

Prognostic value of histone chaperone FACT subunits expression in breast cancer

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Abstract: Understanding the underlying reasons for tumor aggressiveness, such as why some tumors grow slowly and locally, while others rapidly progress to a lethal metastatic disease, is still limited. This is especially critical in breast cancer (BrCa) due to its high prevalence and also due to the possibility that it can be detected early. Several oncogenes and tumor suppressors have been identified and are used in the prognosis and treatment of BrCa. However, even with these markers, the outcome within BrCa subtypes is highly variable. Chromatin organization has long been acknowledged as a factor that plays an important role in tumor progression, but molecular mechanisms defining chromatin dynamics are largely missing. We have recently found that histone chaperone FACT (facilitates chromatin transcription) is overexpressed in ~18–20% of BrCa cases. FACT is elevated upon transformation of mammary epithelial cells and is essential for viability of tumor cells. BrCa cells with high FACT have a more aggressive transcriptional program than those with low FACT cells. Based on this we propose that FACT may be a marker of aggressive BrCa. In this study, we aimed to comprehensively characterize the pattern of FACT expression in BrCa in relation to other molecular and clinical prognostic markers. We developed and tested an assay for the detection and quantitation of protein levels of both FACT subunits, SSRP1, and SPT16, in clinical samples. We compared the value of mRNA and protein as potential markers of disease aggressiveness using a large cohort of patients (n=1092). We demonstrated that only SSRP1 immunohistochemical staining is a reliable indicator of FACT levels in tumor samples. High SSRP1 correlated with known markers of poor prognosis, such as negative hormone receptor status, presence of Her2, high-grade tumors, and tumors of later clinical stage. At the same time, no strong correlation between SSRP1 expression and survival was detected when all samples were analyzed together. Clear trend toward longer survival of patients with low or no SSRP1 expression in tumor samples was seen in several subgroups of patients, and most importantly significant association of high SSRP1 expression with shorter disease-free survival was detected in patients with early-stage and low-grade BrCa, the category of patients with the highest demand in predictive marker of disease progression.

Keywords: SSRP1, SPT16, breast cancer, predictive marker, prognosis

Introduction

Breast cancer (BrCa) is a highly heterogeneous group of neoplastic disorders that shows tremendous variability in clinical, pathological, and molecular features which, therefore, must not be treated as a single disease. Some cases of BrCa may be indolent, requiring almost no intervention and minimally affecting life expectancy or health of a patient, while other cases of BrCa rapidly progress to a highly aggressive lethal metastatic stage. Since the introduction of mammography as a routine screening for

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women, detection of early-stage neoplastic lesions of the mammary gland has been significantly increased. At the early stage, future disease progression is not evident and, therefore, decision about treatment strategy is difficult.

The use of molecular markers, such as hormone receptors, Her2, and Ki67, has revolutionized the way clinicians approach BrCa, which categorizes newly diagnosed breast tumors into specific subtypes dictating treatment and prognosis. Such a prediction is based on the average outcome within subtypes, which limits assessment of risk in individual patients due to the variability of clinical outcomes in patients with similar molecular features. This results in the overtreatment or undertreatment of patients with tumors that do not progress as projected by their subtype. Though this heterogeneity adds a level of complexity to the treatment of BrCa, it does leave open opportunity for the customization of therapies for individual patients in the future. Unfortunately, there is a current lack of appropriate biomarkers able to accurately distinguish between high-risk and low-risk tumors within subtypes. This leaves little justification for prescribing cytotoxic chemotherapy to patients of less aggressive subtypes, such as Luminal A, or forgoing potentially lifesaving chemotherapy in patients with notoriously aggressive subtypes such as basal-like. Thus, there arises a clear need for biomarkers that accurately distinguish between tumors with high or low metastatic potential at an early stage of assessment.

In our previous study on a small cohort ($n=167$) of BrCa patients, we found that expression of structure specific recognition protein 1 (SSRP1) subunit of the histone chaperone “facilitates chromatin transcription” (FACT) correlated with several clinicopathological markers, including high-grade, triple negative status, absence of hormone receptors, and Her2 amplification.¹ FACT is a heterodimeric complex of two highly conserved subunits—suppressor of Ty 16 (SPT16) and SSRP1. FACT was initially identified via its ability to promote RNA polymerase II transcriptional elongation through nucleosomal DNA templates by binding to H2A/H2B histone dimers, thereby weakening their contact with DNA.^{2–4} FACT has also been shown to play an important role in replication, recombination, and DNA damage repair.^{5–8}

Contrary to the previous belief that FACT is a ubiquitous housekeeping factor, several years ago, our laboratory reported that FACT was detectable at protein level in a very limited number of normal adult cells. It is highly expressed at early stages of embryonic development with gradual reduction toward birth.⁹ Of great importance, it was also observed that FACT, at the protein level, is expressed to varying degrees in many breast tumors but not in normal breast tissues.¹⁰ Upon

comparison of BrCa cells of the same subtype, we saw that cells with high levels of FACT were more aggressive than those with low levels of FACT, and upon equalization of FACT levels via short hairpin RNA, the cells with high levels of FACT were converted to a less aggressive phenotype.¹¹ In the same cohort of BrCa patients described earlier, we observed a greater correlation of SSRP1 level with grade than with the stage of the disease and a strong correlation of SSRP1 status between matched primary and metastatic lesions.¹ This suggests that the expression of FACT in tumors does not change significantly with the disease development and, therefore, high FACT expression is observed even in patients with early-stage disease. Thus, we propose FACT as a driver of tumor aggressiveness and hence as a potential biomarker capable of distinguishing high-risk from low-risk tumors within the subtypes of BrCa. Importantly, regulation of FACT is complex and occurs most probably not only via gene expression but also through protein and mRNA stability. In this study, we aimed to assess the value of FACT as a potential predictive marker of BrCa by assessing accurate levels of protein and mRNA of both subunits.

Materials and methods

Ethics, consent, and permissions

This study was reviewed by Roswell Park Cancer Institute Institutional Review Board Ethical Committee, protocol number NHR014210. Biospecimens used in this study were collected from patients that provided written informed consent to use of their remnant tissue for research purposes. The aforementioned research activity has been reviewed and determined to be nonhuman subject research. US Department of Health and Human Services does not consider research involving data and specimens to involve human subjects as defined under 45 CFR 46.102(f) if the following conditions are both met:

1. Private information or specimens were not collected specifically for the currently proposed research project by interacting or intervening with living individuals and
2. The investigator(s) cannot readily ascertain the identity of the individual(s) to whom the coded private information or specimens pertain.

In accordance with 45 CFR 46.102 of the Common Rule, this study did not involve human subjects. In addition, this research activity did not involve any US FDA-regulated product and, therefore, the data collected need not be submitted to or held for inspection by the FDA in support of marketing application. Therefore, the research is not subject to review by the Institutional Review Board (IRB).

Patient population

Patients included in this study (total 1092) were diagnosed with BrCa and treated between 1996 and 2011 at Roswell Park Cancer Institute (RPCI). The RPCI IRB approved this study. Selection of patients included all BrCa patients at that time period with adequate data in the RPCI archival bank for immunohistochemistry and follow-up in the RPCI Tumor Registry or various RPCI Research Program Databases. This study cohort included 1092 patients with the following subtypes: luminal A (ER [estrogen receptor] + and/or PR [progesterone receptor] +, Her2-), n=596; luminal B (ER+ and/or PR+, Her2+), n=151; triple negative (ER-, PR-, Her2-), n=222; Her2 type (ER-, PR-, Her2+), n=91; and unknown, n=32.

Patient demographic and clinical characteristics are reported by group using mean and standard deviation (SD) for continuous variables and using frequencies and relative frequencies for categorical variables.

Tissue microarrays (TMAs)

SSRP1 and SPT16 protein expression in the clinical cohort was assessed using 24 TMAs. All RPCI TMAs are built in a standardized fashion with three 0.6-mm tissue cores from formalin-fixed paraffin-embedded donor blocks arrayed into a recipient paraffin block, including tumor specimens and controls. For some TMAs, 3 cores of matching normal tissue were also evaluated. Controls within the TMA consisted of multiple cores of normal tissue from ten different organs including heart, colon, kidney, adrenal, ovary, myometrium, brain, thyroid, lung, and prostate.

Immunohistochemical staining (IHC)

IHC assays and automated digital pathology analysis for SPT16 and SSRP1 were performed at the Pathology Network Shared Resource at RPCI. Formalin-fixed paraffin-embedded sections were cut at 4 μ m, placed on charged slides, and dried at 60°C for 1 hour. Slides were cooled to room temperature, deparaffinized in three changes of xylene, and rehydrated using graded alcohols. For antigen retrieval, slides were heated in a steamer with either citrate buffer or ethylenediaminetetraacetic acid buffer and allowed to cool for several minutes (see details for each antibody in [Table S1](#)). Endogenous peroxidase was quenched with aqueous 0.3% H₂O₂ for 10 minutes and washed with phosphate buffered saline with 0.2% Tween 20. Slides were loaded on a Dako autostainer (Dako, Glostrup, Denmark) and serum-free protein block was applied for 5 minutes, blown off, and then the primary antibody was applied for 60 minutes. A matched isotype was

also applied on a replicate slide instead of primary antibody as a negative control. The EnVision + horseradish peroxidase system (Dako) was applied for 30 minutes followed by the diaminobenzidine (DAB) chromogen for 10 minutes for visualization. Finally, the slides were counterstained with hematoxylin, rinsed, and cover-slipped.

Slide scanning and image analysis

TMA slides were digitally scanned using Aperio ScanScope XT (Leica Biosystems, Inc., Buffalo Grove, IL, USA) with 20 \times bright-field microscopy. These images are accessible using eSlide Manager (Leica Biosystems, Inc.). Aperio ImageScope version 12.2.1.5005 (Leica Biosystems, Inc.) was used for image analysis. Slide image data field were populated, and images were examined for quality and amended as necessary. An annotation layer was created for each core of interest in the TMA. Invasive tumor cell-only regions were circled using free-form pen tool and areas to be excluded were marked using negative free-form pen in order to eliminate irrelevant regions from image analysis calculations. Care was taken appropriately to represent the heterogeneity of staining of each TMA core for image analysis and also to avoid including regions with staining artifacts. When possible, representative areas of tumor were selected for analysis with a minimum target of 30 cells per TMA core.

A unique analysis macro was created for each target (SPT16 and SSRP1). The Nuclear Algorithm is calibrated to analyze DAB nuclear staining for the individual tumor cells and quantifies their staining intensity. A counterstain hematoxylin, blue stain, was applied for morphologic detail of the surrounding tissue to help identify nuclear and cytoplasmic compartments of cells for analysis. Positivity thresholds were set for the positive stain and the scores for average nuclear intensity of the selected regions were calculated based on these thresholds. Cell nuclei were individually classified as follows: 0, none; 1+, weak; 2+, moderate; and 3+, strong. The results showed the total number of detected cells, the percentage of cells per class (0, 1+, 2+, and 3+), and the percentage of positive stained cells along with the average staining intensity of the positive nuclei as a score of 0, 1+, 2+, and 3+. Image analysis data were exported from Spectrum as a tab delimited ".csv" file and converted to a ".xls" file and formatted using Microsoft Excel 2010. H-score was calculated, which is an intensity score derived from the average intensity of the staining of the cytoplasm according to the threshold intervals set in the algorithm macro. This score equals = 1*(%1+) + 2*(%2+) + 3*(%3+) with the score is between 0 and 300, where 300 represents 100% of cells being 3+.

RNA extraction and expression measurement

RNA was isolated using a standard Trizol (Invitrogen, Carlsbad, CA, USA) application. Tissues were homogenized with Trizol reagent. RNA was then precipitated from the aqueous phase using isopropanol. For QC, 260/280 ratios were examined to confirm preparation purity and an RNA aliquot was run on an Agilent 2100 bioanalyzer to confirm RNA integrity. RNA integrity number values ranged from 7 to 9.4.

RNA was reverted to cDNA by iSCRIPT kit (cat# 170-8891, Bio-Rad) and PCR was conducted using Taq master mix (cat# 71162, USB-affymetrix). Primers were synthesized by Integrated DNA Technologies (Coralville, IA, USA). Sequence details and experimental conditions are provided in [Table S2](#).

Intensity of PCR product bands were analyzed by Image J (software available at imagej.nih.gov) and all samples were scored relative to the intensity of the corresponding band of housekeeping gene *GAPDH*.

The association between the mRNA and protein expressions of both SSRP1 and SPT16 was evaluated using scatter plots and the Spearman correlation coefficient (R).

Protein extraction and immunoblotting

Cytoplasmic and nuclear extracts of Hela cells were obtained using ReadyPrep™ Protein Extraction Kit (cytoplasmic/nuclear) from Bio-Rad (cat# 163-2089; Bio-Rad, Hercules, CA, USA). Protein concentrations were measured using DC™ Protein Assay kit from Bio-Rad (cat# 5000111; Bio-Rad). Western blotting was run using standard method. The following antibodies were used: SSRP1 mouse monoclonal (10D1; Biolegend, Inc., San Diego, CA, USA), SPT16 mouse monoclonal (8D2; Biolegend, Inc.), rabbit polyclonal (A302-492A; Bethyl Laboratories, Montgomery, TX, USA) and (H-300, sc-28734, Santa-Cruz Biotechnology, Santa Cruz, CA, USA), HSP70 mouse monoclonal (5A5; cat# MA3-007, Thermo-Fisher, Grand Island, NY, USA), histone H3—mouse monoclonal (MAB1 0301, cat#39763; Active Motif, Carlsbad, CA, USA).

Statistical analyses

Reproducibility of staining

In cases where multiple tissue samples were obtained from a patient, the SSRP1 or SPT16 scores were combined using a weighted average. Each tissue sample was weighted based on the number of cells examined. Since some patients only had a single tissue sample analyzed, the consistency of the SSRP1 and SPT16 scores is a concern. The reliability of the scores was assessed in patients with multiple tissue samples using intra-class correlation coefficient (ICC). *A priori*, an ICC of

at least 0.6 will be considered sufficient for demonstrating a reasonable level of reliability. The scores were summarized using means and SDs and graphically using histograms.

Identification of thresholds for SSRP1 and SPT16 scores

Previous studies that emphasized FACT as a marker of aggressive cancers did not utilize automatic scoring and it was done by qualified pathologists semi quantitatively;¹ hence, previously identified thresholds would not be valid in this dataset. Therefore, we identified new thresholds for SSRP1 and SPT16 based on disease-specific survival (DSS) and the maximal concordance criterion proposed by Gonen and Sima.¹² This criterion is selected since it concurs with the familiar Youden's index and is appropriate when trying to identify "high-risk" cases, which in this case would refer to patients with poor survival outcomes. Patients with an SSRP1 score above the identified threshold are classified as patients with high SSRP1 expression (*hSSRP1*) and patient with SSRP1 low expression (*/SSRP1*) otherwise. Similarly for SPT16, patients with a score above the identified threshold are classified as patients with high SPT16 expression (*hSPT16*) and patients with low SPT16 expression (*/SPT16*) otherwise. Characteristics of patients with low and high expression were compared using *t*-test and Fisher's exact test for continuous and categorical variables, respectively. For continuous variables, normalizing transformations (e.g., Box-Cox) were applied as appropriate.

There was a subset of patients that also had samples taken from metastatic tissue. In these patients, the differences in SSRP1 and SPT16 scores were summarized using means and SDs and graphically by histograms. Comparisons were made using permutation paired *t*-tests.

Survival analysis

The survival outcomes (e.g., overall, disease-specific, recurrence-free, and progression-free) were reported by the level of protein expression using standard Kaplan–Meier methods. Univariate comparisons were made using log-rank test. Propensity-adjusted analyses were conducted using stratified Cox regression models, where the survival outcomes were modeled as a function of SSRP1/SPT16 expression and a propensity score. The models were fit using Firth's method, and hazard ratios (HR) for SSRP1/SPT16 expression, with corresponding confidence intervals, were obtained from model estimates. The propensity scores were obtained using logistic regression models and correspond to the probability of being classified as high SSRP1 or SPT16 expression based on the demographic and clinical characteristics in Table 1 (except for clinical American Joint Committee on Cancer

Table I Patient characteristics based on SSRPI expression

Characteristic	Subcategory	ISSRPI	hSSRPI	p-value
Overall	N	487 (59.3%)	334 (40.7%)	
Age	Mean (SD)	56.8 (13.0)	55.5 (14.0)	0.14
Gender	Female	485 (99.6%)	331 (99.1%)	0.40
	Male	2 (0.4%)	3 (0.9%)	
Race	White	432 (88.7%)	283 (84.7%)	0.24
	Black	46 (9.4%)	42 (12.6%)	
	Other	9 (1.8%)	9 (2.7%)	
Histology	Ductal carcinoma	390 (80.1%)	272 (81.4%)	0.49
	Lobular carcinoma	10 (2.1%)	11 (3.3%)	
	Ductal & lobular carcinoma	42 (8.6%)	28 (8.4%)	
	Mixed subtypes	20 (4.1%)	13 (3.9%)	
	Other subtypes	25 (5.1%)	10 (3.0%)	
Grade	Grade I/II	215 (45.7%)	115 (35.6%)	<0.01
	Grade III	255 (54.3%)	208 (64.4%)	
AJCC—clinical	Stage 0/I	120 (57.1%)	80 (38.1%)	<0.001
	Stage 2	68 (32.4%)	95 (45.2%)	
	Stage 3/4	22 (10.5%)	35 (16.7%)	
AJCC—path	Stage 0/I	179 (40.0%)	104 (33.3%)	<0.01
	Stage 2	219 (49.0%)	146 (46.8%)	
	Stage 3/4	49 (11.0%)	62 (19.9%)	
Radiation therapy	No	135 (28.4%)	91 (27.6%)	0.81
	Yes	340 (71.6%)	239 (72.4%)	
Hormonal therapy	No	112 (24.8%)	120 (39.3%)	<0.001
	Yes	340 (75.2%)	185 (60.7%)	
Chemotherapy	No	155 (34.4%)	76 (24.8%)	<0.01
	Yes	296 (65.6%)	231 (75.2%)	
Surgery	Lumpectomy	302 (62.1%)	183 (54.8%)	0.07
	Total mast	183 (37.7%)	151 (45.2%)	
	Needle biopsy	1 (0.2%)		
Tumor size (cm)	Mean (SD)	2.3 (1.5)	2.7 (2.2)	0.06
ER	Negative	142 (30.0%)	145 (43.8%)	<0.001
	Positive	331 (70.0%)	186 (56.2%)	
PR	Negative	210 (44.5%)	187 (56.5%)	<0.001
	Positive	262 (55.5%)	144 (43.5%)	
Her2	Negative	333 (71.3%)	227 (69.6%)	0.61
	Positive	134 (28.7%)	99 (30.4%)	
Necrosis	No	329 (72.0%)	188 (58.8%)	<0.001
	Yes	128 (28.0%)	132 (41.3%)	

Note: Data presented as n (%) unless stated otherwise.

Abbreviations: ISSRPI, low SSRPI expression; hSSRPI, high SSRPI expression; SD, standard deviation; AJCC, American Joint Committee on Cancer; clinical, clinical stage; path, pathological stage; ER, estrogen receptor; PR, progesterone receptor.

[AJCC] stage and tumor size, due to the degree of missing values). Overall survival was defined as the time from definitive surgery until death (from any cause) or last follow-up. DSS was defined as the time from surgery until cancer-related death or last follow-up (death from other causes are treated as censored). Recurrence-free survival (RFS) is defined as the time from surgery until recurrence, death from disease, or last follow-up. RFS is only calculated for patients who were free of disease after treatment. Progression-free survival (PFS)

is the time from surgery until treatment failure, recurrence, death from disease, or last follow-up. The median follow-up and corresponding range are calculated using the follow-up times of patients alive (or censored) at last follow-up.

All statistical analyses were conducted using SAS version 9.4 (SAS, Cary, NC, USA) at a significance level of 0.05.

Results

Development of assays for the detection of FACT subunits levels in tumor samples

IHC assay for the staining of SSRPI was previously developed.^{1,9} A small cohort (n=167) of samples were analyzed in 2010 using a different lot of antibody. To compare the data between studies, we restained the same TMAs using a new antibody lot. SSRPI signal was exclusively nuclear as before; however, the intensity of the recent staining was in general stronger, resulting in a higher proportion of weakly positive cells and samples ([Figure S1A and S1B](#)). In general the pattern was similar between the two sets; stromal cells as well as a significant proportion of samples were still negative. Several dilutions of antibodies were tested; however, difference still persisted. Therefore, we reanalyzed TMAs that were used in 2010 in parallel with new TMAs.

We tested several different antibodies to SPT16 to establish IHC assay. All SPT16 antibodies produced different degree of cytoplasmic staining and in some cases only cytoplasmic without nuclear signal ([Figure S2](#)). The same was observed when these antibodies were used for immunofluorescence (data not shown). To test the specificity of cytoplasmic signal, we purified nuclear and cytoplasmic fractions of cells and used them for Western blotting. Immunostaining of these samples showed no cytoplasmic signal of expected size (~140 kDa). Moreover, when these cells were treated with CBL0137, which removed FACT from the nucleoplasm of cells due to the precipitation that occurred in chromatin pellet, specificity of antibodies was confirmed ([Figure S3](#)). Thus, we concluded that cytoplasmic staining is nonspecific and used antibodies with minimal cytoplasmic signal (H300 from Santa Cruz Biotechnology). We also subtracted cytoplasmic signal while scoring nuclear SPT16 signal.

Analysis of patient population

A total of 821 and 836 SSRPI and SPT16 scores, respectively, were obtained from 921 BrCa patients. The difference might be due to poor quality or absence of some specimens on the slides. The average age of the patients was 56.8 years (range: 25–97), among whom 6 (0.7%) were male. The population was predominately Caucasian (87.3%). The patients had mostly low clinical AJCC staging (86.9%, ≤stage 2) and

grade III disease (56.8%). The ER, PR, and Her2 positivity rates were 67.5%, 53.2%, and 28.0%, respectively. As expected, patients who were negative for ER or PR tended to have poorer survival outcomes (Figure S4). Those positive for Her2 had poorer survival outcomes compared to those negative for Her2 regardless of ER or PR status (Figure S5). Thus, the cohort of patients used in the study presented typical BrCa population with no deviation from expected correlation between established markers expression and clinical outcome.

Expression of FACT subunits, SSRP1, and SPT16 in BrCa samples

The distribution of SSRP1 scores is slightly skewed (Figure 1A), with an average of 51.2 (SD=57.6) covering the full range of possible values (range: 0–300). The distribution of SPT16 scores is symmetric (Figure 1B), with an average

of 121.7 (SD=47.9) covering most of the possible values (range: 0–261). The observed heterogeneity of the SSRP1 was high, and the coefficient of variation (CV) was greater than 1, whereas the CV for SPT16 was only 0.4. This is an indication that the FACT subunits may be associated with different clusters or subgroups (e.g., histology and grade) of the patient populations or that IHC staining of SPT16 is not reliable.

The reliability of the SSRP1 and SPT16 scores was of concern since some patients only had a single tissue sample. The ICC for patients with multiple tissue samples was 0.67 and 0.51 for SSRP1 and SPT16, respectively. The SSRP1 scores met our *a priori* threshold for reliability (see section “Material and methods”). However, SPT16 scores did not meet our standard for reliability and this should be kept in mind when interpreting results associated with this FACT subunit.

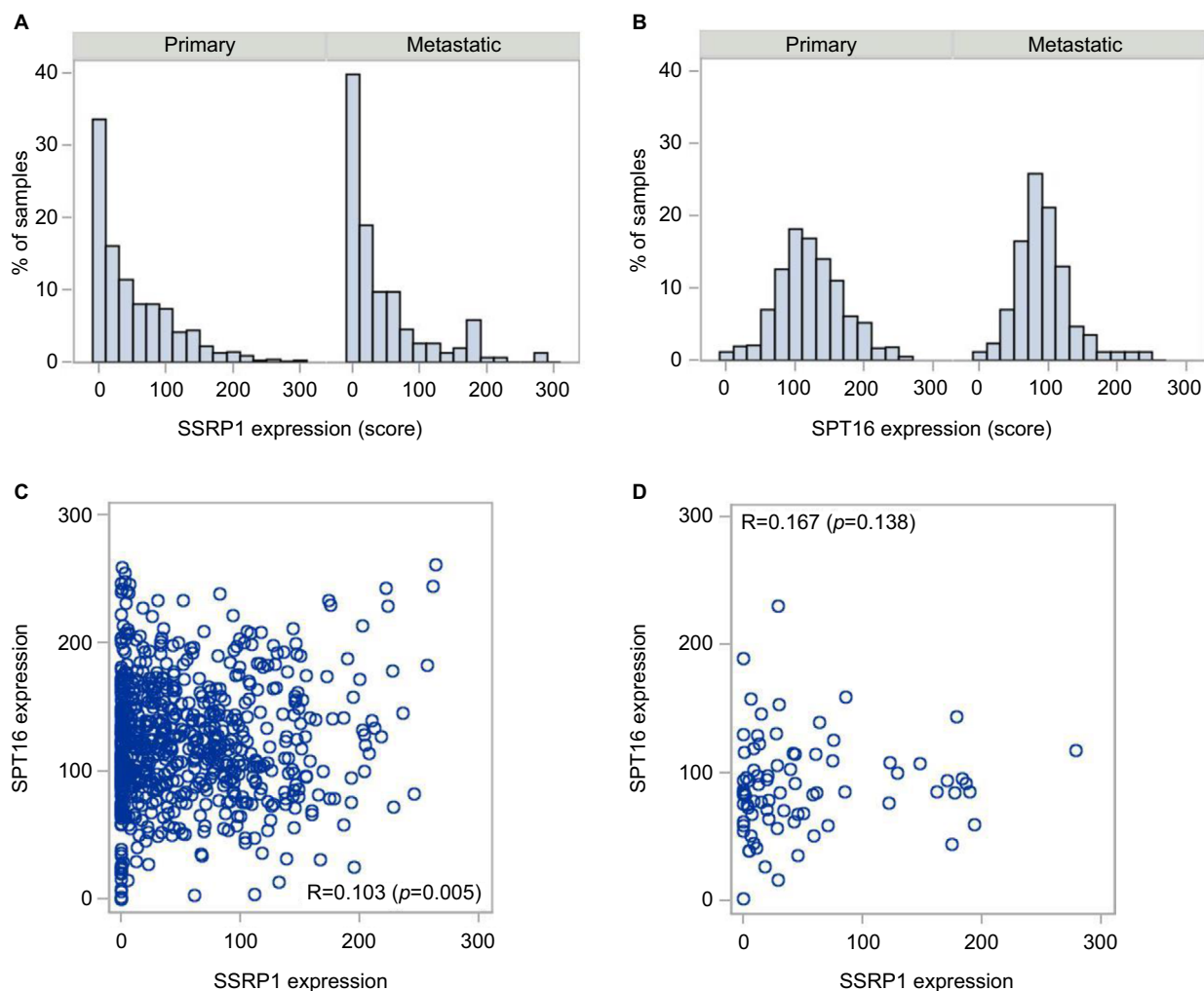


Figure 1 Distribution of SSRP1 and SPT16 IHC scores of primary and metastatic samples of BrCa patients. Histograms of SSRP1 scores (A) and SPT16 scores (B) of primary and metastatic samples. Correlations between SPT16 and SSRP1 scores of primary (C) and metastatic (D) samples are shown by dot plots.

Abbreviations: BrCa, breast cancer; IHC, immunohistochemistry.

SSRP1 and SPT16 are coregulated: stability of each protein subunit depends on the presence of another subunit in the complex with their own mRNA.¹³ Therefore, we expected high correlation between SSRP1 and SPT16 scores. However, correlation between the subunits was very low (primary samples, Figure 1C) or insignificant (metastatic samples, Figure 1D).

The maximal concordance criterion approach was used to identify SSRP1 and SPT16 score that would best separate patients with respect to DSS. For SSRP1, the threshold for dichotomizing patients into low and high SSRP1 expression was determined to be 45. That is, patients with SSRP1 score above 45 were classified as *h*SSRP1 and *l*SSRP1 otherwise. For SPT16, the threshold was determined to be 70; patients with SPT16 scores above 70 are classified as *h*SPT16 and *l*SPT16 otherwise. A total of 334 (40.7%) and 734 (87.8%) of tissue samples were identified as having high SSRP1 and SPT16 expression, respectively.

The demographic characteristics of patients with available SSRP1 and SPT16 scores are reported in Tables 1 and 2, respectively. Neither SSRP1 nor SPT16 expression was significantly associated with gender or race, and *h*SPT16 patients tended to be older ($p=0.04$) than *l*SPT16 patients.

Significant associations between SSRP1 expression and grade ($p<0.01$), AJCC clinical ($p<0.001$) and pathologic ($p<0.01$) stage, ER ($p<0.001$) and PR ($p<0.001$) status, and necrosis ($p<0.001$) were found (Table 1). The *h*SSRP1 patients tended to have higher grade disease and higher clinical/pathological staging compared to *l*SSRP1 patients. They were also more likely to be ER or PR negative. High SSRP1 expression was also linked with increased necrosis. No difference in the distribution of SSRP1 between ductal and lobular carcinomas was found.

Significant associations observed between SPT16 expression and hormonal therapy ($p=0.04$), ER status ($p=0.04$), and PR status ($p<0.01$) are shown in Table 2. The *h*SPT16 patients were more likely to be ER or PR positive and to receive hormonal therapy. The results associated with the SPT16 scores should be carefully interpreted, as we have previously emphasized that they lack reliability.

The results of the analyses of SSRP1 staining and grade, stage, and ER/PR status appear to support the previously published conclusions that FACT expression may be a marker of aggressive cancer.¹

Association between expression of FACT subunits and survival outcomes

The median follow up time of patients with available SSRP1 scores was 97.9 months (range: 1.0–220.3 months). There were

Table 2 Patient characteristics based on SPT16 expression

Characteristic	Subcategory	<i>l</i> SPT16	<i>h</i> SPT16	<i>p</i> -value
Overall	N	102 (12.2%)	734 (87.8%)	
Age	Mean (SD)	54.5 (11.9)	57.5 (13.7)	0.04
Gender	Female	102 (100.0%)	730 (99.5%)	1.00
	Male		4 (0.5%)	
Race	White	94 (92.2%)	639 (87.1%)	0.31
	Black	6 (5.9%)	78 (10.6%)	
	Other	2 (2.0%)	17 (2.3%)	
Histology	Ductal carcinoma	87 (85.3%)	595 (81.1%)	0.28
	Lobular carcinoma		14 (1.9%)	
	Duct. & lob. carcinoma	5 (4.9%)	71 (9.7%)	
	Mixed sub-types	5 (4.9%)	28 (3.8%)	
	Other sub-types	5 (4.9%)	26 (3.5%)	
Grade	Grade I/II	41 (40.6%)	319 (44.9%)	0.41
	Grade III	60 (59.4%)	391 (55.1%)	
AJCC—clinical	Stage 0/I	24 (44.4%)	196 (54.0%)	0.17
	Stage 2	21 (38.9%)	134 (36.9%)	
	Stage 3/4	9 (16.7%)	33 (9.1%)	
AJCC—path	Stage 0/I	38 (39.6%)	306 (45.1%)	0.14
	Stage 2	41 (42.7%)	299 (44.0%)	
	Stage 3/4	17 (17.7%)	74 (10.9%)	
Radiation therapy	No	22 (22.2%)	206 (28.5%)	0.23
	Yes	77 (77.8%)	516 (71.5%)	
Hormonal therapy	No	35 (36.8%)	179 (26.5%)	0.04
	Yes	60 (63.2%)	497 (73.5%)	
Chemotherapy	No	28 (29.5%)	249 (36.8%)	0.17
	Yes	67 (70.5%)	428 (63.2%)	
Surgery	Lumpectomy	61 (59.8%)	479 (65.3%)	0.50
	Total mast	41 (40.2%)	253 (34.5%)	
	Needle biopsy		1 (0.1%)	
Tumor size (cm)	Mean (SD)	2.2 (1.5)	2.3 (1.6)	0.81
ER	Negative	41 (41.0%)	219 (30.5%)	0.04
	Positive	59 (59.0%)	499 (69.5%)	
PR	Negative	59 (59.0%)	314 (43.8%)	<0.01
	Positive	41 (41.0%)	403 (56.2%)	
Her2	Negative	68 (68.7%)	518 (73.0%)	0.37
	Positive	31 (31.3%)	192 (27.0%)	
Necrosis	No	61 (61.6%)	489 (70.5%)	0.08
	Yes	38 (38.4%)	205 (29.5%)	

Note: Data presented as n (%) unless stated otherwise.

Abbreviations: *l*SPT16, low SSRP1 expression; *h*SPT16, high SSRP1 expression; SD, standard deviation.

a total of 228 deaths (121 were due to disease) and 143 recurrences, and an additional 50 patients were never free of disease. Though not statistically significant (all $p>0.05$), Figure 2 and Table 3 demonstrate that *h*SSRP1 patients tended to have poorer survival outcomes (lower survival rates and median survival times). The propensity adjusted analyses were based on only 720 observations (due to missing values for some covariates), and all found a nonsignificant association between increased SSRP1 expression and poorer survival outcomes (Table S3).

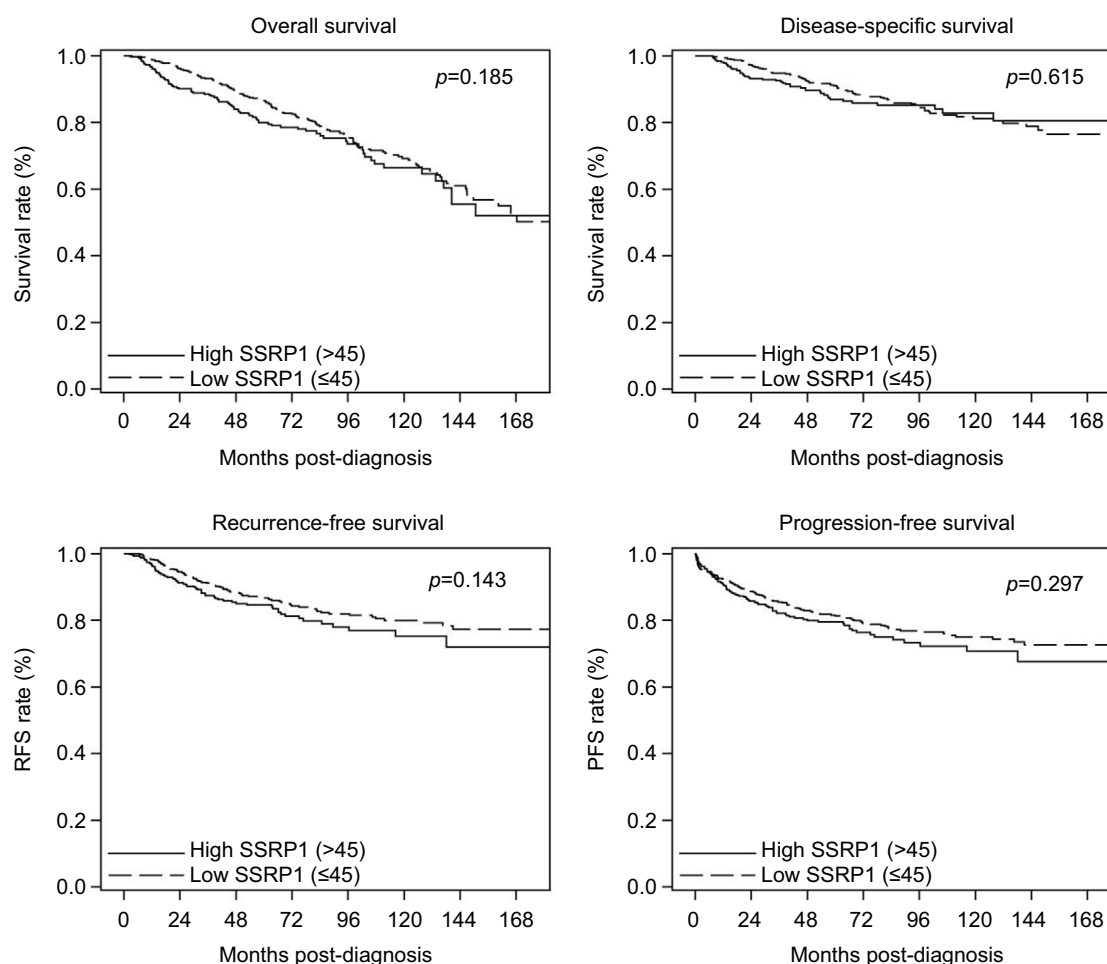


Figure 2 SSRP1 protein levels did not demonstrate any significant correlation with survival of breast cancer patients. Kaplan-Meier curves of survival outcomes for the primary sample.

Abbreviations: RFS, recurrence-free survival; PFS, progression-free survival.

Table 3 Survival outcomes based on SSRP1 expression

FACT		5 year rate (95% CI)	10 year rate (95% CI)	Median time, months (95% CI)	Log rank p-value
SSRP1	Overall survival	hSSRP1 0.80 (0.75, 0.84)	0.66 (0.59, 0.73)	NE (138.2, NE)	0.20
		lSSRP1 0.86 (0.83, 0.89)	0.69 (0.63, 0.73)	183.0 (146.9, NE)	
	Disease specific	hSSRP1 0.87 (0.83, 0.90)	0.83 (0.77, 0.88)	NE (NE, NE)	0.67
		lSSRP1 0.91 (0.88, 0.93)	0.81 (0.76, 0.85)	NE (NE, NE)	
	Recurrence free	hSSRP1 0.80 (0.75, 0.84)	0.66 (0.59, 0.73)	NE (138.2, NE)	0.15
		lSSRP1 0.87 (0.83, 0.90)	0.80 (0.75, 0.84)	NE (NE, NE)	
	Progression free	hSSRP1 0.79 (0.74, 0.83)	0.70 (0.63, 0.76)	NE (NE, NE)	0.23
		lSSRP1 0.81 (0.78, 0.85)	0.75 (0.70, 0.79)	NE (NE, NE)	
SPT16	Overall survival	hSPT16 0.85 (0.83, 0.88)	0.71 (0.67, 0.75)	183.0 (165.2, NE)	0.08
		lSPT16 0.80 (0.71, 0.87)	0.61 (0.47, 0.71)	167.2 (111.0, NE)	
	Disease specific	hSPT16 0.91 (0.89, 0.93)	0.87 (0.84, 0.89)	NE (NE, NE)	0.04
		lSPT16 0.90 (0.82, 0.95)	0.73 (0.58, 0.83)	NE (NE, NE)	
	Recurrence free	hSPT16 0.88 (0.85, 0.90)	0.82 (0.78, 0.85)	NE (NE, NE)	0.47
		lSPT16 0.87 (0.77, 0.92)	0.77 (0.65, 0.86)	NE (NE, NE)	
	Progression free	hSPT16 0.83 (0.80, 0.86)	0.78 (0.74, 0.81)	NE (NE, NE)	0.48
		lSPT16 0.81 (0.72, 0.88)	0.73 (0.61, 0.81)	NE (NE, NE)	

Abbreviations: 95% CI, 95% confidence interval; lSSRP1, low SSRP1 expression; hSSRP1, high SSRP1 expression; SD, standard deviation; FACT, facilitates chromatin transcription; NE, not estimable.

According to the SPT16 scores available, the median follow-up was 97.9 months (range: 1.0–220.3 months). There were a total of 219 deaths (100 were due to disease) and 122

recurrences, and an additional 46 patients were never free of disease. Though only DSS was found to have a statistically significant association with SPT16 expression ($p=0.04$),

Figure 3 and Table 3 demonstrate that *h*SPT16 patients tended to have better survival outcomes (higher survival rates and median survival times). The propensity adjusted analyses were based on 736 observations and were supportive of the univariate analyses, but nonsignificant (Table S1).

In a recent study, we observed that FACT regulates genes expression in a cell-specific manner.¹¹ We hypothesized based on this that FACT may be predictive of survival of patients with certain subtypes of BrCa. Therefore, we analyzed survival outcome for *h*SSRP1 and *l*SSRP1 separately for several subgroups of BrCa (Table S4). Significant association was found between relapse-free survival and SSRP1 expression (HR 2.65, 95% confidence interval 1.20–5.85, $p=0.02$) in patients with low-grade cancer (pathological stage 0/1). Consistent, although nonstatistically significant, trend was observed between *h*SSRP1 and shorter relapse free/PFS for patients with early clinical stage (0/1), HR 1.9–2.7, and surprisingly with longer survival for patients with hormone

receptor positive cancers, HR 0.3–0.8, independently on Her2 status (Table S4).

The overall results of the survival analyses for SSRP1 lend support to the previous observation made on a small cohort of BrCa samples¹ that FACT overexpression is associated with poor survival outcomes in cancer patients. However, the results observed for SPT16 are in conflict with those previous conclusions and could possibly be attributed to the cytoplasmic staining issues and lack of reliability associated with SPT16 scoring.

Comparison of FACT subunit expression in primary and metastatic samples

Both primary and metastatic samples were available from 120 patients with SSRP1 scores and 67 patients with SPT16 scores. A significant positive correlation was observed for subunits expression between primary and metastatic samples, that is, staining of primary and metastatic lesions were similar for SSRP1 or SPT16 in most of the patients.

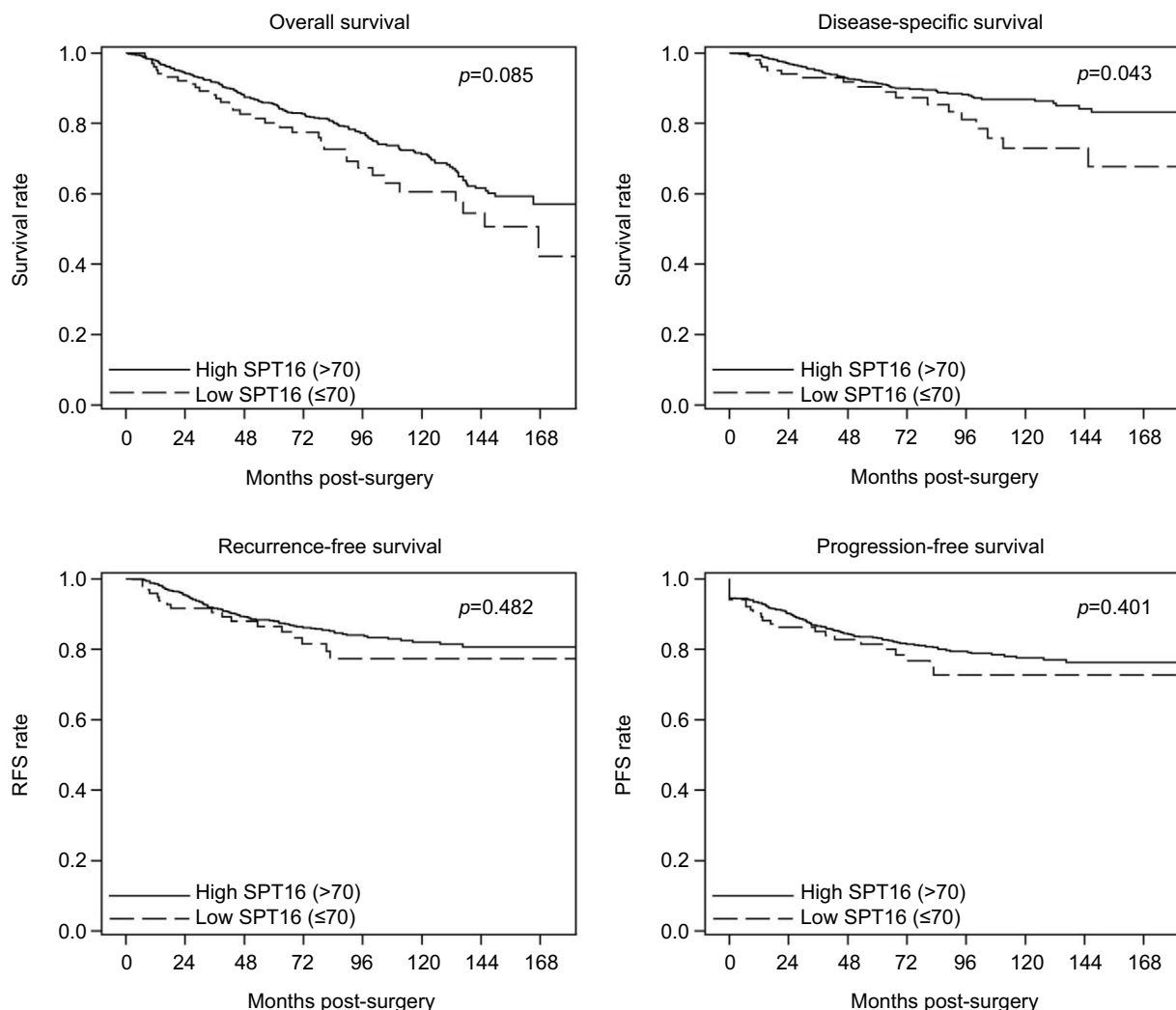


Figure 3 Kaplan-Meier curves of survival outcomes based on SPT16 expression. **Abbreviations:** RFS, recurrence-free survival; PFS, progression-free survival.

Surprisingly we observed small but significant prevalence in the subunit expression in primary versus metastatic samples. The mean difference (primary – metastatic) of SSRP1 and SPT16 expression was 11.0 (SD=57.5) and 23.4 (SD=53.4), respectively, which were both statistically significant (SSRP1: $p=0.05$; SPT16: $p<0.001$) (Figure S6).

Protein and mRNA expression

SSRP1 protein and mRNA expression data were available for 68 patients, whereas SPT16 protein and mRNA expression data for 61 patients. A statistically significant positive correlation ($R=0.30$, $p=0.02$) between SSRP1 protein and mRNA expression was observed. Despite being statistically significant, the correlation coefficient is low and no tight linear relationship as expected was observed (Figure S7A). A low negative correlation between the SPT16 protein and mRNA expressions was observed ($R=-0.15$, $p=0.02$) (Figure S7B).

Discussion

In the previous study with a small cohort of BrCa patients, we noticed that positive staining of TMA samples for SSRP1 subunit of FACT is associated with established clinical and molecular markers of more aggressive BrCa. In addition, using publically available data of SSRP1 mRNA expression, we observed the similar trend—association between higher levels of SSRP1 and more aggressive subtypes of BrCa.¹ FACT subunits are rarely mutated in cancer (TCGA data,^{14,15} Figure S8). Although the mechanism of FACT elevation in tumors is not yet known, using cell lines, we observed that the amount of FACT in cells is regulated via complex mechanism: by stability of SSRP1 and SPT16 proteins and by mutually dependent mRNAs.¹³ Thus, we hypothesized that simultaneous assessment of the protein and mRNA levels of both subunits may make prediction of survival more accurate.

First, we have to conclude that with all our attempts we were unable to develop accurate assay for SPT16 IHC using available commercial antibodies, although these antibodies specifically stain SPT16 on Western blotting (Figure S3). Most probably these antibodies recognize SPT16 only in denatured form. We do not believe that the data obtained for SPT16 with commercial antibodies are reliable as they were not reproducible between replicates of the same samples, there is very weak correlation with SSRP1 level (Figure 1C and 1D), and they negatively correlate with SPT16 mRNA data (Figure S7B). Most importantly, distribution of SPT16 scores was close to normal (Figure 1B), which is difficult to

expect from proteins that are not expressed in normal samples and significant proportion of tumors.

Data of mRNA expression were available only for limited number of patients and did not show high correlation with protein levels of either SSRP1 or SPT16 subunit (Figure S7). This is not completely surprising since regulation of FACT is complex with a significant role of protein stability.¹³ Therefore, for the accuracy of interpretation, we based our discussion only on SSRP1 IHC scores.

Association of hSSRP1 and known prognostic markers of poor survival was confirmed in this study. The data did not show any significant association between SSRP1 expression and survival outcome when all the samples were analyzed together (Figure 2). This cannot be explained based on nonrepresentative population, since established predictive markers (stage, grade, hormone receptor, and Her2 status) demonstrated expected correlations (Figures S4 and S5). Functional studies with BrCa cells differing in the basal level of FACT or upon genetic knockdown of FACT¹¹ are in line with the correlation of high SSRP1 and the presence of established markers of poor prognosis. Weak correlation with overall survival may suggest that input of FACT into aggressive behavior of all types of BrCa is weak but may become more significant within specific subgroups. Thus, we observed that patients with low-grade and/or early-stage cancer with hSSRP1 had a shorter survival time. This serves as an important fact for further studies, as this category of patients is in the highest demand for predictive markers to avoid under- or overtreatment. Alternatively, the problem may be technical. Our analysis was limited by the small pieces of tumors available from TMAs. Many of these samples were negative for SSRP1. In a parallel study of surgical specimens of BrCa, no sample was completely negative among the 20 samples analyzed. This might be due to the fact that many surgical samples appeared very heterogeneous in nature during SSRP1 staining, with some areas being positive and some negative. Clustered localization of SSRP1-positive cells in tumors may be explained by its possible role as stem cell marker and its similar pattern of distribution in some normal organs.^{9,16,17} Thus, TMA samples may not be a good material for the analysis.

Prospective studies that focus on disease progression in early-stage BrCa patients with small, low-grade tumors differing in SSRP1 expression are highly desirable, since they can explore the question as to whether SSRP1 positivity can be used for deciding the treatment options for these patients.

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Disclosure

The authors report no conflicts of interest in this work.

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