Journal of Health Sciences

REVIEW ARTICLE Open Access

Serum levels of oxidative stress marker malondialdehyde in breast cancer patients in relation to pathohistological factors, estrogen receptors, menopausal status, and age

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ABSTRACT

Introduction: The aim of this study was to determine the serum levels of malondialdehyde (MDA) in patients with invasive breast cancer in relation to its serum levels in patients with benign breast disease, and to investigate correlation between MDA serum levels with pathohistological prognostic factors (tumor size, lymph node involvement, and histologic grade [HG]), estrogen receptor (ER) status, and with breast cancer patient's age and menopausal status.

Methods: A total of 43 with well-documented invasive breast cancer were included in this study: 27 with positive axillary's lymph nodes, and 16 with negative axillary's lymph nodes, and 39 patients with findings of benign breast diseases. MDA determination in serum of breast cancer and benign breast disease patients was performed by the fluorimetric method, immunohistochemical staining was performed for ER, and routine pathohistological examination was conducted for pathohistological factors.

Results: MDA serum levels in breast cancer patients were significantly higher than MDA serum levels in benign breast disease patients ($p = 0.042$). No statistically significant difference between MDA serum levels in breast cancer patients with and without lymph node metastases was found ($p = 0.238$). No statistically significant correlations between MDA serum levels and tumor size ($p = 0.256$), HG ($p = 0.124$), or number of positive lymph nodes (0.113) were found. A statistically significant correlation between serum MDA levels and ages of breast cancer patients with lymph node metastases was found ($p = 0.006$).

Conclusion: Obtained results support the importance of MDA in the carcinogenesis of breast cancer. According to our findings, serum level of MDA could not be a useful prognostic factor in breast cancer.

Key words: Breast cancer; benign breast disease; oxidative stress; malondialdehyde; pathohistological factors; estrogen receptors

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Submitted: 14 September 2018/Accepted: 20 November 2018

DOI: https://doi.org/10.17532/jhsci.2018.263

INTRODUCTION

Breast cancer is the most common cancer in women (1), accounting for 25% of all female cancers worldwide (2). Etiology of breast cancer is multifactorial which includes genetic, environmental,

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social, demographic, and hormonal factors (3). Most of the risk factors for breast cancer development and progression are to some extent implicated with reactive species (RS) generation (4,5). Breast tumors are embedded in very pro-oxidative environment, as the mammary gland is plenty in surrounding adipose tissue. Therefore, the exceeding RS acts on the lipid neighborhood yielding several active metabolites that can regulate a wide range of cellular processes (6-8). Some of the consequences of carcinoma cell oxidative stress are: Accelerated tumor progression due to RS mediated inactivation of additional tumor suppressor genes within tumor cells and increasing expression of proto-oncogenes (9-11), activation of growth-promoting signaling pathways due to RS mediated promotion of cell proliferation *in vitro* (12), increasing blood supply to tumor cells (13), and consequently increasing risk of metastasis (14). In addition, oxygen radicals may augment tumor cells migration, increasing the risk of invasion and metastasis (14).

Several markers of the oxidative stress in patients with breast cancer are currently available. One of the most important of these is malondialdehyde (MDA), low-molecular-weight aldehydes derived from lipid peroxidation processes, which has been used as a marker of lipid peroxidation (15).

Various studies were conducted with the relation of oxidative stress and human breast cancer. Nevertheless, previous studies regarding pathohistological factors are poorly understood. The aim of this study was to determine the serum levels of MDA in patients with invasive breast cancer in relation to its serum levels in patients with benign breast disease, and to investigate correlation between MDA serum levels with pathohistologic prognostic factors such as tumor size, lymph node involvement, histologic grade (HG), estrogen receptor (ER) status, and with breast cancer patient's age and menopausal status.

METHODS

Patients

This study was approved by the Ethic Committee of the University Clinical Centre Tuzla. Informed consent was obtained from all of the patients. It was a prospective case–control study with well-defined including criteria: Histologically proven invasive breast cancer, no distant metastases, no previous adjuvant therapy, currently under no treatment, and no other major illnesses.

According to the PHD findings, a total of 43 with well documented invasive breast cancer were included in this study: 27 with positive axillary's lymph nodes, 16 with negative axillary's lymph nodes, and 39 patients with findings of benign breast diseases were also included in the study. All patients were subjected to the appropriate breast surgery at the Department of Surgery, University Clinical Centre of Tuzla (all of them were females, Caucasians, residents of narrow region in Tuzla surrounding, Bosnia and Herzegovina). Tumor tissue and serum sample from patients with primary invasive breast cancer were used, as well as breast tissue and serum samples from patients with benign breast disease.

Routine pathological examination was performed with hematoxylin-eosin staining. Tumors were classified according to the criteria of the World Health Organization (16). Histological grade was obtained in accordance with a modified Scarff-Bloom-Richardson histological grading system. The staging was based on tumor-node-metastasis (TNM) system. Tumor size was evaluated separately (0.5–1.0 cm, ≤2 cm, 2–5 cm, >5 cm).

Blood samples for MDA level determination were collected at the Department of Surgery, University Clinical Centre. Venous blood samples were obtained 24 h before surgery by venepuncture (7 mL of blood for analysis from each patient). After ½ h, samples were processed at room temperature by centrifuging at 3000 rpm for 10 min. Serum samples were stored in plastic microtubes (0.5 mL/microtube) in the freezer at –80°C. Extracting and sample storing were carried out in Polyclinic for Laboratory Diagnostic, University Clinical Centre Tuzla.

Immunohistochemistry

Immunohistochemical staining for ER was performed on 4 μm thick formalin-fixed paraffin embedded sections. Deparaffinization and rehydration were performed in xylene and ethanol solutions (reducing concentration 96–70%). Sections were incubated in H_2O_2 solution (1.5% H_2O_2 in methanol) for 15 min to block endogenous peroxidases.

Antigen retrieval was performed in procedure with the retrieval buffer (pH = 9.0, TRIS 20 mmol/L, EDTA 0.05 mmol/L, 0.05% Tween 20) in a microwave oven by heating the slides for 15 min. After rinsing with the phosphate buffered saline (PBS) buffer (100 mmol/L NaCl and 6 mmol/L $Na_2HPO_4 \times 2H_2O$), normal goat serum (Dako, Golstrup, Denmark) was applied for 15 min to block non-specific antibody binding. Subsequently, sections were incubated with primary antibody (a mouse anti-human monoclonal antibody against ER, clone NCL-ER-6F11, Newcastle Upon Tyne, UK, 1: 50 diluted in PBS/Bovine serum albumin buffer, pH = 7.2) at 37°C. A three-step technique was used for visualization with biotin-conjugated secondary antibody (Dako, Golstrup, Denmark) and diaminobenzidine (Fluka Chemie, GmbH, Buchs, Switzerland). Slides were preserved with Canada balsam (turpentine).

Immunohistochemical evaluation

The evaluation of the immunohistochemical staining for ER was performed by pathologist through a light microscopic observation (Olympus BX-50 light microscope, Olympus Medical System Corp, Tokyo, Japan). The evaluation was performed using Remmeles immunoreactivity score (17) for immunoreactivity analyzing.

MDA determination

MDA determination in serum of breast cancer and benign breast disease patients was performed by the fluorimetric method (18,19). MDA reacts with thiobarbituric acid (TBA) in an acid medium to form a fluorescent complex that is extracted with butanol and measured by fluorescence (20-23). Fluorescence spectra of MDA-TBA fluorescent complex were recorded at spectrofluorimeter RF-5301 PC (Shimadzu, Japan). Fluorescence emission was recorded at 530 nm after excitation 516 nm. The entrance and exit slits for the excitation light beam were both 1.5 nm. Sample and standard preparation was performed using Ohkawa's modified Yagi's method (20-24).

Statistical analysis

The results were evaluated by Mann–Whitney *U*-test for independent samples and with Spearman's correlation. For all performed tests, $p < 0.05$ was considered as statistically significant. For statistical analyses, we used SPSS 17.0 software (SPSS Inc., USA).

RESULTS

MDA levels were determined in 43 patients with breast cancer and 39 patients with benign breast disease, which is considered as a control group. MDA serum levels in breast cancer patients were significantly higher than MDA serum levels in benign breast disease patients ($p = 0.042$) (Table 1). No statistically significant difference between MDA serum levels in breast cancer patients with and without lymph node metastases was found ($p = 0.238$) (Table 2). Pathohistological factors (tumor size, histological grade, and number of positive lymph nodes) were determined in all breast cancer patients, and according to the

TABLE 1. MDA serum levels in patients with breast cancer and with benign breast disease

Group	Number	MDA levels Mean±SD error. Min-Max [nmol/mL]
1. Patients with breast cancer	43	29.09±19.32 [5.04−95.14]
2. Patients with benign breast disease	39	21.38±15.01 [5.34−65.17]

Differences in MDA serum levels between breast cancer and benign breast disease patients Mann-Whitney U=619,00; *P*=0.042*, MDA: Malondialdehyde

TABLE 2. MDA serum levels in breast cancer patients with and without lymph node metastases

Group	Number	MDA levels Mean±SD error. Min-Max [nmol/mL]
1. Patients with lymph node metastases	27	30.77±17.83 [5.04−63.42]
2. Patients without lymph node metastases	16	26.26±21.92 [5.38−95.14]

Differences in MDA serum levels between breast cancer and benign breast disease patients Mann-Whitney U=169,00; *P*=0.238, MDA: Malondialdehyde

results, the pathological TNM status was obtained (Table 3). In breast cancer patients, no statistically significant correlations between MDA serum levels and tumor size (p = 0.256), HG (p = 0.124) or number of positive lymph nodes (0.113) in breast cancer patients with lymph node metastases were found. A statistically significant difference in MDA serum levels between ER-positive and ER-negative breast cancer patients was not found (p = 0.726) (Table 4). Regarding menopausal status, breast cancer patients were classified into two groups, breast cancer patients with premenopausal status and with postmenopausal status (Table 5). No statistically significant difference in the serum level of MDA between these groups was found (p = 0.130). Regarding ages of breast cancer patients, no significant correlation between serum MDA levels and ages of patients was found, but statistically significant correlation between serum MDA levels and ages of breast cancer patients with lymph node metastases was found (p = 0.006) (Figure 1).

DISCUSSION

Increased MDA levels have been reported in breast, ovarian cancer, gastric and lung cancer, and colorectal adenomas (25-30). MDA, as low molecular weight aldehyde, can be produced from the free radical attack on polyunsaturated fatty acids. The process of lipid peroxidation is one of the oxidative conversions of polyunsaturated fatty acids to MDA, the main sensitive parameter of lipid peroxidation (15). Increased MDA levels have been reported in breast, ovarian cancer, gastric and lung cancer, and colorectal adenomas (25-30). Several studies present evidence that reactive oxygen species (ROS) are involved in the etiology and progression of breast cancer (31). Results in this study showed an increase in MDA serum level in invasive breast cancer patients as compared to MDA serum level in benign breast disease patients (Table 1). Previous studies report increased plasma levels of MDA in breast cancer patients compared to healthy control (26), as well as higher MDA concentration in malignant tissue when compared to normal tissue samples from healthy controls (32,33). Thus, previous findings and our results suggest that oxidative stress is present not only in cancer cells but also in the whole organism affected by the tumor. Furthermore, there was found a trend toward increased MDA levels in malignant breast cancer tissue as the TNM stage increases (32). Qebesy et al. found negative relationship between the tissue level of MDA and HG in breast cancer patients suggesting that the defect in the antioxidant mechanism may contribute to tumor development and progression (33) regarding that HG is considered a highly valuable prognostic factor for breast cancer as poorly differentiated lesions are associated with significantly poor clinical outcome (34). In this study, we have not found a significant correlation between MDA level and HG, as well as between MDA level and tumor size and axillary lymph node involvement, which are also considered as a very important prognostic indicator (35). Differences in MDA level between breast cancer patients with positive and negative lymph nodes were tested in this study, since the presence or absence of axillary lymph node involvement is considered as the most significant prognostic indicator for patients with early-stage breast cancer (35) and that there is a direct relationship between the number of involved axillary nodes and the risk for distant recurrence (36). Although *in vitro* studies have shown that oxidative stress significantly motivate the migratory potential of poorly invasive breast cancer cells MCF-7 through Erk signaling activation (37), we have not found significant difference in MDA levels between patients with positive and with negative lymph nodes (Table 2), nor statistically significant correlation between MDA serum level and number of positive lymph nodes in breast cancer patients.

Estrogen and progesterone receptors are a powerful predictive factor for the likelihood of benefit from adjuvant tamoxifen (35), but the prognostic significance of hormone receptors may not persist long-term (38). A few previous studies have shown the relationship between oxidative stress and estrogen-dependent breast cancer (39-42). It was reported that oxidative stress induces activation of Akt/PI3K/mTOR signaling, promoting oncogenesis, and tumor progression in estrogen-dependent breast cancer (39,40). Furthermore, multivariate analysis showed that ROS were predictive for TNM status in patients with estrogen-dependent breast cancer (41). *In vitro* estrogen exposure

TABLE 3. pTNM status, HG, ER status, ages and MDA serum levels in breast cancer patients

MDA [nmol/mL]	pTNM	HG	ER	Ages (years)
8.83	pT1cN0	II	+	60
24.21	pT1cN0	III	+	63
23.27	pT2N3a	III	–	49
26.93	pT2N1a	III	+	42
12.74	pT2(s) N0	II	+	42
24.34	pT4bN1	II	+	missing data
56.42	pT3N3a	III	+	25
8.61	pT2N0	I	–	34
51.93	pT1aN0	III	–	60
14.78	pT3N0	II	missing data	60
32.74	pT2N0	II	+	41
54.18	pT2(m) N3	II	+	55
95.14	pT1N0	II	–	72
63.42	pT2N2	II	missing data	70
51.44	pT2N1	II	+	62
5.04	pT2N3	III	+	73
5.38	pT2N0	II	+	79
34.82	pTa1N0	II	+	58
20.04	pT1cN1	III	missing data	79
19.29	pT2N2a NG3	II	–	60
44.9	pT4bN1a	III	+	60
54.26	pT2N1a	II	+	49
18.52	pT2N2a	III	+	48
21.3	pT4dN2a	III	–	60
32.78	pT2N2	III	–	49
16.82	pT1a (s) N0	II	+	61
14.64	pT3N2a	III	+	60
32.41	pT1bN0	II	+	67
27.82	pT2N2	II	+	58
11.51	pT2N1a	II	+	62
28.13	pT2N3	III	+	45
15.36	pT1cN0	II	+	72
15.33	pT2N1a	II	+	57
55.28	pT3N2a	II	+	47
62.52	pT2N3	III	+	45
35.45	pT2N1a	II	+	45
19.78	pT2mN0	II	+	60
9.46	pT2N1	III	+	69
19.61	pT1c (m) N2	II	+	66
25.82	pT2N0	II	+	35
10.24	pT2N2	II	+	70
24.8	pT2N2	II	–	82
20.74	pT2N0	III	–	61

pTNM: Pathological tumor-node-metastasis, HG: Histologic grade, ER: Estrogen receptor, MDA: Malondialdehyde

TABLE 4. MDA serum levels in breast cancer patients with positive and negative ERs

Group	N	MDA levels Mean±SD error. Min-Max [nmol/mL]
1. ER-positive breast cancer patients	31	28.034±17.447 [5.04−63.42]
2. ER-negative breast cancer patients	9	33.095±26.104 [8.61−95.14]

Differences in MDA serum levels between ER-positive and ER-negative breast cancer Mann-Whitney U=128,00; *P*=0.726,
ER: Estrogen receptor, MDA: Malondialdehyde

TABLE 5. MDA serum levels in breast cancer patients regarding menopausal status

Group	N	Ages Mean±SD (years)	MDA levels Mean±SD. error. Min-Max [nmol/mL]
1. Breast cancer patients with premenopausal status	14	64.35±7.27	26.812±20.203 [5.04−95.14]
2.Breast cancer patients with postmenopausal status	28	45.37±6.98	33.819±17.067 [8.61−62.52]

Differences in MDA serum levels between breast cancer patients with premenopausal status and with postmenopausal status Mann-Whitney U=137,50; *P*=0.130, MDA: Malondialdehyde

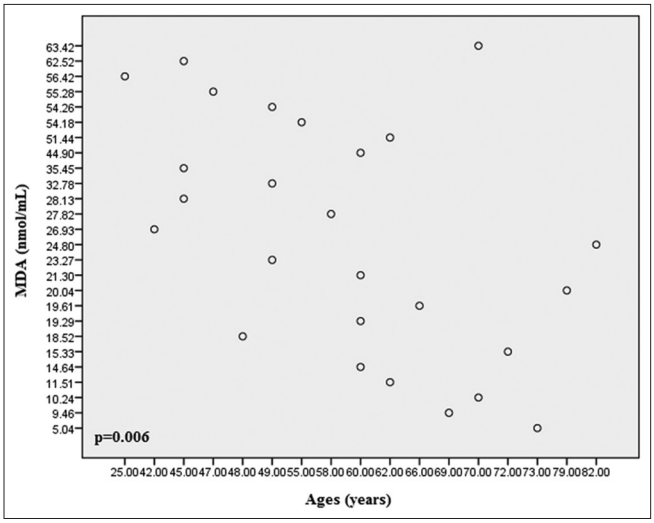

FIGURE 1. Statistically significant correlation between serum malondialdehyde levels and ages of breast cancer patients with positive axillary lymph nodes (Spearman rho=−0.525, $p = 0.006$).

induces ROS production selectively in ER-positive MCF7 cells (42). In our study, we have not obtained a statistically significant difference in MDA serum levels between ER-positive and ER-negative breast cancer patients (Table 4). A case–control study in a wide population of breast cancer patients including both estrogen positive and estrogen negative cancers showed that oxidative stress parameters were positively associated with breast cancer in a postmenopausal woman with higher body mass index (43). Considering results from previous studies, we have tested differences in MDA serum levels between breast cancer patients regarding menopausal status. No statistically significant difference in MDA serum level between breast cancer patients with premenopausal status in relation to patients with postmenopausal status (Table 5). Molecular mechanisms that relate oxidative stress and ERs signaling are very complex and including other molecules such as interleukin-8 (IL-8), vascular endothelial growth factor (37), IL-6, or other processes such as inflammation (41). Thus, further investigation of breast cancer biology from the overview of oxidative stress may be helpful in the understanding of breast cancer etiology and may contribute to the development of new approaches to cancer therapy.

However, we showed statistically significant negative correlation between MDA serum levels in patients with positive lymph nodes and their ages (p = 0.006), which is consistent with findings from two relatively large trials that have demonstrated a worse prognosis for patients <35 years age, even after adjustment for other prognostic factors (44,45).

In conclusion, obtained results support the importance of MDA in the carcinogenesis of breast cancer, although according to our findings serum level of MDA could not be a useful prognostic factor in breast cancer.

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