Apoptosis gene signature of Survivin and its splice variant expression in breast carcinoma

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Abstract

Survivin, an anti-apoptotic protein, was described as strongly expressed in human cancers including breast cancer. However, little is known about the association between Survivin variants (Survivin-2B, Survivin-ΔEx3, Survivin-3B, and Survivin-2α) and the other apoptotic-related genes. In this study, we analyzed the apoptosis gene signature of Survivin and its variant expression in breast cancer. Human Apoptosis Gene Arrays were used to screen genes that could be associated with Survivin variants. Expression of the five transcripts was measured by RT-PCR in 135 breast carcinomas and Cox survival analysis was analyzed according to the patient outcome. Significant associations between Survivin transcripts and apoptotic genes were found. Interestingly, Survivin-3B variant showed major inverse correlations with pro-apoptotic genes. In addition, *in vitro* results indicated that overexpression of Survivin-3B strongly inhibits 5-fluorouracil/epirubicin/cyclophosphamide-induced apoptosis in breast tumor cell lines. In breast carcinomas, uni- and multivariate analysis showed patients with high level of Survivin-3B expression had a shorter overall ($P=0.030$ and $P=0.042$ respectively), and disease-free ($P=0.024$ and $P=0.009$) survival. Our data suggest that Survivin-3B contributes to cell survival through the anti-apoptotic pathway and that its expression level could be an important factor in determining therapeutic strategies for breast carcinoma.

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Introduction

Survivin is a 17 kDa protein belonging to the inhibitor of apoptosis protein (IAP) family and possessing a Baculovirus IAP Repeat (BIR) domain responsible for its anti-apoptotic functions (Ambrosini *et al*. 1997). The Survivin protein is highly expressed in foetal tissues, whereas it is undetectable in most normal adult tissues. The Survivin gene gives rise to a main four exon transcripts and four alternative splice variants (Mahotka *et al*. 1999). Survivin-ΔEx3 results from the skipping of exon 3 leading to the loss of 102 bp and a frameshift. Survivin-2B is derived from the introduction of a new 69 bp exon from intron 2, called exon 2B. Survivin-3B includes a portion of intron 3, called exon 3B (Badran *et al*. 2004). Survivin-2α has an introduction of the 3′ terminus part of intron 2, leading to an early stop codon just before exon 2 giving the shortest Survivin variant (Caldas *et al*. 2005).

The functions of the variants are not well known. Survivin-ΔEx3 was only described as an anti-apoptotic protein (Mahotka *et al*. 1999). Survivin-2B possesses a truncated BIR domain indicating that this variant could have pro-apoptotic functions (Zhu *et al*. 2004). Survivin-3B possesses a complete BIR domain and therefore a potential anti-apoptotic activity (Badran *et al*. 2004). Survivin-2α does not possess the domain involved in inhibition of apoptosis and seems to have opposed activity to Survivin (Caldas *et al*. 2005).

Survivin was described to be strongly expressed in most human cancers such as lung (Bria *et al*. 2008), colon (Fang *et al*. 2009), pancreas (Tonini *et al*. 2005), and breast (Ryan *et al*. 2005). The reasons of this

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re-expression as well as the role of Survivin in carcinogenesis are still questionable. To date, little is known about the association between Survivin splice variants and other apoptosis-related genes. In this study, we analyzed the apoptosis gene signature of Survivin and its four variant expression in breast carcinoma. Our previous observations have shown that expression of anti-apoptotic Survivin variants is higher in p53-mutated breast tumors (Végran *et al.* 2007). Thus, we used breast tumor cell lines expressing either wild type or mutated p53. The breast tumor cell lines were transfected with the transcript of interest and were characterized for their apoptotic response to a combination of 5-fluorouracil/epirubicin/cyclophosphamide (FEC). In addition, the prognostic value of Survivin and its variants was investigated according to the breast cancer patient outcome.

Materials and methods

Patients and samples

We studied retrospectively 135 patients with non-metastasis large tumor (T2, T3N0 or T3N1), unilateral, non-inflammatory, and operable breast cancer requiring mastectomy but who wished to conserve the breast (Supplementary Table 1, see section on supplementary data given at the end of this article). The patients were treated with neoadjuvant chemotherapy in a standard of care clinical setting. Excluded were: patients with previous exposure to chemotherapy, diffuse microcalcifications, a history of cancer and patients likely to be unavailable for appropriate follow-up. Adjuvant hormonal therapy for patients with hormonal receptor-positive tumors was administrated.

The samples used for this study were obtained before any form of treatment, during the period going from 1991 to 1997 at the Centre Georges François Leclerc, Dijon, France. All the examined patients have a well-known clinical history. The study was conducted in accordance with the Declaration of Helsinki and approved by an Ethics Committee, the Comité Consultatif de Protection des Personnes en Recherche Biomédicale de Bourgogne. Written informed consent was obtained from all patients before enrollment. The median clinical follow-up was 12.5 years (range 8.5–16.3 years). The 5 years survival rate for the cohort was 73.8% (confidence interval (CI) 65.5 and 80.4). Breast cancer patients received a chemotherapy treatment (six courses every 21 days) with: 5-fluorouracil (500 mg/m^2), epirubicin (100 mg/m^2), and cyclophosphamide (500 mg/m^2), and were subsequently operated on. In all cases no radiotherapy or hormonotherapy were applied before chemotherapy.

All tissue samples were frozen and stored in liquid nitrogen. A needle core biopsy was performed for initial diagnosis and two more were designed for RNA extraction after controlling the amount of tumor cells with hematoxylin–eosin–safran staining. Only samples with more than 30% of cancer cells were analyzed. Total RNA from a pool of four normal mammary tissues was purchased from Clontech and was used as a control.

Estrogen and progesterone receptor levels were determined in cytosolic tumors by enzyme immunoassay methods (Abbott Laboratories). The cut off level used for estrogen and progesterone was 20 fmol/mg cytosolic proteins.

Real-time quantitative PCR

Total RNA was extracted from cell lines and tissue samples by Qiagen Kit extraction. RT and nucleotide sequences of primers and probes for Survivin transcripts as well as their localization were described previously (Végran *et al.* 2007). The mean value of 18S, TBP, GAPDH, and β-actin genes was used for normalization. The Taqman Gene Expression Assays for CD40, CARD4, and TNFSF8 genes were provided from Applied Biosystems (Foster City, CA, USA).

The quality of PCR is examined by assessing the amplification efficiency on a standard curve established with MCF-7 cell line. Amplification efficiency was in the range of 98%. All samples were amplified in duplicate and results were analyzed by the $2^{-\Delta\Delta C_T}$ method (Livak & Schmittgen 2001). To perform the discrimination between samples, we used the median value obtained with the 135 patients.

Gene expression profiling

All data and explanations concerning gene expression profiling are available on Gene Expression Omnibus (GEO) website with the GEO accession number GSE24556. Hierarchical clustering was performed with Cluster 3.0 Software by complete linkage and Spearman rank correlation.

Cloning and stable transfection of tumor cell lines were reported in Supplementary information, see section on supplementary data given at the end of this article.

Statistical analysis

All analyses were performed with Stata (v10) Software with a bilateral 5% type I error. Comparisons between cell lines were performed with ANOVA test. Comparisons between transfected and vector control cells were

made by Student's *t*-test. In breast carcinomas, qualitative variables were described with frequency and compared with Fisher exact test. The quantitative variables were described with mean (S.D.). Subgroups were compared with Kruskal–Wallis, Mann–Whitney, and/or two-sample Wilcoxon rank-sum tests. The longitudinal changes in Survivin expressions were tested by Wilcoxon matched-pairs signed-rank tests. Tumor size was treated as continuous variable (hazard ratio (HR) for 1 cm). The overall survival was defined as time interval between the diagnosis and death (all causes) or the last follow-up for alive patients. 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 (all causes), whichever came first, or last follow-up. Survival curves were estimated by the Kaplan–Meier method. Univariate relative HR and 95% CI were calculated by Cox's

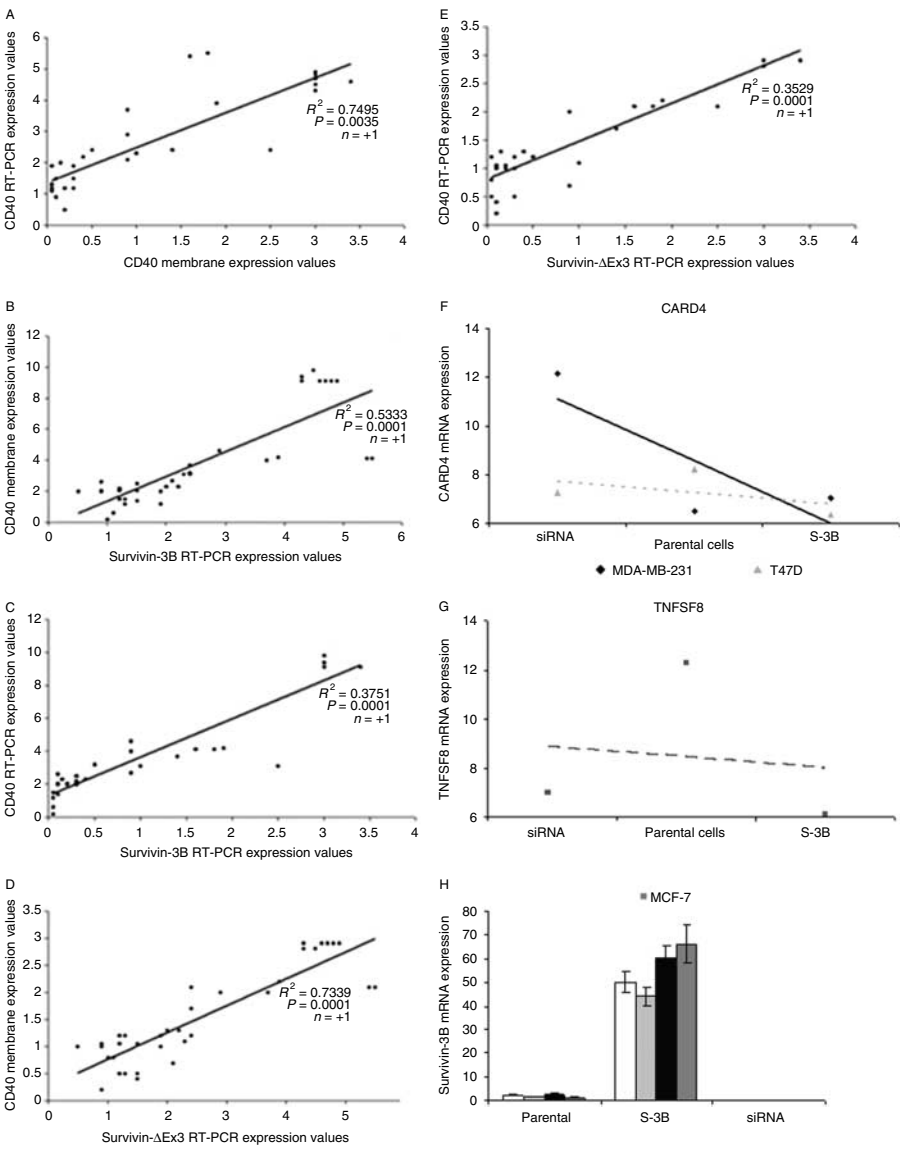

Figure 1 Validation of macroarray data by real-time quantitative RT-PCR. (A–E) Correlation between macroarray analysis in the 41 breast carcinomas and quantitative RT-PCR for CD40 expression. (F) CARD4 expression variation in relation to Survivin-3B expression in mutated p53 MDA-MB-231 (filled diamond and black solid line) and T47D (filled triangle and light gray line) cell lines. (G) TNFSF8 expression variation in relation to Survivin-3B expression in wild-type p53 MCF-7 cell line. (H) Survivin-3B mRNA expression in parental, Survivin-3B and siRNA transfected MCF-7 (open square), MDA-MB-231 (light gray shaded square), T47D (filled square), and HBL100 (dark gray shaded square) cell lines.

proportional hazards model. Multivariate analyses were also performed with Cox's proportional hazards model. Using Harrell (one variable for ten events) rules we have limited number of variables in multivariate model, we have included Survivin variants and variables that were significant in univariate Cox analyses only.

Results

Relationships between Survivin splice variants and apoptosis genes

To screen the genes of apoptosis that could be associated with Survivin splice variants, we used macroarray analysis. As a preliminary work, we determined the median value of the transcriptional expression of the five Survivin transcripts by quantitative RT-PCR analysis in the 135 primary breast tumors. Survivin expression was detected in the 135 examined breast carcinomas, Survivin-2B in 131 (97%), Survivin-ΔEx3 in 130 (96%), Survivin-3B in 82 (60%), and Survivin-2α in 133 (98%; Supplementary Figure 1A, see section on supplementary data given at the end of this article). The median value was calculated for each transcript and was used as a threshold to determine tumor groups as following: 1.57 (0–74.33) for Survivin, 0.27 (0–70.32) for Survivin-2B, 0.47 (0–18.65) for Survivin-ΔEx3, 0.0034 (0–0.41) for Survivin-3B, and 1.03 (0–107.69) for Survivin-2α.

Among the previous analyzed tumors, 41 were selected according to their mRNA quantity and quality (Supplementary Table 1) for 96 apoptosis gene expression analyses. Several associations were found between Survivin transcript expressions and the apoptotic gene array. Only Survivin-2α showed no correlation with any of the macroarray genes. For the main Survivin transcript, only MCL1, member of the Bcl-2 family, was more expressed when Survivin expression was elevated (Supplementary Table 2, see section on supplementary data given at the end of this article). Significant associations were detected between Survivin-2B and low expression of three genes belonging to the TNF-L family, one of the TNF-R family members and one of the Caspases families. Survivin-ΔEx3 was positively associated with a member of the TNF-L family and two of the TNF-R family, and was negatively associated with another two members of the TNF-R family.

Interestingly, five genes of the TNF-R family and five other genes of the TNF-L family were inversely correlated to Survivin-3B expression, whereas two additional genes showed positive correlations. In addition, four members of the TRAF family and one of CARD related genes were inversely correlated with high median Survivin-3B expression levels (Supplementary Figure 1B, see section on supplementary data given at the end of this article). Thus, Survivin-3B expression has the most significant inverse correlations (15 of the 17 associated genes). Among them, only five concerned anti-apoptotic genes, whereas the ten genes had apoptotic functions.

To confirm the efficiency of our threshold, we used the Survivin-3B median value obtained with the 135 tumor samples to classify normal breast tissues and tumor cell lines. All the 18 normal samples were classified as low Survivin-3B expression whereas 8/9 tumor cell lines were classified as high Survivin-3B expression.

Validation of macroarray data by quantitative RT-PCR

In macroarray analysis, the CD40 gene was positively correlated with both Survivin-3B and Survivin-ΔEx3. Thus, it was selected to validate macroarray data by quantitative RT-PCR.

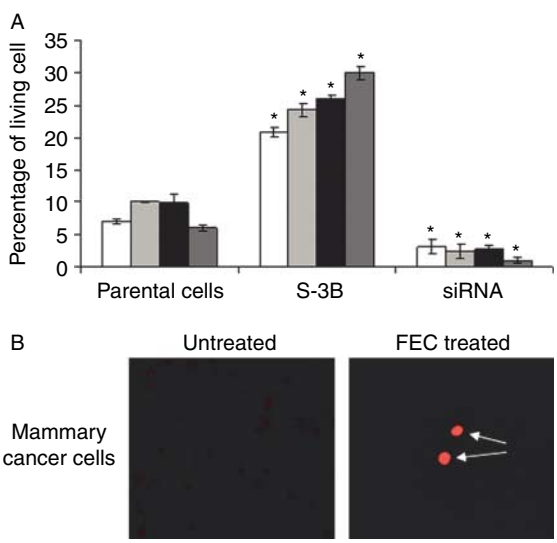

Figure 2 Apoptosis is influenced by Survivin-3B expression level in breast tumor cells treated with FEC for 48 h. (A) The living cell percentage in MCF-7 (open square), MDA-MB-231 (light gray shaded square), T47D (filled square), and HBL100 (dark gray shaded square) was increased in Survivin-3B overexpressing cells and decreased in Survivin-3B down-expressing cells. *Indicates $P<0.05$. (B) *In situ* cell death detection where DNA breaks due to apoptosis were labeled with a red fluorescent dye. Only FEC-treated cells harbored a fluorescent staining.

The CD40 expression detected with the macroarray study was in direct agreement with the quantitative RT-PCR results ($r^2=0.7456$, Spearman rank correlation $P=0.0035$; Fig. 1A). The results also showed a positive correlation between the CD40 expression detected by macroarray and the Survivin-3B and Survivin-ΔEx3 expression detected by RT-PCR ($r^2=0.6833$ and $r^2=0.7839$, respectively, $P<0.0001$; Fig. 1B and D). This correlation was still observed when the CD40 expression was determined by RT-PCR ($r^2=0.8761$ and $r^2=0.8629$, $P<0.0001$; Fig. 1C and E).

Because no RNA material was available for all samples obtained on needle core biopsy, we tested the expression of two another genes (CARD4 and TNFSF8) correlating with Survivin-3B expression in control, Survivin-3B or siRNA transfected breast tumor cell lines (Fig. 1F and G). Survivin-3B expression level in each transfected cell lines is presented in Fig. 1H and Supplementary Table 3, see section on supplementary data given at the end of this article. The expression of CARD4 decreased in mutated p53 MDA-MB-231 and T47D Survivin-3B transfected cells in comparison to parental cells, but was less pronounced in T47D cells. Interestingly, silencing of Survivin-3B increased CARD4 expression. Similarly, the expression of TNFSF8 cells diminished in wild-type p53 MCF-7 Survivin-3B transfected cells in comparison to parental and siRNA silencing cells. These results confirmed that the Survivin-3B expression level correlated with CARD4 and TNFSF8 mRNA expression, which could dependent on the tumor cell P53 status.

Expression variation of Survivin-3B influences the apoptotic induction in breast tumor cell lines

Next, we determined the effect of Survivin-3B overexpression or downexpression on FEC-induced apoptosis in cells transfected with yellow fluorescence protein (YFP)-Survivin-3B or Survivin-3B siRNA (Fig. 1H). In parental cells, after 48 h of treatment, MDA-MB-231, MCF-7, T47D, and HBL100 had 7, 10, 10, and 6% living cell percentage respectively (Fig. 2A). In Survivin-3B overexpressing cells, the living cell percentage increased (MDA-MB-231, 21%; MCF-7, 24%; T47D, 26%; and HBL100, 30%) but when Survivin-3B was downregulated, its decreased dramatically (MDA-MB-231, 3%; MCF-7, 2%; T47D, 3%; and HBL100, 1%; Fig. 2A). These results were confirmed *in situ* (e.g. in Fig. 2B) and also showed that the expression of Survivin-3B influenced the FEC induced cell death by a P53-independent manner.

Table 1 Mean expression levels of Survivin transcripts according to patient's breast cancer clinical subgroups

		Survivin		Survivin-2B		Survivin-ΔEx3		Survivin-3B		Survivin-2α	
Variables	***n***	**Mean (S.D.)**	***P****	**Mean (S.D.)**	***P****	**Mean (S.D.)**	***P****	**Mean (S.D.)**	***P****	**Mean (S.D.)**	***P****
Age (years)											
≤50	57	5.90 (9.12)		1.12 (2.37)		1.43 (3.02)		0.02 (0.05)		3.62 (7.24)	
>50	78	5.73 (11.12)	0.9893	1.70 (8.03)	0.527	0.95 (1.44)	0.561	0.03 (0.07)	0.5456	6.51 (17.3)	0.1433
Histological grade											
1	15	4.53 (9.61)		0.30 (0.64)		0.37 (0.51)		0.02 (0.06)		8.13 (11.0)	
2	63	4.24 (7.97)	**0.008**	0.47 (0.94)	**0.0001**	1.09 (2.60)	**0.0001**	0.02 (0.04)	0.0814	5.83 (14.6)	**0.0401**
3	48	8.88 (13.11)		3.29 (10.3)		1.62 (2.22)		0.04 (0.08)		2.27 (3.55)	
Hormonal receptors											
ER−	45	8.55 (13.84)	0.1104	2.89 (10.50)	**<0.0001**	1.33 (1.41)	**0.0002**	0.03 (0.06)	0.4627	4.11 (11.75)	0.4725
ER+	90	4.44 (7.68)		0.73 (1.93)		1.06 (2.57)		0.02 (0.06)		5.83 (15.80)	
PR−	60	7.18 (12.80)	0.7149	2.22 (9.11)	0.0886	1.05 (1.34)	0.4152	0.03 (0.06)	0.8371	4.94 (11.34)	0.6871
PR+	75	4.70 (7.65)		0.84 (2.10)		1.23 (2.78)		0.03 (0.06)		5.68 (16.17)	
Tumor size (cm)											
<2	12	3.73 (4.88)		0.34 (0.34)		1.19 (1.73)		0.024 (0.04)		12.29 (22.7)	
2–4	121	6.09 (10.73)	0.8035	1.58 (1.62)	0.279	1.16 (2.32)	0.5454	0.03 (0.06)	0.3025	4.65 (13.05)	0.0969
>4	2	1.00 (0.79)		0.06 (0.05)		0.31 (0.33)		0 (0)		4.604 (2.45)	
Nodal status											
Negative	66	3.59 (6.13)		0.64 (2.04)		0.86 (1.64)		0.02 (0.05)		8.43 (19.1)	
Positive	69	7.93 (12.78)	0.2097	2.23 (8.51)	**<0.0001**	1.44 (2.69)	0.0654	0.03 (0.07)	0.3554	2.18 (3.85)	**0.0008**
p53 gene											
Normal	113	5.30 (9.94)		1.48 (6.83)		1.14 (2.43)		0.03 (0.06)		6.01 (15.41)	
Mutated	22	8.42 (11.84)	0.1940	1.32 (1.67)	0.1403	1.22 (0.89)	**0.0042**	0.05 (0.07)	**0.0187**	2.10 (3.67)	0.0578

**P* values related to Kruskal–Wallis, Mann–Whitney, and/or two-sample Wilcoxon rank-sum tests. Significant results are noted in bold.

To confirm that the observed effect on apoptosis is due to Survivin-3B expression variation, expression levels of all Survivin variants, before and after FEC treatment were analyzed. As shown in Supplementary Table 3, the expression of Survivin variants was similar before and after treatment in these cell lines.

Relationship with standard prognostic factors

The mean expression levels of the five Survivin transcripts were compared with the patient's characteristics. Expression of Survivin, Survivin-2B and Survivin-ΔEx3, was higher in high-grade carcinomas. Survivin-2B and Survivin-ΔEx3 presented higher expression in estrogen receptor (ER)-negative carcinomas. In addition, Survivin-2B was higher in carcinomas with nodal invasion contrary to Survivin-2α that was lower in node-positive ones. Moreover, p53 mutations were correlated to Survivin-ΔEx3 and Survivin-3B (Table 1).

Relationship with the patient's survival outcome

At the data cut off, 51 deaths were registered. Univariate Cox analyses showed no significant impact for Survivin, Survivin-ΔEx3, and Survivin-2α expressions in overall and disease-free survivals. However, Survivin-2B and Survivin-3B expressions greater than median were significantly related to the worst overall survival (Table 2 and Fig. 3A). In addition, mutated p53, positive nodal status and tumor size were also associated with shorter overall survival. In contrast, positive hormonal receptors were related to longer overall survival.

Table 2 Univariate and multivariate Cox models for overall survival

	Univariate Cox		**Multivariate Cox** ($n=135$)	
	HR 95% CI	***P***	**HR 95% CI**	***P***
Age (years)				
≤50				
>50	1.37 (0.77–2.44)	0.282		
Tumor size	1.41 (1.11–1.80)	**0.005**	1.30 (0.99–1.71)	0.065
Hormonal receptors				
ER−	1		1	
ER+	0.41 (0.24–0.72)	**0.002**	0.53 (0.24–1.18)	0.119
PR−	1		1	
PR+	0.50 (0.29–0.87)	**0.014**	0.87 (0.38–1.98)	0.742
Histological grade				
1	1			
2	1.31 (0.50–3.47)	0.586		
3	1.73 (0.64–4.67)	0.277		
Nodal status				
Negative	1		1	
Positive	2.41 (1.34–4.32)	**0.003**	2.12 (1.09–4.12)	**0.026**
p53 gene				
Normal	1		1	
Mutated	2.97 (1.62–5.45)	<**0.0001**	2.16 (1.08–4.31)	**0.029**
Survivin				
≤1.57	1		1	
>1.57	0.99 (0.57–1.72)	0.977	0.49 (0.22–1.08)	0.078
Survivin-2B				
≤0.27	1		1	
>0.27	1.84 (1.05–3.22)	**0.034**	1.41 (0.63–3.16)	0.403
Survivin-3B				
≤0.0034	1		1	
>0.0034	1.87 (1.06–3.31)	**0.030**	2.01 (1.02–3.93)	**0.042**
Survivin-ΔEx3				
≤0.47	1		1	
>0.47	1.33 (0.77–2.31)	0.312	0.74 (0.36–1.54)	0.419
Survivin-2α				
≤1.03	1		1	
>1.03	0.93 (0.53–1.60)	0.782	0.77 (0.37–1.60)	0.487
				Harrell's C index=0.74

*Multivariate Cox using stepwise selection rules: $P<0.05$ for adding to the model and $P>0.10$ for removal to the model. Significant results are noted in bold.

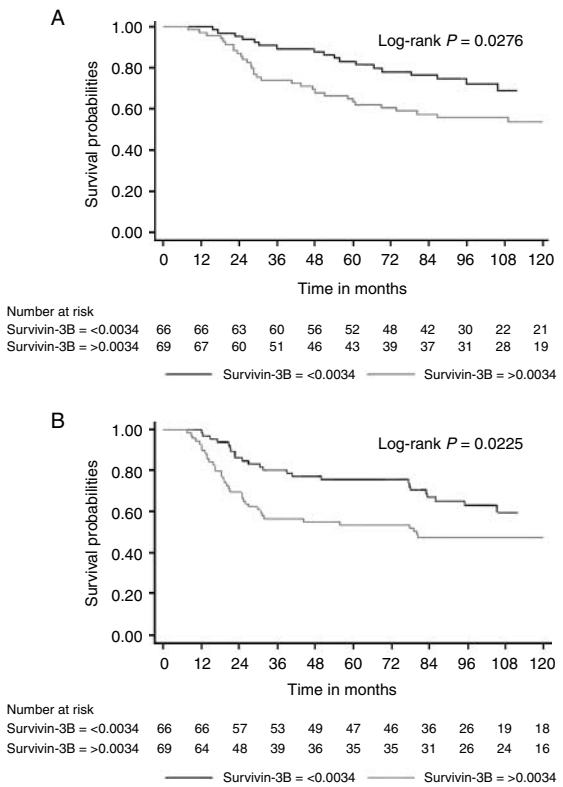

Figure 3 (A) Kaplan–Meier curves of overall survival of patients categorized on Survivin-3B median value. Thus, Survivin-3B subgroup, 0 corresponded to tumors with Survivin-3B levels $\leq$0.0034. Survivin-3B subgroup, 1 corresponded to tumors with Survivin-3B levels $>$0.0034. (B) Kaplan–Meier curves of disease-free survival of patients categorized on Survivin-3B median value. Thus, Survivin-3B subgroup, 0 corresponded to tumors with Survivin-3B levels $\leq$0.0034. Survivin-3B subgroup, 1 corresponded to tumors with Survivin-3B levels $>$0.0034.

Multivariate model highlighted that only positive nodal status (HR = 2.12 (1.09–4.12), P = 0.026), mutated p53 (HR = 2.16 (1.08–4.31), P = 0.029), and Survivin-3B were independent prognostic factors of overall survival (HR = 2.01 (1.02–3.93), P = 0.042).

At the data cut off for DFS analysis, 63 events (distant metastases, local recurrance, or death) were registered. Univariate Cox analyses showed no significant impact for Survivin, Survivin-ΔEx3, Survivin-2α, and Survivin-2B expressions, while Survivin-3B expression greater than median was significantly related to the worst DFS (Table 3 and Fig. 3B). Mutated p53, positive nodal status and tumor size were also associated with shorter DFS. In contrast, positive hormonal receptors were related to longer DFS. Multivariate model highlighted only positive nodal status (HR = 2.98 (1.63–5.43), $P \geq$ 0.0001) and survivin-3B (HR = 2.21 (1.22–4.03), P = 0.009) were independent prognostic factors of DFS.

Discussion

Survivin, an apoptosis inhibitor protein, is highly expressed in human cancers, including breast cancer, and is considered as a new therapeutic target. Recently, a critical role for Survivin in the control of autophagy was also reported (Roca *et al*. 2008). However, its splice variants Survivin-2B, Survivin-ΔEx3, Survivin-3B, and Survivin-2α were less studied. In this report, by macroarrays, we analyzed the apoptosis gene expression profile in breast carcinomas according to Survivin and its splice variant expression levels initially determined by quantitative RT-PCR. To date, only one study reported the gene expression profile analysis of the bladder cancer of Survivin transgenic mice revealing striking changes in gene expression. The clustered genes were extracellular matrix constituents and immune-inflammatory mediators (Altieri 2008). This kind of signature is unknown for human tumors. Our results revealed several significant associations between Survivin transcript (Survivin, Survivin-2B, and Survivin-ΔEx3) expressions and the apoptotic gene array. These differences of Survivin splice variant features might explain how Survivin mediates both apoptosis and cell division regulation (Yang *et al*. 2004). Survivin-3B variant expression showed the major associations, manifested as inverse correlations with ten pro-apoptotic genes. However, Survivin-3B expression has also inverse correlations with five anti-apoptotic genes. These results may suggest a feedback loop at the gene levels with this splice variant. Also, this could be a way for cancer cells to counteract Survivin-3B overexpression.

The data obtained in this study are in accordance with the putative anti-apoptotic role of Survivin-3B initially determined by its amino acid sequence (Badran *et al*. 2004). Moreover, overexpression of Survivin-3B in breast tumor cell lines strongly inhibits FEC toxicity, a combination used in breast carcinoma treatment. Our previous observations have shown that expression of the anti-apoptotic Survivin-ΔEx3 and Survivin-3B is higher in p53-mutated breast tumors (Végran *et al*. 2007). Thus, we used breast tumor cell lines expressing either wild type or mutated p53.

Recently, the cytoprotective effect of Survivin-3B after cisplatin treatment was reported (Knauer *et al*. 2007). Our results also showed for the first time the cytoprotective effect of Survivin-3B after FEC treatment in less aggressive cells as HBL100 and in more aggressive cells as MCF-7, MDA-MB-231, and T47D by a P53-independent manner.

Table 3 Univariate and multivariate Cox models for disease-free survival

	Univariate Cox		Multivariate Cox ($n=135$)	
	HR 95% CI	***P***	**HR 95% CI**	***P***
Age (years)				
≤50				
>50	1.17 (0.71–1.94)	0.536		
Tumor size	1.32 (1.06–1.65)	**0.014**	1.15 (0.89–1.49)	0.288
Hormonal receptors				
ER−	1		1	
ER+	0.48 (0.29–0.79)	**0.004**	0.61 (0.30–1.25)	0.175
PR−	1		1	
PR+	0.57 (0.35–0.94)	**0.028**	0.76 (0.37–1.57)	0.465
Histological grade				
1	1			
2	0.98 (0.45–2.14)	0.958		
3	1.04 (0.46–2.34)	0.927		
Nodal status				
Negative	1		1	
Positive	2.75 (1.62–4.69)	<**0.0001**	2.98 (1.63–5.43)	<**0.0001**
p53 gene				
Normal	1		1	
Mutated	2.11 (1.26–3.54)	**0.005**	1.46 (0.79–2.53)	0.245
Survivin				
≤1.57	1		1	
>1.57	1.03 (0.63–1.70)	0.895	0.57 (0.28–1.17)	0.125
Survivin-2B				
≤0.27	1		1	
>0.27	1.44 (0.88–2.37)	0.150	0.73 (0.37–1.46)	0.375
Survivin-3B				
≤0.0034	1		1	
>0.0034	1.79 (1.08–2.96)	**0.024**	2.21 (1.22–4.03)	**0.009**
Survivin-ΔEx3				
≤0.47	1		1	
>0.47	1.46 (0.89–2.41)	0.137	1.23 (0.64–2.36)	0.534
Survivin-2α				
≤1.03	1		1	
>1.03	1.13 (0.69–1.86)	0.630	0.75 (0.39–1.45)	0.395
				Harrell's C index=0.72

*Multivariate Cox using stepwise selection rules: $P<0.05$ for adding to the model and $P>0.10$ for removal to the model. Significant results are noted in bold.

The implication of Survivin splice variant expression in breast cancer response to chemotherapy is unknown. We earlier reported that expression of Survivin-3B and Survivin-ΔEx3 did not change after an anthracycline-based chemotherapy in breast carcinomas, whereas a significant reduction in the percentage of expression of the remaining variants was observed (Végran *et al*. 2005).

The results also confirm our previous observations that expression of the anti-apoptotic Survivin-ΔEx3 and Survivin-3B is higher in p53-mutated breast tumors (Végran *et al*. 2005, 2007). However, unlike Ryan *et al*. (2005) results but like Span *et al*. (2006) ones, we found Survivin-ΔEx3 expression increases with histological grade and is more expressed in ER-negative tumors. Survivin-2B is overexpressed in high-grade ER-negative and node-invasive tumors. This result is not in accordance with its putative pro-apoptotic role (Krieg *et al*. 2002) but seems to indicate Survivin-2B could be a marker of aggressiveness. Interestingly, Survivin-2α is more present in low-grade and non-invasive tumors, probably due to its potential pro-apoptotic role (Caldas *et al*. 2005).

We have previously showed that expression of Survivin-3B was associated with tumor resistance after one course of FEC treatment (Boidot *et al*. 2009). In addition, increased expression of Survivin-3B after one course of docetaxel/epirubicin treatment was associated with reduced DFS of breast cancer patients. In this study, we found a significant relationship with

either patient's overall or DFS for Survivin-3B expression. Indeed, high Survivin-3B expression tumors had a shorter overall and DFS. Similar to O'Driscoll *et al*. (2003) our results showed Survivin, Survivin-ΔEx3, and Survivin-2B have no prognostic role in breast carcinoma. Indeed, the adverse association between Survivin and poor outcome in breast cancer seems to be quite confusing. This can be explained by the fact that the analysis of Survivin transcripts with specific primers and probe probably differs from studies that do not distinguish the different splice variants.

A pro-apoptotic role for Survivin-2α has been described (Caldas *et al*. 2005); however, it was found to be associated with the worst prognosis of breast cancer patients (Span *et al*. 2006). Our data do not demonstrate this kind of relation but the negative correlation between Survivin-2α expression and the worst clinical parameters suggests it is not implied in aggressive phenotype. However, the relation between Survivin-3B expression and worse survival is in accordance with this study indicating overexpression of Survivin-3B is linked to a decreased relapse-free survival of breast cancer patients. Studies looked at Survivin gene expression, and found associations with outcome, have used RT-PCR method with primers that did not distinguish individually each isoform. The primers were often localized on exon 1, a region that is common to all transcripts. By consequence, the Survivin-main transcript amplified was not representative of this amplicon. In addition, the detection of Survivin protein expression by immunohistochemistry could detect all Survivin isoforms. Thus, the association found between Survivin and outcome might be due to the simultaneous expression of the other isoforms (e.g. Survivin-3B).

Taken together, our results show that Survivin-3B may contribute to cell survival through the anti-apoptotic pathway. The results also indicate that Survivin-3B could play a significant role in breast tumor development and prognosis and could be considered as a new therapeutic target.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/ERC-11-0105.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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