Inducible 70kDa Heat Shock Protein and Autophagy-related Protein P62 Levels in Women With Breast Cancer: an Exploratory Investigation

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Research Article

Keywords: cancer, breast malignancy, Peripheral blood mononuclear cells (PBMCs), intracellular process

Posted Date: April 30th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-475229/v1>

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Abstract

Background:

Peripheral blood mononuclear cells (PBMCs) respond to altered physiological conditions to alleviate the threat. The inducible 70kDa heat shock protein (HSPA1A) protects proteins from degradation. Autophagy is an intracellular process that removes damaged proteins and recycles their components to the cytoplasm. Sequestosome-1 (p62) is a protein consumed during autophagy. We hypothesized that the PBMC response to a malignant breast mass involves elevated production of HSPA1A and a decrease in intracellular p62.

Methods:

In this study 46 women had their breast mass excised. PBMCs were isolated and intracellular levels of HSPA1A and p62 were quantitated by ELISA. Differences between women with a benign or malignant breast mass were determined.

Results:

A breast malignancy was diagnosed in 38 women (82.6%) while 8 had a benign lesion. Mean intracellular HSPA1A levels were 79.3 ng/ml in PBMCs from women with a malignant lesion and 44.2 ng/ml in controls ($p=0.04$). The mean PBMC p62 level was 2.3 ng/ml in women with a benign breast lesion as opposed to 0.6 ng/ml in those with breast cancer ($p<0.001$). Mean p62 levels were also lower in women with invasive carcinoma and a positive lymph node biopsy when compared to those with in-situ carcinoma or absence of lymphadenopathy, respectively.

Conclusion:

Intracellular HSPA1A and p62 levels in PBMCs differ between women with a malignant or benign breast lesion. These measurements may be of value in the preoperative triage of women with a breast mass.

Introduction

The malignant transformation of cells anywhere in the body is almost always accompanied by changes in systemic immunity. Alterations in antigen expression and perturbations in the local environment are detected by the immune system and result in changes in the properties of immune cells [1]. The ability to identify immune cell alterations that predict malignancy will be of value in the initial screening of individuals with a suspected malignancy.

Autophagy is an intracellular mechanism present in almost all organisms to maintain cell functions and optimize survival. Aggregated or non-functional proteins and organelles become bound to a cytoplasmic protein, sequestosome-1 (p62) [2, 3] and the complex is sequestered in a double-membraned structure called an autophagosome [4, 5]. The autophagosome merges with a lysosome and the engulfed

components along with p62 are degraded by lysosomal enzymes. The amino acid, nucleoside, carbohydrate and lipid components are returned to the cytoplasm for reutilization [6–11]. The variation in p62 concentration in the cytoplasm has been used as a biomarker for the extent of autophagy induction [12–14]. However, p62 is also involved in other cellular processes such as apoptosis and inflammatory reactions [15–17]. Therefore, these other properties must be kept in mind when utilizing measurement of p62 to monitor autophagy activity in different systems.

An additional mechanism that promotes cell survival under different conditions is induction of the inducible 70kDa heat shock protein, HSPA1A. When a cell encounters non-physiological conditions, HSPA1A synthesis is greatly up-regulated. The HSPA1A binds to nascent peptides and promotes the formation of functional proteins. It also prevents protein denaturation [18–20]. The HSPA1A is also released from the stressed cell and functions in the extracellular milieu to activate pro-inflammatory immunity to combat the perceived stress [21]. Numerous studies have reported on elevations in circulating HSPA1A in association with malignant transformation, including breast cancer [22–25].

While multiple studies have evaluated changes in autophagy and HSPA1A expression in mammary cells that are associated with malignancy [26–29], concomitant potential alterations in peripheral blood mononuclear cells (PBMCs) have not been evaluated. In this exploratory study we compared the level of p62 and HSPA1A in PBMCs from women with malignant and benign breast lesions. We hypothesized that HSPA1A would be increased, and p62 decreased, in association with malignant transformation and might serve as potential biomarkers for breast cancer.

Material And Methods

Patient enrollment

Women with a breast mass referred to the Section of Breast Surgery at Weill Cornell Medical College who underwent diagnostic breast surgery from 2015 to 2017 and consented to have their PBMCs obtained and evaluated, were enrolled in the study. All patients had preoperative confirmation of a breast mass by sonography, mammogram or magnetic resonance imaging followed by a fine needle aspiration biopsy or a core biopsy. Women with a cancer diagnosis underwent surgery for surgical staging. Women with a benign breast biopsy underwent surgery either for definitive tissue diagnosis, especially for large lesions, or for management of symptoms. All women with a previous history of any type of malignancy or neoadjuvant chemotherapy, with an active infection or a history of a chronic inflammatory disease were excluded. This study was approved by the Institutional Review Board of Weill Cornell Medical College and was conducted according to Declaration of Helsinki. All subjects provided written informed consent at their initial preoperative evaluation.

The following baseline clinicopathologic characteristics were collected from each subject: age, histology, histology subtypes, Estrogen Receptor (ER) status, Progesterone (PR) status, Human Epidermal growth factor Receptor 2 (HER2) status, grade, sentinel lymph node status, and stage. In all cases, the diagnosis

of breast cancer was made by a clinical pathologist based on histological tissue analysis from surgical excision of the breast mass. The personnel of the laboratory were blinded to all clinical and histological data until the completion of all experimental assays.

PBMC isolation and intracellular hsp70 and p62 quantification

Blood was collected from a peripheral vein preoperatively into a sterile heparin-containing tube and sent to the laboratory for analysis. The isolation of PBMCs was performed by Ficoll-Hypaque gradient centrifugation. The PBMC fraction was gently removed and resuspended in Roswell Park Memorial Institute (RPMI) 1640 medium for two cycles of centrifugation and resuspension. The final suspension was adjusted to a concentration of 5×10^6 cells/ml. The PBMCs were centrifuged again and 130 μ l of a cell lysate buffer (1% Triton X 100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 20 mg/mL deoxyribonuclease, 100 mmol/L protease inhibitor cocktail (Sigma, St Louis, Missouri) in 50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 1 mmol/L EDTA, and 1 mmol/L ethylene glycol tetraacetic acid) was added to the pellets. The mixture was incubated for 30 min and the lysed PBMCs were centrifuged at 11000 rpm for 10 min. The supernatant was collected and concentrations of HSPA1A and p62 were quantitated using commercial ELISA kits (HSPA1A ELISA kit from R&D Systems, Minneapolis, MN, p62 ELISA kit from Enzo Life Sciences, Farmingdale, New York). The lower limit of detection was 156 pg/ml for HSPA1A and 100 pg/ml for p62. Values were converted to ng/ml by comparison to a standard curve generated in parallel to each assay.

Statistical analysis

The Mann-Whitney U test was utilized as appropriate to compare continuous variables as the values were not normally distributed. The Pearson chi-square test or Fisher exact test was performed to assess for association between categorical variables. The software GRAPH PAD INSTAT Version 3.0 was utilized for all statistical analyses, with p-values < 0.05 considered as statistically significant.

Results

A total of 38 patients (82.6%) women were diagnosed with a breast malignancy, and 8 (17.4%) had a benign breast lesion. Patient characteristics are summarized in Table 1. There were no differences in age, ethnicity/race, body mass index or reproductive history between the two groups. Table 2 summarizes the histopathologic characteristics of the 38 breast cancer patients. The majority were diagnosed with early stage (IA) ductal carcinoma with positive ER/PR. In 34 women (89.5%) their breast carcinoma was invasive while 4 women (10.5%) had carcinoma-in situ.

Table 1
Characteristics of the study population

Characteristic	Benign (n = 8)	Malignant (n = 38)	P value
Age, median (range)	49.5 (34,71)	54 (31,71)	NS
Race/Ethnicity – n (%)			NS
Non-Hispanic White	3 (37.5)	15 (39.5)	
Non-Hispanic Black	2 (25.0)	9 (18.8)	
Asian	1 (12.5)	4 (10.5)	
Hispanic	2 (25.0)	7 (18.4)	
Other	0 (0.0)	3 (7.9)	
Body mass index (BMI) – mean (SD)	30.2 (6.5)	30.9 (8.3)	NS
Gravidity, median (range)	3 (0,6)	3 (0,10)	NS
Parity, median (range)	2 (0,5)	2 (0,8)	NS
NS, not significant			

Table 2
Histopathologic characteristics of patients diagnosed with breast malignancy

Characteristic	No. women (n = 38)
Histologic Subtype – n (%)	
Ductal	25 (65.8)
Lobular	9 (23.7)
Mixed (Ductal and Lobular)	4 (10.5)
ER status – n (%)	
Positive	33 (86.8)
Negative	5 (13.2)
PR status – n (%)	
Positive	32 (84.2)
Negative	6 (15.8)
HER2 status – n (%)	
Positive	6 (15.8)
Negative	29 (76.3)
Unknown	3 (7.9)
Histologic Grade – n (%)	
1	7 (18.4)
2	16 (42.1)
3	14 (36.8)
Sentinel Lymph Node – n (%)	
Positive	8 (21.1)
Negative	28 (73.7)
Not performed	2 (5.2)
Prognostic stage – n (%)	
0 (in situ)	4 (10.5)
IA	20 (52.6)
IB	0 (0.0)
ER; Estrogen Receptor, PR; Progesterone Receptor, HER2; Human Epidermal growth factor Receptor 2	

Characteristic	No. women (n = 38)
IIA	6 (15.8)
IIB	7 (18.4)
IIIA	0 (0.0)
IIIB	0 (0.0)
IIIC	1 (2.6)
IV	0 (0.0)
ER; Estrogen Receptor, PR; Progesterone Receptor, HER2; Human Epidermal growth factor Receptor 2	

The mean intracellular PBMC HSPA1A and p62 levels in all subjects is shown in Table 3. The median HSPA1A concentration was 79.3 ng/ml in women with breast cancer versus 44.2 ng/ml in the benign group ($p = 0.04$). Conversely, the mean intracellular p62 level was 0.6 ng/ml in women with breast cancer and 2.3 ng/ml in the controls ($p < 0.001$).

Table 3
Comparison of intracellular HSPA1A and p62 in PBMCs from women with malignant or benign breast disease

	Breast malignancy (n = 38)	Benign breast disease (n = 8)	P value
Intracellular HSPA1A, (ng/ml)	79.3 ± 7.4	44.2 ± 8.6	0.04
Intracellular p62, (ng/ml)	0.6 ± 0.1	2.3 ± 0.5	< 0.01
Data are presented as mean ± SEM.			

A subgroup analysis in the women with breast cancer revealed that the mean PBMC p62 level in those with invasive carcinoma (0.30 ng/ml) was significantly lower compared to those with in-situ carcinoma (1.10 ng/ml) ($p = 0.01$). There was no difference in the levels of intracellular HSPA1A between these two groups. A sentinel lymph node biopsy was performed in 36 (94.7%) of the cancer patients and 8 (22.2%) were found to be positive. Women with a positive sentinel lymph node had a significantly lower intracellular p62 level in their PBMCs (0.04 ng/ml) as compared to those with negative lymph nodes (1.4 ng/ml) ($p = 0.008$). There were no differences in the concentrations of intracellular HSPA1A or p62 between women with different histological types or stages of breast cancer.

Discussion

The results of the present study demonstrate that HSPA1A is elevated and the p62 level is decreased in PBMCs from women with breast cancer as compared to their levels in PBMCs from women with benign breast lesions. We also found that the p62 level was decreased in PBMCs from patients with invasive

carcinoma as compared to those with *in situ* carcinoma. This paralleled our observation that p62 was lower in PBMCs from breast cancer patients with a positive sentinel lymph node biopsy as compared to those with a negative sentinel lymph node biopsy.

The influence of HSPA1A levels on autophagy has been investigated previously. Doklandy et al. [30] in an *in vitro* study clearly demonstrated that the extent of HSPA1A expression was inversely related to the level of autophagy in tumor cell lines. The mechanism involved activation of Akt kinase and the mammalian target of rapamycin (mTOR), a kinase that blocks autophagy induction. The authors proposed that an inverse correlation might exist between HSPA1A and autophagy in tumor cells. Additionally, Kanninen et al. [31] in an *in vitro* study provided data suggesting that the intracellular HSPA1A concentration in PBMCs from pregnant women and autophagy induction were inversely proportional. Other studies in yeast cells demonstrated that the chaperone activity of HSPA1A might be the main mechanism to prevent accumulation of misfolded proteins in the endoplasmic reticulum and, thus, limit autophagy [32, 33].

The mechanism(s) responsible for decreased p62 levels and elevated HSPA1A production in PBMCs in women with a malignant mammary tumor remains to be determined. It is likely that tumor-related antigens as well as intracellular macromolecules released from lysed cells appear in the circulation following malignant transformation of mammary cells. Their presence would signal the need for immune system stimulation and an elevated level of autophagy and HSPA1A activation in PBMCs. A probable subsequent increased elevation of these and likely other compounds in the systemic circulation as tumorigenesis progresses is consistent with our observation of decreased p62 and increased HSPA1A in PBMCs as breast cancer became more invasive. Therefore, based on findings from our study, we hypothesize that elevated intracellular levels of HSPA1A and decreased p62 levels in PBMCs could be markers of breast cancer and for advanced disease.

A strength of our study is its unique focus on PBMCs rather than on tumor cells, and specifically on alterations in intracellular levels of compounds associated with elevated immune activation. However, the findings must be interpreted in the context of several limitations. First, the sample size of the study population, especially the group with benign breast lesions, was very small and, therefore, subject to selection bias. The majority of women with benign lesions do not typically undergo surgery which limited our ability to recruit more subjects in this category. In addition, our study was not designed to detect differences in HSPA1A and p62 levels based on histology and stage of breast cancer. Therefore, our study was under-powered to evaluate these differences and the findings must be regarded as preliminary and hypothesis-generating. Further studies are warranted to compare the preoperative and postoperative levels of these markers, since HSPA1A and p62 levels may also fluctuate according to physical conditions and stresses other than breast malignancy. Lastly, utilization of p62 as the sole measure of autophagy is not definitive and optimally requires independent confirmation by measurement of other autophagy-associated biomarkers [31, 34].

Conclusion

Intracellular properties of PBMCs are altered in women with a malignant breast tumor. Detection of elevated levels of HSPA1A and decreased concentrations of p62 in PBMCs may have value as a diagnostic indicator of a breast malignancy or its recurrence. Additional investigations of the observations reported here might be of value in development of clinically useful tests for early detection of invasive breast cancer and/or its differentiation from benign breast masses.

Declarations

Author contributions

TO, EA and SW designed the study, acquired the data and drafted the manuscript. SG, EZ, AA, AMB, GD, TAM, DL, CB, GT, GZ contributed to the interpretation of the data and substantially revised the manuscript. All authors have approved the submitted version and agree to be personally accountable for their own contributions.

Sources of Funding

The authors disclosed receipt of no financial support for the research, authorship, and/or publication of this article.

Disclosures

All other authors have no relationships to disclose.

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