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Research Article
Evaluation of Doxycycline and Cyclophosphamide in Combination with Adjuvant Treatment in Early Breast Cancer Females: Role of Certain Biomarkers

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Abstract

Background and Objectives: Breast cancer is one of the most common cause of cancer death worldwide. Tumor markers have been widely used for assessing the treatment response and early diagnosis of recurrence. The present work was designed to evaluate the new off label use of doxycycline alongside with the ordinary cyclophosphamide medication in combination with the systemic adjuvant treatment protocol in early breast cancer females. **Materials and Methods:** Estrogen (ER), B-cell lymphoma-2 (Bcl-2) and epidermal growth factor (EGF) were estimated. Early diagnostic breast cancer patients treated with adjuvant protocol were additionally orally administered with a dose of 100 mg doxycycline or 600 mg m^{-2} cyclophosphamide and repeat every 3 weeks for 4 cycles. **Results:** The results revealed significant increase in ER, Bcl-2 and EGF levels in serum of breast cancer patients as compared with normal females. Treatments with doxycycline or cyclophosphamide in combination with the adjuvant treatment regimens improved all the measured parameters with variable degrees. **Conclusion:** Doxycycline alongside the adjuvant treatment protocol recorded the most potent effect in ameliorating ER, Bcl-2 and EGF levels.

Key word: Breast cancer, estrogen, doxycycline, cyclophosphamide, cancer death, adjuvant treatment

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Breast cancer is the most frequently diagnosed cancer in women[1]. Breast cancer is frequently hormone-dependent and stimulates the cancer cells to grow. This means that the growth of the cancer cells can be down regulated by the oppositely active hormones or so-called anti-hormones. Therefore, a hormone therapy is possible as an effective agent for treatment[2]. Alterations in different genes are involved in the development of breast cancer[3] and alterations in crucial pathways related to proliferation and apoptosis have been used as targets for treatment[4].

Plasma estrogen levels have been measured in many research studies and have been used to direct clinical management in patients with breast cancer. There is a significant demand in the clinic and in research for highly sensitive, accurate E2 measurements, particularly for samples from postmenopausal women and in relation to treatment usage[5].

Epidermal Growth Factor (EGF), a polypeptide found in human and animal blood and secretions has been found to stimulate a variety of tissues *in vitro* including normal and malignant rodent mammary epithelium and human breast epithelial cells and fibroadenoma[6]. The role of protein in regulating the growth of breast cancer cells *in vivo* is poorly understood. Human breast cancer cells in long-term culture provide a good *in vitro* model system for studying the actions of peptide hormones under defined conditions[7]. They added that the relevance of the serum expression of this protein was significantly higher in patients with breast cancer than in patients with benign breast diseases and in normal control individuals. Apoptosis is a physiological process following which normal cells die after a given number of replications. Tumor cells tend to interfere with this mechanism by activating genes which inhibit apoptosis. One of the main genes limiting apoptosis is Bcl-2. Paradoxically, Bcl-2 expression has been consistently associated with a better prognosis of breast cancer patients[8]. Doxycycline is an antibiotic used to treat various types of infections. It has the ability to inhibit protein synthesis and cell proliferation and also cause cytotoxic effect and activates the apoptotic process which gives a good image to consider the new off label use of doxycycline as a new cancer therapeutic agent.

Therefore, the present work was designed to evaluate the synergistic treatment of doxycycline (antibiotic) or cyclophosphamide (medication used as chemotherapy) with the systemic adjuvant treatment protocol in early breast cancer disease. This evaluation was done through estimation of certain biomarkers as estrogen, EGF and Bcl-2 levels in early stage breast cancer Egyptian women.

MATERIALS AND METHODS

Subjects: Healthy control subjects and patients were recruited from those attending the National Cancer Institute, Cairo University Hospital, Cairo, Egypt. Samples were collected under the National Cancer Institute ethical guidelines and the excess sera were kindly provided to the first author (SAA) for this research with their complete history, clinical examinations and treatment protocol.

Exclusion criteria: The exclusion criteria include pregnancy, lactation, metastasis, radiotherapy, severe infection and uncontrollable diseases.

Patients: Forty females were selected for this study and their ages ranged from 30-35 years. Group 1 consisted of 10 healthy voluntaries. Group 2 was 10 early diagnosis breast cancer women without metastasis. Group 3 was 10 early diagnosis breast cancer women undergo adjuvant treatment protocol beside doxycycline. Group 4 was 10 early diagnosis breast cancer women undergo adjuvant treatment protocol beside cyclophosphamide.

Treatment protocol: Patients were treated by systemic adjuvant therapy according to the NCCN guidelines for treating breast cancer (NCCN Guidelines for breast cancer, 2016). Additionally, the two groups of patients were orally administered with a dose of 100 mg doxycycline or 600 mg m^{-2} cyclophosphamide and repeat every 3 weeks for 4 cycles[9,10].

Clinical examination: All patients were clinically examined at the National Cancer Institute and diagnosed as breast cancer females of grade I. Complete history taking and physical examination were done with special emphasis on family history, contraceptive history, obstetric history, menstrual history and lactational history. Bilateral soft tissue mammography were done for patients. Full laboratory investigations (CBC, SGOT, SGPT, ALP, albumin, bilirubin, creatinine, urea, Ca) were done to all cases.

Biochemical determinations: Bcl-2, estrogen, EGF were estimated by diagnostic ELISA kits (Abcam, UK).

Statistical analysis and calculations: All data were expressed as Mean±SD of 10 women in each group. Statistical analysis was carried out by one-way analysis of variance (ANOVA), Costat Software Computer Program. Significance values between groups were at $p<0.05$.

Pearson's correlation test was done to evaluate the correlation values between ER, EGF and Bcl-2 levels at $p<0.01$ (2-tailed).

$$\text{Changes (\%)} = \frac{\text{Mean treated} - \text{Mean of control group}}{\text{Mean of control}} \times 100$$

$$\text{Improvement (\%)} = \frac{\text{Mean treated} - \text{Mean of BC group}}{\text{Mean of control}} \times 100$$

RESULTS

Effect of treatments on estrogen (ER) level: The present study revealed significant increase in estrogen level by 193.64% in BC females as compared with the normal group. Beside the systemic adjuvant therapy, treatments with doxycycline and cyclophosphamide recorded significant decrease in estrogen level by 45.13 and 33.12% as compared with the BC female patients (Table 1). Therefore, it was observed that treatment with these drugs improved the estrogen level by 132.53 and 97.25%, respectively (Table 2).

Effect of treatments on epidermal growth factor (EGF) level: In case of the epidermal growth factor level in female patients, it was observed significant increase in its level by 285.82% as compared with the normal females. Treatments with doxycycline and cyclophosphamide significantly decreased its level by 48.33 and 41.54%, respectively as compared with the BC females group (Table 1). Hence, treatment with these drugs ameliorated the EGF level by 186.47 and 160.29%, respectively (Table 2).

Effect of treatments on B-cell lymphoma-2 (Bcl-2) level: Significant increase (380.30%) in Bcl-2 level in BC female patients was observed. Treatment with doxycycline and cyclophosphamide recorded significant decrease in Bcl-2 level by 41.73 and 35.50% as compared with BC female patients (Table 1). Therefore, treatments with these drugs improved the Bcl-2 level by 200.47 and 170.53%, respectively (Table 2). Positive correlation was noticed between the parameters under investigation as expressed by Pearson's correlation test (Table 3). The estrogen level was positively correlated with EGF and Bcl-2 levels ($r = 0.924$ and 0.946) and the EGF level was also positively correlated with Bcl-2 ($r = 0.922$).

DISCUSSION

Drastic changes were observed in ER, EGF and Bcl-2 levels in breast cancer patients as compared with the healthy females. Many studies recorded an association between the risk of breast cancer and the elevated estrogen blood levels[11,12]. In accordance with current findings, several endocrine-associated risk factors are regularly associated with an increased relative risk of breast cancer in postmenopausal women[13,14]. One of these factors is obesity, which is probably related to an increased production of estrogen by aromatase activity in breast adipose tissue[15]. Another factor is an elevated blood level of endogenous estrogen[16]. An increased relative risk is also associated with higher-than-normal blood levels of androstenedione and testosterone, androgens that can be directly converted by aromatase to the estrogens estrogen and estradiol, respectively. Elevated urinary levels of estrogens and androgens are also associated with an increased risk of breast cancer in postmenopausal women[17].

Observational epidemiologic studies and a randomized clinical trial[17,18] have investigated the long-term effects of replacement therapy with estrogen alone (estrogen-replacement therapy) or with estrogen plus progestin (hormone-replacement therapy) on various health outcomes,

Table 1: ER, EGF and Bcl-2 levels in breast cancer females patients treated with doxycycline and cyclophosphamide

Groups	ER	EGF	Bcl-2
Control	33.19 ± 4.87^{d}	22.92 ± 3.24^{d}	25.08 ± 4.23^{d}
BC	97.46 ± 15.00^{a}	88.43 ± 12.70^{a}	120.46 ± 14.67^{a}
BC treated with doxycycline	53.47 ± 5.11^{bc}	45.69 ± 4.34^{bc}	66.17 ± 5.13^{bc}
BC treated with cyclophosphamide	65.18 ± 12.31^{b}	51.69 ± 9.54^{b}	75.44 ± 11.50^{b}

Values are Mean±SD of 10 females in each group, data are expressed as ng mL^{-1} for ER and Bcl-2 and pg mL^{-1} for EGF, shared letters between groups represented non-significant level, unshared letters are significant level between groups at $p<0.05$, ER: Estrogen, Bcl-2: B-cell lymphoma-2, EGF: Epidermal growth factor

Table 2: Percentage changes and improvement levels in ER, EGF and Bcl-2 in breast cancer females patients treated with doxycycline and cyclophosphamide

Groups	ER	EGF	Bcl-2
BC	(+193.64)	(+285.82)	(+380.30)
BC treated with doxycycline	[-45.13] {132.53}	[-48.33] {186.47}	[-41.73] {200.47}
BC treated with cyclophosphamide	[-33.12] {97.25}	[-41.54] {160.29}	[-35.50] {170.53}

ER: Estrogen, Bcl-2: B-cell lymphoma-2, EGF: Epidermal growth factor, ( ) are percentage changes over the normal group, [ ] are percentage changes over breast cancer group and { } are improvement percentages of ER, EGF, Bcl-2 after treatment

Table 3: Pearson's correlation values between ER, EGF and Bcl-2 levels

Parameters	ER	EGF	Bcl-2
ER	1	0.924*	0.946*
EGF		1	0.922*
Bcl-2			1

**Correlation is significant at p<0.01 (2-tailed), Bcl-2: B-cell lymphoma-2, EGF: Epidermal growth factor

including breast cancer. The biological and clinical evidence support a key role for estrogen receptor mediated effects; receptor independent pathways involving estrogen metabolites may also contribute to breast cancer initiation[19].

Observational epidemiologic studies and a randomized clinical trial[17,18] have investigated the long-term effects of replacement therapy with estrogen alone (estrogen-replacement therapy) or with estrogen plus progestin (hormone-replacement therapy) on various health outcomes, including breast cancer. The biological and clinical evidence support a key role for estrogen receptor mediated effects; receptor independent pathways involving estrogen metabolites may also contribute to breast cancer initiation[19].

The Epidermal Growth Factor (EGF) family of receptors (EGFR) is overproduced in Estrogen Receptor (ER) negative breast cancer cells. An inverse correlation of the level of EGFR and ER is observed between ER2 and ER positive breast cancer cells. A comparative study with EGFR-overproducing ER2 and low-level producing ER1 breast cancer cells suggested that EGF is a major growth-stimulating factor for ER2 cells. An outline of the pathway for the EGF-induced enhanced proliferation of ER2 human breast cancer cells is proposed. The transmission of mitogenic signal induced by EGF–EGFR interaction is mediated *via* activation of nuclear factor κB (NF-κB)[7]. This is go in line with the present study through the over production of EGF in breast cancer females.

Bcl-2 protein is a member of the Bcl-2 family that regulates apoptosis. The Bcl-2 gene encodes a MR 26000 protein that is mainly localized in the mitochondrial membrane and, to a lesser extent, in the nuclear membrane and the endoplasmic reticulum[1]. Its implication in carcinogenesis and progression makes this gene worthy of investigation. Its tumourigenic potential has been demonstrated: Bcl-2 protein blocks apoptosis and cooperates with c-myc in cell transformation[20]. In addition, in the MCF-7 breast cancer cell line the over expression of Bcl-2 enhances both tumourigenicity and metastatic potential[21]. However, in many solid organ tumours, including breast cancer and in good line with current results, Bcl-2 expression, paradoxically, is associated with favorable prognostic features and good outcome[22]. Interestingly, the expression of Bcl-2 is higher in screen-detected cancers than in symptomatic cancers[23] and in a recent report, Bcl-2 expression was shown to be lower in the stroma of precancerous fibro adenoma lesions than in those of non-cancerous lesions[24].

In the present study, treatment with doxycycline recorded significant decrease in estrogen, EGF and Bcl-2 levels. It was observed that the mitochondrial proteins in breast cancer stem cell (CSCs), defined by their capability to grow in suspension as mammospheres is higher than the bulk of cancer cells that grow under regular conditions[25]. This discovery led to the hypothesis that such increase in mitochondrial protein abundance could be reversed by treating these mammospheres with antibiotics, hence eradicating the CSCs population. Indeed several antibiotics, such as doxycycline were able to inhibit mammosphere formation[26]. However, the mechanism of action of doxycycline remained unidentified and in a further effort to elucidate. Particularly, one of the best doxycycline targets was DNA-PK, the catalytic subunit of the DNA-dependent protein kinase, which is required for proper non-homologous end-joining DNA repair[27], for maintenance of mitochondrial DNA integrity and copy number[28] and it confers resistance to radiation and chemotherapy. Doxycycline treatment reduced not only the general metabolic state of breast cancer cells and their capacity to resist anoikis but also inhibited their anti-oxidant response and several stem related signaling pathways including; the inhibition of which induces anoikis, radio and chemosensitivity[29,30].

Treatment of the present cases by cyclophosphamide recorded significant decrease in E2, EGF and Bcl-2 levels. This was attributed to interfere with DNA replication and DNA crosslinks[31]. The value of this regimen in reducing both recurrence and mortality from early breast cancer has been firmly approved by the overviews of randomized trials of polychemotherapy[32].

In parallel with these observations, it was noticed that the ameliorating effect due to doxycycline treatment on the selected parameters exceed the treatment effect of cyclophosphamide. Therefore, further and detailed studies on its mode of action and its therapeutic effects on different biomarkers are recommended.

CONCLUSION

Adjuvant treatment protocol in parallel with doxycycline therapy recorded the most potent effect for improving estrogen, B-cell lymphoma-2 and epidermal growth factor levels. More detailed studies are needed in this filed.

SIGNIFICANCE STATEMENT

This study explored the new off label use of doxycycline that can be beneficial for being anti-cancer agent beside the

well-established adjuvant treatment protocol in breast cancer patients through certain biomarkers. This study will help the researchers to cover the critical areas of discovering new anticancer drugs that many researchers were not able to explore.

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