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Preclinical study

High survivin predicts a poor response to endocrine therapy, but a good response to chemotherapy in advanced breast cancer

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Summary

Variants of survivin with differing subcellular localizations might mediate the different functions of survivin, i.e. cell-cycle regulation and apoptosis inhibition. Highly proliferative tumors are more sensitive to chemotherapy, whereas apoptosis resistant cells would be refractory to endocrine therapy. Possibly, this explains incongruent data on the association of survivin with prognosis in breast cancer. Survivin levels were measured using ELISA in $800 \times g$ pellets and $100{,}000 \times g$ supernatants of breast cancer tissues from patients that were treated with either chemotherapy or endocrine therapy for advanced disease. These fractions might be enriched with nuclear or cytoplasmatic located survivin variants. Survivin levels were associated with tumors with poor prognostic clinical characteristics. For the patients treated with endocrine therapy, the patients with high survivin levels exhibited a significantly shorter progression free survival (PFS) than those who had low levels (pellet survivin Hazard Ratio (HR) = 2.74, 95% Confidence Interval (CI) = 1.31–5.72, $p = 0.008$ and median PFS 5.8 versus 8.6 months, $p = 0.006$, log-rank; cytosolic survivin HR = 3.03, 95% CI = 1.45–6.35, $p = 0.003$). In contrast, for patients treated with chemotherapy, those with high cytosolic survivin had a significantly longer PFS than those with low levels (median PFS of 6.2 months, versus 4.7 months for patients with low cytosolic concentrations, $p = 0.024$, log-rank). Thus, high levels of survivin are mainly related with a poor response to endocrine therapy, but a good response to chemotherapy. This phenomenon might be related to the different functions of survivin.

Introduction

Survivin (baculoviral IAP repeat-containing protein [Birc]-5) is a member of the inhibitors of apoptosis (IAP) gene family that is implicated in both inhibition of apoptosis and in mitosis regulation (see for a review [1]). Survivin is one of the most uniformly upregulated genes in tumor tissues compared to normal tissues [2]. Uncontrolled growth of cancerous cells requires anti-apoptotic strategies in order to extend an otherwise limited lifespan, and to counter normal apoptotic triggers. In addition, cells that are unresponsive to apoptotic triggers will also be more resistant to cytotoxic treatment. Indeed, high survivin expression in the primary tumor is almost invariably associated with a poor prognosis of the patient in many cancer types. The association of survivin with prognosis of breast cancer patients, however, is ambiguous as previous studies have reported it to be either irrelevant [3], or associated with poor [4], or with good prognosis [5]. Recently, we described that survivin mRNA levels were a strong, independent marker for poor prognosis in early breast cancer [6]. As reported by Li et al. [7], the localization of survivin in nuclei or cytoplasm was determined by immuno-histochemistry (IHC) in all 19 reports that were analyzed in their paper. So far, to the best of our knowledge, no studies have been performed on the prognostic value of survivin protein levels using a quantitative assay.

Besides the originally described survivin form [8], Mahotka et al. [9] described two splice variants, survivin delta-ex3 and survivin-2B. More recently, two additional splice variants, i.e. survivin 3B [10] and survivin-2alpha [11] were described. It has been suggested that these different forms of survivin have different subcellular localizations [12]. In addition, the subcellular localization may

be relevant for its prognostic value [7]. Ryan et al. [13] recently described the occurrence of the mRNAs of several of these survivin variants in breast cancers.

Thus, survivin is involved in both proliferation and apoptosis [1], and its localization is important for its prognostic properties [7]. Highly proliferative tumors are more sensitive to chemotherapy [14], suggesting that survivin expressing tumors might be more sensitive to chemotherapy. Conversely, the anti-apoptotic function of survivin would suggest that these tumors would be refractory to cytotoxic treatments, e.g. endocrine therapy. To assess whether survivin protein levels found in different fractions of breast cancer tissue may predict the efficacy of different anti-tumor treatments (i.e. endocrine therapy or chemotherapy), we measured survivin using a commercially available ELISA in $800 \times g$ pellets and $100{,}000 \times g$ supernatants of tumor tissue from patients treated for advanced breast cancer. Possibly, these different fractions contain specific forms of survivin, or characterize the different functions of survivin in apoptosis and cell proliferation, and as such may have a dissimilar prognostic or predictive value.

Patients and methods

Patients

The current study, in which coded tumor tissues were used, was done in accordance with the Code of Conduct of the Federation of Medical Scientific Societies in the Netherlands ('Code for Proper Secondary Use of Human Tissue in the Netherlands', http://www.fmwv.nl) and adhered to all relevant institutional and national guidelines. It included 162 patients who underwent surgery in the period January 1987 and December 1996 for primary breast cancer, subsequently developed distant metastases during follow-up, and of whom primary tumor tissue $800 \times g$ pellets or cytosolic fractions were available for survivin measurement. The median age was 56 years (range 29–87 years) at the time of surgery of the primary tumor. Ninety patients were treated with first-line endocrine therapy for advanced disease. This therapy consisted of tamoxifen ($n = 73$), ovarian ablation ($n = 13$), aromatase inhibitors ($n = 3$) or medroxy-progesterone acetate ($n = 1$). These patients had a median age of 60 years (31–87 years) at start of first-line endocrine therapy. First-line chemotherapy was given to 72 patients. These patients received classical cyclophosphamide, methotrexate, 5-fluorouracil (CMF, $n = 40$) or 5-fluorouracil, epirubicine/adriamycin, and cyclophosphamide (FEC/FAC, $n = 32$). These patients had a median age of 50 years (29–82 years) at start of first-line chemotherapy.

Tumor tissue processing

At primary surgery, a representative part of the tumor was selected by the pathologist, frozen in liquid nitrogen and sent to our department (Department of Chemical Endocrinology). The primary breast cancer biopsies were stored in liquid nitrogen and pulverized in the frozen state with a microdismembrator as recommended by the European Organization for Research and Treatment of Cancer (EORTC) for analyzing estrogen (ER) and progesterone (PgR) receptor [15]. The tissue powders were suspended in EORTC buffer, containing 20 mM K_2HPO_4/KH_2PO_4, 1.5 mM K_2EDTA, 3 mM sodium azide, 10 mM monothioglycerol, 10% [v/v] glycerol/water, pH 7.4 and centrifuged at $800 \times g$ for 20 min at 4 °C. The pellets were stored at −80 °C until analysis. The supernatants were collected and subjected to further centrifugation for 1 h at $100{,}000 \times g$ (4 °C). A part of the high-speed supernatants obtained ('cytosols') were used for measurement of ER and PgR levels by ligand-binding assay, the remaining cytosols were stored at −80 °C.

Survivin assay

Survivin levels were determined in the primary breast tumor $800 \times g$ pellets and cytosols with a quantitative colorimetric ELISA (Assay Designs, Ann Arbor MI, USA). The $800 \times g$ pellet fractions were lysed, according to the kit insert, in Cell Lysis Buffer (1 mM EDTA, 6 M Urea, 0.5% Triton X-100, 0.005% Tween-20 in Phosphate Buffer Saline). The assay has a sensitivity of 3.6 pg/ml, and a range of 31.25–1000 pg/ml. Intra-assay variation is below 2%, and the inter-assay variation varies from 16% at low concentration to 6% at high concentrations of survivin. The kit uses a monoclonal antibody to survivin immobilized on a microtiter plate to bind the survivin in the standards or samples. Recombinant survivin is used as a standard. After a short incubation the excess sample or standard is washed out and a rabbit polyclonal antibody to survivin is added. After a short incubation the excess antibody is washed out and goat anti-rabbit IgG conjugated to horseradish peroxidase is added, which binds to the polyclonal survivin antibody. Excess conjugate is washed out and substrate (3,3′,5,5′ tetramethylbenzidine, TMB) is added. After a short incubation, the enzyme reaction is stopped and the color generated is read at 450 nm. The measured optical density is directly proportional to the concentration of survivin in either standards or samples.

The protein concentrations in the cytosolic fractions were determined by the method of Lowry [16] using BSA as the standard. The Bradford method [17] was used for the measurement of protein in the $800 \times g$ pellets, as – in contrast to the Lowry assay – this method is not influenced by the ureum in the lysis buffer. All survivin levels are normalized per 1 mg of total protein measured by these methods, and reported as pg/mg protein.

Data analysis

Normality of value distributions were assessed by Kolomogorov–Smirnov testing. Either log-normalized

data were used for analysis using parametric tests, or non-parametric testing was performed. Median levels with minimum and maximum values are shown. For correlation analysis of continuous data, Spearman rank-correlation tests were used. Survivin levels in particular patient categories were compared by either Mann–Whitney *U* or Kruskall–Wallis tests.

Patients were divided in responders and non-responders. Response was defined as complete remission (disappearance of disease), partial remission (more than 50% decrease in product of longest diameter times its perpendicular diameter of measurable parameters) and minor response (more than 25% decrease but, less than 50% decrease in size of measurable parameters), together overall response.

For the survival rate analyses progression-free survival (PFS) time (defined as the time from the start of the first-line therapy until the start of a next therapy or until the time of death) was used as follow-up parameter. Cox univariate regression analysis was used in the analysis of PFS, with survivin levels entered as continuous variables. The method of Kaplan and Meier was used to generate the survival curves of patient groups dichotomized by the median levels of survivin. The log-rank test was used to test for differences. All statistical analyses were performed using the SPSS statistical software package 12.0.1 (SPPS Inc, Chicago IL, USA).

Results

Samples

Clinical data and tumor material could be retrieved from a total of 162 patients that were treated for advanced breast cancer. From these patients, $800 \times g$ pellet material from tumors of 102 patients, and cytosolic material from tumors of 137 patients were available for measurement of survivin protein levels. From 77 of these patients, both fractions were present in our tumor bank and available for analysis of survivin concentrations.

Distribution

After ^{10}log-transformation, the survivin concentrations in both the pellet material and in the cytosolic fraction were normally distributed (Kolomogorov-Smirnov testing). The concentrations were over 10-fold higher in the pellet fraction (median 1800, range 100–23,580 pg/mg protein) than in the cytosolic fraction (median 146, range 12–2605 pg/mg protein). In the tumors from which both pellet and cytosolic material was available, concentrations of survivin were significantly correlated (Spearman correlation $r_s = 0.597$, $p < 0.001$, Figure 1).

Correlations

Associations of survivin concentrations with clinical and pathological characteristics of the patients were assessed

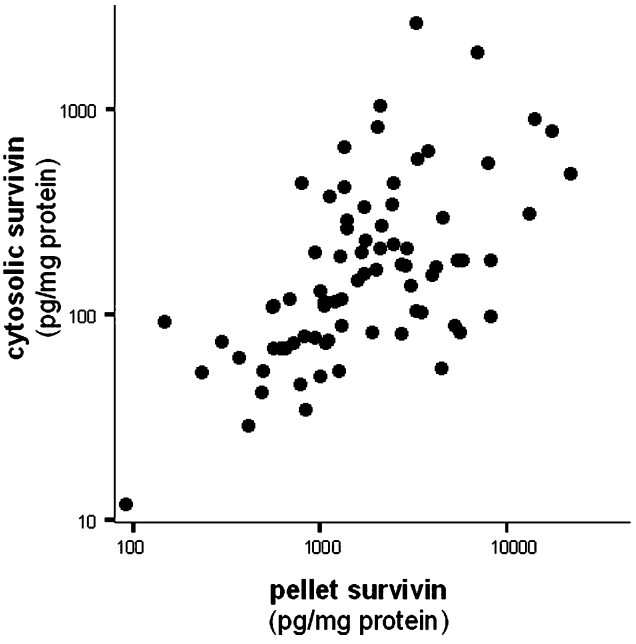

Figure 1. Scatter plot of survivin protein levels found in $800 \times g$ pellets and $100{,}000 \times g$ supernatants of breast tumor tissues ($r_s = 0.597$, $p < 0.001$).

(Table 1). Survivin concentrations in both pellet and cytosolic fractions were negatively correlated with age ($p = 0.004$ and 0.018, respectively). Cytosolic survivin was higher with increasing histological grade ($p = 0.031$). Pellet survivin was significantly lower in primary tumors from patients who later developed bone metastases when compared to those who developed soft tissue or viscera as first site of relapse ($p = 0.013$). No other associations with menopausal status, hormone receptor status, tumor size, lymph node status or PFS were found.

Patients treated with endocrine therapy

From the 90 patients that were treated with first-line endocrine therapy, there were $800 \times g$ pellets from 53, and cytosols from 74 patients available, respectively. The concentrations of survivin in the pellet tumor fraction were lower in the patients that responded to endocrine treatment, with a median pellet survivin of 1955 pg/mg protein in the non-responders (24/53, 45%), and 1170 pg/mg protein in the responders ($p = 0.036$). The median concentrations of survivin in the cytosolic fraction were 175 pg/mg protein in the non-responders (29/74, 39%) and 109 pg/mg protein in the responders ($p = 0.051$).

Initial survival analyses of the patients treated with endocrine therapy were performed with survivin concentration entered as continuous variables. In Cox regression analysis for PFS, high pellet survivin concentrations were significantly associated with a short PFS (Hazard Ratio (HR) = 2.74, 95% CI = 1.31–5.72, $p = 0.008$). Similarly, high cytosolic survivin concentrations were significantly associated with a short PFS (HR = 3.03, 95% CI = 1.45–6.35, $p = 0.003$) in patients treated with endocrine therapy for advanced breast cancer.

Table 1. Categorical distributions of clinicopathological characteristics and survivin protein concentrations in patients treated with first- line therapy for advanced breast cancer

Characteristics	800 × *g* pellet survivin levels (pg/mg protein)			Cytosolic survivin levels (pg/mg protein)		
	n (%)[a]	Median	*p*-values	*n* (%)[b]	Median	*p*-values
Age (years)			0.004[c]			0.018[c]
≤40	21 (21)	3290		29 (21)	175	
41–55	28 (27)	2895		39 (28)	170	
56–70	40 (39)	1510		51 (37)	120	
>70	12 (12)	865		18 (13)	113	
Menopausal status			0.110[d]			0.117[d]
Pre	41 (40)	2610		57 (41)	176	
Post	60 (59)	1615		80 (58)	120	
ER/PgR (fmol/mg protein)			0.202[d]			0.865[d]
Both <10	30 (29)	2830		38 (28)	152	
Both or one ≥ 10	67 (66)	1720		96 (70)	161	
Primary tumor size			0.509[e]			0.571[e]
pT1	26 (25)	1585		40 (29)	152	
pT2	54 (53)	2270		66 (48)	171	
pT3+4	18 (18)	1960		28 (20)	133	
Histological grade			0.353[e]			0.031[e]
I	4 (4)	995		6 (4)	52	
II	15 (15)	2610		27 (20)	158	
III	42 (41)	2695		50 (36)	167	
Lymph node status			0.347[d]			0.998[d]
Negative	36 (35)	1615		49 (36)	146	
Positive	56 (55)	2455		77 (56)	167	
First site of relapse[f]			0.013[e]			0.602[e]
Soft tissue	4 (4)	3630		7 (5)	169	
Bone	41 (40)	1350		62 (45)	122	
Viscera	53 (52)	2950		61 (45)	159	
Progression-free survival			0.273[d]			0.312[d]
<1 year	72 (71)	2180		97 (71)	156	
≥ 1 year	25 (25)	1390		34 (25)	151	
First-line therapy						
Endocrine therapy	57 (56)	1450	0.171[d]	78	146 (57)	0.144[d]
Chemotherapy	44 (43)	2660		59	166 (43)	

[a]Because of missing values, numbers do not always add up to 102 (100%).
[b]Because of missing values, numbers do not always add up to 137 (100%).
[c]Spearman rank correlation.
[d]Mann–Whitney *U*-test.
[e]Kruskall–Wallis test.
[f]In case of multiple sites, the site with the worst prognosis was considered dominant.

After using median levels to dichotomize the patient group for Kaplan–Meier analyses (Figure 2a and b), the patient group with high pellet survivin levels exhibited a significantly shorter PFS than those that had low levels of survivin in the pellet fraction of their primary tumor (median PFS 5.8 versus 8.6 months, $p=0.006$, log-rank). For patients with high cytosolic survivin, median PFS was 5.7 months, versus 8.4 months for patients with low cytosolic concentrations of survivin ($p=0.078$, log-rank).

Patients treated with chemotherapy

Seventy-two patients were treated with first-line chemotherapy for advanced breast cancer. Of these, in 43 pellet survivin could be measured, and in 57 cytosolic survivin. In contrast to patients treated with endocrine therapy, levels were rather higher than lower in those who responded to chemotherapy. However, no statistically significant differences in survivin concentrations could be found, with a median pellet survivin of 2610 pg/mg protein in the non-responders (17/43, 40%), and 3810 pg/mg protein in the responders ($p=0.168$). The median concentrations of survivin in the cytosolic fraction were 159 pg/mg protein in the non-responders (32/57, 56%) and 183 pg/mg protein in the responders ($p=0.700$).

Again, survivin concentrations were entered as continuous variables in survival analyses. The Cox regression analysis for PFS showed that high pellet survivin

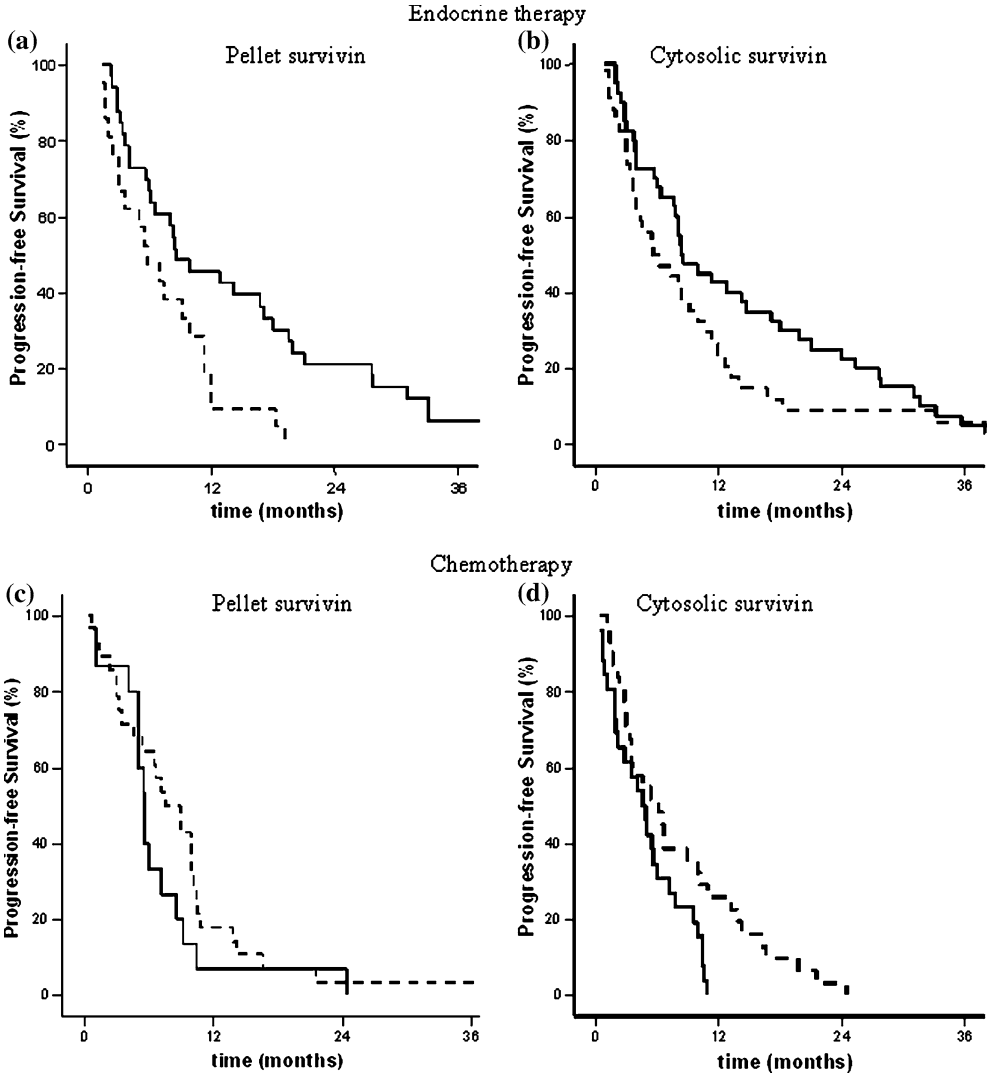

Figure 2. Kaplan–Meier plots of PFS of patients with advanced breast cancer, dichotomized by the median levels of survivin protein levels (low levels denoted by solid lines).

concentrations were – although not significantly – associated with a longer PFS (HR = 0.62, 95% CI = 0.35–1.11, $p = 0.106$). Similarly, high cytosolic survivin concentrations showed a trend towards an association with a longer PFS (HR = 0.53, 95% CI = 0.27–1.06, $p = 0.075$) in patients treated with chemotherapy for advanced breast cancer.

After using median levels to dichotomize the patient group for Kaplan–Meier analyses (Figure 2c and d), pellet survivin levels could not significantly distinguish patients with longer or shorter PFS (median PFS 7.6 versus 5.7 months, $p = 0.231$, log-rank). In contrast, for patients with high cytosolic survivin, median PFS was 6.2 months versus 4.7 months for patients with low cytosolic concentrations of survivin, which was statistically significant ($p = 0.024$, log-rank).

Median survivin in patients with progression free survival of more or less than 1 year

In a final analysis survivin levels were compared between patients with a PFS of more or less than 1 year (Figure 3). Pellet survivin levels did not differ between patients who had a PFS of more or less than 1 year after first line chemotherapy ($p = 0.262$) or endocrine therapy ($p = 0.077$) or. In contrast, patients who had a PFS ≥ 1 year had higher (437 versus 149 pg/mg protein) cytosolic survivin when on chemotherapy ($p = 0.003$), but lower (80 versus 172 pg/mg protein) cytosolic survivin when on endocrine therapy ($p = 0.008$).

Discussion

Here we find that, similar to the data on survivin mRNA levels we reported earlier [6], survivin protein levels were associated with clinicopathological characteristics of patients with a poor prognosis, i.e. higher in high-grade tumors and in tumors from younger patients. Survivin protein levels in breast cancer tissue fractions, as quantified by ELISA, can predict the efficacy of cytotoxic treatment for advanced disease. Remarkably, high levels of survivin were mainly related to a poor response to endocrine therapy, but a good response to

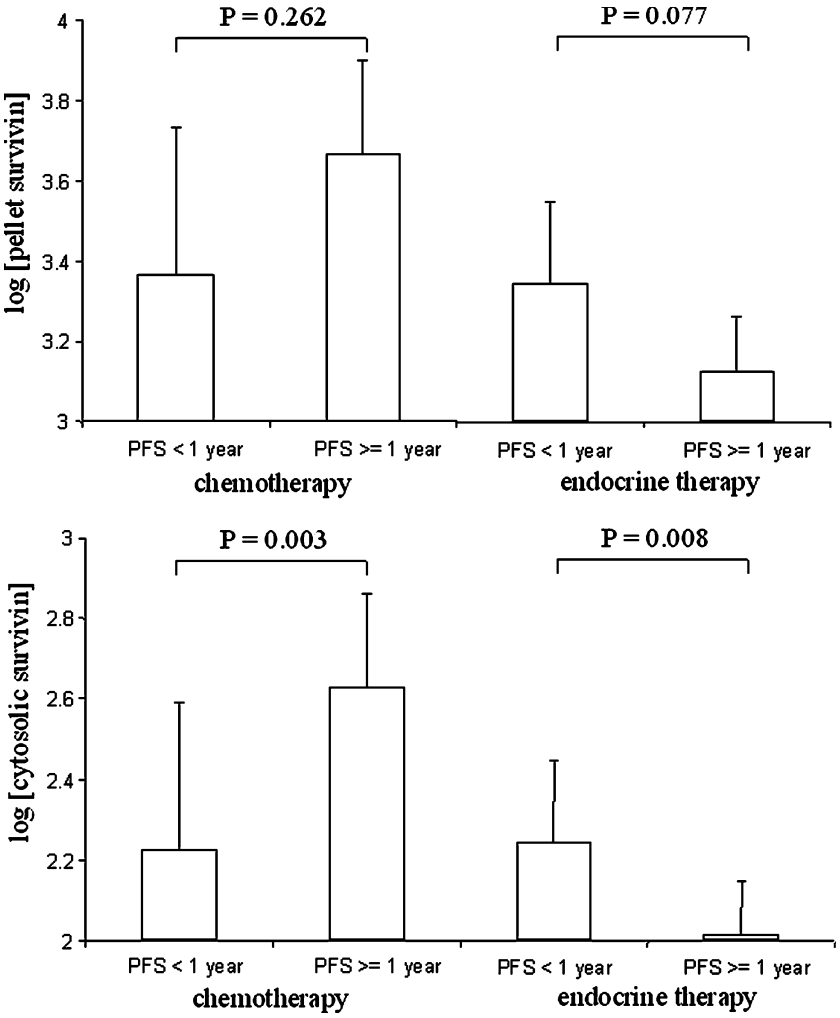

Figure 3. Log [survivin] levels (pg/mg protein) in pellet or cytosol, in patients groups dichotomized by relapse-free interval more or less than 1 year. Pellet survivin levels did not differ between patients who had a PFS of more or less than 1 year after first-line chemotherapy ($p = 0.262$) or endocrine therapy ($p = 0.077$) or. In contrast, patients who had a PFS ≥ 1 year had higher cytosolic survivin when on chemotherapy ($p = 0.003$), but lower cytosolic survivin when on endocrine therapy ($p = 0.008$).

chemotherapy. Some distinction can be made between survivin found in $800 \times g$ pellets ('pellet') or found in $100{,}000 \times g$ supernatant ('cytosolic'), although survivin levels in these fractions are significantly correlated.

This is the first report to measure survivin protein levels using a quantitative ELISA in different breast cancer tissue fractions. Other reports, especially those that distinguish between nuclear and cytoplasmatic localized survivin, use IHC [7], which is semi-quantitative at best. The confirmation of our results, and the possible eventual use of this assay in a clinical setting in other institutions, is probably more feasible as the assay used is quantitative, not based on subjective interpretation of results, and commercially available. We measured survivin using an ELISA in the $800 \times g$ pellet and $100{,}000 \times g$ supernatant fractions that remained after routine ER and PgR measurement using dextran-coated charcoal ligand binding assay. These fractions were not prepared for the purpose of isolating nuclear and cytoplasmatic fractions, but we nonetheless expected that the $800 \times g$ pellet fraction would be enriched with nuclear components. In this fraction, we found levels that were over 10-fold higher than in the cytosolic fraction. Although there was a significant correlation in levels between the two fractions, the enrichment of survivin in the pellet fraction argues against the possibility that pellet survivin is merely comprised of trapped cytosolic survivin.

Data on whether nuclear survivin is a marker for good or poor prognosis is unclear [7]. For breast cancer, however, it has been reported that specifically nuclear-localized survivin, as assessed by IHC, predicts a good prognosis [5]. Our results do not concur with those of Kennedy et al. [5] on the premise that $800 \times g$ pellet survivin is indeed primarily composed of nucleus-associated survivin, as we find that high pellet survivin predicts a poor response to endocrine therapy (Figures 2a and 3). For patients receiving chemotherapy as first-line treatment for advanced disease, no significant results were obtained for pellet survivin in our study.

A source of variability in previous results on the prognostic value of survivin in breast cancer [3–6] might be related to its contribution to treatment success (predictive value). Some biomarkers can be related to

treatment success, e.g. ER and adjuvant endocrine therapy, HER2/neu status and trastuzumab treatment. When assessing the value of a biomarker for estimating prognosis and its association with the natural course of the disease, patients should not receive treatment. Obviously, ethical considerations preclude this as an experiment in patients who are currently accepted candidates for routine adjuvant therapies. The correlation we found of survivin with patient's age and the histological grade of the tumor ([6] and this paper) suggests a relation between high survivin levels in the primary tumor and poor prognosis. In addition, the data reported in the present paper indicate a role for survivin as a predictive biomarker, with high levels being related to response to chemotherapy and resistance to endocrine therapy. Thus, in previous studies, the patient group and in particular the therapy given to these patients will be important for the finding whether survivin is related to good or poor prognosis.

Several splice variants are present in breast cancer tissues [13]. These splice variants have different subcellular localizations and possibly different functions [7,12]. The nuclear localized form might be the so-called delta-ex3 variant, which has an anti-apoptotic function, whereas the classical form and survivin-2B are preferentially localized in the cytoplasm. The survivin-2B splice variant is probably a natural antagonist of survivin [9]. We found high protein levels of pellet survivin to be most strongly related to poor efficacy of endocrine therapy, which would seem to be in concert with an anti-apoptotic function of nuclear localized survivin delta-ex3. Cells that contain increased levels of nuclear localized survivin delta-ex3 would seem to be apoptosis insensitive and thereby resistant to apoptosis induced by endocrine therapy. However, Ryan et al. [13] found that survivin delta-ex3 mRNA levels are in fact positively correlated with apoptosis counts in breast cancer. In addition, we found that high cytosolic survivin protein levels are positively correlated with tumor grade, but also with increased efficacy of chemotherapy. As patients with a high-grade tumor have a poorer prognosis irrespective of therapy, these data emphasize that tumors with high survivin levels respond better to chemotherapy. This could be explained by the fact that highly proliferating cells usually are more sensitive for chemotherapy [14]. Thus, these data support the conclusion that cytosolic survivin is a predictive factor for chemotherapy success.

The data presented here does not prove that survivin itself is responsible for the differential response to systemic therapy, although its established functions are in line with the data presented here. It is also possible that survivin expression is related to a particular breast cancer phenotype that also differs in its sensitivity to systemic treatment regimen, irrespective of survivin expression. As such, it is relevant to consider the so-called Recurrence Score (RS) [18]. Survivin is one of the 21 genes that are assessed by Q-PCR of RNA extracted from formalin-fixed paraffin-embedded tissue. Importantly, the RS predicts the relative insensitivity to tamoxifen [18,19] and sensitivity to chemotherapy [18,20]. Thus survivin protein levels, as assessed by ELISA and as presented in the present paper, give similar predictive information on the response of breast tumors to systemic therapy as does the RS score, which may very well be related to the phenotype of the breast tumors that are represented thereby.

In conclusion, we found quantitative assessment of survivin protein levels in breast cancer tissue fractions to be informative of first line therapy success in patients with advanced breast cancer. High levels of pellet survivin are associated with poor efficacy of endocrine therapy, and high levels of cytosolic survivin possibly with superior efficacy of chemotherapy. These data might relate to the different splice variants of survivin with different subcellular localizations, and with the role of survivin and its splice variants in both anti-apoptosis and cell cycle regulation.

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