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Survivin expression as a prognostic marker for breast cancer

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SUMMARY

OBJECTIVE: Due to the speed of development observed in breast cancer, several studies aimed at discovering new biomarkers have been carried out in order to arrive at an early diagnosis. As survivin plays a fundamental role in the evasion of apoptosis in tumor cells, the aim of this study was to verify the expression profile of the survivin gene in paraffin-embedded breast tumor samples and associate it with the clinical characteristics of the patients.
METHODS: This is a cross-sectional study, for which 100 tumor samples were obtained from cancer patients treated throughout the year 2019 at Instituto de Mama do Cariri (Juazeiro do Norte, in the state of Ceará). This study included women over 30 years old who had confirmed breast cancer through anatomopathological examination but excluded those with non-neoplastic breast comorbidities, other neoplasms, or chronic diseases. Survivin gene expression was assessed by quantitative polymerase chain reaction.
RESULTS: The expression of survivin is associated with the lack of expression of estrogen (p=0.027) and progesterone (p>0.0005) receptors. It means that survivin expression is higher in patients in which labeling was absent for estrogen receptor and progesterone receptor.
CONCLUSION: Our data reinforce that survivin expression is higher in estrogen receptor-patients, thus representing an additional prognostic tool.
KEYWORDS: Survivin. Breast neoplasm. Prognosis. Paraffin embedding. Hormone receptors.

INTRODUCTION

Cancer is highly prevalent worldwide, with breast cancer being the second most common in women[1]. Due to the speed of development observed in breast cancer, several studies aimed at discovering new biomarkers have been carried out in order to arrive at an early diagnosis and quickly initiate treatment, thus increasing the chances of achieving a cure for the patient.

Currently, survivin, a member of the inhibitor of apoptosis (IAP) family of proteins, has been the subject of several studies involving cancer[2]. This 16.5 kDa protein, encoded by the *BIRC5* gene, was cloned for the first time in 1997 by Ambrosini et al.[3] and is detected in many cases of malignant tumors[4], but not in healthy tissue samples, therefore directly relating its presence to oncogenesis[2,4,5].

Survivin participates in several biological pathways[4-6], but one of the main pathways in which this protein is expressed is in escape from apoptosis. Apoptosis, i.e., programmed cell death, can be initiated by extrinsic and intrinsic cell expression pathways[7]; in the extrinsic pathway, an external agent (i.e., radiotherapy, chemotherapy, and so on) stimulates p53, an apoptosis-linked transcription factor that regulates a number of other pro-apoptotic genes that lead to cell death[2]. Survivin acts directly to inactivate the apoptosis cascade. Thus, p53 acts antagonistically to the anti-apoptotic action of surviving[2,4,6]; in this way, research is being carried out to evaluate how the inactivation of survivin would be an effective form of treatment in patients with metastatic cancer[2]. In cells that are being signaled for apoptosis, survivin is more expressed and released into the cytosol, preventing the activation of caspases, and thus, inhibiting apoptosis[2,5].

The intrinsic pathway, in turn, is initiated by signals from mitochondria, a cytoplasmic organelle in eukaryotic cells. After intrinsic stimulation, cytochrome-C and Smac/DIABLO produced in the mitochondria form the apoptosome that activates caspase-9[7]. Then caspase-9 activates other caspases that induce cellular apoptosis. In mammals, IAP proteins inhibit apoptosis by indirectly inhibiting caspases[7].

As survivin plays a fundamental role in the evasion of apoptosis in tumor cells, the aim of this study was to verify the expression profile of the survivin gene in paraffin-embedded breast tumor samples and associate it with the clinical characteristics of the patients.

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METHODS

Patients

This is a cross-sectional study, for which tumor samples were obtained. The samples were collected. A total of 100 breast cancer patients from Instituto de Mama do Cariri (Juazeiro do Norte, in the state of Ceará) were included in this study throughout the year 2019. This study was approved by the Centro Universitário FMABC Research Ethics Committee under research protocol no. 346712/14 of August 2013. A free and informed consent form was signed by each individual included in this study. All experiments were performed in accordance with the Declaration of Helsinki. Women over 30 years of age with breast cancer confirmed by anatomopathological examination were included. Women with other non-neoplastic breast comorbidities or with other neoplasms or chronic diseases such as human immunodeficiency virus, hepatitis, and diabetes were excluded from this study.

The parameters studied were age, staging, and the presence of prognostic markers [i.e., human epidermal growth factor receptor-2 (HER2), Ki-67, estrogen receptor (ER), and progesterone receptor (PR)] in addition to survivin gene expression.

Tumor samples

Breast tumor biopsies obtained from the patients were fixed in 10% buffered formalin, embedded in paraffin (FFPE), and sliced into 10 µm sections using a microtome for histopathological, immunohistochemical, and molecular analyses.

Immunohistochemistry

In slides with paraffin material, the percentage of Ki-67, ER, PR, and HER2 labeling was analyzed to determine the tumor type of each patient.

Survivin gene expression

A total of five 10 µm sections of tumor samples from each patient were subjected to RNA extraction using the RNeasy® FFPE Kit (Qiagen, cat. no. 73504, Hilden, Germany), following the manufacturer's recommendations. RNA concentration was measured by spectrophotometry (NanoVue Plus – GE Health Care, Buckinghamshire, UK).

Complementary DNA synthesis was performed using 100 ng of RNA with the QuantiNova Reverse Transcription Kit (Qiagen, cat. no. 205413, Hilden, Germany), according to the manufacturer's recommendations. Specific primers for target and endogenous genes (GAPDH – glyceraldehyde-3-phosphate dehydrogenase) were designed using the Primer3 Input 0.4.0 software; surviving F-CAGATTTGAATCGCGGGACCC and surviving R-CCAAGTCTGGCTCGTTCTCAG generated an amplicon of 187 bp; GAPDH F-GACCACAGTCCATGCCATGA and GAPDH R-CAGCTCAGGGATGACCTTGC generated an amplicon of 148 bp. Amplifications were performed on an Applied Biosystems 7500 Real-Time PCR Systems thermocycler (Applied Biosystems, Foster City, USA), using the 1× Quantitec SYBR Green PCR fluorophore kit (Qiagen, Cat No. 204143, Hilden, Germany), and 0.2 µM of each specific primer, in a final volume of 15 µL per sample. The thermal conditions were an initial phase with hot start at 95°C for 10 min, followed by 45 cycles of 95°C for 10 s and 60°C for 25 s.

The expression of the target gene for each patient was determined by the $2^{-\Delta^{Cq}}$ [8] method and associated with the characteristics, clinical variables, and anatomopathological results of the patients.

Statistical analysis

Absolute and relative values were used to describe the qualitative variables. Quantitative analyses were performed using the Shapiro-Wilk test, with a significance of $p<0.05$, using medians and the 25th and 75th percentiles. The Kruskal-Wallis and Mann-Whitney tests were also used to study the association between diagnostic variables and the expression of survivin. Spearman's test was also used to observe the correlation between survivin and Ki-67. For all analyses, a confidence level of 95% was used. The program utilized was Stata, version 11.0.

Among the quantitative variables, the markers such as Ki-67, HER2, ERs, and PRs were evaluated and quantified by immunohistochemistry technique. Age and staging were also quantitatively assessed, and the obtained values were expressed as mean±standard deviation, median, maximum, and minimum values. The criteria to include variables involved factors such as theoretical relevance, statistical significance, correlation with the outcome of interest, and adjustment for potential confounders. In addition, the criteria were adjusted to avoid possible confounders. It is common to use stepwise selection (i.e., forward, backward, and stepwise) or statistical significance criteria (e.g., $p<0.05$) approaches for the inclusion or exclusion of variables in the logistic regression model.

RESULTS

The clinical characteristics of the patients included in this study are described in Table 1. Of the 100 samples submitted to gene amplification, adequate quality for molecular analysis was obtained only in 89% of the samples (89 out of 100 samples). Of these, expression of survivin was absent in 13.5% of the samples (n=12); the other 77 samples (86.5%) had detectable expression levels.

Table 1. Clinical characteristics of the patients.

Variables	n	%
Staging*		
IA	15	15.5
IIA	34	35.1
IIB	32	33.0
IIIA	5	5.1
IIIB	11	11.3
HER2		
Negative	62	62.0
+	20	20.0
++	6	6.0
+++	12	12.0
ER*		
Negative	18	18.2
Positive	81	81.8
PR		
Negative	21	21.0
Positive	79	79.0
Chemotherapy		
No	4	4.0
Yes	96	96.0
	Median	**p.25–p.75**
Age	56.0	49.0–68.0
Survivin	1.6	0.008–23.6
Ki-67 (%)	5.0	0.0–20.0

HER2: human epidermal growth factor receptor 2; ER: estrogen receptor; PR: progesterone receptor *without information for some samples.

Table 2. Association between survivin expression values and clinical characteristics of the patients.

Variables	Survivin Median (95%CI)	p[a]
Staging		
0	136.2 (0.0; 272.3)	0.469
IA	3.7 (0.14; 145.0)	
IIA	0.1 (0.01; 7.2)	
IIB	1.4 (0.2; 4.1)	
IIIA	0.5 (0.0; 30.1)	
IIIB	23.6 (0.0; 182.6)	
HER2		
Negative	1.6 (0.1; 10.5)	0.835
+	0.2 (0.02; 4.4)	
++	3.8 (0.0; 25.1)	
+++	2.5 (0.2; 8.2)	
	Median (95%CI)	**p[b]**
ER		
Negative	18.0 (0.5; 134.4)	0.027
Positive	0.8 (0.06; 2.8)	
PR		
Negative	26.5 (2.9; 139.4)	0.0005
Positive	0.2 (0.04; 2.1)	
Chemotherapy		
No	136.2 (0.0; 1528.5)	0.623
Yes	1.6 (0.2; 3.9)	

ER: estrogen receptor; PR: progesterone receptor. [a]Kruskal-Wallis. [b]Mann-Whitney.

The association between survivin expression values and the clinical characteristics of patients is described in Table 2. As the expression of survivin is associated with the expression of ER and PR (higher in patients in which labeling was absent for ER and PR), samples were divided according to the tumor subtype in which survivin expression was analyzed (Figure 1). As can be seen, patients with triple negative tumors have the highest expression of survivin, while those who are positive for hormone receptors and HER2 have the lowest expression of this marker.

There was no correlation between survivin expression and patient age or between survivin and Ki-67 marker status.

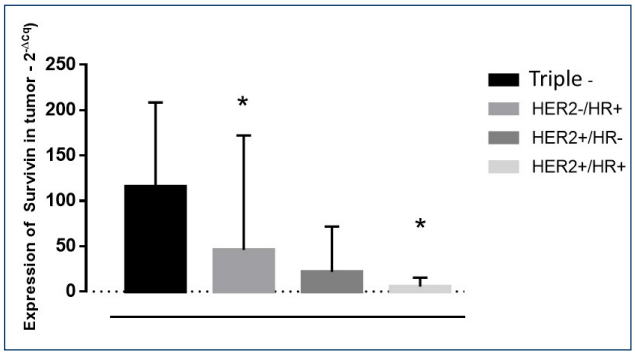

Figure 1. Representation of survivin gene expression in breast cancer tumor subtypes: triple- (n=7), HER2-/HR+ (n=36), HER2+/HR- (n=10), and triple+ (n=21). One-way ANOVA test. *p<0.05.

DISCUSSION

In this study, the expression of survivin in the tumor of patients with breast cancer without hormone markers is greater than that found in patients whose tumors express hormone receptors. Survivin is an apoptosis-inhibiting protein whose expression is associated with a poor prognosis in breast cancer patients[5]. In these patients, staging is very important to inform the mastologist on the correct therapeutic approach in order for the treatment to be as effective as possible[9]. Increased cytoplasmic expression of survivin has already been correlated with the stage and histological grade of the tumor and the development of metastasis[10]. Although, in this study, an association was not found between the expression of survivin and the staging of the patients, it was possible to establish an association between the increased expression of survivin and the absence of a hormonal biomarker (i.e., ERs, PRs, and the HER2 protein) in paraffinized tumor samples. According to our data, patients with triple-negative tumors have a significantly higher expression of survivin compared with that found in other subtypes, and the lowest expression of this gene was found in triple-positive tumors. Thus, by separating patients into groups (HER2-/HR- or Triple N, HER2-/HR+, HER2+/HR, HER2+/HR+, or Triple+, where HR stands for hormone receptor), it was possible to clearly visualize the difference in expression levels between these groups.

Triple-negative breast cancer (TNBC) are a tumor subtype that does not express HER2 or ERs and PRs; as they do not respond to hormonal or anti-HER2 therapies, TNBC tend to be more aggressive and develop metastases[11]. In addition to the lack of a therapeutic target, this tumor subtype lacks an adequate prognostic marker[12]. According to the authors, survivin and epidermal growth factor receptor expression evaluated by immunohistochemistry were considered of prognostic value for TNBC. Likewise, survivin expression together with zinc finger of the cerebellum 1 also showed prognostic potential[13]; in this case, the expression of these was evaluated in frozen tumor samples using quantitative polymerase chain reaction (qPCR) and in paraffin-embedded samples by immunohistochemistry. Our results show that the isolated expression of survivin obtained by qPCR can be considered a prognostic marker for all breast tumor subtypes, and that this evaluation can be performed on stored paraffin-embedded samples.

In breast cancer, HER2 is overexpressed in 15–30% of tumors[14]; HER2 is a trans-membrane tyrosine kinase receptor that participates in the control of cell proliferation and tumorigenesis[15]. As this protein has the function of suppressing apoptosis and promoting tumor growth, its expression has been used as a prognostic biomarker[16]. Increased expression of HER2 induces the activation of the PI3K/Akt pathway which, in turn, leads to increased surviving expression[17]. However, although HER2 expression is accompanied by that of survivin, our results indicate that the triple-negative tumor samples had increased survivin expression compared with the triple-positive ones.

Breast cancer is a hormone-dependent tumor, and the analysis of hormone receptors, such as progesterone and estrogen, is a widely accepted prognostic marker for predicting treatment response[18]. According to the authors, survivin expression in invasive ductal carcinoma tumors is negatively correlated with PR and is independent of ER. Our data show that lower expression of survivin was indeed obtained in the samples with hormone expression. Elevated levels of progesterone have an antiproliferative effect on breast cancer[19], having, therefore, contrary action to survivin, a fact that may explain this negative correlation between these biomarkers.

The absence of ER expression indicates a worse prognosis for the patient. Thus, additional markers that can complement this assessment and indicate a more effective treatment are of great importance. Our data reinforce that, in ER– patients, increased survivin expression is present; therefore, this protein has great potential as a therapeutic target, and as such, it has been widely tested in different carcinomas by several groups[20,21].

This study has some limitations: the study of gene expression in paraffin material, even using adequate methodology for this purpose, may have loss of information associated with the quality of the isolated genetic material. In addition, the beginning of the pandemic that occurred shortly after the collection of the material did not allow us to follow up the patients in order to associate the data obtained with clinical outcomes. Even so, the data described here demonstrate that survivin mRNA expression in samples is higher in negative hormone-receptor tumors. Thus, the evaluation of surviving mRNA expression in tumors immersed in paraffin can be considered a tool to aid prognosis, as this profile is different for breast cancer subtypes.

AUTHORS' CONTRIBUTIONS

PQ: Conceptualization, Investigation, Writing – review & editing. **RQ:** Conceptualization, Investigation, Writing – review & editing. **MMP:** Methodology, Formal Analysis, Writing – review & editing. **GLV:** Methodology, Formal Analysis, Writing – review & editing. **BCAA:** Validation, Writing – original draft, Writing – review & editing. **ECP:** Validation, Writing – original draft, Writing – review & editing. **FLAF:** Supervision, Writing – review & editing.

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