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Hsp27 expression and ratio between cell proliferation rate and apoptosis in breast cancer with and without axillary metastases

Expressão de Hsp27 e razão entre os índices de proliferação celular e apoptose em carcinoma de mama com e sem metástases axilares

Clarice M. Feyh Birnfeld[1]; Vinicius D. Silva[2]

1. Serviço de Oncologia do Hospital Moinhos de Vento, Rio Grande do Sul, Brazil. 2. Hospital do Câncer de Barretos, São Paulo, Brazil.

ABSTRACT

Introduction: The involvement of the immune system in the process of neoplasms has been increasingly studied due to its potential in antitumor therapy. **Objectives:** To evaluate the expression of heat shock protein 27 (Hsp27) in tissue samples from invasive ductal carcinomas with and without the presence of axillary sentinel lymph node metastasis, as well as to study the relationship of cell proliferation rate (Ki-76) and apoptosis (caspase 3) compared with samples of fibrocystic change in breast tissue. **Methods:** Cross-sectional study with tissue samples collected at the Hospital São Lucas, between September 2001 and October 2009, divided into three groups: 1. fibrocystic breast change (control group); 2. invasive ductal carcinoma of the breast with presence of lymph node metastasis; and 3. absence of lymph node metastasis. **Results:** Increased Hsp27 expression was observed in the group of non-metastasis carcinomas when compared to the other groups. There was difference in Ki-67 protein expression and in the ratio between cell proliferation and apoptosis among the carcinoma groups compared to the control group. However, there was no significant difference between the carcinoma groups or between the expressions of caspase 3 in the three groups, when compared to each other. **Conclusion:** Quantitative analysis of Hsp27 demonstrated increased protein expression in primary neoplasm tissues and in lymph nodes in carcinomas without axillary sentinel lymph node metastasis. Although the methodology and the number of cases do not allow concluding that this behavior in metastatic disease is a constant finding, this justifies the need to evaluate this finding in future studies.

Key words: ductal carcinoma of the breast; cell proliferation; apoptosis.

INTRODUCTION

According to the Brazilian National Cancer Institute, breast cancer is the second most common type of cancer worldwide and accounts for about 22% of new cases of cancer in women in Brazil each year[1]. This type of cancer mainly affects women from 40 years of age, and presents a higher incidence in the sixth decade of life[2, 3]. Currently, more than 60% of the cases are identified in advanced stages, which is clearly reflected in the mortality curves[1].

The knowledge of the relevance factors on the biological behavior of breast cancer, the diagnostic classifications and its treatment has been of increasing concern to several studies. At the time of diagnosis, precise staging is essential to determine prognosis and therapeutic response possibilities[1]. Initially, the biopsy is performed to define the tumor type by anatomopathological and immunohistochemical examination[4-6]. In addition, other tests are performed to establish staging of the disease, and axillary lymph node status is still a strong prognostic factor in breast cancer[7].

Most non-surgical antitumor therapies have limiting adverse events. Thus, new therapies are being studied to provide the best treatment, such as mediating antitumor immune responses[8, 9]. Nevertheless, several factors may be responsible for an ineffective response, such as tolerance to the immune response[10].

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Although these results suggest that immunotherapy against malignant neoplasms may be very promising, a better understanding of the whole process involved in the immune response to tumor is necessary in order to improve the proposed strategies and provide important information for the development of new therapies[11].

GENES AND PROTEINS INVOLVED IN CARCINOGENESIS

It is known that the onset of neoplasms has relations with mutations that occur in genes that control growth and survival. In general, if the mutation is irreparable, the trigger of the process of cell death by apoptosis occurs[12]. Apoptosis is a type of cell death that occurs in a physiological way, as a mechanism of removal of damaged cells and cell and tissue renewal[13]. This is an innate mechanism of defense, and for this reason, several medicinal agents act by inducing this process as a treatment method[14].

The apoptosis process involves several agents, in particular certain proteases, called caspases, which have the ability to recognize and cleave substrates leading to condensation, nuclear fragmentation and exposure of cell membrane phospholipids that will signal for these cells to be phagocytosed by macrophages[15, 16].

Other agents are also involved in the development of cancer. The heat shock proteins (Hsp) are a class of proteins that provide protection in the process of protein biogenesis, avoiding incorrect interactions, without being part of their final structure[17-19]. They are called stress proteins, since their expression is stimulated after cellular stress, especially Hsp27, Hsp70 and Hsp90[20, 21]. However, studies show an increased expression in some types of neoplasms, a fact that may be correlated with increased carcinogenic potential, metastatic potential and resistance to chemotherapeutic agents[22]. In several studies Hsp27 has been associated with increased aggressiveness pattern of certain primary tumors and decreased survival in patients with breast cancer and melanoma[23-25]. However, their roles in the later stage of tumor progression, especially in the stage of metastatic dissemination, are still unknown[26].

There are also other genes involved with the carcinogenesis, such as TP53 (tumor suppressor), responsible for the synthesis of p53 protein, which is often found mutated in most types of cancer[27]. Mutant cells that do not encode this gene do not undergo apoptosis, end up surviving longer and accumulating more mutations, multiplying out of control and generating neoplasms[12]. Cellular stress conditions [ultraviolet light, X-rays, deoxyribonucleic acid (DNA) damage, low extracellular pH, hypoxia, and thermal stress] may also influence the direct interaction between p53 and DNA repair, since this protein is inductive of cell cycle arrest and apoptosis, thus avoiding the proliferation of mutated cells[12, 28, 29].

OBJECTIVE

To investigate the expression of Hsp27 in tissue samples from patients diagnosed with invasive ductal breast cancer with and without axillary lymph node metastases using the immunohistochemical method, relating this expression to the proliferative activity of the neoplasm through the expression of the Ki- 67 protein, and apoptosis by caspase 3.

METHODS

A cross-sectional study was carried out. Patients with a diagnosis of invasive ductal breast cancer with and without metastases in axillary lymph nodes were selected in the period between September 2001 and October 2009.

Adult women with invasive ductal breast cancer of any histological grade, who had clinical staging II and III, with or without axillary metastasis, were included in the sample. We excluded patients who did not have material for immunohistochemical study or scarce tumor tissue and those with a known history of breast cancer in first-degree relatives and syndromes related to increased incidence of breast cancer.

The sample calculation estimated a minimum of 25 cases per group to detect a significant difference ($p < 0.005$). Altogether 75 samples were selected and equally divided into three groups with the following diagnoses: invasive ductal breast cancer with presence of metastasis in axillary sentinel lymph node (TUMOR+LN+); invasive ductal breast cancer without presence of metastasis in the axillary sentinel lymph node (TUMOR+LN-); fibrocystic breast change (control group), which is a benign process of high prevalence.

Preparation of histological slides

The slides were prepared in the pathology laboratory of the Hospital São Lucas of the Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS). The paraffin blocks were taken from the service material file and cut 3 µm thickness with a rotary microtome (Leica, RM2165, Nussloch, Germany). One slide was

prepared for each marker. Slide readings were performed on Zeiss Axioskop 40 optical microscope with 40× neofluar objective. For the capture of the images, we used the Retiga 2000R camera (Qimaging, Surrey, BD, Canada) coupled to the microscope and to an IBM-PC standard microcomputer. Each image was captured under ordinary halogen lamp with voltage control, for further quantification of the cells with expression of the markers studied by the immunohistochemical method.

The sections for immunohistochemical analysis were placed on positively charged slides (Dako, Glostrup, Denmark) and taken to the oven at 60°C for 1 hour. Exposure of the antigen by heat-induced antigen retrieval method performed on pTLink (Dako, Glostrup, Denmark) programmed with 40 minutes incubation time at 98°C with high pH buffer. After that time, the slides were washed in phosphate buffered saline (PBS), pH 7.2. Endogenous peroxidase blocking was performed with 3% de H_2O_2 solution in methyl alcohol in two 15 minutes incubations followed by three wash cycles with PBS buffer, pH 7.2.

The sections were incubated with anti-HspBp1 (clone ab3858, ABCAM, Cambridge, MA, USA) diluted 1:100 with background reducing components, (antibody diluent with background reducing components – Dako), Ki-67 (clone NCL- KI67-MM1, Novocastra, UK) diluted 1: 250 and caspase 3 (clone 3 CSD01, Neomarkers, USA), diluted 1: 100. The incubation process was carried out by the capillary method through the Dako Autostainer immunostaining station, (Dako, Glostrup, Denmark). After incubation with the primary antibody, the sections were washed three times with PBS buffer, pH 7.2. For amplification of the antigen-antibody reaction, the validated Envision Flex HRP system (Dako, Glostrup, Denmark) was used. Thereafter the slides were washed with PBS buffer, pH 7.2 and incubated in diaminobenzidine (DAB) solution (Dako Liquid DAB Substrate Chromogen System, Dako, Glostrup, Denmark) for 5 minutes. After washing in distilled water, the slides were counterstained with Harris hematoxylin for 1 minute, followed by washing in running water until complete removal of the dye, and incubated in 37 mM ammonia solution for 15 seconds. To conclude, they were dehydrated in absolute ethyl alcohol (four incubations of 2 minutes) and then performed two Xylene treatments for 5 minutes. The slides were mounted with synthetic Entellan (Merck Millipore, Billerica, MA, USA). Hsp27, Ki-67 and caspase 3 expressions were evaluated after preparing the slides.

Analysis of images

Ten random and non-overlapping images were obtained at 400 magnifications of each slide, as shown in **Figure**. The capture system was calibrated prior to each session and had the illumination voltage controlled and the microscope optics adjusted by the Koehler method. Measurements were performed with a standardized color palette and stored by the Image Pro Plus software, assuring a uniform color selection.

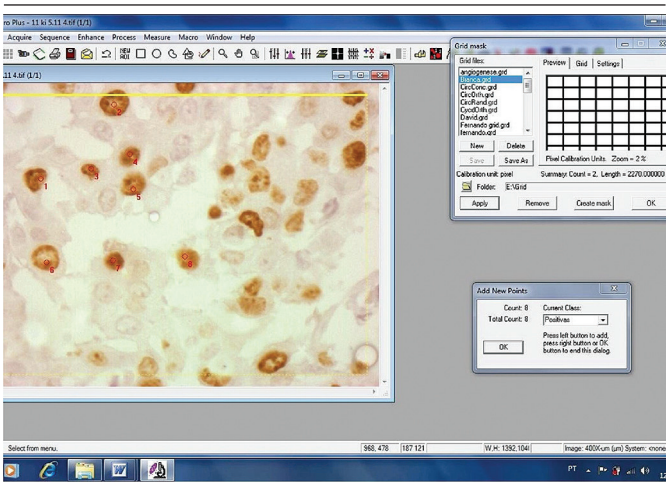

FIGURE – *Image of screen capture of Image Pro Plus 6.0 software with the file of cells brown stained with Ki-67 antibody. Each field had the number of cells and the stained area counted. DAB dye, 400×*

DAB: diaminobenzidine.

Statistical analysis

The descriptive analysis was performed by the dispersion measures (standard deviation and magnitude) of central tendency (mean and median) and by frequency distribution, $n(\%)$. For the study of data distribution, the Kolmogorov-Smirnov test ($n > 50$) was used. On the comparison of the expression of positivity, the Kruskal-Wallys test – post-hoc Dunn test and the Mann-Whitney test were used.

For the analysis that involved the comparisons on the categorical variables between the groups, the Pearson Chi-square test was used; for statistical decision criteria, a significance level of 5% ($\alpha = 0.05$) was adopted. Data were analyzed using the Statistical Package for the Social Science® (SPSS) software, version 17.0.

The present study was submitted to and approved by the Institutional Research Ethics Committee of the Hospital São Lucas at PUCRS, under number (CAAE) 34425514.6.0000.533.

RESULTS

The TUMOR+LN+ group presented 52% prevalence of involvement in the left breast, while the TUMOR+LN- group,

56% in the left breast. The mean age for the TUMOR+LN+ group was 54.2 years, ranging from 22 to 76 years. In the TUMOR+LN- group, the mean age was 55.8 years, aged between 30 and 81 years. In the control group, mean age was 25.9 years, ranging from 18 and 30 years.

The results presented in **Table 1** describe the evaluation of Hsp27 protein expression in the three groups. It was found that in both groups, most lesions presented Hsp27 positive expression, but more consistent in the TUMOR+LN+ group, as observed in **Tables 1** and **2**.

The results of the quantitative evaluation are presented in Table 2. Comparing the three groups, a significant statistical difference ($p < 0.001$) was detected: TUMOR+LN- presented a significantly higher mean in comparison to the TUMOR+LN+ and control groups. No significant differences were detected between these last two groups. Comparing the positivity expressions detected in the lymph nodes between the TUMOR+LN+ and TUMOR+LN- groups, the results showed a significantly higher expression in the TUMOR+LN+ ($p = 0.003$) group, when compared to the TUMOR+ LN- group.

TABLE 1 – Absolute and relative distribution of anti-Hsp27 antibody positivity for the three groups considering the number of cases where presence or absence (zero) of expression was detected ($n = 75$)

		Tumor		Lymph node	
Group	Total	Cases	%	Cases	%
TUMOR+LN+	25				
Expression detected		21	84%	25	100%
Expression not detected		4	16%	0	0%
TUMOR+LN-	25				
Expression detected		25	100%	20	80%
Expression not detected		0	0%	4	20%
Control	25				
Expression detected		24	96%	-	-
Expression not detected		1	4%	-	-
Total	75				

TABLE 2 – Hsp27 expression in mammary tissue and sentinel lymph node for TUMOR+LNF+ and TUMOR+LNF- groups and the control group by quantitative analysis (values in µm²)

Tissue	Group	*n*	Mean	Standard Deviation	*p*
Mammary tissue	TUMOR+LN+	25	5995.7_a	8291.2	$< 0.001_§$
	TUMOR+LN-	25	34124.6_b	30909.5	
	Control	25	4755.9_a	6346.6	
Lymph node	TUMOR+LN+	25	17729.2	14333.2	$= 0.003_¥$
	TUMOR+LN-	25	15420.5	31283.3	

§: Kruskal-Wallis test – post-hoc Dunn test, in which the values followed by equal letters do not differ by more than 5% of significance; ¥: Mann-Whitney test.

Table 3 presents the results of the quantitative analysis of the Ki-67 protein in the mammary tissue of the three groups, with no significant difference between the breast tissue of patients with or without axillary metastases, while protein expression was significantly higher in the neoplastic tissues compared with the tissues of the control group. The results were consistent with the expected biological behavior in the different groups.

Table 4 shows the results of the evaluation of caspase 3 expression by quantitative method in the mammary tissue, which presented no significant difference between the three groups.

Table 5 shows the ratio between Ki-67 and caspase 3 expressions, estimating the ratio between proliferation rate and apoptosis among the three groups. The results show that there was difference between the TUMOR+LN- and TUMOR+/LN+ groups when compared to the control group. There was no difference in the comparison between the two groups of tumor tissue (TUMOR+LN- and TUMOR+LN+).

TABLE 3 – Results of the quantitative data analysis of the Ki-67 expression in the mammary tissue among the different groups studied (values in µm²)

Tissue	Group	*n*	Mean	Standard deviation	*p*
Mammary tissue	TUMOR+LN+	25	2140.2_a	1559.4	$< 0.001_§$
	TUMOR+LN-	25	2589.3_a	1666.2	
	Control	25	755.9_b	346.5	

§: Kruskal-Wallis test – post-hoc Dunn test, in which the values followed by equal letters do not differ by more than 5% of significance.

TABLE 4 – Results of the quantitative data analysis of the caspase 3 expression in the mammary tissue among the different groups studied (values in µm²)

Tissue	Group	*n*	Mean	Standard deviation	*p*
Mammary tissue	TUMOR+LN+	25	23895.2	1094.0	0.1
	TUMOR+LN-	25	1945.3_a	1579.2	
	Control	25	2299.7_a	1354.6	

TABLE 5 – Evaluation of ratio between cell proliferation by Ki-67 (cell proliferation) and caspase 3 (apoptosis) in the mammary tissue among the different groups studied

Tissue	Group	*n*	Mean	Standard deviation	*p*
Mammary tissue	TUMOR+LN+	25	0.3051_a	0.1914	$0.0301_§$
	Control	25	0.0463_b	0.0304	
	TUMOR+LN-	25	0.2858_a	0.1896	$0.0441_§$
	Control	25	0.0455_b	0.0297	
Lymph node	TUMOR+LN+	25	0.2834	0.2030	$0.9950_§$
	TUMOR+LN-	25	0.2799	0.1865	

§: Kruskal-Wallis test – post-hoc Dunn test, in which the values followed by equal letters do not differ by more than 5% of significance.

DISCUSSION

The Hsp27 has different roles in cell signaling in carcinomas, along with Hspb5[30]. This feature possibly arises from the ability of these proteins to bind, to play the role of molecular chaperones, and to modulate the activity or half-life of various protein targets involving cell regulation, such as apoptosis, stages of carcinogenesis, and metastasis formation. These proteins have complex oligomeric structures, which allow the formation of homo and hetero-oligomeric structures of dynamic sizes. In addition, Hsp27, HspB4 and HspB5 contain several serine sites that can be phosphorylated by specific kinases, including stress kinases and MAP kinases. Both the phosphorylation and the possibilities of oligomeric organization of these proteins are dynamic and modified by changes in the cellular environment. One example is the reorganization of the phosphorylation and oligomerization status of Hsp27, depending on the apoptosis inducer, which suggests that this protein can be modified in various ways to inhibit the apoptosis process[31].

This structural plasticity could facilitate the recognition of ''client'' proteins that would favor new aggressive characteristics of neoplastic cells, thus promoting the growth and spread of neoplasms. In the literature, they have been consistently associated with different cytoplasmic receptors, such as vascular endothelial growth factor (VEGF), FGF-2 and HER2, enhancing the MAPK kinase/MEK/ERK pathway activity[31].

The first report of interaction between a Hsp and a receptor was with the Hsp27, which acts as a estrogen receptor beta (ER-beta) associated protein, as a possible corepressor in estrogen signaling[32]. More recently, it has been shown that Hsp27 in breast carcinoma increases the stability of HER2/neu protein, which results in an increase in resistance to trastuzumab[30]. The so-called epithelial to mesenchymal transition of carcinomas remains a poorly understood process, but the role of Hsp27 in maintaining the viability of breast cancer tumor stem cells through the regulation of EMT and the transcription factor nuclear factor-kappa B (NF-kB) has already been described[33].

The findings related to the expression of Ki-67 and caspase 3 and the ratio between the expression of these proteins were consistent with the literature and also with the natural history of breast carcinoma[34]. However, it is possible that the method used to evaluate apoptosis in this study has low sensitivity to detect apoptosis and its pathways, since there was no difference in the expression of caspase 3 among the three groups. Indeed, a method of adequate cost and good sensitivity and specificity to determine the rate of apoptosis in formalin-fixed and paraffin-preserved material has not yet been developed. Simultaneously, other forms of cell death, such as necroptosis and pyroptosis, have been described in recent years, requiring new approaches, methods and models for analysis[35, 36].

CONCLUSION

The present study demonstrated increased expression of Hsp27 in invasive ductal carcinomas. The quantitative analysis of Hsp27 demonstrated increased expression of the protein in TUMOR+LN-, whereas there was no difference in the expression of this protein between the group of carcinomas with axillary sentinel lymph node metastases and the control group. Although the methodology used and the number of cases studied in this study do not allow concluding that this behavior in metastatic disease is a constant finding, the need to explore this finding better in future studies is clear.

Likewise, studying the relationship between the Hsp27 expression with hormone receptors, HER2/neu and p53 in a much larger number of patients, among several other possibilities, is an interesting future perspective from the findings of this work. This change in the immunophenotyping of the neoplasia may result from the emergence of new sub-clones with different immunomodulatory abilities during the natural history of the disease, and there are evidences that suppression of Hsp27 decreases the rate of cell proliferation of breast cancer[23], which makes the findings of this work, particularly the reduction of Hsp27 expression in the primary neoplasia tissue in the group with axillary metastases, interesting for future investigation.

RESUMO

Introdução: *O envolvimento do sistema imunológico no processo de neoplasias vem sendo cada vez mais estudado devido ao seu potencial em terapia antitumorais.* ***Objetivos:*** *Avaliar a expressão de* heat shock protein *27 (Hsp27) em amostras teciduais de carcinomas ductais invasores com e sem presença de metástase em linfonodo sentinela axilar, bem como estudar a relação da taxa de proliferação celular (Ki-76) e apoptose (caspase 3) em comparação com amostras de alteração fibrocística no tecido mamário.* ***Métodos:*** *Estudo transversal com amostras de tecidos coletados no Hospital São Lucas, entre setembro de 2001 e outubro de 2009,*

divididas em três grupos: 1. alteração fibrocística da mama (grupo-controle); 2. carcinoma ductal invasor de mama com presença de metástase linfonodal; e 3. sem presença de metástase linfonodal. ***Resultados:*** *Observou-se expressão aumentada da Hsp27 no grupo de carcinomas sem metástase, quando comparado com os demais grupos. Houve diferença na expressão da proteína Ki-67 e na razão entre proliferação celular e apoptose entre os grupos com carcinoma quando comparados com o grupo-controle. Contudo, não houve diferença significativa entre os grupos com carcinoma ou entre a expressão de caspase 3 nos três grupos, quando comparados entre si.* ***Conclusão:*** *A análise quantitativa da Hsp27 demonstrou maior expressão da proteína nos tecidos da neoplasia primária e nos linfonodos em carcinomas sem metástase em linfonodo sentinela axilar. Embora a metodologia e o número de casos não permitam concluir que esse comportamento na doença metastática seja um achado constante, justifica-se a necessidade de avaliar esse achado em estudos futuros.*

Unitermos: *carcinoma ductal de mama; proliferação de células; apoptose.*

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CORRESPONDING AUTHOR

Clarice Martins Feyh Birnfeld
Rua dos Burgueses 295, apto 402; Partenon; CEP: 91530-020; Porto Alegre-RS, Brasil; Phone: +55 (51) 98412-0417; e-mail: clafeyh@hotmail.com.