Role of Caspase-3, IL-1β and oxidative stress in Iraqi women with breast cancer

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Abstract. Breast Cancer is caused by malignant tissue cells and has become one of the world's biggest medical problems. The goal of this research was to determine the serum concentration of markers of oxidative stress that play an important role in the multiple factors involved in breast cancer development, growth, and invasion. Serum of 90 women patients (45 breast cancer and 45 benign breast tumors) and 42 healthy individuals as control group were used in this study. Serum level of MDA, PC, 8-OHdG, IL-1β and Caspase-3 were measured by ELISA. Highly significantly increased levels (p<0.01) of MDA, PC, 8-OHdG, IL-1β and Caspase-3 were found in breast cancer and benign breast tumor when compared to healthy controls. The MDA and Caspase-3 level are considered strong parameters to diagnose and detection for breast cancer using the ROC curve. High lipid peroxidation is a significant risk factor for breast cancer and the activation of apoptosis and pro-inflammatory activity may be due to elevated levels of IL-1β & Caspase-3 in breast cancer cells. Nonetheless, lipid peroxidation and Caspase-3 are major factors in breast cancer growth and progression.

Keywords: Breast Cancer, Lipid peroxidation, Caspase-3, IL-1β, 8OHDG, and Protein Carbonyl.

1. Introduction
Breast cancer is a big concern for women all around the country. Breast cancer is the most common cancer in women in the United States and the second most common cause of death from cancer [1]. Oxidative stress plays an important part in breast cancer development, growth, and invasion [2,3]. Inflammation has been shown to play a crucial role in the growth and progression of tumors, as many of the main molecular pathways are now revealed, highlighting the central role of cytokines, notably interleukins (ILs), in the initiation, migration, and progression pathway of breast cancer [4]. ILs are secretory immunomodulatory proteins which belong to the cytokine superfamily and present complex immunological function as cytokines. Interleukin-1 is a group of cytokines which have a molecular weight of around 17-20 kD. The key representatives are the proinflammatory cytokines IL-1α and IL-1β, interleukin-1 receptor antagonist, both of which are involved in the initiation and progress of inflammatory processes. The key goal of ILs is to mediate intercellular contact in the immune system, which plays a crucial role in inflammatory response, including cell migration, proliferation, maturation, and adherence [5]. Apoptosis is a type of cellular death that can be thought of as a controlled form of cell suicide. Also known as caspases, apoptosis is the mechanism by which cells are sent off-course on their path to become dead cells (the main executor of both intrinsic and extrinsic variants of apoptosis) [6]. In 1993, with their function well
established in apoptosis, the role of these enzymes in programmed cell death was first described, which occurs widely during development and to sustain cell homeostasis during life, activation of caspase means that the cellular components are killed in a controlled manner, with a marginal effect on the surrounding tissue, causing cell death. 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 lipid peroxidation marker [8], which is frequently used to estimate oxidative stress condition [9]. Excessive free radicals can affect proteins oxidatively [10]. Carbonyl modification caused by protein oxidative stress has many structural and functional effects, including loss of protein structure, abnormal protein cleaning, cellular redox balance alteration, interruption of the cell cycle, and canceling progression (11,12). The formation of carbonyl groups may be involved in the process of carcinogenesis, the side chain oxidation of amino acids such as threonine, proline, arginine, and lysine in the protein structure or the oxidative degradation of the peptide bonds may form protein carbonyl (PC) groups [9,13]. The main products formed when DNA strands are oxidized are 8-hydroxy-deoxyguanosine (8-OHdG) which is a biomarker for assessing the harm caused by endogenous oxidative DNA and is a risk factor for many diseases, including cancer [14]. The early detection and evaluation of women at high risk of cancer for breast cancer screening, treatment and prognosis may provide a significant discriminatory biomarker due to oxidative stress [15]. In certain cases, cells bearing DNA mutations undergo programmed cell death when they are unable to completely repair these DNA lesions, but in certain circumstances, these cells survive as they produce offspring that are immune to certain DNA lesions [16,17]. During oxidative stress conditions, single or double strand breaks within the DNA are also produced [18]. DNA disruption is thought to play a major role in the initiation of carcinogenesis [19]. The ultimate aim of our research is to determine the best parameter to forecast the prognosis and monitor the success of breast cancer treatment.

2. Experimental Section

2.1. Study Group

The present study was conducted in Baghdad Oncology Teaching Hospital Medical in Baghdad- Iraq, from June 2018 to February 2020, carried on 132 breast tumors patients at different age groups. The data of breast cancer patients (all of them were females and diagnosed as invasive ductal carcinoma) with their Histopathology reports and immunohistochemical results including ER and PgR (positive or negative) were obtained from the laboratory of the Baghdad Oncology Teaching Hospital and from cancer research department of the hospital which included the histopathological reports that confirm their diagnosis regarding breast subtypes, grading and staging, with informed consent obtained from each patient. Group I consisted of 45 newly diagnosed women with breast cancer their ages ranging 31-65 years, while group II involved 45 women with benign breast tumors (all of them were females and diagnosed as fibroadenoma), their ages ranging 26-60 years. A total of 42 healthy female volunteers with ages ranging from 25-64 years served as controls (group III).

2.2. Blood Collection

Five milliliters of venous blood samples were obtained before surgical operation and oncological treatment by using 5 mL plastic disposable syringes, the sample let to coagulated for 15 minutes at room temperature 25°C in Gel Clot activator tube, after the centrifugation at 1500 xg for 15 minutes, the serum was collected and stored at -20°C until assayed the time of analysis. Pregnancy, smoking, and alcohol-dependent women were excluded from this study.

2.3. Anthropometric measurements
The Body Mass Index (BMI) was estimated by dividing weight (kg) by height\(^2\) (m\(^2\)). Women were deemed obese when their body mass index was 29.9 (kg/m\(^2\)) or higher [20].

2.4. Laboratory analysis

Serum levels of IL-1β, Caspase-3, MDA, 8-OHdG, and PC were determined by a double-antibody Sandwich Enzyme-Linked Immunosorbent Assay (ELISA) kits obtained from (Melsin Medical Co., Limited, China) according to the provided assay procedure (www.melsin.com), each assay was calibrated using its standard curve following the manufacturer's instructions and using the instruments (SAGA Reader and GEA Washer, LiNEAR Chemical, Spain). VIDAS CA15-3 and VIDAS CEA were determined by combining a 2-step enzyme immunoassay sandwich method with a final fluorescent detection (ELFA) kit obtained from (bioMerieux SA, Marcy -L’Étoile, France).

2.5. Statistical Analysis

All study data are shown as mean±standard division (SD) and analysed statistically with social sciences (SPSS) Statistics version 25 program. One-way variance analysis (ANOVA) was used to measure the difference among groups. In addition, MedCalc Version 19.4.1 used ROC to evaluate the AUC. The best cut off point of the studied markers, sensitivity, specificity, PPV and NPV were also calculated. A P value of <0.05 was considered as statistically significant.

3. Results

Table 1. showed the demographic characteristics of women patients with breast tumors and control groups which participated in this study. No significant differences (P>0.05) were observed in age, weight, length and BMI. Table 2 represented the clinico-pathological parameters in breast cancer cases.

| Parameters | Group I, N=42 | Group II, N=45 | Group III, N=45 | P Value |
|------------|---------------|----------------|----------------|---------|
| Age (Years) | 40.357 ± 11.916 | 42.977 ± 9.218 | 45.556 ± 9.314 | NS      |
| Weight (Kg) | 75.119 ± 7.232  | 70.744 ± 7.538 | 78.0667 ± 7.362 | 0.0001* |
| Length (Cm) | 170.666 ± 4.760 | 173.133 ± 5.809 | 170.82 ± 4.914 | 0.047*  |
| BMI (Kg/m\(^2\)) | 25.825 ± 2.682  | 23.630 ± 2.561 | 26.792 ± 2.771 | 0.0001* |

Group I: Breast Cancer; Group II: Benign breast tumor; Group III: Control group; NS: non-significant at P<0.05; *

In the current study, breast cancer tissues expressing positive immunohistochemical ER and PgR values were observed in 60% and 69% respectively, while breast cancer tissues expressing negative immunohistochemical ER and PgR values were observed in 40% and 31% respectively, as seen in Table2. In regards of the tumor markers CEA and CA15-3 showed positive 80% and 47% respectively, while a negative value was observed in 18% and 47% respectively.
Table 2. Hormonal receptor (ER and PgR), Tumor markers (CA15-3 and CEA) status, and grades in women with breast cancer.

| Clinico-pathological Factors | Characters | Number of BC(%) |
|-----------------------------|------------|----------------|
| Grade                       | II         | 29 (64.44%)    |
|                             | III        | 16 (35.56%)    |
| ER                          | Positive   | 27 (60%)       |
|                             | Negative   | 18 (40%)       |
| PgR                         | Positive   | 31 (69%)       |
|                             | Negative   | 14 (31%)       |
| CEA                         | Positive*  | 37 (82%)       |
|                             | Negative   | 8 (18%)        |
| CA15-3                      | Positive** | 24 (53%)       |
|                             | Negative   | 21 (47%)       |
| Total                       | -          | 45 (100%)      |

* Normal Value of CEA is 0-3 ng/mL, ** Normal Value of CA15-3 is ≤ 30 U/mL

A highly significant increased (P<0.01) can be seen in all parameters which included MDA, 8-OHDG, PC, IL-1β, and Caspase-3 levels in women patients with breast cancer or benign breast tumors in comparison with control groups.

Table 3. The Parameters for Control, Benign, and Breast Cancer characteristics of patients participating in the study and P Value.

| Parameters       | Group I Mean ± SD | Group II Mean ± SD | Group III Mean ± SD | P Value |
|------------------|-------------------|--------------------|---------------------|---------|
| MDA (nmol/mL)    | 0.570 ± 0.117     | 0.834 ± 0.156      | 2.686 ± 0.191       | 0.0001* |
| 8-OHDG (ng/mL)   | 0.978 ± 0.423     | 1.364 ± 0.532      | 1.875 ± 0.999       | 0.0001* |
| PC (nmol/L)      | 0.671 ± 0.401     | 0.791 ± 0.441      | 1.027 ± 0.447       | 0.001*  |
| IL-1β (pg/mL)    | 6.928 ± 2.325     | 8.420 ± 2.708      | 10.195 ± 4.424      | 0.0001* |
| Caspase-3 (pmol/L)| 3.998 ± 0.423     | 5.113 ± 0.925      | 7.952 ± 2.536       | 0.0001* |

*: significant at P<0.01.

The recipient operational characteristic (ROC) curve is a plot of the y-axis sensitivity (true positive rate) against the x-axis 100 accuracy (false positive rate) for the various potential diagnostic test cut-off points. The measure is more accurate where the blue curve is closest to the left-hand boundary and then the top border of the ROC space(21). All ROC curve results for women with breast cancer and benign breast tumors have been seen in Table 4 and Table 5, respectively.
Table 4. Diagnostic dates of serum levels of MDA, 8 OHDG, PC, IL-1β, and Caspase-3 using ROC curve for Breast Cancer.

| Parameters | AUC  | SE   | Asymptotic Significance | Asymptotic 95% Confidence Interval | Sensitivity (%) | Specificity (%) |
|------------|------|------|-------------------------|------------------------------------|----------------|----------------|
| MDA        | 1.000| 0.000| <0.0001                 | 0.958 1.000                         | 100            | 100            |
| 8OHDG      | 0.844| 0.0429| <0.0001                | 0.750 0.913                         | 73.33          | 88.10          |
| PC         | 0.737| 0.0545| <0.0001               | 0.631 0.825                        | 88.89          | 57.14          |
| IL-1β      | 0.766| 0.0506| <0.0001               | 0.663 0.850                        | 77.78          | 64.29          |
| Caspase-3  | 1.000| 0.000| <0.0001               | 0.958 1.000                         | 100            | 100            |

Table 5. Diagnostic dates of serum levels of MDA, 8 OHDG, PC, IL-1β, and Caspase-3 using ROC curve for Benign Breast Tumors.

| Parameters | AUC  | SE   | Asymptotic Significance | Asymptotic 95% Confidence Interval | Sensitivity (%) | Specificity (%) |
|------------|------|------|-------------------------|------------------------------------|----------------|----------------|
| MDA        | 0.922| 0.0272| <0.0001                | 0.844 0.968                        | 97.78          | 73.81          |
| 8OHDG      | 0.768| 0.0509| <0.0001                | 0.665 0.852                        | 57.78          | 88.10          |
| PC         | 0.610| 0.0613| 0.0738                 | 0.499 0.712                        | 68.89          | 57.17          |
| IL-1β      | 0.682| 0.0598| 0.0023                 | 0.574 0.778                        | 95.56          | 42.86          |
| Caspase-3  | 0.918| 0.0282| <0.0001                | 0.839 0.966                        | 82.22          | 90.48          |

The MDA and Caspase-3 level are considered strong parameters to diagnose and detection for Malignant breast cancer because the area under the ROC curve (AUC) for both analyses are (1.00). The best cut-off point derived from the ROC curve show a sensitivity of (1.00) and specificity of (1.00) in value of. Accordingly, the test value above (nmol/mL) for MDA and (pmol/L) for Caspase-3 are considered the abnormal case (breast cancer) and the values below represents the healthy condition, as shown in Figure 1. The significance level is very important (P<0.0001).
In our current research highly diagnostic success in breast cancer was recorded (AUC=1.00, sensitivity=100%, specificity=100% at cutoff value >0.848ng/mL) in the MDA level and (AUC=1.00, sensitivity=100%, specificity=100% at cutoff value >4.971 ng/mL) in Caspase-3 level (Table 4).

4. Discussion
Oxidative stress is an important factor in cancer devolvement previous researches, elevated oxidative stress has been confirmed in multiple type of cancer forms, including breast cancer [22,23]. The study indicates that MDA has a negative impact on the risk of breast cancer, in women. In breast cancer, elevated MDA levels have been identified as an indication of lipid peroxidation [24,25]. Several studies have found evidence that reactive oxygen species (ROS) are implicated in breast cancer etiology and development [24,26]. 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 [27]. Anticipated mechanisms for amplified oxidative stress in breast cancer is assumed to have the ability to induce genetic changes in antioxidant enzymes, estrogen treatment, intemperance generation of reactive oxygen species, as well as impaired antioxidant system [28]. There is ample evidence demonstrating a contributory role of free radicals, oxidative disruption, and lipid peroxidation in incidence and growth of the forms of cancer [29]. 8-OHDG is a sensitive biomarker that is widely used to quantify DNA damage caused by oxidative stress. Some data from studies suggests that 8-OHdG is related to cell proliferation and that measurement of this marker may shed light on the time to recurrence of a given tumor [19,30]. Guo et al 2017 reported substantially elevated levels of 8-OHdG (a measure of oxidative stress) in benign breast lesions as well as an early stage of breast cancer [31]. There are many studies that confirm our findings and show that, when tumor tissue is measured, 8-OHdG concentrations are substantially dependent on tumor type, and could serve as a possible biomarker for evaluating women with elevated risk of breast cancer [32-34]. Oxidative damage to proteins results in decreased enzymatic functions and increased protein catabolism, an increase in protein carbonyl levels is not only indicative of oxidative stress but also indicates protein dysfunction [35]. The basal level of protein carbonyls under non-stressed circumstances is about one for every ten
proteins. This rises to around one in three during oxidative stress, with deleterious effects on cell function [36]. Several studies have found a highly significantly increased level of PC (p< 0.001) in patients with breast cancer than healthy individuals [13,37,38]. Recently, the association between inflammation and cancer development has been generally accepted as a cancer hallmark, permanent inflammation leads to cancer progression by fostering tumor survival, proliferation, angiogenesis, metastasis, and immune outflow, and the mechanisms involved in cancer-associated inflammation include complex interactions between tumor cells and tumor microenvironment [39,40]. IL-1 is one of the most common and most active pro-inflammatory cytokines at tumor sites, and can modulate the growth and invasive properties of tumor cells [41]. In breast cancer and its pro-inflammatory cytokines, IL-1β is a key regulator of systemic inflammatory response, involving innate immune responses by inflammatory cell infiltration (e.g., neutrophils, macrophages, and monocytes) through infection sites [42]. In breast cancer patients, serum levels of the representative pro-inflammatory cytokine IL-1β were substantially elevated contrast to healthy controls [43]. When the inflammasomes have been activated, IL-1β can cause the invasion of mesenchymal and endothelial cells in inflammation and immune cells in compromised and weakened regions as well as the release of inflammatory mediators [44-47]. Although IL-1β is involved in the mechanism of host defense, aberrant inflammasome activation and subsequently prolonged IL-1β production are closely linked to autoimmune and inflammatory disease pathogenesis [46]. Caspase-3 cascades play essential roles in apoptosis and cancer development and prognosis. Caspase inactivation also occurs in cancer cells, rendering the cells immune to external pressures and therapies [48,49]. Xuan et al. 2017 reveals that elevated expression of caspase-3 is strongly associated with adverse survival specific to breast cancer [50]. Therefore, increased Caspase-3 level (AUC= 1.00, sensitivity=100%, specificity= 100% at cutoff value >4.971 ng/ml) in breast cancer would be of clinical benefit.

5. Conclusion
Oxidative stress and Caspase-3 could be involved in predicting the risk of breast cancer. The patients with breast cancer had a high level of both MDA and Caspase-3 in the early and advanced stage of disease. Our data showed that Caspase-3 was found to be a marker for diagnosis and differentiate between benign and malignant breast tumors, and we confirmed that breast cancer is not age dependent.

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