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RESEARCH

Tumour Topoisomerase II Alpha Protein Expression and Outcome After Adjuvant Dose-Dense Anthracycline-Based Chemotherapy

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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.

Keywords 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

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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

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 phase II dose-dense sequential adriamycin (A)-docetaxel (D)-cyclophosphamide (C) chemotherapy study (ADC group), 34 breast cancer patients were recruited between 08/2004 and 08/2009. The study had been approved by the Institutional Review Board of the University of Szeged, and all enrolled patients gave a written informed consent before being registered on the study. Eligible patients had invasive breast cancer showing casting-type microcalcifications with or without an associated tumour mass in the mammogram. The primary tumor had to had been resected with a clear margin of at least 5 mm. The presence of distant metastases had to be excluded by conventional methods. Most patients had tumour excision (n=24) and axillary blockdissection (n=23), while less had mastectomy (n=10) and sentinel node biopsy (n=11). Nine of the cancers were T2 and 11 were N1, whereas the others were T1 and/or N0. The 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 accessable 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 (≤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.

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

	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	43 (78.2)	30 (88.3)	35 (81.4)	108 (81.8)
ILC	6 (10.9)	1 (2.9)	4 (9.3)	11 (8.3)
Medullary	1 (1.8)	0 (0)	3 (7.0)	4 (3.0)
Other	5 (9.1)	3 (8.8)	1 (2.3)	9 (6.9)
LVI present (%)	38 (69.1)	30 (88.3)	35 (81.4)	108 (81.8)
Grade 1	3 (5.4)	0 (0)	2 (4.7)	5 (3.8)
Grade 2	10 (18.2)	12 (35.3)	18 (41.9)	40 (30.3)
Grade 3	27 (49.1)	17 (50)	22 (51.2)	66 (50.0)
Grade unknown	15 (27.3)	5 (14.7)	1 (2.3)	21 (15.9)
ER+(%)	25 (45.5)	17 (50)	16 (37.2)	33 (25)
PR+(%)	23 (41.8)	15 (44.1)	15 (34.9)	30 (22.7)
HER2+ (%)	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 2 TOP2A IHC status in the study groups and the overall population

	ATC	ADC	CECOG	Overall
TOP2 A IHC (n)	40	27	39	106
TOP2A (mean±SE,%)	18.3±3.4	17.33.5	24.5±5.0	21.02±2.3
TOP2A (median,%)	10	15	10	10
TOP2A+(%)	16 (40)	14 (51.9)	21 (53.8)	51 (48.1)

Results

The patient- and tumour-related characteristics within the 3 study cohorts and in the overall population are included in Table 1. 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). 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). 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). The outcome in the hormone receptor-positive and hormone receptor-negative subgroups was analysed separately (Table 5, Fig. 2). 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). Fig. 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.

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

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

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

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. In the 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 anthracycline 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

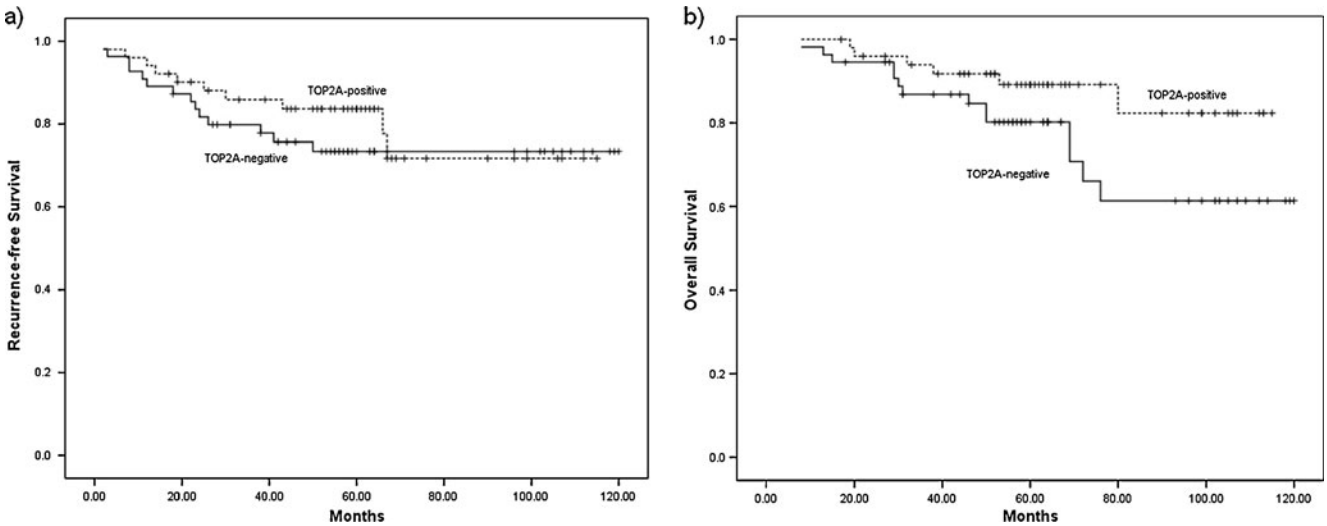

Fig. 1 Effects of the TOP2A protein expression status on the recurrence-free (**a**) and overall survival (**b**)

Table 5 Survival (OS and RFS) according to the TOP2A and ER/PR status of the tumor

	ER-negative		ER-positive		PR-negative		PR-positive	
	TOP2A−	TOP2A+	TOP2A−	TOP2A+	TOP2A−	TOP2A+	TOP2A−	TOP2A+
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	

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

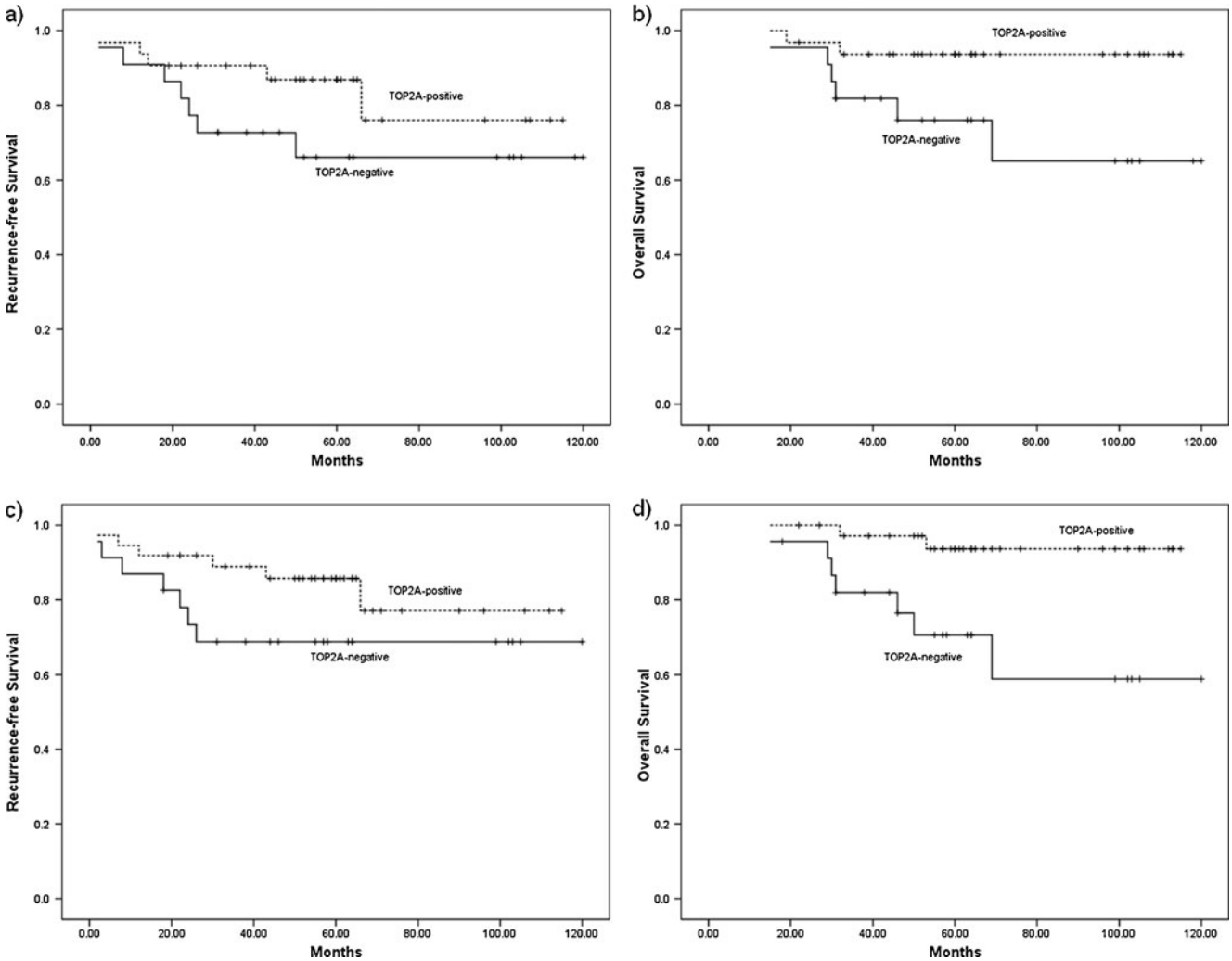

Fig. 2 Effects of the TOP2A protein expression status of ER-negative (**a**, **b**) and PR-negative (**c**, **d**) cancers on the recurrence-free (**a**, **c**) and overall survival (**b**, **d**)

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.

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