SPANDIDOS PUBLICATIONS

Predictive value of survivin alternative transcript expression in locally advanced breast cancer patients treated with neoadjuvant chemotherapy

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Abstract. Survivin, a member of the apoptosis inhibitor protein family, is expressed in numerous human tumours, and its expression is described as a negative prognostic marker. Four alternative splice variants (survivin-ΔEx3, survivin-3B, survivin-2B and survivin-2α) have been described. To date, little is known about the prognostic or predictive role of all five survivin transcripts in breast cancer. In this study, we analysed, by means of real-time quantitative PCR, the five survivin transcripts in a population of 60 breast carcinoma patients treated with 5-fluorouracil + epirubicin + cyclophosphamide (FEC, n=32) or with docetaxel + epirubicin (Tax-Epi, n=28). For each patient, samples were obtained before and after one course of chemotherapy. Before treatment, the ratio of survivin-2α was significantly higher in resistant than in sensitive tumours treated by the FEC regimen (p=0.0161), while the ratio of survivin-ΔEx3 was higher in sensitive than in resistant samples treated with Tax-Epi (p=0.0234). After one course of chemotherapy, expression of survivin-3B was significantly associated with resistance (p=0.0448) in the FEC treatment group, and the ratios of survivin-ΔEx3 (p=0.0071) and survivin-2B (p=0.0380) were significantly higher in sensitive than in resistant tumours in the Tax-Epi treatment group. Notably, increased expression and ratio of survivin-3B after one course of Tax-Epi was associated with reduced disease-free survival (p=0.0299 and 0.0277, respectively) and with reduced overall survival (p=0.0145 and <0.0001, respectively) of the patients. These results indicate that an imbalance in the alternative transcript ratios may make the cells resistant or sensitive to apoptosis. They also demonstrate for the first time that alternative survivin transcript expression levels may be predictive markers in FEC and Tax-Epi treatment in breast carcinoma.

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Introduction

In adult tissues, apoptosis participates in homeostasis and assures the stability of tissue integrity. An imbalance between pro-apoptotic elements such as caspases and anti-apoptotic genes such as inhibitors of apoptosis proteins (IAP) can inhibit apoptosis or activate it. The inhibition of apoptosis is one of the main mechanisms implied in cancer generation.

Survivin is the smallest member of the IAP family and is periodically expressed during the cell cycle; undetectable in phase G1, its expression is multiplied by 6 in phase S and by more than 40 in G2/M (1,2). Survivin is expressed in foetal tissues and regulated in a developmentally dependent manner (3,4). Survivin is not expressed in human adult tissues but is re-expressed in numerous cancers such as breast, colon and lung (1).

By alternative splicing, the survivin gene encodes 5 transcripts. The main transcript is survivin which is formed by the 4 exons for a length of 431 nt. This transcript encodes a 17-kDa protein possessing a BIR (Bacculovirus IAP repeat) domain responsible for its anti-apoptotic functions (5). Survivin inhibits extrinsic and intrinsic apoptotic pathways (6) by direct or indirect interactions with caspases-3, -7 and -9 (7-9). Survivin is necessary for mitosis especially in G2/M transition (2). This protein controls chromosome compaction and mitotic spindle formation and regulates microtubule dynamics (10).

Survivin-ΔEx3, with a length of 329 nt, results from the skipping of exon 3 which leads to a loss of 102 bp and a frameshift. Survivin-ΔEx3 protein, with a molecular weight of 15.9 kDa, engages in the same anti-apoptotic activities as survivin although no role in the cell cycle has been identified (11-14). Survivin-2B results from the introduction of a new 69-bp exon called exon 2B, which is part of intron 2 (11). The survivin-2B protein, with a molecular weight of 18.5 kDa, possesses a truncated BIR domain but is believed to have pro-apoptotic functions with no evidence for a role in cell cycle regulation (15). Survivin-3B includes a 165-nt part of intron 3 corresponding to exon 3B. Survivin-3B may have a molecular weight of 12.5 kDa. This variant is unable to interact either with tubulin or with chromosomes and may have no role in cell cycle regulation. Moreover, survivin-3B possesses a

complete BIR domain and therefore a potential anti-apoptotic activity (16). Survivin-2α corresponds to the introduction of 197 nt of the 3' terminus part of intron 2. This inclusion results in an early stop codon just before exon 3, giving rise to the shortest survivin variant. Survivin-2α does not possess the BIR domain, and it seems to have an antagonistic activity in relation to survivin (17).

Numerous studies have explored survivin expression in many types of cancer, but most of them have focused on the main survivin transcript. Deregulated survivin expression has been reported at both the mRNA and protein levels. Survivin mRNA overexpression has been described as a negative prognostic marker in breast cancer (18,19). At the protein level, survivin is localised to the nucleus and cytoplasm or both, and a number of studies have implied that differences in patient prognosis correlate with differences in nuclear or cytoplasmic compartmentalisation (20-22). It has been reported that cytoplasmic survivin is associated with a poor prognosis (23), whereas nuclear survivin was a significant independent prognostic indicator of favourable outcome in both the disease-free and overall survival of breast cancer patients (24). Recently, nuclear survivin was reported to be a poor prognostic marker in breast cancer (25). Thus, no clear consensus emerges from these studies.

To date, very little is known about the prognostic or predictive role of all five survivin transcripts in breast cancer. One study analysing three of the survivin transcript expression levels showed a lack of prognostic significance of survivin, survivin-ΔEx3 and survivin-2B (26). In another study, the level of both survivin and survivin-ΔEx3 but not survivin-2B was found to correlate significantly with apoptosis (27). To date, only one study has analysed the five survivin transcripts in breast carcinomas. However, the amplicon amplified for survivin-wt was not representative of this transcript, since it localised on exon 1, a region that is common to all transcripts (28). In the present study, we investigated the predictive values of the five transcripts individually in locally advanced breast cancer patients treated by neoadjuvant chemotherapy.

Materials and methods

Patients and samples. We studied a series of 60 patients with non-metastatic large-tumour, unilateral, non-inflammatory, operable breast cancer requiring mastectomy (but who wished to conserve the breast) treated with neoadjuvant chemotherapy between 1999 and 2000 at the anticancer centres in Dijon, Reims, Nancy and Strasbourg, France, and enrolled in a multicenter, phase II GIREC 01 trial (29). Criteria for the selection of these patients included the availability of tumour tissue from biopsies removed before and after one cycle of chemotherapy for mRNA analysis. Of these patients, 32 received 5-fluorouracil-epirubicin-cyclophosphamide (FEC): 5-fluorouracil (500 mg/m^2), epirubicin (100 mg/m^2) and cyclophosphamide (500 mg/m^2) (6 courses every 21 days). The remaining 28 patients received docetaxel-epirubicin (Tax-Epi) treatment: docetaxel (75mg/m^2) and epirubicin (100 mg/m^2) (6 courses every 21 days).

Response evaluation. Assessment of histological response (HR) in the surgical specimens was based on a classification proposed by Sataloff *et al* (30). This classification allows the evaluation of the extent of therapeutic effect on the primary tumour site and axillary lymph nodes. HR was graded as complete if a total or near total therapeutic effect on the tumour and negative nodes were present. Carcinomas were classified as partially resistant to the treatment if a >50% therapeutic effect on the tumour and negative or positive nodes with the therapeutic effect were present. Carcinomas were classified as resistant to the treatment if a <50% therapeutic effect in the tumour, regardless of node status, was present. In the FEC subset, patients with complete (5 cases) or partial (9 cases) histological response were classified as sensitive (14 cases) and patients with no histological response (18 cases) were classified as resistant to chemotherapy. In the Tax-Epi group, the same classification was used, resulting in 7 complete and 9 partial histological responses (16 sensitive tumours) and 12 subjects with no histological response to this regimen.

All tissue samples, taken before and after one course of chemotherapy, were frozen and stored in liquid nitrogen. A needle core biopsy was performed for initial diagnosis and two more were designed for RNA extraction. All biopsies were controlled for tumour cell frequencies by a pathologist after HES staining. Only cases with at least 30% of tumour cells were studied. This research was carried out with the approval of the local boards governing research on human subjects. Total RNA from a pool of 4 normal mammary tissues (NMT) was purchased from Clontech (Palo Alto, CA) and was used as a normal sample.

RNA extraction and cDNA synthesis. Total RNA was extracted from the needle core biopsies by using the Qiagen® RNA/DNA Mini Kit (Qiagen, Hilden, Germany) or the TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA). The quantity and purity of RNA were assessed spectrophotometrically at 260 and 280 nm (the A_{260}/A_{280} ratio of pure RNA is >1.8). The quality of RNA extracts was determined by electrophoresis through agarose gel, staining with ethidium bromide, and visualisation of the 18S and 28S bands under UV light. One microgram of total RNA was reverse transcribed in 20 μl of reverse transcriptase reaction as previously described (31).

Quantitative RT-PCR. Real-time quantitative PCR was performed on ABI PRISM 7300 (Applied Biosystems, Foster City, CA, USA) by using the Taqman® method. As we analysed tumours before and after one course of chemotherapy, we tested expression variation in 4 housekeeping genes (18S, GAPDH, ß-actin and TBP). Of these 4 genes, *18S* was the only gene that was stable after one course of chemotherapy. The analysis of the *18S* gene was thus used to assess cDNA quality and served as the reference control gene.

The nucleotide sequences of primers and probes for the *18S* gene and survivin transcripts, as well as the location of primers and probes for survivin transcripts have been described previously (34).

The probes were labelled at the 5' end with VIC for the *18S* gene or FAM for survivin transcripts, and at the 3' end with Tamra. Amplification was performed in a total volume of 25 μl in the presence of 12.5 μl of Universal Master Mix (Applied Biosystems), 80 nM of each primer and 50 nM of probe for the 18S gene, 150 nM primers and 200 nM probe

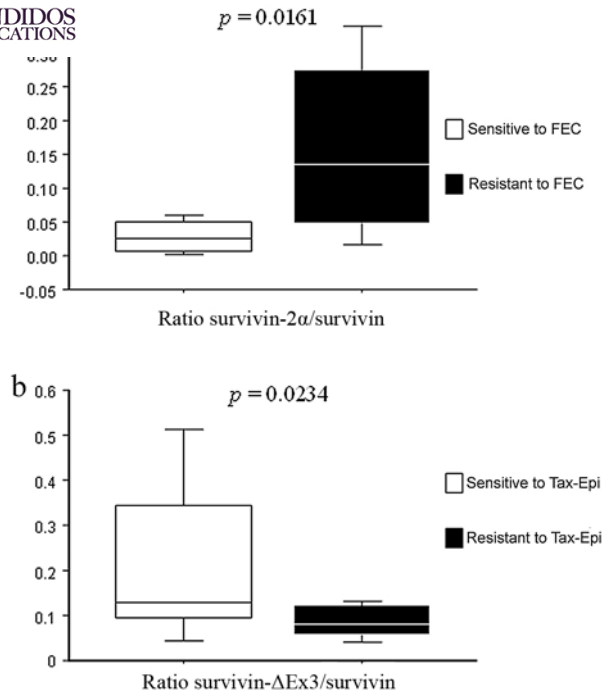

Figure 1. Distribution and comparison of survivin variant ratio expression in tumours analysed before treatment according to histological response. (a) Survivin-2α/survivin ratio was significantly correlated with response to FEC. (b) Survivin-ΔEx3/survivin ratio was significantly correlated with response to Tax-Epi treatment.

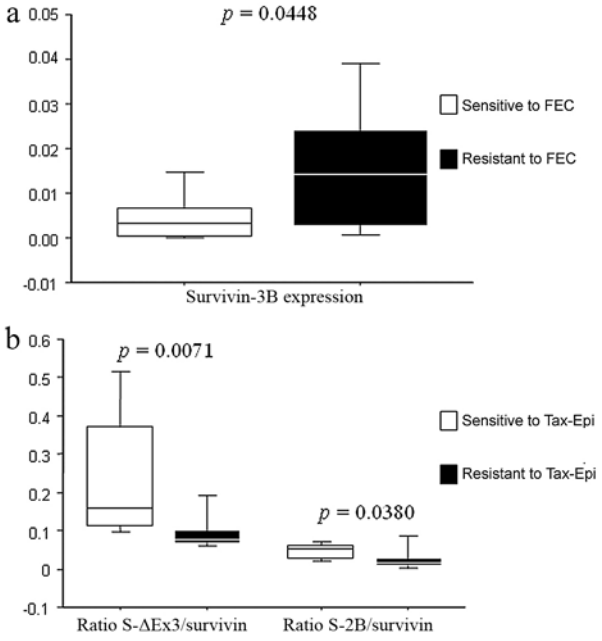

Figure 2. Distribution and comparison of survivin variant expression or ratio in tumours analysed after one course of chemotherapy according to histological response. (a) Survivin-3B expression was significantly lower in responders to FEC treatment. (b) In Tax-Epi-treated tumours, an increase in the survivin-ΔEx3/survivin or the survivin-2B/survivin ratio was correlated with a favourable response.

for survivin, 300 nM primers and 150 nM probe for survivin-ΔEx3, 300 nM primers and 200 nM probe for survivin-2B, 300 nM primers and 150 nM probe for survivin-3B, 600 nM primers and 200 nM probe for survivin-2α, and 12.5 ng of cDNA (or water as a negative control). The PCR program consisted of a 10-min initial denaturation step at 95˚C, followed by 40 cycles of 15 sec at 95˚C and 1 min at 60˚C. The specificity of the 18S and survivin PCR amplifications was verified by the sequencing of PCR products. The quality of PCR primers was verified by assessing the amplification efficiency on a standard curve established with the MCF-7 cell line. Amplification efficiency was measured in the range of 98% (±1%). All samples were amplified in duplicate, and results were analysed by the $2^{-\Delta Ct}$ method (32).

Statistical analysis. All analyses were performed with Statview 5.0 software. Histological response was correlated with survivin transcript expression and variations using the Mann-Whitney U test. The overall survival (OS) was defined as the interval between the diagnosis and the last follow-up or death. Disease-free survival (DFS) was defined as the time between the date of diagnosis and the date of distant metastases or local recurrence or death, whichever came first, or the last follow-up. Survival curves were generated using the Kaplan and Meier method, and the significance of differences between dichotomized patient groups was obtained by the Mantel-Cox log rank test. Dichotomization of the patient groups was carried out according to the median level of survivin transcripts in each treatment subset. Only tests with $p<0.05$ were considered significant.

Results

Survivin expression and histological response in tumours analysed before treatment. In the entire population, survivin and survivin-2α expression was detected in all samples before treatment (100%); survivin-ΔEx3 in 54/55 (98%), survivin-3B in 43/52 (83%), and survivin-2B in 53/55 (96%) samples. Correlations between the five survivin transcript expression levels and histological response to FEC or Tax-Epi treatment revealed no significant links. As the ratio between alternative transcripts and the main transcript of a gene could influence cell response to apoptosis (33), ratios between each variant and survivin were explored. The ratio of survivin-2α was significantly higher in resistant than in sensitive tumours treated with the FEC regimen (Fig. 1a), while the ratio of survivin-ΔEx3 was higher in sensitive than in resistant samples treated with Tax-Epi (Fig. 1b).

Survivin expression and histological response in tumours obtained after one course of chemotherapy. After one course of chemotherapy, survivin, survivin-ΔEx3 and survivin-2α transcripts were present in all tumours; survivin-3B in 30/38 (79%), and survivin-2B in 40/42 (95%). In the FEC treatment group, expression of survivin-3B was significantly associated with resistance (Fig. 2a). In the Tax-Epi treatment group, the ratios of survivin-ΔEx3 and survivin-2B were significantly higher in sensitive than in resistant tumours (Fig. 2b).

Expression variations between non-treated (NT) and chemotherapy-treated (TT) tumours and response to chemotherapy. We investigated the correlation between the variations in

Table I. Details of events for disease-free and overall survival of the studied breast cancer patients.

Patient no.	Treatment	Disease-free survival		Overall survival	
		Months	Events	Months	Events
1	FEC	56.4	Non-censured	62.4	Non-censured
2	FEC	72.0	Censured	72.0	Censured
3	FEC	62.4	Non-censured	74.4	Censured
4	FEC	13.2	Non-censured	13.2	Non-censured
5	FEC	12.0	Non-censured	20.4	Non-censured
6	FEC	84.0	Censured	84.0	Censured
7	FEC	79.2	Censured	79.2	Censured
8	FEC	84.0	Censured	84.0	Censured
9	FEC	30.0	Non-censured	36.0	Non-censured
10	FEC	51.6	Non-censured	72.0	Censured
11	FEC	64.8	Non-censured	76.8	Censured
12	FEC	28.8	Non-censured	60.0	Non-censured
13	FEC	60.0	Censured	60.0	Censured
14	FEC	67.2	Non-censured	68.4	Non-censured
15	FEC	84.0	Censured	84.0	Censured
16	FEC	85.2	Censured	85.2	Censured
17	FEC	84.0	Censured	84.0	Censured
18	FEC	80.0	Censured	80.0	Censured
19	FEC	51.0	Non-censured	72.0	Censured
20	FEC	72.0	Censured	72.0	Censured
21	FEC	91.0	Censured	91.0	Censured
22	FEC	29.0	Non-censured	35.0	Non-censured
23	FEC	60.0	Censured	60.0	Censured
24	FEC	55.0	Non-censured	63.0	Non-censured
25	FEC	87.0	Censured	87.0	Censured
26	FEC	61.0	Censured	61.0	Censured
27	FEC	85.0	Censured	85.0	Censured
28	FEC	69.0	Censured	69.0	Censured
29	FEC	30.0	Non-censured	54.0	Non-censured
30	FEC	67.0	Censured	67.0	Censured
31	FEC	50.0	Non-censured	89.0	Censured
32	FEC			n/a	
33	Tax-Epi	67.2	Non-censured	79.2	Censured
34	Tax-Epi	84.0	Censured	84.0	Censured
35	Tax-Epi	78.0	Censured	78.0	Censured
36	Tax-Epi	84.0	Censured	84.0	Censured
37	Tax-Epi	51.6	Non-censured	80.4	Non-censured
38	Tax-Epi	32.4	Non-censured	36.0	Non-censured
39	Tax-Epi	38.4	Non-censured	78.0	Non-censured
40	Tax-Epi	91.2	Censured	91.2	Censured
41	Tax-Epi	21.6	Non-censured	36.0	Non-censured
42	Tax-Epi	61.0	Censured	61.0	Censured
43	Tax-Epi	60.0	Censured	60.0	Censured
44	Tax-Epi	84.0	Censured	84.0	Censured
45	Tax-Epi	26.4	Non-censured	48.0	Non-censured
46	Tax-Epi	90.0	Censured	90.0	Censured
47	Tax-Epi	41.0	Non-censured	62.0	Non-censured
48	Tax-Epi	70.0	Censured	70.0	Censured
49	Tax-Epi	45.0	Non-censured	50.0	Censured
50	Tax-Epi	90.0	Censured	90.0	Censured
51	Tax-Epi	76.0	Censured	76.0	Censured
52	Tax-Epi	39.0	Non-censured	65.0	Non-censured
53	Tax-Epi	88.0	Censured	88.0	Censured
54	Tax-Epi	78.0	Censured	78.0	Censured
55	Tax-Epi	83.0	Censured	83.0	Censured
56	Tax-Epi	66.0	Non-censured	69.0	Censured
57	Tax-Epi	73.0	Censured	73.0	Censured
58	Tax-Epi	82.0	Censured	82.0	Censured
59	Tax-Epi			n/a	
60	Tax-Epi			n/a	

n/a, not available.

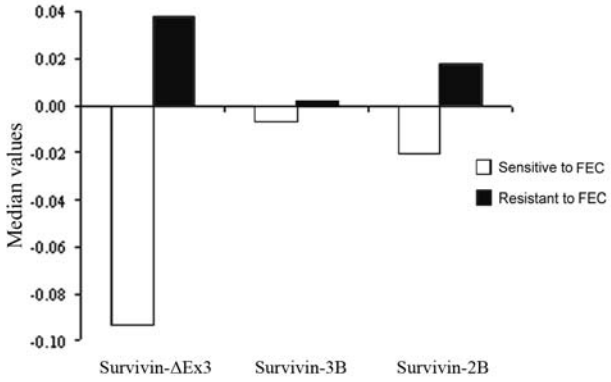

Figure 3. Variations of survivin alternative transcripts after one course of chemotherapy in the FEC treatment group. The values were obtained by subtraction of survivin transcript expression in chemotherapy-treated (TT) tumours by survivin transcript expression in non-treated (NT) tumours. Positive values correspond to overexpression and negative values to a decrease in expression. Decrease in survivin-ΔEx3, survivin-3B or survivin-2B expression was highly correlated with response (■) in comparison to no response (□).

survivin transcript expression levels in tumours analysed before and after one course of chemotherapy and the histological response. The results revealed that a decrease in survivin-ΔEx3, survivin-3B and survivin-2B after one course of FEC chemotherapy was statistically related with responsive tumours, whereas an increase in this transcript expression was associated with no response (p=0.0070, 0.0164 and 0.0129, respectively) (Fig. 3).

Increased expression of survivin-3B after one course of Tax-Epi treatment is associated with both disease-free and overall survival in patients. All survivin transcript expression and ratios were analysed before and after one course of treatment according to patient survival (Table I). The median disease-free survival (DFS) was 64.8 months (range 12-91) for FEC and 71.5 (21.6-91.2) for Tax-Epi treatment. The median overall survival (OS) was 72 months (13.2-91) in the FEC and 78 (36-91.2) in the Tax-Epi group. Details of censured and non-censured events are presented in Table I.

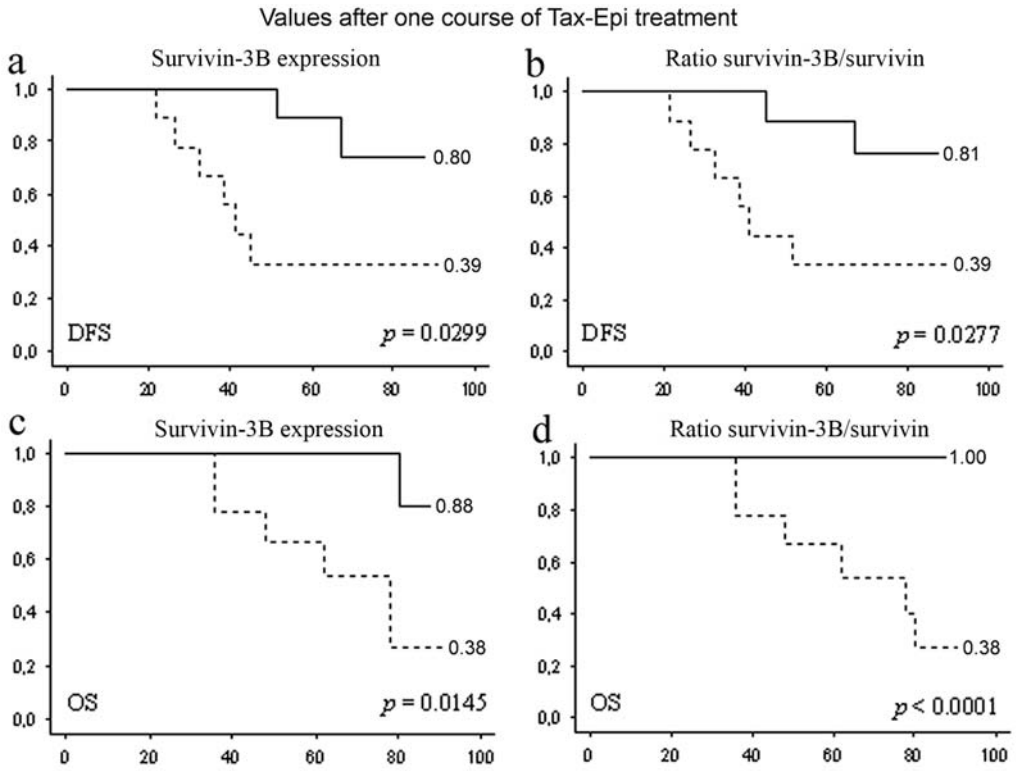

Figure 4. Correlation of survivin-3B expression and its ratio in tumours analysed after one course of Tax-Epi treatment with disease-free survival (DFS) and overall survival (OS). Patients with survivin-3B expression (a) or ratio (b) inferior to median values (solid line) had an increased DFS than patients with superior values (dotted line). Identical findings were obtained for survivin-3B expression (c) or ratio (d) and OS.

Before treatment, no relations were found between survivin transcript expression or ratios and DFS or OS in either the FEC or the Tax-Epi group. After one course of treatment neither survivin transcripts nor ratios were correlated with any survival in patients of the FEC group. In contrast, in the Tax-Epi group, survivin-3B expression and ratio were significantly associated with DFS and OS. Patients with high survivin-3B expression or ratio showed a reduced survival (p=0.0299 and 0.0277, respectively) (Fig. 4a). The same findings were obtained for overall survival (p=0.0145 and <0.0001, respectively) (Fig. 4b).

Discussion

In this study, five of the survivin transcripts were specifically analysed at the mRNA level by means of quantitative RT-PCR in breast tumours sampled before chemotherapy and in samples obtained after one course of FEC or Tax-Epi regimen. Our results revealed that ratios between alternative transcripts and survivin were significantly associated with histological response. In the FEC group, the survivin-2α/survivin ratio was higher in resistant tumours, suggesting for the first time that survivin-2α may be involved in resistance to FEC treatment. This result may be consistent with the finding that survivin can heterodimerise with its splice variants causing specific subcellular localisation patterns leading to formation of the functionally distinct survivin complex (12).

In the Tax-Epi subset, the survivin-ΔEx3/survivin ratio was associated with sensitivity, suggesting that high expression of this variant may sensitise tumours to a docetaxel-based regimen. As survivin-ΔEx3 is a nuclear protein (34) and docetaxel acts on microtubules in the cytoplasm (35), survivin-ΔEx3 is unable to protect cells from docetaxel-mediated cytoplasm aggressions.

Our study showed that increased survivin transcript expression or ratios after one course of chemotherapy were also linked to the histological response. After one course of FEC treatment, survivin-3B was overexpressed in resistant tumours, suggesting that survivin-3B could be useful in determining, after one course of FEC, whether patients are sensitive to the treatment. These data are in accordance with the putative anti-apoptotic function of survivin-3B (16) and may corroborate a recent study describing survivin-3B as a cytoprotective protein (36).

In both samples taken before and after one course of Tax-Epi chemotherapy, the survivin-ΔEx3/survivin ratio indicated significantly higher expression in sensitive than in resistant tumours. Moreover, after one course of Tax-Epi treatment, a high survivin-2B/survivin ratio was statistically associated with tumour sensitivity. This latter result is in accordance with most studies describing survivin-2B with a pro-apoptotic function (11,15). Notably, a recent study demonstrated that forced expression of survivin-2B sensitises cells to taxol-induced cell growth inhibition and cell death (37). However, new alternative transcripts of survivin have been described recently (38). Among the primers and probes we used in the present study, only detection of the survivin-2B transcript could be influenced by the presence of the newly described

survivin transcripts. Nevertheless, the expression of these transcripts has not yet been proven in breast carcinoma.

Before treatment, neither survivin transcript expression nor ratio was correlated with DFS or OS in any group. However, after one course of treatment, both survivin-3B expression and ratio were significantly associated with reduced DFS and OS in Tax-Epi-treated patients. Thus, it is important to determine the mechanisms by which survivin-3B could be implicated in the resistance to Tax-Epi treatment.

Our study characterised for the first time the predictive value of survivin variants and their ratios in breast cancer patients treated with specific regimens. Our results further showed that expression variations could be important for response prediction, since observed variations between samples taken before and after chemotherapy were significantly associated with sensitivity or resistance to chemotherapy.

In conclusion, the present study demonstrated that survivin transcript ratios were informative in predicting histological response to FEC or Tax-Epi treatments. This finding may reflect previous studies demonstrating that an imbalance in the alternative transcript ratios may make cells resistant or sensitive to apoptosis (33). Based on our results, it is necessary to study a large population of breast carcinoma patients treated with FEC or docetaxel-based regimens to check a) the usefulness of a biopsy after one course of chemotherapy to analyse survivin-3B expression, variation and ratio as well as survivin-ΔEx3 and survivin-2B; and b) the prognostic value of the survivin-3B transcript after one course of chemotherapy. These points may help to determine whether the continuation of treatment is beneficial for the patient.

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