Clinical Translational Research

Oncology

Oncology 2009;77:378–384
DOI: 10.1159/000276765

Received: May 8, 2009
Accepted after revision: July 20, 2009
Published online: January 18, 2010

Elevated Expression of the Tyrosine Phosphatase SHP-1 Defines a Subset of High-Grade Breast Tumors

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Key Words
Breast cancer · Tissue microarray · Tyrosine phosphatase · Clinical setting · SHP-1

Abstract
Objectives: Protein tyrosine phosphatases are key regulators of intracellular signaling that contribute to determining cancer cell growth, which thus makes them attractive targets for therapeutic and diagnostic agents. SHP-1 phosphotyrosine phosphatase is rarely expressed in epithelial tumor cells, but expression has been found in several breast cancer cell lines and tumors. To determine the potential significance of SHP-1 as a prognostic marker in the clinical setting, we examined SHP-1 protein expression in breast tumors. ***Methods:*** We analyzed SHP-1 expression by immunohistochemistry in a breast tissue microarray composed of 2,081 cores, either alone or in combination with known prognostic markers. ***Results:*** Our data showed that SHP-1 expression was confined to a well-defined subset of high-grade tumors characterized by unique biological parameters. SHP-1 expression correlated directly with expression of the tyrosine kinase receptor HER-2 and inversely with expression of the estrogen receptor, while it was weakly associated with Bcl-2 expression. ***Conclusions:*** Levels of SHP-1 were correlated with conventional pathologic parameters of tumor aggressiveness and were associated with reduced patient survival, suggesting that elevated expression of SHP-1 is a common molecular abnormality in a defined subset of breast tumors and might be used in routine diagnosis to identify patients with high-risk tumors.

Introduction

Tyrosine phosphorylation through the concerted action of protein tyrosine kinases and protein tyrosine phosphatases (PTPs) regulates the activity of key proteins involved in cell proliferation, differentiation and the cell cycle. Consequently, aberrant activation of protein tyrosine kinases by somatic mutation, amplification and/or overexpression is a frequent event in human tumors. In-

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creased tyrosine phosphatase activity has been implicated in human breast cancer and is regarded as an important diagnostic parameter [1]. PTPs do not merely attenuate kinase signaling but may directly determine cancer cell growth, which thus makes them interesting targets for therapeutic and diagnostic agents. A paradigmatic example is the SH2-containing tyrosine phosphatase SHP-2, which is implicated in various solid cancers and is found to be mutated in several leukemias [2, 3]. SHP-2 and its homolog, SHP-1, are non-transmembrane phosphotyrosine phosphatases that play a key role in regulating cytokine/protein tyrosine kinase-mediated signaling and are counted among the molecular determinants that regulate cell proliferation in normal and neoplastic cells [3–6]. SHP-1 (previously named SHPTP1, HCP or PTP1C) is predominantly expressed in hematopoietic cells and likely serves as a negative regulator of most hematopoietic-specific signaling systems [7–12]. In tumors of epithelial origin, little is known about the expression of SHP-1 [13, 14]. However, the presence of high levels of SHP-1 mRNA in some breast cancer cell lines and primary breast tumors [13] suggests an active function for SHP-1 in these tumors. On the other hand, in the human breast cancer cell line MCF-7, a role for SHP-1 as a positive regulator of cell death has been suggested. Indeed, the resistance of MCF-7 cells to trichostatin A-induced apoptosis is decreased by stable overexpression of SHP-1, likely by a mechanism that involves the phosphatidylinositol 3-kinase pathway [15]. A function of SHP-1 as a positive regulator of cell death is further supported by the finding that it may act as a TrkA phosphatase, balancing the level of TrkA-induced cell survival in cultured neurons and PC12 cells [16].

To evaluate the prognostic role of SHP-1 in breast cancer, we analyzed SHP-1 protein expression in the clinical setting, using a large number of breast tumors in tissue microarray (TMA) format. TMA data were compared with molecular features, demonstrating that SHP-1, which is not expressed in normal breast tissues, is consistently present in a subset of high-grade breast cancer. High expression levels of SHP-1 correlate significantly with tumor aggressiveness, indicating that SHP-1 protein might help to make a more accurate prediction of progression of the disease.

Materials and Methods

Breast Cancer TMA and Immunohistochemistry

Four-micrometer-thick sections of TMA blocks were transferred to an adhesive-coated slide system (Instrumedics Inc., Hackensack, N.J., USA) supporting the cohesion of 0.6-mm array elements on glass. Standard indirect immunoperoxidase procedures were used for immunohistochemistry. After pretreatment with proteinase K (Dako, Carpinteria, Calif., USA), the anti-SHP-1 antibody (ab2020; Abcam plc, Cambridge, UK) was used at a dilution of 1:500. A diaminobenzidine chromogen was used. Nuclei were counterstained with hematoxylin. The percentage of positive neoplastic cells was estimated. For SHP-1, we arbitrarily used 20% as a cutoff of positivity for the purpose of statistical analysis. The scoring was performed by a single pathologist (L.I.). All slides from all tumors were reviewed by 1 of 2 pathologists to define the histologic grade according to Elston and Ellis [17] and the histologic tumor type.

Cells and Culture Conditions

MCF-7 and MCF-7 siRNA-SHP-1 cell lines were grown in DMEM (Invitrogen Corporation, San Diego, Calif., USA) supplemented with 5% fetal bovine serum (Invitrogen), 2 mM L-glutamine, 1% penicillin/streptomycin, 3.75 ng/ml epidermal growth factor (Sigma, St. Louis, Mo., USA), 10 μg/ml insulin and 500 ng/ml hydrocortisone. T47D and MDA-MB-231 cell lines were grown in RPMI-1640 (Invitrogen) supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 1% penicillin/streptomycin. The SKBr-3 cell line was grown in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 1% penicillin/streptomycin.

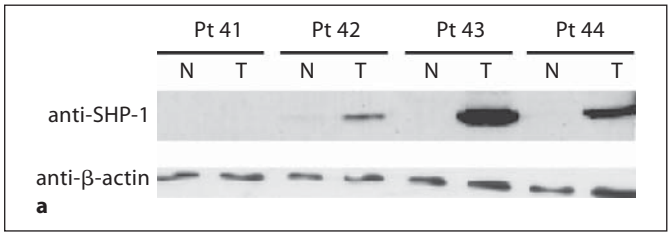
MCF-7 siRNA-SHP-1 is an MCF-7 derivative cell clone stably expressing the shRNA for SHP-1 as an insert into pcDNA 6.2-GW/miR (Invitrogen). The sequence of the siRNA that specifically knocks down expression of human SHP-1 is as follows: 5′-TACTCCACCAGCTCTGTCAGA-3′. This sequence is followed by 19 nucleotides derived from miR-155 to form a terminal loop and sense target sequence nucleotides 1–8 and 11–21 (nucleotides 9 and 10 are removed to form a short internal loop in the mature miRNA, which results in more efficient knockdown). MCF-7 siRNA- SHP-1 cells stably expressing miRNA were established by selection with medium containing 5 ng/ml blasticidin S HCl (Invitrogen). The medium was renewed every 3 days. After 2 weeks, resistant colonies were trypsinized, isolated and characterized.

Synchronization and Cell Cycle Analysis

Cells were rendered quiescent by serum deprivation for 24 h and then stimulated to re-enter the cell cycle by the addition of 10% serum. When 70–80% confluence was reached, cells were detached with trypsin and fixed with methanol overnight. Samples were resuspended in 0.5 ml of PBS and stained with propidium iodide in the dark for 30 min, and the DNA content was measured by fluorescence-activated cell sorting on a Becton-Dickinson FACScan flow cytometry system. The data were analyzed using Mod-FIT software.

Protein Isolation from Human Breast Tissues

Breast tissue specimens were collected from surgical mastectomy of 25 patients affected by human breast cancer. All of our studies were carried out after fulfilling all the required ethical standards of the responsible institution (Committee on Human Experimentation). Tissues were minced with a mortar and pestle (Morgan Technical Ceramics, Waldkraiburg, Germany) and then homogenized in 1 ml of harvest buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1 mM sodium orthovanadate, 1% Nonidet P40 (Sigma)] containing protease inhibitor cocktail



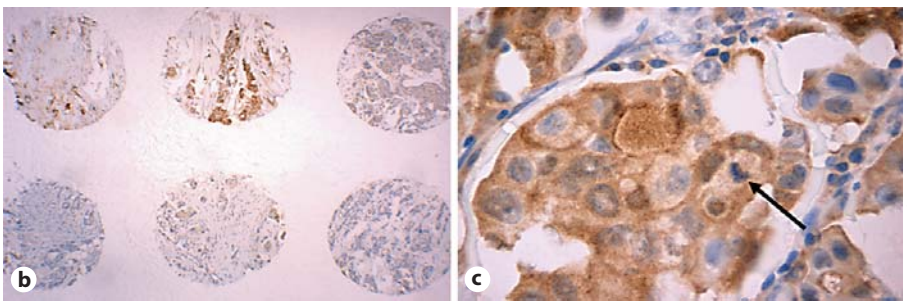


Fig. 1. SHP-1 protein expression in human breast tissues. **a** SHP-1 expression levels were analyzed by immunoblot with anti-SHP-1 antibody in different breast tumors (T) or adjacent normal tissue (N) obtained from 4 patients (Pt). Equal loading of protein was confirmed using anti-β-actin antibody. **b, c** Immunoperoxidase staining for SHP-1. **b** Representative TMA with negative and positive cores for SHP-1. Original magnification ×40. **c** High-grade breast carcinoma with strongly positive tumor cells. Mitotic activity is evident (arrow). Original magnification ×400.

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(Roche Diagnostic, Montreal, Canada), with a Dounce tissue grinder (Wheaton, Millville, N.J., USA). Solubilized proteins were incubated for 30 min on ice. After centrifugation at 10 000 *g* for 30 min at 4°C, supernatants were collected.

Immunoblot Analysis

Protein concentrations from tissues (100–200 mg each) or cell extracts were determined by Bradford assay using bovine serum albumin as standard. Thirty micrograms of each sample of protein extracts were prepared in Laemmli sample buffer, denatured for 5 min at 100°C, then subjected to SDS-PAGE. Gels were electroblotted into polyvinylidene difluoride membranes (Millipore Co., Bedford, Mass., USA), and the signals were detected with the indicated primary antibodies and peroxidase-conjugated secondary antibodies using an enhanced chemiluminescence system (Amersham-Pharmacia Biosciences Ltd.). Primary antibodies used were anti-SHP-1 (C-19; Santa Cruz Biotechnology, Santa Cruz, Calif., USA) and anti-β-actin (Sigma). Secondary antibodies used were anti-mouse (Amersham-Pharmacia Biosciences) and anti-rabbit (Santa Cruz Biotechnology).

[^{3}H]Thymidine Incorporation Assay

The plasmids pCEFL-hemagglutinin (HA), pCEFL-HA SHP-1(wildtype) and pCEFL-HA SHP-1 Cys453 to Ser mutant (C/S), containing the SHP-1 gene, fused to the HA epitope. The mutated human SHP-1 (C/S) is still able to bind substrates, but it is catalytically inactive. Transient transfections (15 μg of each plasmid) were carried out in 100-mm cell culture dishes with Lipofectamine 2000 reagent (Life Technologies Inc.), and cells were grown in the absence of serum for 5 h. After 16 h, 50,000 cells were replated in triplicate in 24-well tissue culture dishes. For each transfection, a cell aliquot was lysed and protein was analyzed by immunoblot with anti-HA antibodies; equal transfection efficiency was confirmed (not shown). Six, 30 and 54 h after replating, cells were treated with 4-hour pulses of 1 μCi/ml [^{3}H]thymidine (45 Ci/mmol; Amersham-Pharmacia Biosciences) added in complete growth medium and incubated at 37°C. At the end of each pulse, dishes were placed on ice, and cells were washed with ice-cold PBS, 5% trichloroacetic acid and, finally, with methanol. Cells were then dissolved in 0.3 N NaOH, and [^{3}H]thymidine incorporation was analyzed by a Beckman LS 1701 Liquid Scintillation Counter.

Statistics

The associations of some molecular features with SHP-1 expression were evaluated using the χ^2 test. To investigate the effect of high expression of SHP-1 on patient prognosis, we performed a univariate survival analysis using the Kaplan-Meier method and a log-rank test. Statistical analysis was performed using Stata software (Stata Corp., College Station, Tex., USA).

Results

SHP-1 Expression in Human Breast Tumors

We first determined the expression levels of SHP-1 in human breast cancer specimens from 4 patients for whom the corresponding adjacent normal tissue was available. None of the patients were receiving medical treatment at the time of the operation. Immunoblot analysis showed that SHP-1 was never expressed in normal breast, while it was expressed at various intensities in the adjacent breast tumor samples from 3 patients (fig. 1a).

We then analyzed the frequency of SHP-1 expression in breast tumors by using a breast cancer TMA composed of 2,081 samples (2,048 tumors and 33 normal breast controls) [18]. The large majority of tumors belonged to the ductal (1,426 cores) and lobular carcinoma (297 cores) histotypes. In line with the results obtained by immunoblot, all epithelial cells from normal breast controls were

Table 1. Staining levels of SHP-1 in the different breast cancer histotypes of the TMA

Tumor histotype	SHP-1 staining		Total
	negative	positive	
Adenocystic carcinoma	1	0	1
Apocrine carcinoma	13	1	14
Atypical medullary carcinoma	8	1	9
Carcinosarcoma	2	0	2
Clear cell carcinoma	8	4	12
Cribriform carcinoma	58	2	60
Ductal carcinoma	1,307	119	1,426
Histocytic carcinoma	2	0	2
Lipid-rich carcinoma	1	1	2
Lipid-rich or histioc	2	0	2
Lobular carcinoma	291	6	297
Medullary carcinoma	53	2	55
Metaplastic carcinoma	5	0	5
Mucinous carcinoma	65	2	67
Neuroendocrine carcinoma	1	1	2
Papillary carcinoma	23	4	27
Signet ring carcinoma	0	1	1
Small cell carcinoma	2	0	2
Lobular carcinoma	47	5	52
Ductal carcinoma in situ	9	0	9
Lobular carcinoma in situ	1	0	1
Total	1,932	149	2,081
Normal breast	33	0	33

Results for negative and positive SHP-1 expression were obtained by grouping negative and positive SHP-1 staining as described in Materials and Methods.

Table 2. Association between staining of SHP-1 and 3 clinical parameters: tumor grade, HER-2 overexpression and ER status

		SHP-1 staining		p value
		total, n	positive, %	
Tumor grade	G1	532	5	<0.001
	G2	866	6	
	G3	667	11	
HER-2	0+1	1,637	6	<0.001
	2	61	11	
	3	197	18	
ER	negative	438	13	<0.001
	positive	1,480	5	

For each parameter (tumor grade, HER-2 staining intensity and ER status), the total number of tumors and the percentage of tumors that stained positive for SHP-1 are indicated. The p values were calculated with Stata software.

immunohistochemically negative for SHP-1, while a group of tumors showed cytoplasmic positivity (table 1).

Since nuclear SHP-1 staining was observed in only a small fraction of the SHP-1-positive samples (2.2%), only cytoplasmic staining was considered positive in this setting (fig. 1b). Overall, approximately 7.2% of all breast tumors analyzed showed positive staining for SHP-1. The ductal histotype, which constituted 68% of the TMA, had a similar distribution (8.3% of cores positive for SHP-1), while SHP-1-positive staining in the lobular carcinoma cores was much rarer, with only 2.0% of such tumors expressing SHP-1 (table 1), thus suggesting that SHP-1 expression is not homogenously distributed among all types of breast cancers.

SHP-1 Defines a Subset of High-Grade Breast Cancers

In order to test the significance of SHP-1 expression in determining the biological behavior of the disease, we analyzed the relationship between SHP-1 expression and 3 common parameters of aggressiveness, i.e. histological grade [17] and expression levels of the HER-2 gene and the estrogen receptor (ER). As shown in table 2, 11% of high-grade tumors (G3) expressed SHP-1, while only 5% of low-grade tumors (G1) were positive for this phosphatase. In addition, 18% of the tumors that expressed high levels of HER-2 also expressed SHP-1. Accordingly, SHP-1 was expressed in 13% of tumors characterized by the absence of a functional ER (ER-negative tumors).

Furthermore, by introducing a cutoff to exclude values less than 10%, we tested the correlation between SHP-1 expression and the Ki67 labeling index, a marker of cell proliferation that was recently analyzed on the same TMA [18]. The Ki67 labeling index was shown to be strongly associated with high-grade tumors and was strongly linked to poor prognosis in this same cohort [18]. Here, we found that, although expression of SHP-1 correlates with molecular indicators of aggressiveness (see above), it did not correlate with high Ki67 (not shown). Taken together, these results indicate that high expression of SHP-1 identifies a subset of high-grade breast tumors, as assessed by independent parameters.

High Levels of SHP-1 Expression Are Associated with the Absence of Bcl-2 and the Presence of p16/Ink4a.

In order to further characterize tumors expressing high levels of SHP-1, we analyzed expression levels of 2 key proteins involved in cell survival and proliferation,

Table 3. Association between SHP-1-positive tumors and Bcl-2 or p16 expression in TMA samples

		SHP-1 staining		
		total, n	positive, %	p value
Bcl-2	0+1	311	12	<0.01
	2+3	1,551	7	
p16	negative	1,235	6	<0.001
	positive	634	10	

For each parameter (Bcl-2 and p16 expression intensity), the total number of tumors and the percentage of tumors that stained positive for SHP-1 are indicated. The p values were calculated with Stata software.

i.e. Bcl-2, which is involved both in counteracting apoptosis and cell cycle control [19], and the tumor suppressor gene, p16/Ink4a, which acts by inhibiting the phosphorylation of retinoblastoma protein family members [20]. As shown in table 3, expression of SHP-1 was significantly higher in tumors with absent or low Bcl-2 expression (p = 0.01) and in tumors expressing p16/Ink4a (p = 0.001).

Survival Analysis

We further analyzed the relationship between high expression of SHP-1 and patient survival. As expected, survival estimates in the whole TMA (fig. 2a) decreased with increasing tumor grade, with a value of 50% for overall survival at approximately 86 months for high-grade tumors (p value = 0.05). Values and trends for survival of patients with tumors positive for SHP-1 staining (fig. 2b; p value = 0.022) were similar to those of high-grade tumors, reaching a value of 50% for overall survival at 100 months, with a similarly shaped curve, while the control population (tumors negative for SHP-1 staining) reached 50% survival at 145 months. A similar trend for survival was obtained if only the ductal histotype was analyzed, as expected with the high frequency of this tumor type in the TMA (data not shown).

Expression of SHP-1 Does Not Promote Cell Proliferation

Given the lack of correlation of SHP-1 levels with the Ki67 labeling index as a measure of cell proliferation (see above), we determined the ability of SHP-1 to promote DNA synthesis in breast cancer-derived stable cell lines.

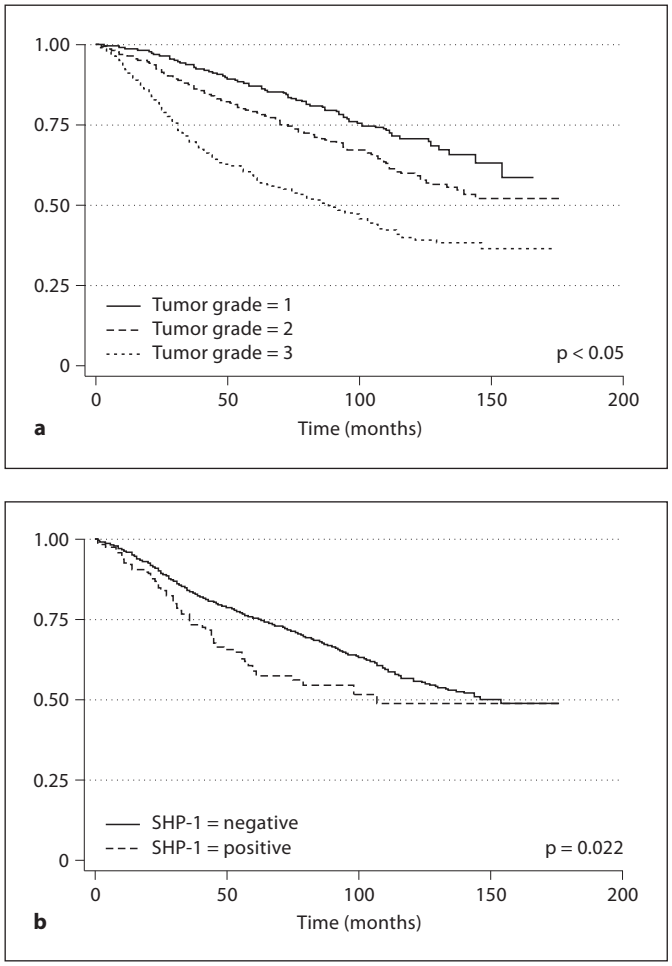

Fig. 2. Survival analysis. Survival analysis of the TMA cohort, showing Kaplan-Meier curves according to tumor grade (**a**) and SHP-1 expression (**b**).

SHP-1 was expressed in only 2 of the 4 cell lines analyzed, i.e. MCF-7 and SKBr-3, whilst T47D and MDA-MB-231 were negative (fig. 3a).

As shown in figure 3b, forced expression of SHP-1 (wildtype) in SHP-1-negative T47D cells had low inhibitory effects on thymidine incorporation (of less than 10% at 54 h), while inhibition of endogenous SHP-1 activity in MCF-7 cells by expression of the interfering mutant SHP-1 (C/S) had no effect (fig. 3c). Likewise, using an MCF-7 derivative cell line (MCF-7 siRNA-SHP-1) in which the expression of SHP-1 is reduced by stable expression of a specific siRNA (not shown), the percentage of cells in S phase increased by around 5% (fig. 3d). These data indicate that SHP-1 is unlikely to be functionally involved in determining the proliferation rate of those tumors in which it is expressed.

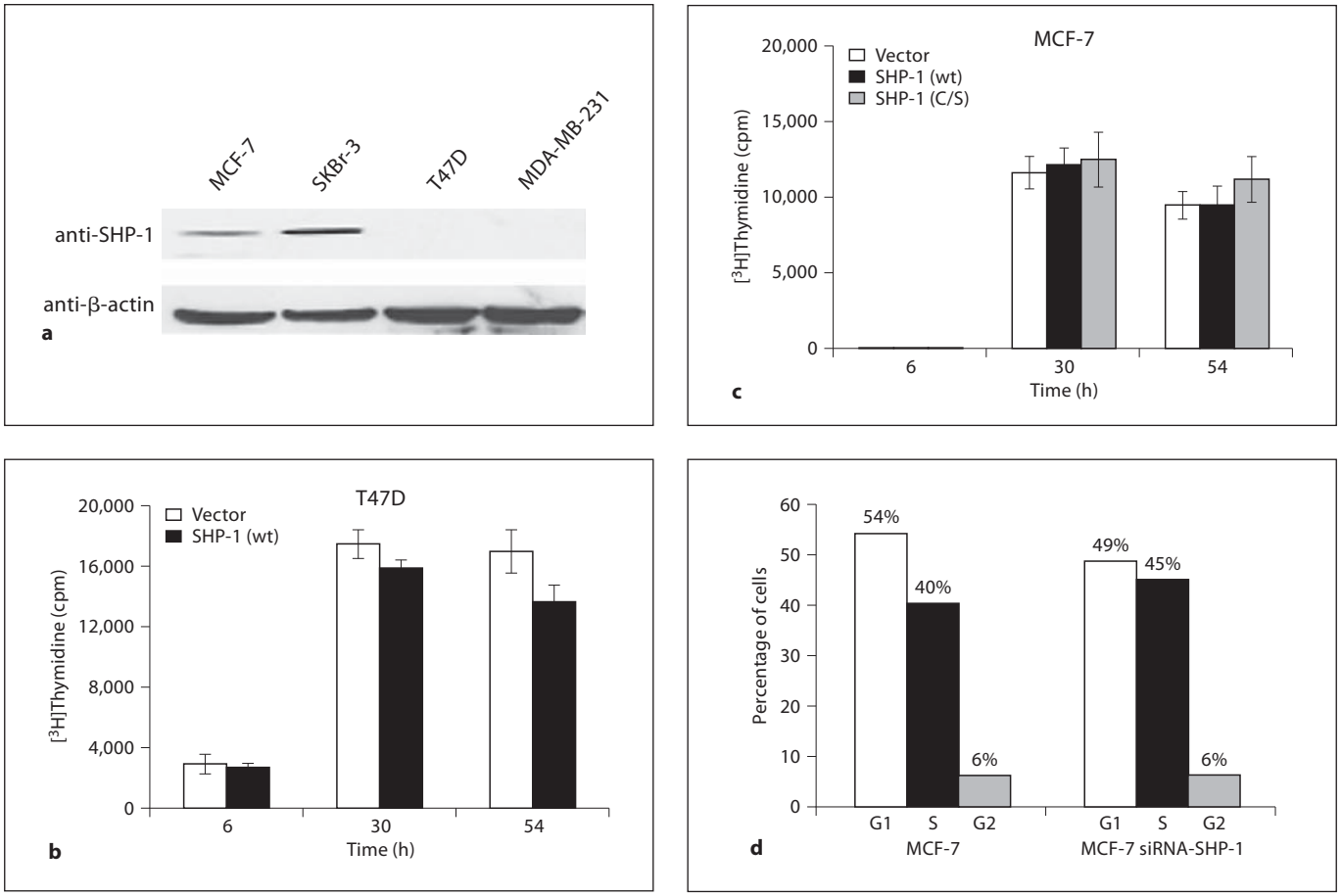

Fig. 3. SHP-1 expression does not affect thymidine incorporation in breast cancer cell lines. **a** SHP-1 expression levels were analyzed by immunoblot with an anti-SHP-1 antibody in exponentially growing MCF-7, SKBr-3, T47D and MDA-MB-231 breast carcinoma cell lines. Equal loading of samples was confirmed on the same blots with anti-β-actin antibody. **b** T47D cells were transfected with the vector pCEFL-HA and with pCEFL-HA SHP-1(wildtype; wt), and [^{3}H]thymidine incorporation was monitored (as described in Materials and Methods) at the indicated times. **c** MCF7 cells were transfected with pCEFL-HA SHP-1 (wildtype; wt) and with pCEFL-HA SHP-1 C/S, and [^{3}H]thymidine incorporation was monitored at the indicated times. Values are the means of triplicate reactions, with vertical bars representing the SD. **d** MCF-7 and MCF-7 siRNA-SHP-1 cells were analyzed by optical cytofluorimetry analysis to determine the percentage of cells in the G1, S and G2 phases of the cell cycle. The coefficient of variation of the peaks, which represents the ratio of the standard deviation and average values, was measured to confirm the significance of measurements and was 4.5% for the MCF-7 cell line and 5.2% for the MCF-7 siRNA-SHP-1 cell line. Values are results of a representative experiment.

Discussion

In the present study, we used a TMA-based approach to investigate the expression of the tyrosine phosphatase SHP-1 in human breast cancer in association with several clinical and biological parameters. Our data demonstrate that SHP-1 protein is expressed in tumors whilst it is absent in normal breast tissue, indicating that SHP-1 expression is frequently deregulated in breast tumors. They further indicate that high expression of SHP-1 identifies a particular subset of breast tumors. Tumors expressing SHP-1 were in fact predominantly of the ductal type and were characterized by aggressive biological behavior. Accordingly, high expression of SHP-1 correlated directly with high HER-2 expression and inversely with ER expression. Importantly, high SHP-1 expression was significantly associated with reduced survival. Furthermore, by using independent measurable parameters present in our TMA records [18], we assigned SHP-1-expressing tumors to a rather homogeneous subset of high-grade

tumors. Indeed, we show that, in contrast to the large majority of high-grade tumors in the TMA (data not shown), those expressing SHP-1 are weakly associated with high Bcl-2 expression. Moreover, this subset is characterized by the frequent expression of the tumor suppressor gene p16/Ink4a.

Our results indicate that even though it seems unlikely that SHP-1 has a functional involvement in determining the highly malignant phenotype of tumors in which it is expressed, some of the effects that have previously been reported to be induced by SHP-1 in the breast cell line MCF-7 are reflected in the subset of high-grade breast tumors expressing SHP-1. Considering that SHP-1 generally opposes oncogenic signaling pathways [14], it is plausible that the overexpression of SHP-1 in tumors of advanced stage and poor clinical outcome may act as a compensatory and opposing mechanism to more pronounced oncogenic signaling.

Although SHP-1 seems to be implicated at the molecular level in regulating cell signals in hematopoietic cell lines, little is known about its involvement in cancer. SHP-1 protein and mRNA levels are downregulated in various leukemia and lymphoma cell lines, but not in epithelial cell lines, including breast cancer cell lines, in which SHP-1 levels are normal or overexpressed [13, 14]. To our knowledge, this is the first study on a large group of solid tumors demonstrating that elevated expression of SHP-1 may serve as a marker of poor prognosis for a subset of high-grade breast cancers characterized by unique biological parameters and with a poor clinical course that makes this set of tumors of interest for therapeutic approaches.

Acknowledgements

We wish to thank S. Del Vecchio for suggestions and comments. This work was partially supported by funds from: Associazione Italiana Ricerca sul Cancro (grants to L.C. and G.C.), MIUR-FIRB (RBIN04J4J7) and an EU grant from the European Molecular Imaging Laboratories Network (contract No. 503569). I.A. and F.T. were supported by the MIUR-FIRB grant.

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