Original Article

The Significance of Heat Shock Protein 27 in Breast Cancer: A Signature to Predict the Outcome

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Abstract

Introduction: Heat shock protein (HSP) 27, if overexpressed in breast cancers, affects the disease outcome and sensitivity of tumors to chemotherapy and radiotherapy. There are limited studies on actual patients in India, so the present study was designed to contribute our experiences to the literature regarding HSP 27 expression and its significance in breast carcinoma. **Materials and Methods:** Thirty diagnosed cases of invasive duct carcinoma are evaluated for histopathology grade, stage, and expression of immune markers, namely, ER, PR, HER2, VEGF, and HSP 27, followed up to 2 years after primary management, especially if treated with Herceptin. The staining was evaluated, and data were analyzed using the χ^2 and Fisher's exact tests. **Results:** HSP 27 is expressed strongly in most ER- and PR-positive cases, but there is no correlation with Her2 and VEGF. The findings suggest more advanced disease, adverse outcomes, and lymph node metastasis. Moreover, these findings are directly related to strong HSP 27 expressions and shorter overall survival in the group of ER/PR-positive breast carcinoma cases, but this relation can be relied upon up to stage II disease. After that, in advance cases, HSP 27 loses its significance slowly and could not establish any link between HSP 27 and Herceptin resistance because of a short follow-up. **Conclusion:** HSP 27 proved a promising prognostic marker in ER/PR-positive breast carcinoma cases.

Keywords: Breast cancer, ER-PR, heat shock protein, Her2, VEGF

Introduction

In recent years, significant advances in understanding molecular biology in the development of breast cancer have led to the discovery of new immunological markers. More efforts are also being made to develop better molecular targets, crucial for tumorigenesis and metastasis and tentative candidates for target therapy. Despite significant progress in recent years to strengthen breast cancer diagnosis and treatment, it is still the most commonly diagnosed cancer in women and the second leading cause of cancer mortality globally.[1] It has also become the most common cancer among Indian women, accounting for 57.0% of India's 1,392,179 cancer cases by 2020.[2]

Heat shock proteins (HSPs) are a family of highly conserved proteins functioning as molecular chaperones in stress, such as carcinogenesis.[3] HSPs interact with a wide variety of unfolded, misfolded, and semi-native proteins, assisting in acquiring their active structures and preventing unintended intermolecular interactions and protein aggregates.[4] HSPs are overexpressed in human tumors, including breast, bladder, ovarian, colon, lung, and prostate cancers.[5] Most of these studies correlated high HSP expression with cancer aggressiveness and prognosis.[3] In average persons, these proteins have also been found to have a role in cell survival during stress conditions.[6] In cancer cells, this network is remodeled to evade cell death, refashion cell signaling, and bypass senescence in helping cancer cells to survive.[4] A Hippo pathway is hypothesized to play a role in tumor initiation, metastases,

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epithelial-mesenchymal transition, and overall prognosis through HSPs. Furthermore, it was recently discovered that HSPs play a role in cancer cell evolution, resulting in tumor heterogeneity. Chemotherapy could accelerate such expansion and contribute to the development of chemoresistance.[4,7] However, contradictory to this, a few studies have shown that reduced expression of HSPs is linked with poor prognosis in carcinoma patients.[8,9]

HSP 27 regulates cancer growth, progression, metastasis, cell death, and drug resistance, indicating a poor prognosis. HSP 27 is overexpressed in several malignancies and can be exploited to diagnose prognostic cancer. As a result, future research on disorders linked to HSP 27 and medication development is required. Recent studies[10,11] have suggested that inhibiting HSP 27 could be a cancer treatment target. Researchers should focus on discovering potential novel cancer therapeutics because antisense oligonucleotide medications like OGX-427 are still in clinical trials. Small compounds that cross-link HSP 27 could help suppress the function of HSP 27. HSP 27 is commonly overexpressed in various malignancies and has been linked to the development of anti-cancer agent resistance. As a result, HSP27 inhibitors may improve cancer treatment when combined with anti-cancer therapies.

The proposed study aimed to evaluate the significance of HSP 27 in carcinoma and specifically to know the relationship of HSP 27 expression with other prognostic markers such as between grades, stage of carcinoma breast, and expressions of ER, PR, Her2, and VEGF. Our study is the first to present the results for expression and secretion of Hsp 27 and VEGF in breast cancer in the central rural Indian population.

Materials and Methods

Study design and patient selection criteria

The study was carried out at the Mahatma Gandhi Institute of Medical Sciences (MGIMS), a medical school in Sevagram in Central India. It is attached to Kasturba Hospital, a nearly 1000-bedded tertiary care rural hospital. The Histopathology section of the Department of Pathology receives approximately 6500 specimens each year. The prospective cross-sectional study was conducted from May 2013 to September 2015 in the Department of Pathology of Histopathology section. The tissue was obtained during modified radical mastectomy (MRM) and lumpectomy specimens.

(i) *Study cases*: Thirty diagnosed cases of invasive duct carcinoma are evaluated for histopathology grade, clinical stage, and expression of prognostic markers, namely, ER, PR, HER2, VEGF, and HSP 27. These were followed up to 2 years after primary management, especially if treated with Herceptin. A detailed history is obtained from the patient and information from the clinical case records.

(ii) *Comparative cases*: Ten cases of benign proliferative breast lesion (mainly fibroadenomas) were collected from patients of the same age groups to compare the rates of VEGF, ER, PR, and HER2/neu immunoexpression with malignant breast tissues.

(iii) *Control cases*: Ten cases of normal breast tissue were selected from patients of the same age group presented with breast biopsy other than the tumor and regarded as a control group. Each step was done for the control group in parallel with the study and comparative groups.

(iv) *Positive control slides*: Parallel positive control sections were processed with each set of immunostaining. Positive controls of breast carcinoma sections that express ER, PR and HER2/neu, VEGF, and HSP 27, respectively, were used with each run.

(v) *Negative control slides*: Sections untreated with primary antibodies for ER, PR and HER2/neu, VEGF, and HSP 27 tumor markers were considered negative controls for each set of slides.

Exclusion criteria

Cases with full clinical details and follow-up were unavailable; those with insufficient representative tissue for immunohistochemistry (IHC), male patients with breast cancer and history of recurrence, and neoadjuvant therapy were excluded from the study.

Histopathology and immunohistochemistry

The selected formalin-fixed paraffin-embedded tissue block has proceeded for histopathology and IHC. Sections of 3–5 μm in thickness were obtained and stained with hematoxylin and eosin. For IHC, hematoxylin and eosin-stained slides will be screened to get the best section and rescreened to find the best section. IHC was carried out on a 3–5 μm thick formalin-fixed, paraffin-embedded section mounted on positively charged glass slides. Immunohistochemical staining was evaluated independently by two pathologists blinded to the clinicopathological data. Light microscopy at 40× magnification for at least 10 high-power fields was done [Figure 1].

Ready-to-use primary antibodies for immunohistochemical detection of VEGF, HER2/neu, ER, and PR using monoclonal mouse anti-human vascular endothelial growth factor, Code No. AR483-5R, BioGenex, The Netherlands, polyclonal rabbit anti-human c-erbB-2 oncoprotein, Code No. RTU-CB11, Leica, UK, monoclonal mouse anti-human estrogen receptor α, Code No. IS084, Dako, Denmark, and monoclonal mouse antihuman progesterone receptor, Code No. IS068, Dako, Denmark, were used as primary antibodies for the detection. Anti-human HSP 27 (Monoclonal, Immunogen) Balb/c mice were immunized with "24K" protein isolated from the cytosol of MCF-7 cells; spleen

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cells from immunized mice were fused with NS-1 myeloma cells. The clone was G3.1, species: mouse, Ig class: IgG1, protein concentration 10–15 mg/mL, 932-171M-4 Rev. F (BioGenex, USA) and was applied to cover the tissue sections.

Evaluation of immunohistochemical staining for ER, PR, and HER2 was done by template for reporting results of biomarker testing specimens from patients with breast carcinoma.[12] The immune reactive score (IRS) was applied in assessing VEGF and HSP 27 expression by Schlüter *et al.*[13] and Drexler *et al.*,[14] respectively. VEGF expression was scored as the intensity of the staining reaction in the samples, and the IRS was calculated as the mean IRS score of the two independent observers. VEGF was scored as negative, weak, medium, and strong and subsequently categorized as negative and positive.[13] The intensity of staining was scored as negative (0), mild (1), moderate (2), and intense (3). The percentage of positive cells was evaluated as 0 (no positive cells), 1 (< 10% positive cells), 2 (10–50% positive cells), 3 (51–80% positive cells), and 4 (> 80% positive cells). The IRS score was obtained by multiplying these two individual scores. As a result, every tissue sample was classified as negative (IRS points 0–1), weak (2–3), mild (4–8), or strong (9–12).[14]

After primary management, patients' outdoor and indoor visits to the hospital were recorded using departmental records, the hospital information system, and our population-based cancer registry. For the next 2 years, follow-up data were collected for the disease course for recurrence or swelling at the operated site, lump in the opposite breast, or any other clinically significant event.

Statistical analysis

Statistical analysis was done using the χ^2 test and Fisher's exact test using descriptive and inferential statistics. The software used is SPSS17.0 and GraphPad PRISM 5.0; $P < 0.05$ is considered a significant level.

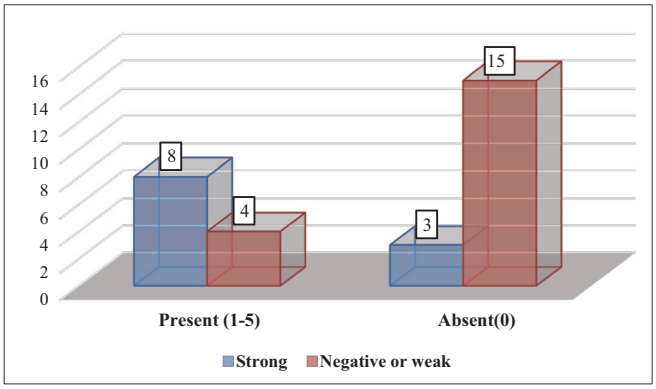

Figure 1: (a) HSP 27 staining 1–10%, 1 + (low intensity) [total score: 1] (×400, IHC); (b) HSP 27 staining 50%, 2 + (moderate) [total score: 4] (×400, IHC); (c) HSP 27 staining 100%, 3 + (intense) [total score: 12] (×400, IHC); and (d VEGF-positive [score 4+] (×400, IHC)

Ethical considerations

The Institutional Ethics Committee approved this study of Mahatma Gandhi Institute of Medical Sciences, Sevagram (MGIMS/IEC/PATH/71/2013;13-12-2013). Informed consent was taken from every patient in their language regarding willingness to participate in the study. Patient confidentiality was maintained during all research procedures.

Results

The clinicopathological parameters were studied separately—their association with each other and immunological markers to find all possible relationships in the present study. The age of the patients ranged from 24 to 66 years old, and the commonest age group of breast carcinoma was 41–50 years, comprising 53.33%. Histological grade 2 tumors showed stronger expression of HSP 27 than that of grade 3 tumors. Similarly, Tumor, Node, Metastases stage II tumors showed stronger HSP 27 expressions than the more advanced stage. These findings are statistically significant and mean that these prognostic markers correlate negatively with HSP 27. However, lymph node status showed an insignificant relationship with HSP 27.

The association of ER and PR positivity with HSP 27 expression is statistically highly significant ($P = 0.002$) as 17/21 ER/PR-positive cases and 2/9 ER/PR-negative cases showed strong HSP 27 expression. However, HSP 27 expression did not correlate significantly with Her2 and VEGF expression in the present study. VEGF was expressed in most of the study cases and found non-specific [Table 1]. Strong expression of HSP 27 predicted the possibility of adverse outcomes in 8 out of 11 cases, which is statistically significant. Further, 15 out of 19 negative weakly positive HSP 27 expression cases show no adversities, reinforcing the hypothesis that positive expression induces a bad disease course ($P = 0.005$) [Figure 2].

Table 1: Correlation between HSP 27 and other markers

	Number	HSP 27 expression		*P*-value
		IRS 0–4	IRS 6–12	
ER/PR				
Positive	21	4	17	0.002, S
Negative	9	7	2	
HER2				
Positive	12	5	7	0.643, NS
Negative	18	6	12	
VEGF				
Positive	27	9	18	0.256, NS
Negative	3	2	1	
Total	30	11	19	

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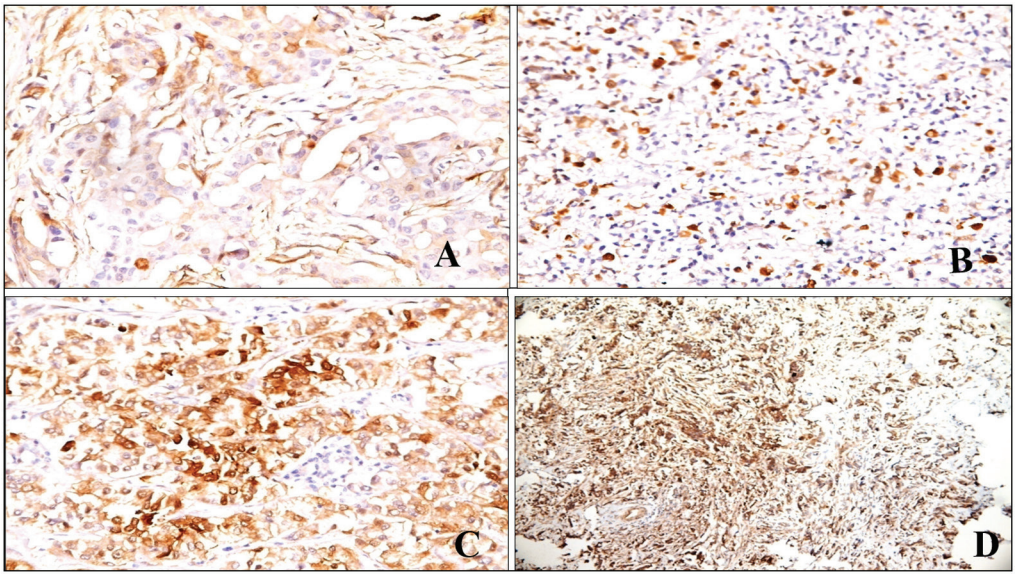

Figure 2: Adverse outcome of HSP 27 expression

Table 2: Correlation between HSP27 expression with Tumor, Node, Metastases staging, invasive duct carcinoma grading, and lymph node status

	Total number	Hsp27 expression		*P*-value
		IRS 0–4	IRS 6–12	
TNM staging				
I	0	0	0	0.016, S
II	**18**	**3**	**15**	
III	11	7	4	
IV	1	1	0	
IDC grading				
1	0	0	0	0.003, S
2	**16**	**2**	**14**	
3	14	9	5	
Lymph node				
Positive	19	5	14	0.12, NS
Negative	11	6	5	
Total	30	11	19	

IDS = invasive duct carcinoma, IRS = immunoreactive score, TNM = tumor, node, metastases

Table 3: Correlation of treatment plan and completeness of treatment in cases with adverse outcome

	Treatment plan			Total
	I*	II*	III*	
T/t completed	0	3	1	4
T/t not completed	0	7	1	8

I*—surgery followed by chemotherapy, II*—surgery followed by adjuvant chemotherapy and radiotherapy, III*—neoadjuvant chemotherapy followed by surgery and radiotherapy

Table 4: Correlation of treatment completed cases with Hsp 27 expression with adverse outcomes

Hsp27 expression	Adverse outcomes		Total	*P*-value
	Present	Absent		
Strong	1	1	2	0.32, NS
Negative or weak	2	9	11	

Discussion

Our study's association of Tumor, Node, Metastases staging with HSP 27 expression was statistically significant (P = 0.016). Association of invasive duct carcinoma histological grading with HSP 27 expression is statistically significant (P = 0.016), which means that stronger HSP 27 expressions presented with the mid-advanced disease. In higher histological grades, the intensity of expression starts diminishing. We found only one study analyzing this aspect[15] [Table 2].

ER and PR expressions significantly correlate with HSP 27 in the present study (P = 0.002). Grzegrzolka *et al.*[15] showed no relation between ER and PR with HSP 27 expression (P-value =1.000 for ER and 0.8317 for PR). This discrepancy needs further investigation with a larger sample size. HSP 27 expression did not correlate with Her2 expression significantly in the present study cases. A study by Grzegrzolka *et al.*[15] on the ER and PR relationship with HSP 27 is inconsistent with our findings as there is a significant correlation in the present study (P = 0.002) [Table 1].

The study showed that 11 of 27 negative cases of HSPs completed the treatment. Follow-up of these patients was essential to look for any effect of HSP 27 negativity on the disease course after treatment. No such effect has been seen (P > 0.05), and only two of these patients presented with adverse events later. The finding suggests again that HSP 27 weak or negative cases have a better prognosis than strong positive cases (9/11). However, more than half

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(n = 17) of the patients did not complete the treatment plan due to personal or financial reasons. This aspect also compromised our study's aims to find the relationship between different markers with this dreaded disease in all cases. We had to divide study groups into patients with completed treatment therapy and those with treatment non-completion, which lowered our sample size.

Incomplete treatment logically should end in more complications and so are the present study's findings in which 8 of 12 patients with adverse outcomes were those who did not complete their treatment [Table 3]. The sites of adverse outcomes in our series involved recurrence at MRM scar, secondary metastasis to the brain, lymph nodes, liver, bone, and lungs. The same findings are well recognized in literature, mentioning the sites of adverse outcomes as local, bone, lymph nodes, liver, lung and skin, and subcutaneous tissue.[16]

Out of the 13 treatment cases, we found that 2 cases show strong HSP 27 expressions, with one with and another without adverse outcomes. Weak or negative HSP expression was present in two cases with adverse outcomes and nine cases with absent adverse effects [Table 4]. These findings suggest that HSP 27-negative cases have better prognoses than positive cases after completion of treatment. These findings correlate with Thanner *et al.*,[17] who found significantly decreased overall survival and survival after the first recurrence with HSP 27-positive tumor.

Upregulation of HSP 27 in human breast cancer cells can reduce Herceptin susceptibility by increasing Her2 protein stability.[18] Hsp 27 levels in the human breast tumor microenvironment can be highly elevated and increase the tumor-associated macrophages level, negatively impacting patients' clinical outcomes.[19] HSP 27 is highly up-regulated in angiogenic breast cancer cells.[20] It proved that HSP 27 plays a vital role in balancing tumor dormancy and expansive tumor growth associated with the onset of angiogenesis.[20] Phenethyl isothiocyanate significantly inhibited the expression of HSPs (mainly HSP 90) and HSF1. Molecular consequences due to HSP inhibition were downregulation of cell-cycle regulatory proteins such as Cyclin B1, CDK1, Cdc25C, and PLK-1 and upregulation of p21 irrespective of p53 status.[21]

Limitations of the study

Some of the findings could not be analyzed by statistical methods because of the small sample size due to financial constraints, a short period of patient follow-up, and non-compliance of around half of the cases to the planned treatment. Moreover, some present study findings could not be compared with other studies because there were smaller studies on the clinical significance of HSP 27, especially from the Indian subcontinent.

Conclusions

HSP 27 predicts bad outcomes in ER/PR-positive breast carcinomas. HSP 27 is an important biological marker in breast carcinoma in overall survival, prognosis, and outcome prediction and provides a new insight role in cancer development. More extensive studies should achieve independent and interdependent functions in tumor cells.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

Authors' contribution

The authors' contributions are as follows:

- Conceptualization: AG
- Methodology: MS, BUP, AG
- Formal analysis: MS, BUP
- Data curation: MS, BUP
- Software: MS, BUP
- Validation: AG
- Investigation: MS, BUP
- Writing—original draft preparation: MS, BUP, PG, AG
- Writing—review and editing: MS, BUP, AG
- Approval of final manuscript: MS, BUP, PG, AG

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