the top and bottom of the target breast in the prone position, which is consistent with the experience of Mahe *et al.* The application of intensity-modulated beams in our study may have played a role in the apparent lack of a relation between dose uniformity and breast size.

Because of the different shape of the chest wall when the patient is positioned prone, the lung volume included in the tangent fields is considerably less. All authors agree that lung doses are dramatically reduced if breast radiotherapy is performed with the patient prone (5–9). The beneficial effect of prone positioning on the protection of the ipsilateral lung is further enhanced if the almost absent intrafractional motion of the chest wall is taken into account for the calculation of safety margins around the CTV (20, 24, 25).

When left-sided irradiation is performed, the irradiated volume of the heart is not reduced, despite the fact that less intrathoracic volume is exposed to radiation in the prone than in the supine position. Reports on heart doses are not concordant, however. Some studies suggest a reduction in heart doses as a result of prone positioning but do not provide direct comparisons with supine positioning (5, 8). Others are consistent with our results in showing no significant difference in doses to the heart as a function of the treatment position (6, 7, 9). This finding may be accepted if the change in position of the heart by treatment position is taken into consideration. In fact, the prone position causes an anterior displacement of the heart within the thorax by 19 mm on average, as demonstrated by CT and MRI measurements in breast cancer patients receiving radiotherapy (26).

Because breast radiotherapy increases the risk of late contralateral breast cancer by 18%–34%, special attention is necessary to protect the opposite breast (3, 4). Although some studies allude to the radiation dose to the opposite breast in the prone position, detailed dose–volume histogram data have not been provided (5, 6). No widely accepted dose constraints exist for the contralateral breast. We registered V_{5Gy} and the mean dose to the healthy breast. In the first phase of the study, we detected higher doses to the opposite breast in the prone than supine position, a consequence of suboptimal positioning in the prone state. Following revision of the positioning method, no difference was observed in the second phase of the study. We consider careful application of the polyfoam wedge in the prone position, and of mask fixation in the supine position, to be important in removing the opposite breast from the radiation fields.

The largest prospective Phase I–II study on prone breast irradiation is that of Formenti *et al.* (8). Accelerated whole breast radiotherapy was feasible in 90 patients, with high setup reproducibility, although numerical data were not provided. In another feasibility study (10), prolonged adequate immobilization could not be achieved in 3 of 35 patients with large pendulous breasts in the prone position. In one retrospective study (11), 5% of the patients during prone breast
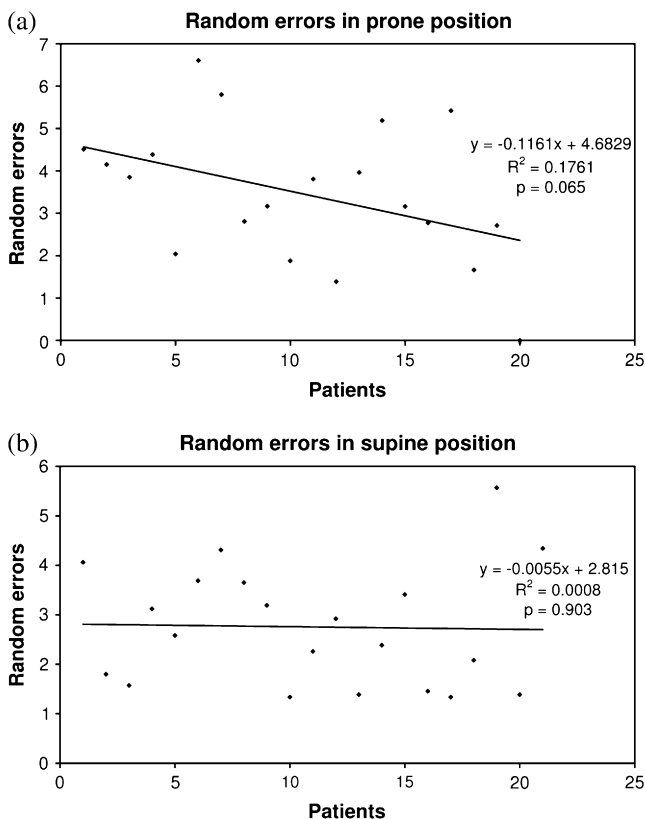

Fig. 2. Random errors for repositioning among the patients who received radiotherapy prone (a) and those received radiotherapy supine (b) by sequence of enrolment in the study.

radiotherapy complained of chest wall or rib pain, and 2 of 248 patients suffered a rib fracture (11), as did 1 of 35 in the previous study (10). All our patients considered the prone radiotherapy convenient and completed the course of radiotherapy. We believe that the comfortable positioning system was essential to achieve such good adherence to the protocol. It is our view that repositioning accuracy is a key condition for radiotherapy, particularly if inverse or forward intensity modulation is applied (24, 25). During simulation in 308 patients with various cancer sites, Schüller *et al.* (27) found that the repositioning accuracy was improved in the entire patient population if positioning aids or mask fixation were used, but this was not affected by prone or supine positioning. Breast irradiation was performed without mask fixation in the supine position for 64 patients. Of the various tumor sites, the breast exhibited the poorest repositioning accuracy. Displacement was carried out in 27 patients (42.2%) and exceeded 1 cm in many cases. In another study of 25 breast cancer patients irradiated in the supine position (28), the isocenter displacement on simulation was 5.7 mm on average. Morrow *et al.* (20) studied interfractional error in repositioning in 15 patients and recommended image guidance during prone breast radiotherapy because of the need for frequent and large displacements. In agreement with our results, they observed no relation between the breast size and the repositioning accuracy. Interestingly, however, we found that the repositioning accuracy in the supine position is significantly worse in obese patients. To the best of our knowledge, no such data have been published previously. If confirmed, they indicate that increased attention must be paid to the position of overweight patients during breast radiotherapy. We believe that the relatively good repositioning accuracy in our study, was related to the comfortable positioning device used

for both the prone and the supine position and to the mask fixation used in the supine position. Repositioning accuracy in the prone position improved over time, indicating the need for experience and expertise. Furthermore, our study warrants the development of mask fixation in the prone position, which would reduce setup uncertainty.

In other publications (10, 11), acute skin reactions after breast radiotherapy in the prone position were reported in similar incidences as among our patients. Mahe *et al.* (10) found that acute skin reactions were most frequent at the top and the bottom of the fields, in accordance with the high dose regions. In our study, radiodermatitis in the prone position was not related to the size of the breast or the dose inhomogeneity in it.

Merchant and McCormick (5) recommend breast radiotherapy in the prone position if the supine position is likely to result in unacceptable dose inhomogeneity or significant doses to normal tissues. We hoped to identify those patients who would benefit most from the prone position during breast radiotherapy. Because we could not detect any advantage of prone radiotherapy other than the absence of radiation exposure of the lung, we set out to identify those patient-related parameters that are associated with a higher lung dose if the patient is irradiated in a supine position. Consideration of breast volume, breast separation, and CLD as measures of the PTV shape indicated that only CLD was related to the dose to the ipsilateral lung. Thus, we recommend monitoring of the CLD as a primary measure for the indication of prone radiotherapy. Moreover, because the risk of early and late lung sequelae is strongly related to patient age (13), the presence of lung disease, and possibly to certain systemic therapies, these factors should be taken into account when a decision is made concerning the position used during breast radiotherapy.

REFERENCES

1. Kwa SLS, Lebesque JV, Theuws JC, *et al.* Radiation pneumonitis as a function of mean lung dose: An analysis of pooled data of 540 patients. *Int J Radiat Oncol Biol Phys* 1998;42: 1–9.
2. Darby S, McGale P, Taylor CW, Peto R. Long-term mortality from heart disease and lung cancer after radiotherapy for early breast cancer: Prospective cohort study of about 300 000 women in US SEER cancer registries. *Lancet Oncol* 2005;6: 557–565.
3. Early Breast Cancer Trialists' Collaborative Group (EBCTCG). Effects of radiotherapy and of differences in the extent of surgery for early breast cancer on local recurrence and 15-year survival: An overview of the randomised trials. *Lancet* 2005;366: 2087–2106.
4. Levi F, Randimbison L, Te VC, La Vecchia C. Cancer risk after radiotherapy for breast cancer. *Br J Cancer* 2006;95:390–392.
5. Merchant TE, McCormick B. Prone position breast irradiation. *Int J Radiat Oncol Biol Phys* 1994;30:197–203.
6. Griem KL, Fetherston P, Kuznetsova M, *et al.* Three-dimensional photon dosimetry: A comparison of treatment of the intact breast in the supine and prone position. *Int J Radiation Oncology Biol Phys* 2003;57:891–899.
7. Kurtman C, Andrieu MN, Hicsönmez A, Celebioglu B. Three-dimensional conformal breast irradiation in the prone position. *Brazilian J Med Biol Res* 2003;36:1441–1446.

8. Formenti SC, Gidea-Addeo D, Goldberg JD, *et al.* Phase I–II trial of prone accelerated intensity modulated radiation therapy to the breast to optimally spare normal tissue. *J Clin Oncol* 2007;25:2236–2242.
9. Buijsen J, Jager JJ, Bovendeerd J, *et al.* Prone breast irradiation for pendulous breasts. *Radiother Oncol* 2007;82:337–340.
10. Mahe M-A, Classe J-M, Dravet F, *et al.* Preliminary results for prone-position breast irradiation. *Int J Radiation Oncology Biol Phys* 2002;52:156–160.
11. Stegman LD, Beal KP, Hunt MA, *et al.* Long-term clinical outcomes of whole-breast irradiation delivered in the prone position. *Int J Radiation Oncology Biol Phys* 2007;68:73–81.
12. Grann A, McCormick B, Chabner ES, *et al.* Prone breast radiotherapy in early-stage breast cancer: A preliminary analysis. *Int J Radiation Oncology Biol Phys* 2000;47:319–325.
13. Kahán Z, Csenki M, Varga Z, *et al.* The risk of early and late lung sequelae after conformal radiotherapy in breast cancer patients. *Int J Radiat Oncol Biol Phys* 2007;68:673–681.
14. Fein DA, McGee KP, Schultheiss TE, *et al.* Intra- and interfractional reproducibility of tangential breast fields: A prospective on-line portal imaging study. *Int J Radiation Oncology Biol Phys* 1996;34:733–740.
15. Lirette A, Pouliot J, Aubin M, Larochelle M. The role of electronic portal imaging in tangential breast irradiation: A prospective study. *Radiother Oncol* 1995;37:241–245.

16. Hurkmans CW, Remeijer P, Lebesque JV, Mijnheer B. Set-up verification using portal imaging; review of current clinical practice. *Radiother Oncol* 2001;58:105–120.

17. Stroom JC, Heijmen BJM. Geometrical uncertainties, radiotherapy planning margins, and the ICRU-62 report. *Radiother Oncol* 2002;64:75–83.

18. Goodman KA, Hong L, Wagman R, *et al*. Dosimetric analysis of a simplified intensity modulation technique for prone breast radiotherapy. *Int J Radiation Oncology Biol Phys* 2004;60:95–102.

19. Algan Ö, Fowble B, McNeeley S, Fein D. Use of the prone position in radiation treatment for women with early stage breast cancer. *Int J Radiation Oncology Biol Phys* 1998;40:1137–1140.

20. Morrow NV, Stepaniak C, White J, *et al*. Intra- and interfractional variations for prone breast irradiation: An indication for image-guided radiotherapy. *Int J Radiation Oncology Biol Phys* 2007;69:910–917.

21. Moody AM, Mayles WP, Bliss JM, *et al*. The influence of breast size on late radiation effects and association with radiotherapy dose inhomogeneity. *Int J Radiation Oncology Biol Phys* 1994;33:106–112.

22. Johansen J, Overgaard J, Rose C, *et al*. Cosmetic outcome and breast morbidity in breast-conserving treatment. Results from the Danish DBCG-82TM national randomized trial in breast cancer. *Acta Oncol* 2002;41:369–380.

23. International Commission on Radiation Units and Measurements. Prescribing, recording and reporting photon beam therapy (supplement to ICRU Report 50) (ICRU Report 62). Bethesda, MD: ICRU Publications; 1999.

24. van Herk M. Errors and margins in radiotherapy. *Semin Radiat Oncol* 2004;14:52–64.

25. Saliou MG, Giraud P, Simon L, *et al*. Radiotherapy for breast cancer: Respiratory and set-up uncertainties. *Cancer Radiother* 2005;9:414–421.

26. Chino JP, Marks LB. Prone positioning causes the heart to be displaced anteriorly within the thorax: Implications for breast cancer treatment. *Int J Radiation Oncology Biol Phys* 2008;70:916–920.

27. Schüller P, Bruns F, Hesselmann S, *et al*. Simulator verification of the accuracy of patient repositioning after virtual simulation. *Strahlenther Onkol* 2002;178:715–721.

28. Horst E, Schuck A, Moustakis C, *et al*. CT simulation in nodal positive breast cancer. *Strahlenther Onkol* 2001;177:511–516.