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Depression enhanced the production of autoantibodies against 16α-hydroxyestrone-estrogen receptor adduct in breast cancer

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ABSTRACT

Mentally depressed breast cancer (MDBC) patients expressed estrogen receptor (ER) and 16α-hydroxyestrone (16α-OHE_1) is directly responsible for causing breast cancer (BC). This study aimed to identify whether depression in breast cancer patients enhanced the production of autoantibodies against 16α-OHE_1-ER adduct in breast cancer patients. The antibodies in the serum of 65 breast cancer patients (including 35 MDBC) and 40 control subjects were screened by direct binding, inhibition enzyme-linked immunosorbent assay (ELISA), and quantitative precipitin titration. Competition ELISA was also utilized for the estimation of 16α-OHE_1 in the serum of 30 cancer patients. Autoantibodies from MDBC showed strong recognition to 16α-OHE_1-ER in comparison to overall breast cancer patients ($p < 0.05$) and control subjects ($p < 0.001$). Although breast cancer sera showed high binding to 16α-OHE_1-ER in comparison to ER ($p < 0.05$) or 16α-OHE_1 ($p < 0.001$), the relative affinities of autoantibodies for 16α-OHE_1-ER were found to be 1.38×10^{-7} and 1.23×10^{-7} for breast cancer and MDBC patients respectively. No significant difference, either in the level of 16α-OHE_1 or 2-hydroxyestrone/16α-OHE_1 ratio, was observed in the serum of cancer patients compared with controls, although inflammatory cytokines (IL-6, TNF-α) were significantly high in these patients. Depression in breast cancer patients augments the production of autoantibodies against 16α-OHE_1-ER through the generation of inflammatory conditions. Depression in these patients increased the release of pro-inflammatory cytokines that generate more autoantibodies and show strong binding with 16α-OHE_1-ER.

1. Introduction

It has been well established that certain estrogen metabolites are biologically active and known to cause cancer [1]. 16α-Hydroxyestrone exerts its effects through covalent binding to the estrogen receptor [2] while 2-hydroxyestrone (2-OHE_1) has anti-estrogen effects and binds to the estrogen receptor with the same affinity as estradiol [3]. Circulating estrogen levels are positively associated with breast cancer [4] and their metabolism potentially yield active metabolites that are estrogenic and genotoxic [5,6]. The production of genotoxic or antiestrogenic metabolites might depend on the oxidation of estrogen, which might produce a variety of metabolites with various activities. Oxidation of estrogen occurs mainly at two locations, one at C-2 and another one at C-16, yielding 2-hydroxyestrone and 16α-hydroxyestrone respectively [5,7]. However, oxidation also occurs at C-4, yielding 4-hydroxyestrogen. 16α-hydroxyestrone stimulates cell proliferation in estrogen receptor positive breast cancer cell lines and has greater estrogenic activities than 2-hydroxyestrone [8,9]. Earlier studies have shown that hydroxyestrogen, such as 4-hydroxy and 16-hydroxyestrogen, are capable of inducing DNA damaged directly through the formation of semiquinone and quinone, which make adducts with DNA or indirectly through the redox cycling, thereby producing more reactive oxygen species (ROS) that alter or modify DNA [7,10]. In addition, the genotoxicity of 16α-hydroxyestrone has been addressed by two studies: one *in vitro* study shows an increment in unscheduled DNA repair and cell proliferation in mouse mammary epithelial cells [11] and another that reports that the process of 16α-hydroxylation of estrogen was strongly related with an increased rate of the mammary tumor in multiple mouse strains [12]. Other human studies also explain the association of extent of 16α-hydroxylation of estradiol with the breast cancer in which this process was found to be greater in cancer patients compared to controls in the blood and terminal duct lobular

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units [13]. However, other investigators have assumed that catecholestrogen rather than 16α-OHE_1 increases the risk of breast cancer [10,14].

16α-Hydroxyestrone is one of the important estrogen metabolites formed during the oxidative estrogen metabolic pathway. Such metabolism not only promotes unscheduled DNA in mouse mammary epithelial cycle [11] but is also associated with increased proliferation of mammary cells [15]. Urinary 16α-hydroxyestrone is also associated (directly or indirectly) with breast cancer as it increased proliferation of mammary cells [15], expression of the *Ras* gene [16], and increased mammary tumor occurrence [12]. Antibodies against HER-2/neu in the sera of patients with breast cancer have been described in early-stage tumors [17]. Antibodies have also been described against p53 and related family member p73 in the serum of these breast cancer patients [18,19]. Monoclonal antibodies against HER-2/neu have demonstrated considerable efficacy in breast cancer [20]. Trastuzumab, the only humanized anti-HER-2 antibody approved by the U.S. Food and Drug Administration, used as a single agent in first-line treatment of metastatic breast cancer [21]. Recently, an antibody fragment, LH8, which is specific for breast cancer cells, have been generated to bind to specific breast cancer subpopulations [22]. Regarding the efficacy of trastuzumab on animal models of breast cancer, it is found that trastuzumab treatment caused a substantial reduction in tumor growth in treated animals [23]. Autoantibodies from sera of breast cancer patients activate muscarinic acetylcholine receptors in tumor cells [24]. These autoantibodies in breast cancer patients promote tumor progression by activating muscarinic acetylcholine receptor (mAChR) in tumor cells. Therefore, a deficient functional activity of dendritic cells in cancer was observed [25].

Many breast cancer patients suffer from major depression, which may cause functional impairment or treatment adherence [26] and low immunity. Individual differences in the depressive symptoms were observed in these breast cancer patients. There is a positive correlation between hormone receptor status and depressive symptoms in breast cancer patients [27]. Approximately 60–75% of breast cancer patients are hormone receptor-positive at the time of diagnosis, where the hormone receptor is either estrogen receptor, progesterone receptor or both [28]. A recent study has reported that breast cancer patients show considerable distress and depression symptoms [29]. This depression in cancer patients is influenced by the diagnosis of cancer, the choice of drug, and therapy side effects [30]. The level of anxiety, depression and risk factors increased after the diagnosis of breast cancer [31]. Endocrine treatment, particularly tamoxifen, was strongly correlated with depressive symptoms in multiple breast cancer patients [32].

In this study, we investigate the affinity of breast cancer autoantibodies to 16α-OHE_1-ER, taking advantage of the fact that MDBC patients express the estrogen receptor and 16α-OHE_1 is directly responsible for causing breast cancer. This creates an opportunity to screen breast cancer (and MDBC) sera with 16α-OHE_1-ER adduct to elucidate their potential role. 16α-OHE_1-ER was also used as a challenge antigen to produce antibodies that can be used as a probe for the estimation of 16α-OHE_1 and 2-hydroxyestrone/16α-OHE_1 (2-OHE_1/16α-OHE_1) ratio in breast cancer patients. The combined effect of 16α-OHE_1 and ER have been evaluated and discussed.

2. Material and methods

2.1. Patients and controls

Blood samples were collected from 65 breast cancer patients (including 35 MDBC) and 40 normal subjects, who served as negative controls and were without symptoms of cancer. The clinical and laboratory data of these subjects are given in Table I (age, disease duration, menopausal status, etc.). The control group consisted of women coming to the hospital for routine checkup or hospital staff free from breast cancer. All patients and controls underwent the same diagnostic procedure, and the patients were confirmed by the physician based on the various procedures for breast cancer (e.g., physical examination, mammography, histopathology, etc.). Exclusion criteria for these patients include pregnancy or lactation, patients with tamoxifen therapy, thyroid medication, antibiotics or alcohol drinker. The Self-Rating Depression Scale questionnaire was administered to screen the patients and determine their level of depression. We adopt a modified version of the previously used questionnaire [33] to screen the patients' level of depression. The depression index score was evaluated as the total score from 20 questions divided by 80 (the maximum possible score). Spot urine samples were also obtained from 40 patients. Serum samples were isolated from all participants and heated at 56 °C for 30 min to deactivate complement protein, and were then stored at −80 °C with 0.2% sodium azide as a preservative. This work has been approved by the Institutional Ethical Committee, and prior consent of the patients and controls subjects was obtained.

2.2. 16α-OHE_1-ER adducts formation

16α-OHE_1-ER adducts were formed as mentioned earlier [34] with slight modification. 16α-OHE_1 (1–10 mM) was incubated with 1 mg of estrogen receptor (ER) in 0.1 M potassium phosphate buffer pH 6. Sodium cyanoborohydride (1 μM) was incubated at 37 °C for 48 h with gentle shaking. 16α-hydroxyestrone was dissolved in ethanol, in which the concentration of ethanol was 0.1% of the total reaction mixture. Excess of 16α-OHE_1 and ER, which were not bound to each other, was removed by dialyzing the whole mixture with phosphate buffer saline (PBS), pH 7.4.

2.3. Antibodies against 16α-OHE_1-ER

Antibodies against 16α-OHE_1-ER and their controls were induced in experimental animals (female rabbits) as described elsewhere [35]. Briefly, 50 μg of respective antigen were emulsified with an equal volume of complete Freund's adjuvant, and the mixture injected intramuscularly. Later, injections were given when the antigens were emulsified with incomplete Freund's adjuvant. Each animal received a total dose of 400 μg given in eight injections, weekly. Blood was collected and stored at −20 °C with 0.1% sodium azide. Pre-immune sera were collected prior to immunization, which serves as negative controls.

2.4. Enzyme-linked immunosorbent assay

Direct binding ELISA was performed to detect the presence of antibodies against 16α-OHE_1-ER, ER, and 16α-OHE_1 in breast cancer sera/immunized animal, as described elsewhere [26]. Competition ELISA was also done to evaluate specific recognition of antibodies (breast cancer patients/immunized antibodies) to 16α-OHE_1-ER, ER, 16α-OHE_1 [36]. Briefly, 100 μl of various antigens (16α-OHE_1-ER, ER, 16α-OHE_1) with a concentration of 2.5 μg/ml were coated onto microtiter plates, incubated for 3 h at 25 °C and then overnight at 4 °C. After overnight incubation, the plates were washed with TBS-T (Tris Buffer Saline with Tween 20) and blocked by 150 μl of BSA (Bovine Serum Albumin) (1.5%). Immune complexes were formed by incubating 100 μl of 1:100 dilutions of breast cancer/immunized sera with an increasing amount of antigens (0–20 μg/ml). The reaction mixture was incubated at 37 °C for 2 h and then at 4 °C for 12 h. Immune complexes (100 μl) were added, followed by addition of anti-human IgG-alkaline phosphate conjugate in each well. We then waited until the reaction was developed by adding *p*-nitrophenyl phosphate as substrate and, finally, a reading was taken at 410 nm on to the microtiter plate. For estimation of 16α-OHE_1 and detection of 2-OHE_1/16α-OHE_1 ratio, we used ELISA Kit (Glory Science Co. Lt, USA) and Estramet 2-OHE_1/16α-OHE_1 ELISA Kit (CD Diagnostics, NY, USA). Human IL-6 ELISA Kit and Human IL-1 ELISA Kit (Sigma-Aldrich,

Table I
Clinical data and estimation of 16α-OHE_1 by anti-16α-OHE_1-ER antibodies in breast cancer patients.

Characteristics	Breast cancer (n = 65)	MDBC (n = 35)	Controls (n = 40)
Age (years)	63 ± 8	62 ± 7	61 ± 5
Disease duration (years)	8.3 ± 3.1	8.4 ± 4.2	_
Estrogen receptor			
Positive (n)	37 (56.9%)	19 (54.2%)	_
Negative (n)	28 (43%)	16 (45.7%)	_
Menopausal status			
Premenopausal (n)	30 (46%)	17 (48.5%)	_
Postmenopausal (n)	35 (53.8%)	18 (51.4%)	_
BMI, kg/m^2 (mean)	27.2	28.3	26.3
Family history of breast cancer (%)	20.3	22.8	14
16α-Hydroxyestrone estimation in serum (n = 30) by[a]			
Anti-16α-OHE_1-ER antibodies	354 pg/ml[c]	365 pg/ml	351 pg/ml[e]
Human 16α-hydroxyestrone ELISA kits	350 pg/ml	361 pg/ml	–
2-OHE_1/16α-OHE_1 ratio	0.36	0.37	0.35
Inflammatory cytokines estimation[b]			
IL-6	39.3 ± 18.3 pg/ml[d]	38.9 ± 17.1 pg/ml	2.1 ± 1.3 pg/ml
IL-1	3.8 ± 2.9 pg/ml	3.9 ± 2.1 pg/ml	2.8 ± 1.5 pg/ml
TNF-α	1.99 ± 0.39 pg/ml[#]	2.2 ± 0.23 pg/ml	0.93 ± 0.29 pg/ml
IL-12	140.2 ± 15.1 pg/ml	144.8 ± 20.3 pg/ml	138.9 ± 30.3 pg/ml

2-OHE1/16α-OHE_1; 2-hydroxyestrone/16α-hydroxyestrone ratio and measured by commercially available kit.
[a] The amount of 16α-OHE_1 level was measured by ELISA and the values are presented in median. The concentrations of IL-6 and IL-1 are given in mean ± SD.
[b] n = 30.
[c] Correlation coefficient r = 0.94 ($p < 0.001$).
[d] $p < 0.001$.
[e] n = 20.
[#] $p < 0.01$.

Chemie GmbH, Germany) were used for IL-6 and IL-1 estimation. TNF-alpha (TNF-α) High Sensitivity ELISA Kit (IBL International GmbH, Germany) and Interleukin 12 alpha ELISA Kit (Biocompare CA, USA) were used for TNF-α and IL-12 estimation.

2.5. Purification of anti-16α-OHE_1-ER antibodies

IgG was isolated from breast cancer/immune sera on Protein A-Agarose [37]. Homogeneity of the purified IgG was checked on 7.5% SDS-PAGE. The concentration of IgG was evaluated by considering 1.40 OD_{280} = 1.0 mg/ml.

2.6. Quantitation of affinity of antibodies from breast cancer patients

Formation and quantitation of immune complexes between the antigens and their respective antibodies from the breast cancer patients (MDBC) were done as described previously [38]. Briefly, 100 µg of immunoglobulin from breast cancer patients were incubated with various amounts (0–40 µg) of antigens (16α-OHE_1, ER, 16α-OHE_1) in a total volume of 500 µl. Normal human IgG serves as a negative control. The reaction mixture was incubated at 25 °C for 2 h and overnight at 4 °C. Immune complexes were pelleted, washed with PBS and dissolved in 250 µl NaCl (1 N). Free and bound protein was estimated by colorimetric methods [39]. The date was used to calculate the affinity of the antibodies from the breast cancer patients [40].

2.7. Statistical analysis

Statistical significance was determined using the student's *t*-test. A p-value of < 0.05 was considered statistical significance.

3. Results

3.1. Characterization of 16α-OHE_1-ER

Fig. 1 shows the electrophoretic pattern of 16α-OHE_1-ER, ER, and 16α-OHE_1 separately. Incubation of 16α-OHE_1 with ER resulted in the formation of high molecular weight complex (Fig. 1, insert: Lane 3) that showed less migration compared to either ER or 16α-OHE_1 (Fig. 1, insert: Lane 1, 2). The molecular weight of 16α-OHE_1-ER is close to ≈68 kDa, as revealed in the SDS-PAGE. 16α-OHE_1-ER showed high absorbance, and about 38.3% UV hyperchromicity at 280 nm was observed, demonstrating the formation of a high molecular adduct (Fig. 1).

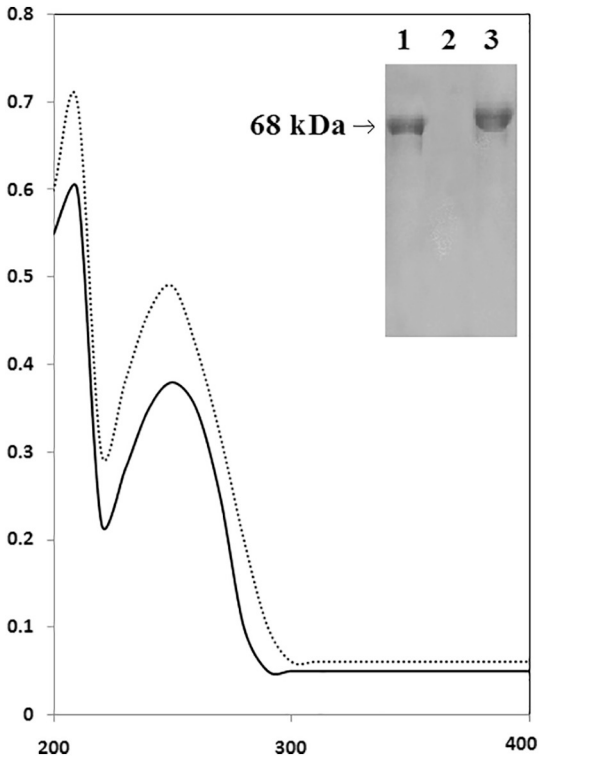

Fig. 1. UV absorption spectra of 16α-OHE_1-ER adduct (·····) and ER (———). Insert: SDS–polyacrylamide gel electrophoresis of 16α-OHE_1-ER adduct and controls. Lanes: 1- ER, 2- 16α-OHE_1 & 3- 16α-OHE_1-ER adduct.

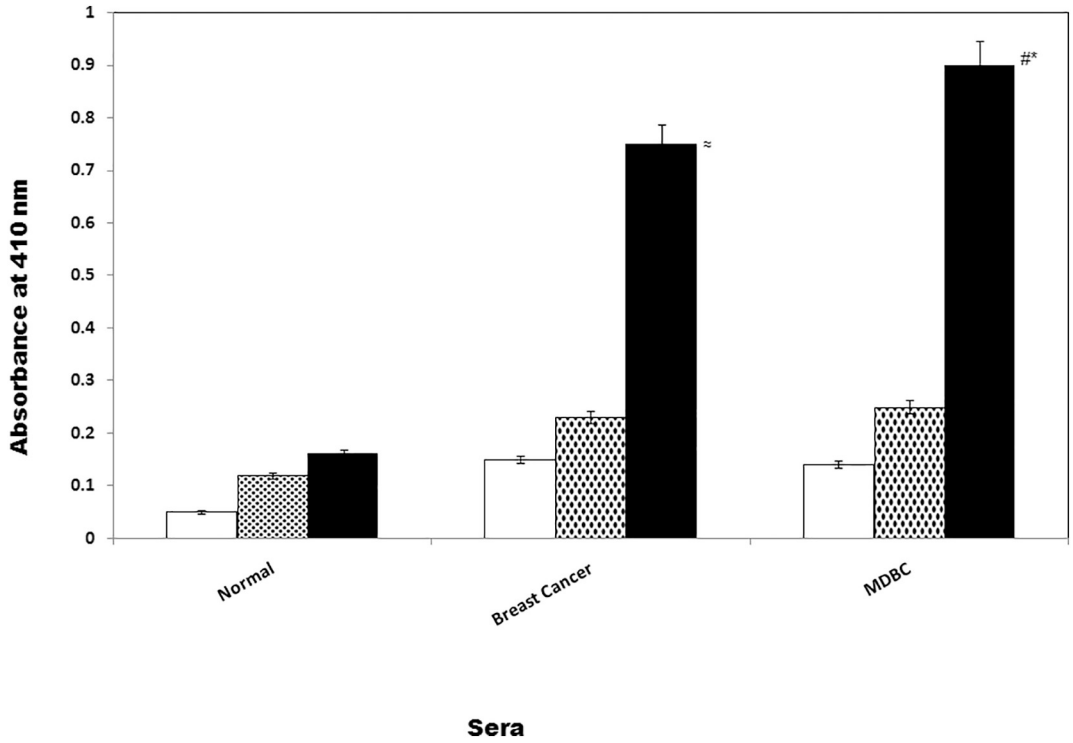

Fig. 2. Direct binding ELISA of control, breast cancer and MDBC patients. Direct binding enzyme-linked immunosorbent assay of control (n = 40), breast cancer (n = 65) and MDBC patient's antibodies (n = 35) to 16α-OHE$_1$-ER (■), ER (▒) and 16α-OHE$_1$ (□). Microtitre plates were coated with 100 µl of respective antigen (2.5 µg/ml). The reaction was developed with *p*-nitrophenyl phosphate as the substrate and the absorbance was recorded at 410 nm as describe in "Material and methods" section. Each histogram represents the mean ± SD (Bar represent ± SD). $^{\#}p < 0.001$, significantly higher binding than normal sera, $^{*}p < 0.05$, significantly higher binding than breast cancer sera, $^{\approx}p < 0.001$, significantly higher binding than normal sera.

3.2. Anti-16α-OHE$_1$-ER antibodies in the sera of breast cancer patients

Breast cancer sera (n = 65) were checked for the presence of antibodies against 16α-OHE$_1$-ER, ER, and 16α-OHE$_1$ by direct binding ELISA. We have chosen 65 sera out of 115 breast cancer sera that show high recognition to 16α-OHE$_1$-ER in comparison to ER ($p < 0.05$) or 16α-OHE$_1$ ($p < 0.001$) (Fig. 2). The recognition was highest for MDBC patients with 16α-OHE$_1$-ER, which was even higher than for the overall breast cancer sera ($p < 0.05$).

Competition inhibition ELISA was used to evaluate the binding specificity of cancer autoantibodies towards 16α-OHE$_1$-ER, ER, and 16α-OHE$_1$. The 16α-OHE$_1$-ER had shown an inhibition of about 60.3 ± 8.5% (35.8% to 80.5%) in 65 breast cancer sera, while ER and 16α-OHE$_1$ showed lower inhibition ranging from 18.3–49.8% (31.3 ± 5.4%) for ER and 4.5% to 18.1% (9.3 ± 3.6%) for the 16α-OHE$_1$ (Fig. 3a). MDBC patient's sera (n = 35) showed an inhibition of 65.8 ± 4.8 (36.8% to 88.5%) with 16α-OHE$_1$-ER as an inhibitor, which was higher than the overall breast cancer sera.

Breast cancer IgG was isolated and purified by affinity chromatography on Protein A-Agarose Column (Sigma, USA). The purity of the IgG obtained from BC patient's sera was checked on SDS-PAGE under non-reducing conditions, which showed a single homogenous band on the gel, and purified IgG was eluted as a single symmetrical peak on the column. In competition ELISA, the cancer IgG showed an inhibition ranging from 42.3% to 86.3% (68.3 ± 6.3%) for 16α-OHE$_1$-ER. ER showed an inhibition of about 36.3 ± 4.1% (20.8% to 57.3%) (Fig. 3b). 16α-OHE$_1$ showed inhibition of about 11.3 ± 3.1% (5.4% to 24.2%). Again, IgG from MDBC patients showed an inhibition of about 74.8 ± 6.5%, which was the highest among all other groups of cancer IgGs (Fig. 3b). We have also tested the inhibition values according to the different groups in breast cancer patients (Table II). Among all, as described previously, MDBC patients showed the highest inhibition (i.e., 74.8 ± 6.5%), followed by patients with chemotherapy (73.4 ± 8.1%), surgery + chemotherapy (70.8 ± 6.3%), and positive estrogen receptor (70.3 ± 4.5%). While for other groups, such as menopausal status and smoking, follow-up periods have no major difference in the inhibition values (Table II).

The antigen-antibodies interactions were also evaluated by quantitative precipitation titration. In this procedure, an increasing amount of antigens (16α-OHE$_1$-ER, ER and 16α-OHE$_1$) were added to the constant amount of breast cancer IgG (100 µg, n = 15). Normal human IgGs served as a negative control. We found that 23 µg of 16α-OHE$_1$-ER was bound to about 75 µg of breast cancer IgG and 21 µg of 16α-OHE$_1$-ER was bound about 78 µg of MDBC patient's IgG. With ER, 41 µg of ER was bound to approximately 60 µg of cancer IgG. For 16α-OHE$_1$, a maximum of about 48 µg of antigen was bound to about 58 µg of cancer IgG. A Langmuir plot was used to determine the apparent association constant (Fig. 4). The constants, as estimated by Langmuir plot, were 1.38×10^{-7} M, 1.71×10^{-6} M, and 1.21×10^{-6} M for 16α-OHE$_1$-ER, ER, and 16α-OHE$_1$ with cancer IgG respectively. Again, for MDBC patients, the constant was found to be of the order of 1.23×10^{-7} M, which showed the maximum affinity of IgG with 16α-OHE$_1$-ER.

3.3. Anti-16α-OHE$_1$-ER antibodies and their characterization

The antigenicity of antigens (16α-OHE$_1$-ER, ER and 16α-OHE$_1$) was also evaluated by trigging antibodies in experimental animals (female rabbits) against these antigens. Direct binding and competition ELISA were used to assay antigenic specificity of the induced antibodies. Specific immune responses against 16α-OHE$_1$-ER, ER, and 16α-OHE$_1$ were characterized by direct binding ELISA. The 16α-OHE$_1$-ER was highly immunogenic, trigging high titer antibodies (≥1:25600). Pre-immune sera served as control and did not show any appreciable binding to 16α-OHE$_1$-ER. After immunization with ER, the titer shown by this antigen was almost the same (but low) as 16α-OHE$_1$-ER. 16α-OHE$_1$ showed very low titer after immunization and is considered

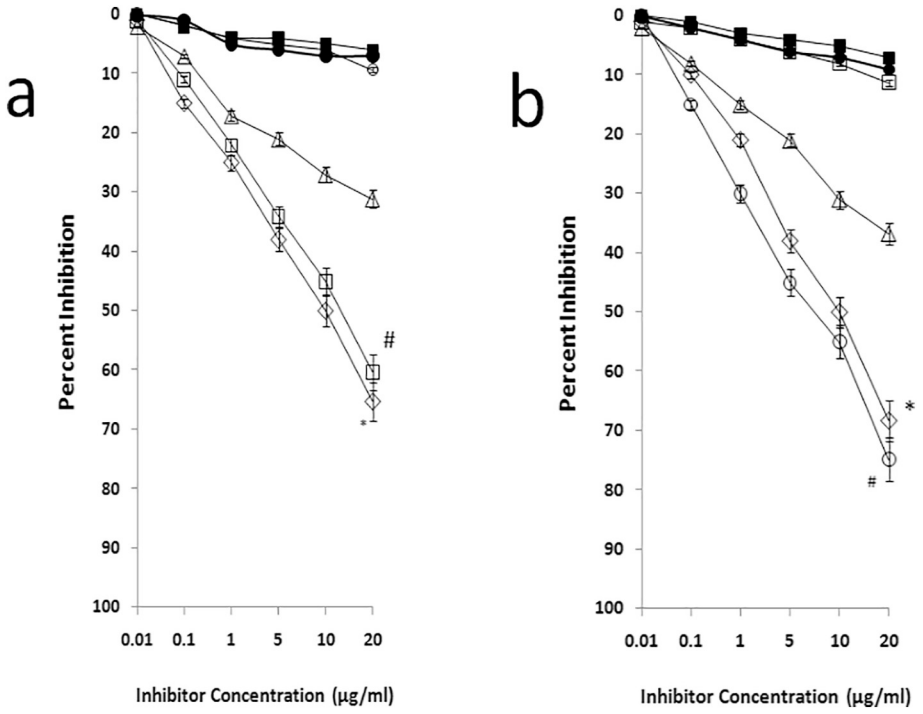

Fig. 3. Inhibition ELISA of control, breast cancer and MDBC patients. a) Inhibition ELISA of anti-(16α-OHE_1-ER, ER, 16α-OHE_1) breast cancer & MDBC (-□-, -Δ-, -○- & -◊-) and Control (-●-, -■-) sera with 16α-OHE_1-ER, ER, 16α-OHE_1. b) Inhibition of breast cancer & MDBC anti-(16α-OHE_1-ER, ER, 16α-OHE_1) IgG binding to 16α-OHE_1-ER (-◊- & -○-), ER (-Δ-), 16α-OHE_1 (-□-). (-●-, -■-) Represent the inhibition of normal anti-16α-OHE_1-ER and ER IgG binding to 16α-OHE_1-ER and ER. Microtitre plates were coated with respective antigens (2.5 µg/ml). Immune complexes were prepared by mixing 100 ml of 1:100 dilution of serum antibodies from breast cancer patients and control individuals, with the increasing amount (0–20 mg/ml) of respective antigens at 37 °C. *Note*: inhibition values for control sera and IgG with 16α-OHE_1 were negligible and are not shown. *# Significantly higher inhibition than ER ($p < 0.05$, $p < 0.05$) and 16α-OHE_1 ($p < 0.001$, $p < 0.001$). Each points represent mean ± SD for 63 BC sera/IgGs, 35 MDBC sera/IgGs and 40 controls sera/IgGs.

Table II
Immunological data (antibodies) of different breast cancer patients and healthy controls.

Breast cancer patients (n = 65)	Maximum percent (%) inhibition at 20 µg/ml		
	16α-OHE_1-ER[a]	ER[b]	16α-OHE_1[c]
Overall	68.3 ± 8.3	36.3 ± 4.1	11.3 ± 3.1
MDBC patients (n = 35)	74.8 ± 6.5	37.5 ± 8.5	11.9 ± 3.8
Menopausal status			
Premenopausal (n = 30)	67.4 ± 8.1	38.5 ± 5.4	10.9 ± 2.1
Postmenopausal (n = 35)	69.3 ± 7.3	34.3 ± 7.1	9.8 ± 3.2
Estrogen receptor			
Positive (n = 37)	70.3 ± 4.5	37.8 ± 4.1	7.8 ± 3.1
Negative (n = 28)	65.8 ± 8.2	36.7 ± 3.3	8.3 ± 4.1
Smoking at baseline			
Current (14)	67.3 ± 4.5	38.3 ± 3.5	11.9 ± 4.2
Past/never (50)	66.3 ± 7.1	37.8 ± 4.1	10.8 ± 5.1
Treatment			
None (n = 20)	67.8 ± 5.8	37.9 ± 3.4	9.4 ± 3.2
Surgery (n = 21)	68.9 ± 7.1	38.5 ± 4.1	12.8 ± 5.7
Chemotherapy (n = 10)	73.4 ± 8.1	39.5 ± 3.8	11.3 ± 6.2
Surgery + radiotherapy (n = 9)	69.3 ± 5.1	35.8 ± 5.2	12.1 ± 3.3
Surgery + chemotherapy (n = 5)	70.8 ± 6.3	33.7 ± 4.1	12.9 ± 8.5
Follow-up period			
6 month–2 years (n = 15)	67.3 ± 8.1	37.3 ± 5.4	11.9 ± 4.2
2–5 years (n = 20)	65.8 ± 7.3	36.4 ± 3.5	12.8 ± 3.1
>5 years (n = 30)	66.9 ± 9.1	38.3 ± 4.1	11.7 ± 3.9
Depressed normal subjects (n = 25)	10.8 ± 5.8	9.3 ± 5.8	8.4 ± 4.1
Control*	9.3 ± 3.1	10.8 ± 3.4	9.4 ± 1.3

The experiments were carried out by incubating ELISA plate with 100 µl of different antigens (2.5 µg/ml) as described in "Material and methods" section; mean ± SD.

* 16α-OHE_1-ER vs ER or 16α-OHE_1 ($p < 0.05$, $p < 0.001$).

[a] 16α-OHE_1-ER as inhibitor.

[b] ER as inhibitor.

[c] 16α-OHE_1 as inhibitor.

to be negligible. The competition ELISA further confirmed the specificity of induced IgG. An inhibition of about 75.3% in the antibody was observed at a 16α-OHE_1-ER concentration of 20 µg/ml. The concentration of 16α-OHE_1-ER required for 50% inhibition was 7.7 µg/ml (Fig. 5a). For ER, an inhibition of about 71.8% was observed in the antibody activity at an immunogen concentration of 20 µg/ml, and 50% inhibition was achieved at about 13.8 µg/ml of immunogen (Fig. 5a). Induced IgG from the pre-immune and immune sera was isolated by affinity chromatography on a Protein A-Agarose column, as described previously. The purity of the isolated IgG was checked on SDS-PAGE under the non-reducing condition, and it was found that purified IgG migrated as a single band. Direct binding ELISA showed strong recognition to 16α-OHE_1-ER. Pre-immune IgG served as a negative control. The specific recognition of purified IgG with their respective antigens was also evaluated with competition inhibition assays. In competition ELISA, anti-16α-OHE_1-ER antibodies showed strong binding to the immunogen (16α-OHE_1-ER) cause inhibition of 95% inhibition in the antibody binding at 20 µg/ml of the immunogen and 50% inhibition was observed only at 3.4 µg/ml (Fig. 5b). While for ER, an inhibition of about 91% in the antibody binding was observed with ER as the inhibitor. The concentration for 50% inhibition was 8.3 µg/ml.

The specific antigenicity of the purified induced IgG (against 16α-OHE_1-ER and ER) was also characterized by competition inhibition assays using similar molecules. The anti-16α-OHE_1-ER antibodies showed a low degree of specificity towards all other similar molecules, except 16α-OHE_1 (Table III). In contrast, anti-ER antibodies demonstrated a high degree of binding towards 16α-OHE_1-ER, showing that some of the epitopes of this antigen are common for this antibody. Although anti-16α-OHE_1 antibodies do not show any reactivity with any other antigen, except 16α-OHE_1-ER, anti-ER antibodies also showed cross-reactivity with 16α-OHE_1-ER, indicating the presence of some common epitopes on this antigen.

As anti-16α-OHE_1-ER antibodies showed cross-reactivity with 16α-OHE_1, this gives us the opportunity to estimate 16α-OHE_1 in the sera of breast cancer patients. This also allows us to check whether anti-16α-OHE_1-ER antibodies have sufficient specificity to estimate 16α-OHE_1 in the sera of these patients, which was comparable with the

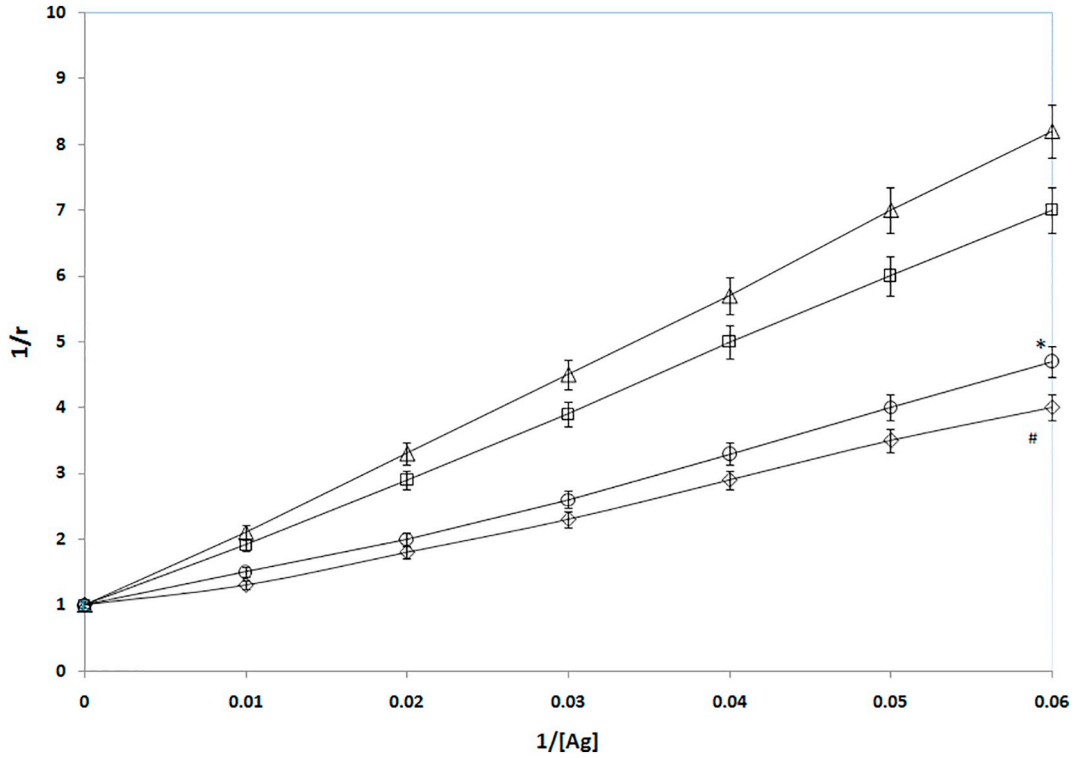

Fig. 4. Determination of an apparent association constant by Langmuir plot. Antigens were 16α-OHE_1-ER (-○- & -◊-), ER (-□-) and 16α-OHE_1 (-Δ-). Immune complexes were prepared by incubating 100 mg of IgG (breast cancer, MDBC and controls) with varying amount of different antigens (0–100 mg) in an assay volume of 500 ml for 2 h at room temperature and overnight at 4 °C. The binding data were analyzed for antibody affinity as described in Material and methods section. #Significantly higher binding than ER ($p < 0.05$) and 16α-OHE_1 ($p < 0.001$). *Significantly higher binding than ER ($p < 0.05$) and 16α-OHE_1 ($p < 0.001$). Each points represent mean ± SD for 15 BC IgGs and 15 MDBC IgGs.

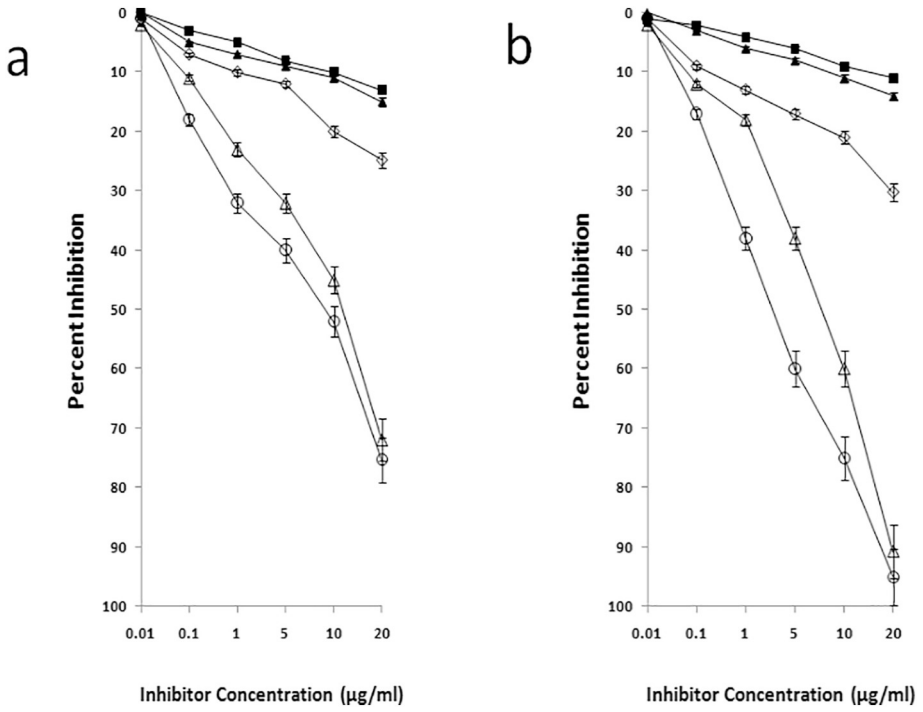

Fig. 5. Inhibition ELISA of immunized antigens. a) Inhibition ELISA of immune sera (-○-,-Δ-, -◊-) and pre-immune sera (-▲-, -■-) pre-incubated with 16α-OHE_1-ER, ER, 16α-OHE_1, on binding to antigen coated plate. b) Inhibition ELISA of immune IgG pre-incubated with 16α-OHE1-ER (-○-), ER (-Δ-), 16α-OHE_1 (-◊-), on the binding to antigen coated plates. (-▲-, -■-) represent the inhibition of pre-immune anti-16α-OHE_1-ER and ER IgG binding to 16α-OHE_1-ER and ER. The experiments were carried out by incubating ELISA plate with 100 µl of respective antigens (2.5 µg/ml) as described in "Material and methods" section. Each points represent mean ± SD for 6 independent inhibition values for immune sera/IgGs and pre-immune sera/IgGs.

concentration estimated with commercially available kits (ELISA kits, Glory Science Co. Lt., USA). The level of 16α-OHE_1 in the sera of breast cancer patients (n = 30) and MDBC (n = 35), as estimated by anti-16α-OHE_1-ER antibodies, was found to be 354 pg/ml and 365 pg/ml, which was comparable with the data obtained by using a commercially available kit (350 pg/ml and 361 pg/ml). In healthy controls (n = 15), the mean value of 16α-OHE_1 in the sera was found to be 351 pg/ml (Table I). There is no significant difference either in 16α-OHE_1 concentration or 2-OHE_1/16α-OHE_1 ratio when comparing breast cancer patients and controls. The pro-inflammatory cytokines, such as IL-6 and

Table III
Relative affinity of induced antibodies towards different inhibitors.

Inhibitors	Maximum % inhibition at 20 µg/ml	Concentration for 50% inhibition (µg/ml)	Percent relative affinity
Anti-16α-OHE_1-ER antibodies			
16α-OHE_1-ER	95	3.4	100
ER	21.8	[b]	-
16α-OHE_1	72.8	5.8	58.6
PR[a]	19.8	-	
2-OHE_1	10.9	-	-
Anti-ER antibodies			
ER	91	8.3	100
16α-OHE_1-ER	68.8	10.5	79
16α-OHE_1	10.3	-	-
PR	9.4	-	-
2-OHE_1	10.2	-	-
Anti-16α-OHE_1 antibodies			
16α-OHE_1	82.9	12.5	100
16α-OHE_1-ER	71.8	13.8	90.5
ER	10.8	-	-
PR	9.7	-	-
2-OHE_1	10.3	-	-

The experiments were carried out by incubating ELISA plate with 100 µl of different antigens (16α-OHE_1-ER, ER, 16α-OHE_1) at 2.5 µg/ml. Rest of the experimental conditions were explained in "Material and methods" section.

[a] PR: progesterone receptor, 2-OHE_1: 2-hydroxyestrone.

[b] 50% inhibition was not achieved.

TNF-α, were found to be significantly higher in the cancer patients compared to controls ($p < 0.001$, $p < 0.01$) (breast cancer patients = 39.3 ± 18.3 pg/ml, 1.99 ± 0.39 pg/ml, control subjects = 2.1 ± 1.3 pg/ml, 0.93 ± 0.29 pg/ml), although, there is no significant difference in the concentration of IL-1 and IL-12 in breast cancer patients compared to controls. This further shows the generation of pro-inflammatory conditions in MDBC patients.

4. Discussion

Breast cancer is the most common and leading cause of death among women worldwide. In Latin America, breast cancer is responsible for the highest number of diagnosis and death [41]. Epidemiological data has shown that breast cancer causes about 33% of all neoplasms and is responsible for about 20% of death caused by cancer [42]. However, the mortality rate has been decreasing because of new treatments and early detection techniques [43]. Previous studies have shown that breast cancer patients experience more stress and depression in comparison to the general female population [44]. Initially, patients with breast cancer experience more stress, especially during the various diagnostic processes and early medication. Even after a long period of treatment, patients continue to suffer from stress after one or more years, and even after completion of various treatments [45]. Long-term stress is associated with depression in these cancer patients [29]. Depression also shows a small but marginally significant association with overall cancer risk [30]. Estrogen is linked to breast cancer by activating its receptor (estrogen receptor), leading to various processes in the earlier stages of breast cancer, such as increased cell division, inhibition of cell death, and increased blood vessels formation [46]. Therefore, by blocking estrogen, it is possible to treat breast cancer for those patients with a hormone-expressing tumor, thereby preventing reoccurrence. Hormone therapy along tamoxifen (a selective estrogen receptor modulator) has been widely used for the last three decades. This therapy is used in patients with breast cancer and is based on the antagonist action of estrogen receptors in the breast tissue [47].

Considering the above as well as the considering the role played by estrogen and its receptor, this study was designed to evaluate whether depression in breast cancer might trigger the production of autoantibodies. We used 16α-OHE_1-ER adduct as an antigen because most of the breast cells express estrogen receptor and 16α-OHE_1 is directly or indirectly linked with breast cancer [15,28]. A high molecular weight adduct is formed between 16α-OHE_1 and ER. To test its etiology in this disease, the specificity and binding affinities of the breast cancer autoantibodies against 16α-OHE_1-ER have been evaluated in breast cancer patients. These adducts had a molecular weight close to about ≈68 kDa and showed about 38.3% UV hyperchromicity at 280 nm. The formation of the adduct is almost the same as in previous studies demonstrating the covalent binding of the estrogen receptor to 16α-hydroxyestrone in human breast cancer cell lines [2].

Autoantibodies against tumor-associated antigen have been well-described in the early stages of breast cancer [48]. These autoantibodies against a variety of antigens would be used in mammography for the detection and diagnosis of breast cancer especially in those women who are at high risk of breast cancer, where mammography is not sufficiently sensitive or specific [48]. Breast cancer is a heterogeneous disease in which cancer cells express a variety of antigenic protein. Sometimes, they are too insensitive to be detected in the test, and this property makes them vulnerable for early detection and diagnosis of primary breast cancer [49]. Autoantibodies against these kinds of antigens might provide a valuable tool for the early detection of the carcinogenic signal and allow us to detect cancer from current methods. Groups of autoantibodies with various specificities provide better sensitivity for the detection of breast cancer [50]. Autoantibodies specific to estrogen receptor were also found in breast cancer, where they can act as estrogen agonists, playing a pathogenic role and could function as possible peripheral blood biomarker indicative of breast cancer. The binding of antibodies from sera of 65 cancer patients and 40 controls subjects to 16α-OHE_1-ER was studied by direct binding and inhibition ELISA. The 16α-OHE_1-ER adduct demonstrates high binding with cancer sera as compared to control subjects ($p < 0.001$). These data indicate that 16α-OHE_1-ER can function as an effective inhibitor demonstrating a substantial difference in recognition of 16α-OHE_1-ER over ER or 16α-OHE_1. The data shown here is consistent with previous studies from our lab that show higher recognition of estrogen modified DNA by breast cancer autoantibodies [51]. Formation of adduct might expose unique epitopes on the antigen molecule (16α-OHE_1-ER) that can be well recognized and show a higher binding by breast cancer autoantibodies. Such recognition was not shown either by ER or 16α-OHE_1. It is interesting to note that if the cancer patients were somehow depressed, the autoantibodies showed higher recognition to this antigen, thus showing that these patients produce more antibodies that are binding more vigorously to this antigen. Although the exact mechanism is unknown, it is somehow linked with pro-inflammatory conditions. Depression in these patients increased pro-inflammatory cytokines (e.g., TNF-α, IL-6), which might arise as a result of cancer treatments [52,53]. Pro-inflammatory cytokines might be elevated even after 3–5 years of breast cancer treatment [53]. These pro-inflammatory cytokines somehow produce more autoantibodies that might bind to this antigen more vigorously. A high concentration of these cytokines further confirmed the generation of pro-inflammatory conditions in MDBC patients. However, the function of Th2 cytokines should be taken into consideration in breast cancer. Th2 cytokines include IL-4, IL-5, IL-10 that are involved in allergy and M2 polarization of macrophages, which are important in mediating humoral immunity and promote mammary tumor progression [54,55]. Th2 response mediated by IL-4 can potentiate M2-bioactivity of tumor-associated macrophages, leading to growth promotion and metastasis [55,56]. IL-10 inhibits tumor growth in a murine model of breast cancer by elaboration of chemokines, inhibition of angiogenesis, and stimulation of NO production [57]. Binding was also good for two group of cancer patients. For those cancer patients that are estrogen receptor positive, we detected higher recognition to 16α-OHE_1-ER. More than 70% of the breast cancer tumors express estrogen receptor and selective estrogen receptor

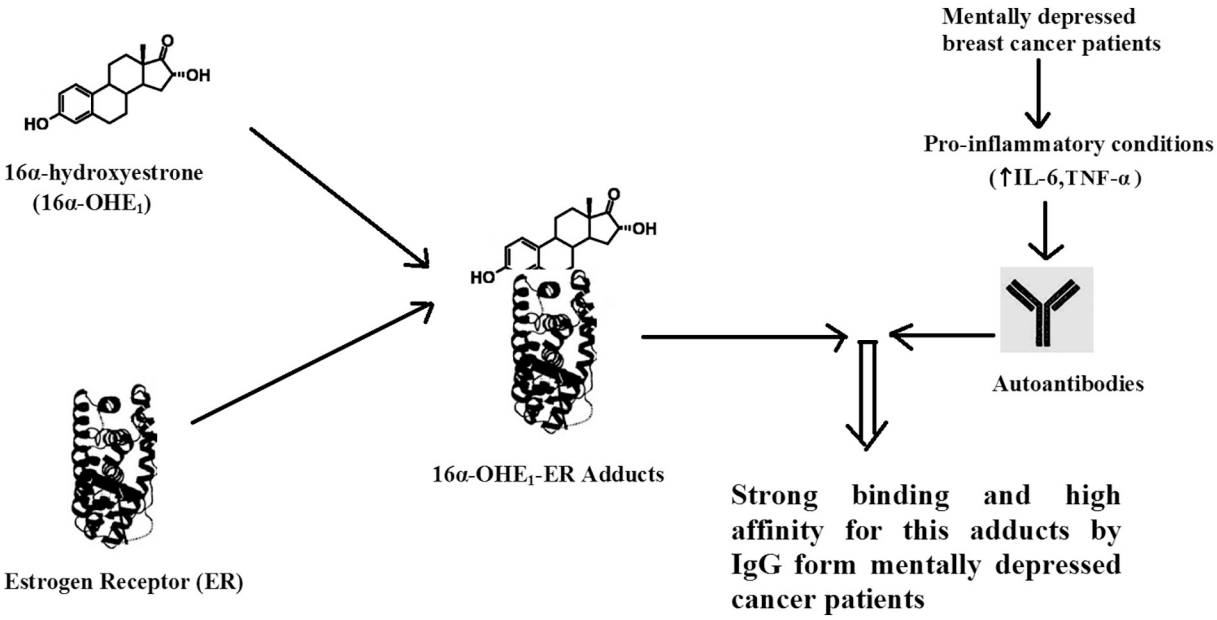

Fig. 6. The proposed mechanism for the generation of high affinity autoantibodies in MDBC patients.

modulator tamoxifen, which neutralize estrogen receptor, the most commonly used treatment for breast cancer [58]. Such binding might be because these patients already had autoantibodies against ER, which in combination with 16α-OHE_1 trigger more vigorous immunological responses. This is what is observed in case of induced antibodies against ER, in which anti-ER antibodies cross-react with 16α-OHE_1-ER, showing the relative affinity to about 79%. Patients who had taken chemotherapy also showed higher binding relative to general breast cancer patients. This might be because chemotherapy stimulates various immunological responses and, during this process, various types of autoantibodies have been produced that might show higher binding with 16α-OHE_1-ER. Effective and curative chemotherapy is a goal for modern-day cancer medicine. The immune system of the patient regulates malignant progression, where immune cell and cytokine/chemokine are expressed within tumors [59]. However, chemotherapy eliminates immune cells and, thus, might limit the effectiveness of immunotherapy. The target of the immune response in patients undergoing chemotherapy was not known and cannot be measured. Therefore, the relation between effective cancer chemotherapy and stimulation of host-protective immunity is unknown. Chemotherapeutic agents induced autophagy-dependent anticancer immune responses, and autophagy-competent cancer attracted dendritic cells and T lymphocytes in the tumor bed [60]. It also inhibits cancer development by orchestrating inflammation and immunity. Apoptosis is a key process in immune homeostasis and prevents the appearance of lymphomas or autoimmunity [61]. Cells derived from hematopoietic progenitor cells induced cell death via the extrinsic pathway. Menopausal status, smoking, and disease duration have no relation in the binding of 16α-OHE_1-ER with the cancer autoantibodies. Their binding is almost the same as overall binding in different cancer patients. Data have revealed that breast cancer patients who were depressed show the highest binding with this antigen. This means that depression somehow augments the production of autoantibodies against 16α-OHE_1-ER in breast cancer patients.

To further investigate the recognition of 16α-OHE_1-ER by autoantibodies in breast cancer, we checked the specificity of these antibodies by quantitative precipitation titration. Affinity constant showed that affinity of autoantibodies from the cancer patients was highest for mentally depressed patients with 16α-OHE_1-ER. The affinity of breast cancer autoantibodies was also high for 16α-OHE_1-ER, but with another antigen (ER, 16α-OHE_1), the affinity was relatively low. This recognition of 16α-OHE_1-ER over ER or 16α-OHE_1 might be due to the generation of unique epitopes on the formation of this adduct. Better recognition of 16α-OHE_1-ER by breast cancer IgG is an indication of the participation of 16α-OHE_1-ER in breast cancer etiopathogenesis. The induced antibodies (anti-16α-OHE_1-ER antibodies) showed cross-reactivity with 16α-OHE_1. Similarly, for anti-16α-OHE_1 antibodies, the induced antibodies showed binding with 16α-OHE_1-ER, indicating that the epitopes on 16α-OHE_1-ER were efficiently recognized by anti-16α-OHE_1 antibodies. Because of the immune-cross reactivity shown by anti-16α-OHE_1-ER and anti-16α-OHE_1 antibodies towards 16α-OHE_1 and 16α-OHE_1-ER, anti-16α-OHE_1-ER antibodies can be used as an immunological probe for the estimation of 16α-OHE_1 in the sera of breast cancer patients. The antibodies were also used to evaluate 2-OHE_1/16α-OHE_1 ratio in various cancer patients. No significant difference, either in the concentration of 16α-OHE_1 or 2OHE_1/16α-OHE_1 ratio of the breast cancer patients, was observed relative to the controls.

In conclusion, we have presented evidence to show the possible antigenic role of 16α-OHE_1-ER in the generation of autoantibodies in patients with breast cancer. Our data imply that the combined action of 16α-OHE_1 and ER generate discriminating epitopes that are efficiently recognized by cancer antibodies. Furthermore, depressed patients with breast cancer recognized these discriminating epitopes more efficiently, indicating that depression somehow augments the production of autoantibodies against this antigen in breast cancer patients. The possible mechanism involves the generation of multiple types of autoantibodies due to the production of pro-inflammatory cytokines (IL-6, TNF-α) in MDBC patients. These autoantibodies bind the 16α-OHE_1-ER adduct more efficiently and show strong recognition (Fig. 6). This study indicates the possible role of 16α-OHE_1-ER in generating unique neo-epitopes that might form one of the factors in the induction of breast cancer autoantibodies.

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Conflict of interest

All authors declare that they have no conflict of interest.

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