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ORIGINAL ARTICLE

Early Effect of Radiotherapy on Serum Levels of HSP70 and S100B in Patients with Breast Cancer

Elif Eda ÖZER,[1] Nilgün IŞIKSAÇAN,[2] Gülşen Pınar SOYDEMİR,[1] Gülçin ŞAHİNGÖZ ERDAL,[3] Asuman GEDİKBAŞI,[4] Meltem KIRLI BÖLÜKBAŞ[1]

[1]Department of Radiation Oncology, University of Health Sciences, Bakırköy Dr. Sadi Konuk Training and Research Hospital, Istanbul-*Turkey*
[2]Department of Biochemistry, University of Health Sciences, Bakırköy Dr. Sadi Konuk Training and Research Hospital, Istanbul-*Turkey*
[3]Department of Medical Oncology, University of Health Sciences, Bakırköy Dr. Sadi Konuk Training and Research Hospital, Istanbul-*Turkey*
[4]Department of Biochemistry, Istanbul University Faculty of Medicine, Istanbul-*Turkey*

OBJECTIVE
This study aimed to evaluate the difference between serum levels of S100B and HSP70 in the non-metastatic, Luminal A breast cancer (BC) patients with the healthy population, and determine the impact of post-operative radiotherapy (RT) on serum markers.

METHODS
21 BC patients who underwent chest wall/breast±axillary RT after adjuvant chemotherapy and twenty-one healthy individuals were included in the study group. The changes in serum HSP70 and S100B levels were observed in the study and control groups.

RESULTS
A total of 42 participants were included. A significant difference was found between the HSP70 and S100B measurements of the study and control groups before and after RT ($P=0.001$ and $p<0.01$, respectively). At the same time, the increase in HSP70 after RT was statistically significant ($p=0.025$ and $p<0.05$, respectively). However, the change in S100B measurements after RT was not statistically significant in the study group patients compared to before RT ($p>0.627$).

CONCLUSION
S100B and HSP70 levels are higher than the healthy population before and after RT in non-metastatic, luminal A BC patients. The significant increase in Hsp70 after RT may be due to the release from dying tumor cells in the microenvironment. Therefore, HSP70 levels in the blood may be useful for microscopic tumor focus detection or evaluation of treatment response.

Keywords: Breast cancer; HSP70; radiotherapy; S100B.

Introduction

Breast Cancer (BC) is the most frequently diagnosed cancer and the leading cause of cancer-related death in women worldwide.[1] Non-metastatic BC is treated with a multidisciplinary approach, including breast surgery, radiation oncology, and medical oncology. [2] The surgical option is decided on a patient basis, considering tumor location, size, cancer stage, and patient-related factors. Adjuvant treatments are added to tumor-free patients with proven non-metastatic to prevent local and systemic recurrences after breast and

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Dr. Elif Eda ÖZER
Sağlık Bilimleri Üniversitesi,
Bakırköy Dr. Sadi Konuk Eğitim ve Araştırma Hastanesi,
Radyasyon Onkolojisi Anabilim Dalı,
Istanbul-Turkey
E-mail: drelifeda@gmail.com

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axillary surgery because of the possibility of cellular micro-tumors. Adjuvant systemic treatment options in BC are endocrine therapy, chemotherapy, or biological therapies. Radiotherapy (RT) is added for adjuvant regional and lymphatic therapy.[3,4]

Patients whose adjuvant treatment process is completed are followed up.[5] While full screening is performed every 3 months for the first 2 years, then the frequency of follow-up is reduced. Although BC recurrences are common in the first 1-2 years, they may occur within 5 years after diagnosis, especially in hormone receptor-negative disease[6]. Unfortunately, it has been reported that 10-20% recurrence is observed in early-stage BC even after 5-10 years, despite complete surgical resection (R0) and successful adjuvant treatments.[7] Recurrence or metastases developing after 5 years in these patients who received adjuvant treatments following R0 tumor surgery and were followed up completely tumor-free draw attention to the presence of cellular micro-tumors.[8]

BC is not only caused by neoplastic cells but also caused by the stroma surrounding the tumor or changes in the tumor microenvironment. The BC microenvironment encompasses fibroblasts, leukocytes, adipocytes, and myoepithelial and endothelial cells, as well as the extracellular matrix, cytokines, hormones, proteins, and enzymes. The tumor microenvironment is regarded as a critical factor for tumor growth, progression, and therapy response.[9]

Many studies on cancer biology have revealed many potential biomarkers for tumor development, progression, treatment, and follow-up. Two of these biomarkers associated with malignancy, metastasis, and survival are calcium-binding protein B (S100B) and Heat shock proteins (HSP). S100 calcium-binding protein family, of which S100B is a member, is a protein group that contains a multigene group consisting of 21 low molecular weight proteins. S100B plays a role in cell proliferation and is secreted from various inflammatory cells, neurons, adipocytes, melanocytes, and chondrocytes. There is a direct relationship between S100B expression and the degree of malignancy and survival time.[10] HSP are mainly involved in protein folding, protein transport, and protein targeting for lysosomal degradation.[11] HSP27, HSP70, and HSP90 have been previously reported in BC.[12] Serum HSP70 is expressed through the plasma membrane and released into the bloodstream in many different tumor types such as pancreatic cancer, colorectal cancer, BC, brain, and lung cancer. Recent studies have indicated that HSP70 plays a role in cancer development, tumor cell proliferation, differentiation, metastases, and death.[13]

Nevertheless, in the selected population with non-metastatic, Luminal-A disease there is no clear information regarding the difference of serum levels of S100B and HSP70 from the healthy population and the effects of post-operative RT on early serum markers. This is the first study to present the acute impact of RT on S100B and HSP70 change in selected BC patients. Our study was designed to compare biomarker levels and investigate the effect of RT. We compared the biomarkers in patients with non-metastatic, Luminal-A BC who completed adjuvant systemic therapy with the healthy population. In addition, we have detailed the impact of RT on early biomarker changes below.

Materials and Methods

Twenty-one BC patients who received chestwall/breast±axilla RT in our clinic between February 2017 and December 2019 were included in the study group. Twenty-one healthy individuals whose ages and body mass index (BMI) was compatible with the study group were randomly recruited into the control group. The study group consisted of patients who received adjuvant chemotherapy after R0 breast surgery and local-regional 50 Gy RT. The age, height, weight, BMI, performance status (PS), comorbid diseases, surgery type, histopathological tumor type, tumor and lymph node stage, tumor grade, and hormone receptor group data of the patients were recorded. The patients had no comorbid kidney disease, cardiovascular disease, inflammatory disease, diabetes, and none of them had a previous cancer diagnosis.

The pathological tumor stage was defined according to the eighth edition of the International Union Against Cancer's tumor-lymph node-metastasis classification. Both tumor size and lymph node metastasis status were evaluated separately. Estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor (HER2) data were obtained from the pathology records of the patients. Biological subclassification was made using ER, PR, HER2. Patients with Luminal A disease (ER-positive, PR-positive, and HER2-negative) were included in the study. Performance scoring was performed according to the Eastern Cooperative Oncology Group PS (ECOG-PS).

Blood samples of tumor-free patients whose adjuvant systemic treatments were completed after surgery were taken into biochemistry tubes on the 1st day of RT and in the 1st week after RT, centrifuged and separated into serum and stored at -80°C until the day of

evaluation. S100B and HSP70 levels were measured in the Biotek instruments USA ELISA device using Abbkine Human HSP70 and Cusabio S100B ELISA kits. Briefly, the microtiter plate provided in these kits has been pre-coated with an antibody. Standards or samples are added to the appropriate microtiter plate wells with Horseradish Peroxidase conjugated detection antibody and incubated. Then, chromogen solutions are added to each well, respectively. The enzyme-substrate reaction is terminated by adding a stop solution, and the color change is measured spectrophotometrically at a wavelength of 450 nm±2 nm. The concentration of the samples is then determined by comparing the O.D. of the samples to the standard curve.

Statistical Analysis

Number Cruncher Statistical System 2007 (Kaysville, Utah, USA) program was used for statistical analysis. Descriptive statistical methods (mean, standard deviation, median, frequency, rate, minimum, and maximum values) evaluated the study data. The suitability of quantitative data for normal distribution was tested by Kolmogorov-Smirnov, Shapiro-Wilk test, and graphical evaluations. Student's t-test compared two groups' data with normal distribution, while the Mann Whitney U test was used to compare two groups of non-normally distributed data. Pearson Chi-square test evaluated qualitative data. A paired sample t-test compared the data measured before and after RT. The Kruskal-Wallis test was used for comparisons of three or more groups of data that did not show normal distribution. Wilcoxon Signed Ranks Test evaluated the data measured before and after RT. Statistical significance level was considered as a $p<0.05$.

Ethics

This study was conducted with permission from Local Institutional Ethics Committee (Ethics Committee Decision Number: 2020-06-18).

Results

A total of 42 participants, 21 patients and 21 healthy, were included in this study. The ECOG PS was 0-1. The mean age of the study group was 57.43±12.54 years, and the control group was 48.81±16.27. There was no statistically significant difference in mean age between the two groups (p=0.062). Both groups had a BMI between 20 and 30, and there was no obese person with a BMI of >30. When the histology types were examined, it was determined that 76.2% (n=16) were invasive ductal carcinoma, and 23.8% (n = 5) were invasive lobular carcinoma. Tumor differentiation (grades) of the 9.5% (n=2) were Grade 1, 76.1% (n=16) were Grade 2 and 14.4% (n=3) were Grade 3. All patients diagnosed with BC in the study group were from the Luminal A biological subgroup. There were not any T4 patients and N3 diseases (Table 1).

Table 1 Disease characteristics distribution regarding the patient group

Features	n	%
Surgery Type		
BCS	9	42.9
MRM	12	57.1
Histology		
Invasive ductal carcinoma IDC	16	76.2
Invasive lobular carcinoma ILC	5	23.8
Grade		
Grade 1	2	9.5
Grade 2	16	76.1
Grade 3	3	14.4
T stage		
T1	9	42.9
T2	9	42.9
T3	3	14.3
T4	0	0
N stage		
N0	11	52.4
N1	6	28.6
N2	4	19.0
N3	0	0
Total	21	100

BCS: Breast conserving surgery; MRM: Modified radical mastectomy; IDC: Invasive ductal carsinom; ILC: Invasive lobular carcinom; T: Tumor; N: Nodal

A statistically significant difference was found between the HSP70 measurements of the study and control groups before and after RT (p=0.001 and p<0.01, respectively). At the same time, the increase in HSP70 measurements after RT was found to be statistically significant in the study group patients compared to before RT (p=0.025 and p<0.05, respectively). A statistically significant difference was found between the S100B measurements of the study and control groups before and after RT (p=0.001 and p<0.01, respectively). However, the change in S100B measurements after RT was not statistically significant in the study group patients compared to before RT (p>0.627) (Table 2). Both HSP70 and S100B measurements were significantly higher in the study group than in the control group (Figs. 1, 2).

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Table 2 Evaluation of HSP70 and S100B measurements by groups

	Group 1 (Control group) (n=21)	Group 2 and 3 (Study group) (n=21)		p (Group 1 vs. 2)	p (Group 1 vs. 3)	p (Group 2 vs. 3)
		Group 2 Before RT	Group 3 After RT			
HSP70						
Median (min-max)	27 (16.3-35.4)	38.2 (25.4-71.1)	43.6 (22.4-102.3)	0.001	0.001	0.025
S100B						
Median (min-max)	4.2 (0-10.2)	50.9 (16.8-90.9)	62.8 (18-88)	0.001	0.001	0.627

RT: Radiotherapy

The relationship between serum levels of S100B and HSP70 and pathological features of BC was also examined. HSP70 and S100B measurements of the patients according to tumor differentiation Grade 1, 2, and 3 levels were not statistically evaluated because 73% of the patients were Grade 2. HSP70 and S100B measurements before and after RT did not show statistically significant differences according to T and N stages ($p>0.05$). As a result, no relationship was observed between pathological features of BC and S100B and HSP70 measurements.

Discussion

Ionizing radiation can indirectly cause deoxyribonucleic acid (DNA) damage by generating reactive oxygen species. Chromosomal deletions, translocations, or inversions, as well as single and double-stranded DNA breakage and base-pair mismatch during replication, are potential mechanisms of radiation-induced cellular damage. If genomic damage is too great, p53 initiates programmed cell death by activating the apoptotic cascade. In the apoptosis pathway, p53 activates calcium-dependent endonucleases and proteases such as interleukin 1 converting enzyme, DNAse I, and caspases. Activation of these enzymes leads to sequential DNA cleavage, an irreversible step in apoptosis. The genetic determinants and molecular mechanisms of therapeutic radiation sensitivity are not fully understood. Few reports document endogenous inhibitors of radiation-induced apoptosis.[14] Considering the radiation-induced apoptosis mechanism, we examined acute biomarker changes after RT. When the HSP70 and S100B results before and after BC RT were compared in our study, HSP70 levels were significantly higher in the acute period after RT. However, there was no significant change in S100B.

Moore described S100B as a calcium-binding protein from brain tissue in 1965.[15] S100 proteins show a certain tissue and cellular distribution, and S100B is secreted from nervous system glial cells, melanocytes, adipocytes, and chondrocytes. Serum S100B protein plays a role in cell proliferation. S100B levels have also been found to be elevated in malignant melanoma, glioma and neuroblastoma, progressive BC, and many other cancers. The strength of S100B expression was

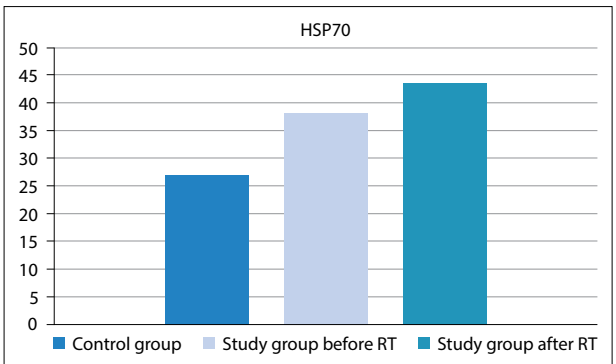

Fig. 1. The distribution of HSP70 measurements.
RT: Radiotherapy.

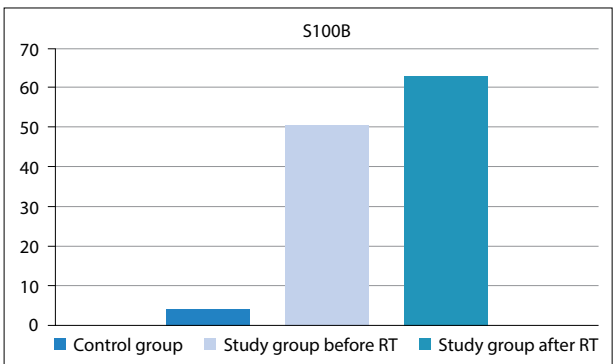

Fig. 2. The distribution of S100B measurements.
RT: Radiotherapy.

directly related to the degree of malignancy.[8,16] Many articles in the literature aimed to use S100B as a biomarker in the diagnosis, treatment, or follow-up of BC.[17-19] Charmsaz et al.[20] investigated the inhibition of the S100B signaling network in endocrine-resistant BC patients as a biomarker. They examined the S100B expression in tissue and serum and determined that tissue and serum S100B levels predicted poor disease-free survival in patients receiving endocrine therapy. The marker was proven to have the potential to be a new surveillance tool for monitoring the ongoing response to endocrine therapy for ER-positive BC patients. There was no relationship between serum S100B levels and clinical-pathological data. However, when they evaluated S100B levels before and after surgery, elevated serum S100B levels returned to normal following surgical resection of the tumor. These data suggest that high S100B levels may indicate the presence of tumor burden in BC patients and have the potential to predict disease progression. Nonetheless, even in the selected tumor-free group with good prognostic factors who completed adjuvant systemic therapy in our study, the S100B values before and after RT were significantly higher than in the healthy population.

HSPs are among the target molecular chaperonesin cancer treatment. HSP70 is actively released by viable, intact tumor cells and also at a lower level by dying tumor cells. It functions to induce mitotic signals, suppress stress-induced and apoptosis, as well as oncogene-induced aging.[21] The mechanisms by which HSPs regulate cancer cell proliferation, invasion, metastasis, and avoidance of apoptosis have also been investigated, and they have also been found to increase resistance to anti-cancer treatments such as chemotherapy and RT.[22-24] It has been shown that HSP70 is frequently overexpressed in many different tumor types such as brain, breast, prostate, colon, and lung cancer compared to the healthy group and will cause metastasis development through upregulation of mesenchymal markers.[25,26]

Hurwitz et al.[27] reported on increased levels of circulating sHsp70 up to several days after whole-body irradiation of mice bearing xenograft prostate tumors that might be explained by dying cells. The slight increase in sHSP70 levels after radiation therapy might account for sHSP70 which is released by dying cells. Apart from dying cells viable tumor cells actively secrete large amounts of HSP70 in vesicles.

Gehrmann et al.[28] measured HSP70 levels in biopsy and serum parameters of 21 head and neck cancer patients. HSP70 levels were significantly higher in patients compared to healthy volunteers. They also found that HSP70 levels decreased in patients without tumor recurrence during the follow-up period after surgery and RT. Moreover, in this study, the reduction of HSP70 levels in post-surgery tumor-free patients suggests that HSP70 levels may be beneficial not only for detecting tumors but also for monitoring the therapeutic response to RT. Similarly, in our study, HSP70 levels in the group that completed the adjuvant systemic therapy were significantly higher than in the healthy population. In addition, RT caused a significant increase in HSP70 levels in the early period that might be explained by dying cells.

In our study, the patients were admitted to our clinic with post-operative adjuvant chemotherapy completed and at the post-operative tumor-free 7th month on average. Numerous biomarker studies have been conducted to measure the presence of microtumors. In our study, we demonstrated the significant S100B and HSP70 difference between the healthy group and the study group and evaluated the clinical use of these markers Luminal-A non-metastatic BC.

The limitations of this study are the small number of patients, the absence of S100B and HSP70 levels measured in the long-term after RT, the absence of accompanying pathological and immunohistochemical studies, and the short duration of follow-up. Therefore, a survival analysis could not be performed. In our subsequent study, we aim to demonstrate the long-term follow-up of our patient group and the chronic period marker change after RT, along with the correlation between biomarkers with progression and/or overall survival.

Conclusion

In summary, even if there is no macroscopic tumor focus, S100B and HSP70 levels are higher than the healthy population before and after RT in non-metastatic, Luminal-A BC. Moreover, a significant increase in HSP70 levels was determined in the acute period after RT. The significant increase in HSP70 after RT may be due to the release from dying tumor cells in the microenvironment. Therefore, HSP70 levels in the blood may be useful for microscopic tumor focus detection or evaluation of treatment response.

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