

### DEPRESSION AUGMENTS THE PRODUCTION OF HIGH-AFFINITY ANTIBODIES AGAINST ESTROGEN METABOLITE-RECEPTOR COMPLEX IN PROSTATE CANCER PATIENTS

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#### ABSTRACT

##### Background

Depression is a common symptom associated with prostate cancer (PC), elevated levels of 16 $\alpha$ -hydroxyestrone (16 $\alpha$ -OHE<sub>1</sub>) have been linked to increased risk of PC and estrogen receptor (ER) had been expressed in prostate tissue. This study was carried out to know whether depression augments the production of antibodies against 16 $\alpha$ -OHE<sub>1</sub>-ER in PC patients.

##### Methods

Forty-six depressed PC (DPC) (out of total 60 PC) patients and 40 control subjects (who have normal circulating prostate-specific antigen [PSA] levels) were checked for the presence of antibodies by ELISA (direct binding and competition) and quantitative precipitin titration.

##### Results

Antibodies from DPC patients demonstrate high binding to 16 $\alpha$ -OHE<sub>1</sub>-ER in comparison to overall PC patients ( $p < 0.05$ ) and controls ( $p < 0.001$ ). Although, PC sera showed high binding to 16 $\alpha$ -OHE<sub>1</sub>-ER in comparison to ER ( $p < 0.05$ ) or 16 $\alpha$ -OHE<sub>1</sub> ( $p < 0.001$ ). The relative affinity of IgGs from DPC and PC patients was  $1.01 \times 10^{-7}$  and  $1.19 \times 10^{-7}$  M, respectively.

##### Conclusions

Depression triggers high-affinity antibodies against this antigen in PC patients through inflammatory conditions. It also increased the release of pro-inflammatory cytokine (IL-6) that further enhanced antibodies in PC patients.

**Keywords:** 16 $\alpha$ -hydroxyestrone; antibodies; depression; estrogen receptor; prostate cancer

## INTRODUCTION

Depression is common amongst prostate cancer (PC) patients,<sup>1</sup> with a frequency of approximately one in six patients. Depression is linked to PC by increasing macrophages trafficking and IL-6 releasing into the cells.<sup>2</sup> It promotes myeloid cell infiltration and enhanced IL-6 concentration by a sympathetic-Neuropeptide Y signal.<sup>2</sup> There are different characteristics of PC that makes this cancer a promising approach for immune-based therapy. It grew slowly which allow the immune system to generate an anti-tumor immune response when stimulated.<sup>3</sup> Prostate cancer is immunogenic and capable of inducing autoantibodies in cancer patients.<sup>4</sup> The immune approach is more reliable because of less toxicity compared to chemotherapy.

Prostate cancer is hormone-dependent and common among males worldwide.<sup>5</sup> The estrogen and their metabolites ( $16\alpha$ -OHE<sub>1</sub>, 2-hydroxyestrone; 2-OHE<sub>1</sub>) can also be related to PC.<sup>6</sup> In PC, patients with high urinary 2-OHE<sub>1</sub>/ $16\alpha$ -OHE<sub>1</sub> ratio had a 40% nonsignificant reduction in the risk of PC, with a condition that prostate-specific antigen (PSA) concentration higher than 4 ng/mL was excluded from control subjects.<sup>7</sup> Recently, 15 urinary estrogen metabolites were measured in PC patients and a modest difference in their concentration between the patients and control subjects was observed.<sup>8</sup> Earlier studies from our lab have also shown an important role of estrogen metabolites in the etiopathogenesis of PC.<sup>9</sup> These metabolites are also responsible for caused breast cancer<sup>10</sup> and various autoimmune diseases.<sup>11–13</sup>

The estrogen can also be linked to PC as mentioned in the earlier two studies. One was the presence of estrogen receptor (ER) in the prostate tissues<sup>14</sup> and the other was the response to estrogen therapy by PC patients.<sup>15</sup> Considering that increased depression is common among PC patients, ER had been expressed in prostate tissue and  $16\alpha$ -OHE<sub>1</sub> is linked to PC, we decided to test whether

depression in PC patients trigger more antibodies against  $16\alpha$ -OHE<sub>1</sub>-ER. This gives us the opportunity to screen PC patients' sera with  $16\alpha$ -OHE<sub>1</sub>-ER to probe their role in PC.

## METHODS

### *Prostate cancer patients and controls*

We have taken 60 PC (including 46 depressed PC [DPC]) patient's blood samples to access antibodies against  $16\alpha$ -OHE<sub>1</sub>-ER complex. Their ages are  $65 \pm 7.1$  years, who underwent a prostate biopsy. All the biopsy samples were confirmed by an experienced pathologist in routine histology diagnosis. Among the PC patients, five were taken from patients before definitive radiation treatment or prostatectomy, 20 were collected from patients after definitive treatment without evidence of disease recurrence, and 35 were collected from patients with metastatic disease. The controls ( $n = 40$ ) were the males, normal individuals, who had no symptoms of PC. The normal individuals had a normal circulating PSA level (0.036–1.155 ng/mL) and did not have any prostate problem or disease or inflammation. The baseline characteristics of all the groups were given in Table 1. Spot urine samples from both the groups were also taken for the estimation of estrogen metabolites and their ratio. The Self Rating Depression Scale questionnaire was used to test the patients and their level of depression. We used a modified version of the earlier used questionnaire to test the PC patients with their level of depression.<sup>16</sup> All serum samples were heated at 56°C for 30 min to deactivate complement protein and then stored at –20°C with sodium azide (0.1%) as preservative. Prior consent from all the subjects was taken and, finally, this study was approved by the Institutional Ethical Review Board. The procedures were followed in accordance with the ethical standards of the responsible committee on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008.

**TABLE 1.** Baseline Characteristics of Subjects and Immunological Data of Different Prostate Cancer Patients.

| Characteristics                                      | Prostate cancer (n=60)                            |                       | Controls (n=40)                        |
|--|---|-----------------------|--|
| Age (years)  | 65 ± 7.1  |                       | 62 ± 8.3                               |
| Depressed PC (DPC) patients BMI (kg/m <sup>2</sup> ) | 46 (76.6%)  |                       | –                                      |
| <25  | 15 (25%)  |                       | 9 (22.5%)                              |
| 25–29.9  | 36 (60%)  |                       | 25 (62.5%)                             |
| ≥30  | 9 (15%)   |                       | 6 (15%)                                |
| Smoking status                                       |   |                       |  |
| Never  | 24 (40%)  |                       | 17 (42.5%)                             |
| Past   | 15 (25%)  |                       | 12 (30%)                               |
| Current  | 21 (35%)  |                       | 11 (27.5%)                             |
| Family history of prostate cancer                    |   |                       |  |
| Yes  | 21 (35%)  |                       | 2 (5%)                                 |
| No   | 39 (65%)  |                       | 38 (95%)                               |
| Inflammatory cytokines <sup>a</sup> estimation       |   |                       |  |
| - L-6  | 7.9 ± 1.3 pg/mL <sup>s</sup>                      |                       | 2.3 ± 1.3 pg/mL                        |
| PSA  | 61.3 ± 5.3 ng/mL                                  |                       | 1.0 ± 0.05 ng/mL                       |
| <b>Immunological data</b>                            | <b>Maximum percent (%) inhibition at 20 µg/mL</b> |                       |  |
|  | <b>16α-OHE<sub>1</sub>-ER<sup>a,*</sup></b>       | <b>ER<sup>b</sup></b> | <b>16α-OHE<sub>1</sub><sup>c</sup></b> |
| Overall (n=60)                                       | 69.3 ± 10.3                                       | 46.4 ± 3.2            | 15.9 ± 3.9                             |
| DPC patients (n=46)                                  | 76.8 ± 3.9  | 43.9 ± 8.7            | 12.8 ± 7.1                             |
| Estrogen receptor (ER)                               |   |                       |  |
| Positive (n=35)                                      | 72.4 ± 8.9  | 52.3 ± 4.5            | 14.9 ± 4.1                             |
| Negative (n=25)                                      | 67.9 ± 11.3                                       | 45.8 ± 3.1            | 11.2 ± 3.1                             |
| Smoking at baseline                                  |   |                       |  |
| Current/Past (n=36)                                  | 70.9 ± 3.4  | 45.1 ± 8.1            | 13.5 ± 9.1                             |
| Never (n=24)   | 68.3 ± 11.8                                       | 43.4 ± 3.5            | 11.2 ± 8.1                             |
| PSA (ng/mL)  |   |                       |  |
| <4 (n=15)  | 67.3 ± 8.9  | 45.3 ± 8.4            | 11.5 ± 4.1                             |
| ≥ 4 (n=45)   | 69.8 ± 8.1  | 48.4 ± 4.3            | 15.8 ± 3.9                             |
| 2-OHE <sub>1</sub> /16α-OHE <sub>1</sub> ratio       |   |                       |  |
| High (n=28)  | 67.2 ± 11.4                                       | 47.4 ± 3.1            | 11.4 ± 3.9                             |
| Low (n=32)   | 69.9 ± 10.1                                       | 48.5 ± 5.4            | 12.1 ± 8.9                             |
| Stages   |   |                       |  |
| I  | 67.3 ± 9.8  | 45.3 ± 8.2            | 11.6 ± 2.1                             |
| II   | 69.9 ± 10.1                                       | 48.4 ± 7.1            | 12.4 ± 3.9                             |
| III  | 71.3 ± 8.4  | 48.9 ± 8.2            | 13.1 ± 2.8                             |
| IV   | 73.5 ± 11.4                                       | 49.5 ± 8.9            | 12.5 ± 2.6                             |

(continues)

TABLE 1. Continued.

| Immunological data | Maximum percent (%) inhibition at 20 µg/mL       |                 |  |
|--------------------|--|-----------------|--|
|                    | 16 $\alpha$ -OHE <sub>1</sub> -ER <sup>a,*</sup> | ER <sup>b</sup> | 16 $\alpha$ -OHE <sub>1</sub> <sup>c</sup> |
| Grade              |  |                 |  |
| 1                  | 65.8 ± 8.3                                       | 44.8 ± 2.3      | 12.6 ± 3.1                                 |
| 2                  | 68.5 ± 8.9                                       | 45.7 ± 3.2      | 13.6 ± 4.1                                 |
| 3                  | 70.1 ± 9.1                                       | 46.8 ± 4.6      | 13.8 ± 5.2                                 |
| 4                  | 71.3 ± 7.8                                       | 47.3 ± 3.6      | 13.6 ± 4.3                                 |
| 5                  | 72.8 ± 10.3                                      | 48.9 ± 4.5      | 14.2 ± 4.3                                 |
| NH IgG (n=25)      | 9.1 ± 3.3  | 8.4 ± 3.8       | 6.8 ± 2.8                                  |

<sup>a</sup>n=30, <sup>s</sup>Significantly higher than control ( $p < 0.05$ ).

The experiments were carried out by incubating an ELISA plate with 100 µL of different antigens (2.5 µg/mL) as described in the "Materials and Methods" section; mean ± SD.

NH IgG: normal human IgG.

\* $p < 0.001$  and  $p < 0.05$ , significantly higher inhibition than NH IgG and ER IgG.

<sup>a</sup>16 $\alpha$ -OHE<sub>1</sub>-ER as an inhibitor.

<sup>b</sup>ER as an inhibitor.

<sup>c</sup>16 $\alpha$ -OHE<sub>1</sub> as an inhibitor.

### 16 $\alpha$ -OHE<sub>1</sub>-ER complex formation

The 16 $\alpha$ -OHE<sub>1</sub>-ER complex was formed as described previously.<sup>10</sup> Briefly, 16 $\alpha$ -OHE<sub>1</sub> with a concentration of 1–10 mM was incubated with ER (1 mg) in potassium phosphate buffer (0.1 M, pH 6) and 1 µM sodium cyanoborohydride was added to the reaction mixture. The reaction mixture was kept for 48 h at 37°C with gentle shaking. 16 $\alpha$ -OHE<sub>1</sub> was dissolved in ethanol in such a way that the ethanol concentration was 0.1% of the total volume of the reaction mixture. The reaction mixture was dialyzed with PBS, pH 7.4, to remove excess unbound of 16 $\alpha$ -OHE<sub>1</sub>.

### Antibodies against 16 $\alpha$ -OHE<sub>1</sub>-ER complex

Antibodies against this antigen were induced in female rabbits as described earlier.<sup>11</sup> We have also induced antibodies against the controls (16 $\alpha$ -OHE<sub>1</sub> and ER) to check their immunogenicity in the experimental animals.

### Isolation and purification of anti-16 $\alpha$ -OHE<sub>1</sub>-ER antibodies in prostate cancer patients

Immunoglobulin G was purified from the sera of PC patients (or immunized animal) on a Protein A

Agarose column as mentioned earlier.<sup>17</sup> The purity and homogeneity of the purified IgG were checked on 7.5% PAGE.

### ELISA

Antibodies were detected from the sera by direct binding ELISA as described earlier.<sup>12</sup> Competition ELISA was also used for specific binding of PC/immunized antibodies to 16 $\alpha$ -OHE<sub>1</sub>-ER complex.<sup>12</sup> Briefly, this complex (100 µL, 2.5 µg/mL) was coated onto a microtiter plate for 2 h at 25°C and later for 24 h at 4°C. This plate was washed with Tris buffer saline-Tween 20 and unoccupied sites were blocked with 100 µL of bovine serum albumin (1.5%). Immune complexes were prepared by incubating 100 µL of PC/immunized sera (1:100 dilution) with an increasing concentration of 16 $\alpha$ -OHE<sub>1</sub>-ER complex (or 16 $\alpha$ -OHE<sub>1</sub> or ER) at 37°C for 2 h and overnight at 4°C. Hundred microliters of the immune complex was incubated in each well and anti-human IgG-alkaline phosphatase conjugate was finally added, followed by the addition of p-nitrophenylphosphate as substrate to develop the reaction. The absorbance was taken at 410 nm

on to a microplate reader and data were present as percent inhibition. Human IL-6 ELISA Kit (Sigma-Aldrich, Chemie GmbH, Germany) was used for IL-6 estimation.

### **Quantitation and formation of immune complexes from PC patients**

Quantitation and formation of immune complexes were done as mentioned previously.<sup>13</sup> Briefly, PC IgG (100 µg) was incubated with an increasing amount (0–40 µg) of various antigens (16α-OHE<sub>1</sub>-ER, ER, and 16α-OHE<sub>1</sub>) in a reaction mixture of 400 µL. The reaction mixture was incubated for 4 h at 37°C and overnight at 4°C. Normal human IgG serves as a control that was also treated with the same conditions. The mixture was centrifuged and pelleted, washed with PBS, and finally solubilized in 250 µL NaCl. Free protein and proteins bound in the immune complex, were determined by colorimetric methods.<sup>18</sup> The affinity constant was calculated by determining affinity using Langmuir plot.<sup>19</sup>

### **Statistical analysis**

Statistical significance was determined using the Student's *t*-test. A P-value of <0.05 was taken as statistical significance.

## **RESULTS**

### **Characterization of 16α-OHE<sub>1</sub>-ER complex**

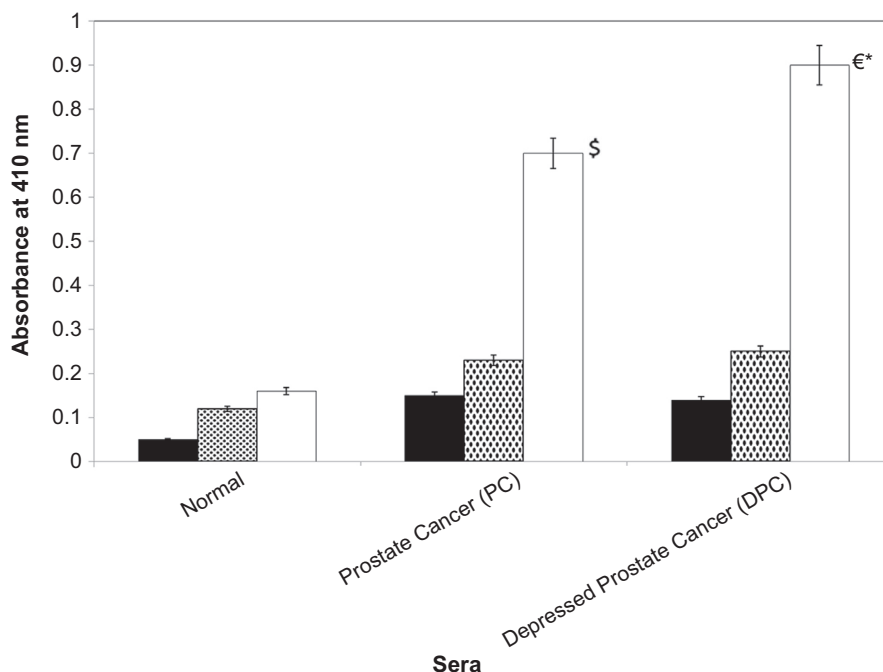
16α-OHE<sub>1</sub> when incubated with ER resulted in the formation of a high molecular weight complex that showed less mobility on the SDS-PAGE as compared to ER.<sup>10</sup> The molecular weight of the newly synthesized complex is closed to 68 kDa and showed 38.3% UV hyperchromicity compared to ER at 280 nm.<sup>10</sup>

### **Detection of anti-16α-OHE<sub>1</sub>-ER antibodies in the sera of prostate cancer patients**

Prostate cancer patients and control subjects were tested for the presence of serum antibodies against 16α-OHE<sub>1</sub>-ER, ER, and 16α-OHE<sub>1</sub> by direct binding ELISA. Nearly all the selected sera demonstrate high

binding to 16α-OHE<sub>1</sub>-ER in comparison to ER or 16α-OHE<sub>1</sub> ( $p < 0.05$  or  $p < 0.001$ ). Normal human sera showed no appreciable binding to either of the antigens (Figure 1). Binding with ER and 16α-OHE<sub>1</sub> was found to be low as compared to 16α-OHE<sub>1</sub>-ER. The binding was highest for DPC (n=46) patients with 16α-OHE<sub>1</sub>-ER, which was found to be higher in comparison to overall PC patients ( $p < 0.05$ ). Competition ELISA was further used to detect binding specificities of PC antibodies to 16α-OHE<sub>1</sub>-ER, ER, and 16α-OHE<sub>1</sub>. 16α-OHE<sub>1</sub>-ER showed inhibition to about  $59.8 \pm 7.3\%$  (37.3–81.9%) in the antibody activity while ER and 16α-OHE<sub>1</sub> showed inhibition of  $42.3 \pm 5.3\%$  (15.5–65.3%) and  $12.4 \pm 3.9\%$ , respectively (Figure 2a). Again, a DPC patient's sera showed the highest inhibition of about  $63.8 \pm 8.1\%$  (25.8–86.8%).

The PC antibodies were isolated and purified by affinity chromatography on a Protein A-Agarose column. The purity of the isolated IgG from PC patients was further confirmed by running on polyacrylamide gel electrophoresis (data not shown). In competition ELISA, 16α-OHE<sub>1</sub>-ER showed inhibition of about  $69.3 \pm 10.3\%$  (41.8–85.3%) in the antibody activity. ER and 16α-OHE<sub>1</sub> showed inhibition of  $46.3 \pm 3.2\%$  (18.1–69.8%) and  $15.9 \pm 3.9\%$ , respectively (Figure 2b). The binding of PC antibodies was also checked in various groups of patients. Accordingly, we divide them into five groups based on different clinical characteristics in these patients. Whether the cancer patients are depressed (DPC), ER-positive or not, PSA less than or greater than 4, smokers, and 12-OHE<sub>1</sub>/16α-OHE<sub>1</sub> ratio. Among all, DPC patients were shown highest inhibition ( $76.8 \pm 3.9\%$ ) followed by cancer patients who were ER-positive ( $72.4 \pm 8.9\%$ ), patients with smoking ( $70.9 \pm 3.4\%$ ), low 12-OHE<sub>1</sub>/16α-OHE<sub>1</sub> ratio ( $69.9 \pm 10.3\%$ ) and PSA level  $\geq 4$  ( $69.8 \pm 8.1\%$ ) (Table 1). While for other groups such as ER-negative, PSA < 4, and high 12-OHE<sub>1</sub>/16α-OHE<sub>1</sub> ratio have no major effects on the inhibition values (Table 1). The inhibition values according to different stages were I:  $67.3 \pm 9.8\%$ , II:  $69.9 \pm 10.1\%$ , III:  $71.3 \pm 8.4\%$ , and IV:  $73.5 \pm 11.4\%$ . According to different grade of PC, the values were



**FIGURE 1.** Direct binding ELISA of control, prostate cancer (PC), and depressed PC (DPC) patients. Direct binding enzyme-linked immunosorbent assay of control (n=40), PC (n=60), and DPC patient’s antibodies (n=46) to 16α-OHE<sub>1</sub>-ER (□), ER (■), and 16α-OHE<sub>1</sub> (▨). Microtiterplates 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 described in the “Materials and Methods” section. Each histogram represents the mean±SD. <sup>\$</sup>p<0.001, p<0.001, significantly higher binding than normal sera and 16α-OHE<sub>1</sub> in PC; <sup>€\*</sup>p<0.05 significantly higher binding of DPC than PC and ER.

1: 65.8 ± 8.3%, 2: 68.5 ± 8.9%, 3: 70.1 ± 9.1%, 4: 71.3 ± 7.8%, and 5: 72.8 ± 10.3%, respectively.

**Binding specificity of IgG against 16α-OHE<sub>1</sub>-ER in PC patients**

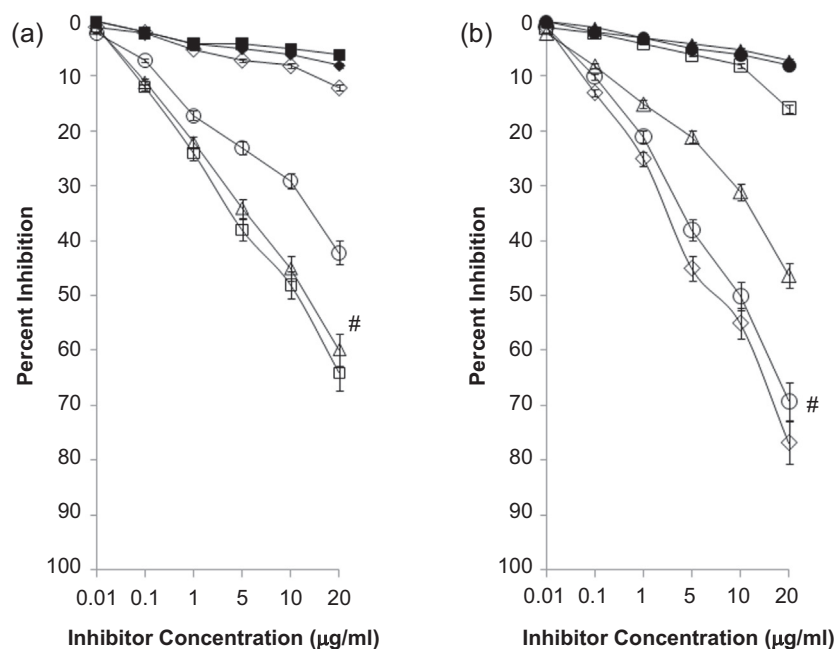
The affinity of the PC IgGs was further characterized by estimating the affinity constant. Here, varying amounts of different antigens were incubated with a constant amount of PC IgG (n = 8). Normal human IgG was a negative control. The results showed that about 24 μg of 16α-OHE<sub>1</sub>-ER complex was bound to about 73 μg of PC IgG and 20 μg of 16α-OHE<sub>1</sub>-ER was bound to about 75 μg of DPC IgG. With ER and 16α-OHE<sub>1</sub>, a maximum of 32 μg of ER was bound to about 61 μg of cancer IgG

and a maximum of 35 μg of 16α-OHE<sub>1</sub> was bound to about 59 μg of PC IgG. The Langmuir plot was used to evaluate the apparent association constant (Figure 3). The affinity constant of PC IgG was found to be of the order of 1.19×10<sup>-7</sup> M, 1.45×10<sup>-6</sup> M, and 1.13×10<sup>-6</sup> M for 16α-OHE<sub>1</sub>-ER, ER, and 16α-OHE<sub>1</sub> respectively. Again for DPC patients, the constant was found to be 1.01×10<sup>-7</sup> M, which showed the highest affinity of DPC IgG with 16α-OHE<sub>1</sub>-ER.

**Antigenicity of 16α-OHE<sub>1</sub>-ER and their characterization**

The antigenicity of 16α-OHE<sub>1</sub>-ER along with suitable controls was checked by inducing antibodies in experimental animals (female rabbits).

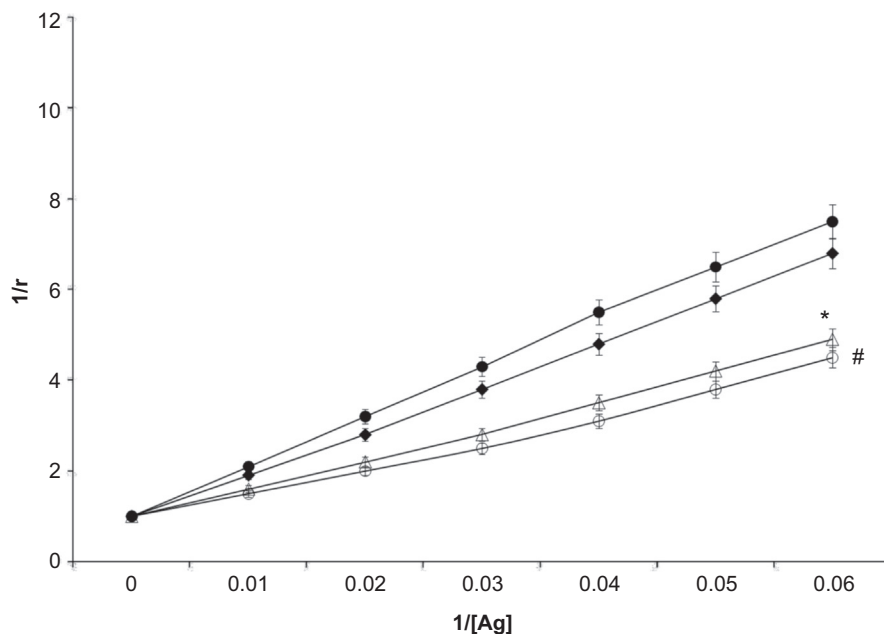




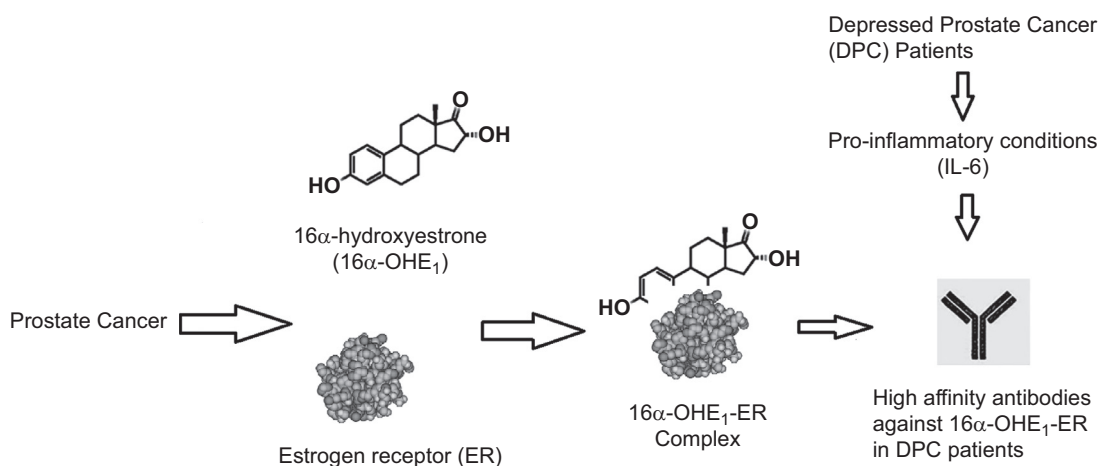
**FIGURE 2.** Inhibition ELISA of control, PC, and DPC patients. (a) Inhibition ELISA of anti-(16 $\alpha$ -OHE<sub>1</sub>-ER, ER, 16 $\alpha$ -OHE<sub>1</sub>) PC and DPC (- $\Delta$ -, - $\circ$ -, - $\diamond$ - & - $\square$ -) and Control (- $\blacklozenge$ -, - $\blacksquare$ -) sera with 16 $\alpha$ -OHE<sub>1</sub>-ER, ER, 16 $\alpha$ -OHE<sub>1</sub>. (b) Inhibition of PC and DPC anti-(16 $\alpha$ -OHE<sub>1</sub>-ER, ER, 16 $\alpha$ -OHE<sub>1</sub>) IgG binding to 16 $\alpha$ -OHE<sub>1</sub>-ER (- $\circ$ - & - $\diamond$ -), ER (- $\Delta$ -), 16 $\alpha$ -OHE<sub>1</sub> (- $\square$ -). (- $\bullet$ -, - $\blacktriangle$ -) Represent the inhibition of normal anti-16 $\alpha$ -OHE<sub>1</sub>-ER and ER IgG binding to 16 $\alpha$ -OHE<sub>1</sub>-ER and ER. Microtiter plates were coated with respective antigens (2.5  $\mu$ g/mL). Immune complexes were prepared by mixing 100  $\mu$ L of 1:100 dilution of serum antibodies from PC, DPC 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 16 $\alpha$ -OHE<sub>1</sub> were negligible and are not shown. #Significantly higher inhibition than ER (p<0.05, p<0.05) and 16 $\alpha$ -OHE<sub>1</sub> (p<0.001, p<0.001).

The 16 $\alpha$ -OHE<sub>1</sub>-ER was found to be highly immunogenic ( $\geq 1:25,600$ ) triggering high titer antibodies in experimental animals.<sup>10</sup> Here, pre-immune sera served as a negative control. The antibodies against ER and 16 $\alpha$ -OHE<sub>1</sub> were also induced but the titer shown by these two antigens was low in comparison to 16 $\alpha$ -OHE<sub>1</sub>-ER. In competition ELISA, induced anti-16 $\alpha$ -OHE<sub>1</sub>-ER antibodies in the serum showed inhibition of about 75.3% in the antibody activity with 16 $\alpha$ -OHE<sub>1</sub>-ER as an inhibitor at 20  $\mu$ g/mL and 50% inhibition was achieved at 7.7  $\mu$ g/mL. For ER and 16 $\alpha$ -OHE<sub>1</sub>, the inhibition values were found to be 71.8 and 64.3%, respectively, and 50% inhibition

was achieved at 13.8 and 17.3  $\mu$ g/mL.<sup>10</sup> The induced antibodies were isolated and purified on protein A-Agarose column and their cross-reactivity was also checked. Immunocross-reactivity of anti-16 $\alpha$ -OHE<sub>1</sub>-ER antibodies was also checked in presence of 16 $\alpha$ -OHE<sub>1</sub>-ER, ER, 16 $\alpha$ -OHE<sub>1</sub>, 2-OHE<sub>1</sub>, progesterone receptor (PR), 4-OHE<sub>1</sub>. The anti-16 $\alpha$ -OHE<sub>1</sub>-ER antibodies cross-react with 16 $\alpha$ -OHE<sub>1</sub>. Similar is the case for anti-16 $\alpha$ -OHE<sub>1</sub> antibodies in which these antibodies showed binding with 16 $\alpha$ -OHE<sub>1</sub>-ER. The concentration of pro-inflammatory IL-6 was found to be 7.9  $\pm$  1.3 pg/mL, which is significantly higher in comparison to controls (2.3  $\pm$  1.3, p<0.05).



**FIGURE 3** Determination of an apparent association constant by Langmuir plot. Antigens were 16 $\alpha$ -OHE<sub>1</sub>-ER (-○- & -Δ-), ER (-◆-), and 16 $\alpha$ -OHE<sub>1</sub> (-●-). Immune complexes were prepared by incubating 100  $\mu$ g of IgG (PC, DPC, and Controls) with varying amount of different antigens (0–100  $\mu$ g) in an assay volume of 400  $\mu$ L for 2 h at room temperature and overnight at 4°C. The binding data were analyzed for antibody affinity as described in the “Materials and Methods” section. #Significantly higher binding than PC (p<0.05). \*Significantly higher binding than ER (p<0.05) and 16 $\alpha$ -OHE<sub>1</sub> (p<0.001).



**FIGURE 4** The proposed mechanism for the production of high-affinity antibodies in depressed prostate cancer (DPC) patients.



## DISCUSSION

Estrogen and their metabolites seem to play an important role in the progression and development of PC.<sup>20</sup> They might play a causative role in PC but the exact mechanism remains elusive. The potential mechanism for different causes includes epigenetic modification and estrogenic imprinting hyperprolactinemia, direct genotoxicity, inflammation, and receptor-mediated actions. Estrogen can be used as potential hormonal therapy in PC but it is also known to cause this cancer.<sup>21</sup> Estrogen mediates its effect through binding to its receptor (ER $\alpha$  and ER $\beta$ ) and it is expressed in normal prostate. Depression is a major problem in men diagnosed with PC that can further complex issues related to diagnostic efforts.<sup>16</sup> This cancer is not only associated with various biochemical changes but also related to psychological stress.<sup>22</sup> Some of the patients suffer some additional problems that may further contribute to depressive symptoms.<sup>23</sup> Depression is a major challenge among PC patients and emerges as a significant issue with a prevalence of 16–30%.<sup>24</sup>

Estrogen acts as a causative factor through their receptors and they also have a genotoxic role. Elevated levels of urinary 16 $\alpha$ -OHE<sub>1</sub> have been associated with increased risk of PC,<sup>7</sup> depression is common among PC patients and ER had been expressed in the prostate. So, considering all these facts, this study was designed to evaluate the combining effect of these parameters on the PC. To know this important fact, the binding affinity of antibodies against 16 $\alpha$ -OHE<sub>1</sub>-ER was measured in DPC patients to check whether this complex (16 $\alpha$ -OHE<sub>1</sub>-ER) has any affinity with the antibodies or not. The binding specificity of antibodies from the sera of DPC patients and controls to 16 $\alpha$ -OHE<sub>1</sub>-ER was checked with direct binding and inhibition ELISA. This complex showed high binding with almost all the chosen sera as compared to controls ( $p < 0.001$ ). The combination of 16 $\alpha$ -OHE<sub>1</sub> and ER exposed to specific groups/molecules that can function as high specificity epitopes for the PC IgGs. It is important to note that PC patients who were depressed showed

much higher binding with PC antibodies. The exact mechanism is unknown but it is somehow related to pro-inflammatory conditions. Depression has been associated with dysregulation of the immune system and promotes tumor progression by releasing IL-6 from the cells in PC patients.<sup>2</sup> Pro-inflammatory cytokine (IL-6) released from cancer patients somehow produces more antibodies that might bound to 16 $\alpha$ -OHE<sub>1</sub>-ER more efficiently. The high concentration of this cytokine (i.e., IL-6) further confirmed the generation of the pro-inflammatory condition in DPC patients. Serum IL-6 was also significantly associated with the clinical stages of PC and can function as a prognostic factor for PC.<sup>25</sup> It can also function as an autocrine growth factor for PC.<sup>26</sup> 16 $\alpha$ -OHE<sub>1</sub> is involved in the release of inflammatory mediators from the human amnion-derived cells<sup>27</sup> and is somehow linked to the inflammation. Again high binding is due to the autoantibodies produced during inflammatory conditions.

The binding specificities of antibodies from PC were also tested according to various characteristics in PC patients. Among them, DPC patients showed the highest inhibition, followed by those PC patients who expressed ER, patients with a history of smoking, low 2-OHE<sub>1</sub>/16 $\alpha$ -OHE<sub>1</sub> ratio, and PSA level  $\geq 4$ . As mentioned already, ER $\alpha$  and ER $\beta$  are expressed in prostate tissues and also present in the prostate during carcinogenesis.<sup>14</sup> High specificities might be observed because PC patients have antibodies against ER that might cross-react with this antigen and caused the immunological response. Cigarette smoking may increase the risk of PC by affecting circulatory hormone or through exposure to various carcinogens.<sup>28</sup> Smoking in cancer patients might increase 16 $\alpha$ -OHE<sub>1</sub> that comes in contact with the ER (already expressed), making a complex and showed high specificity. High binding of patients with low 2-OHE<sub>1</sub>/16 $\alpha$ -OHE<sub>1</sub> ratio was observed because a low ratio means a high concentration of 16 $\alpha$ -OHE<sub>1</sub> and elevated urinary level of this metabolite have been associated with increased risk for PC.<sup>7</sup> High binding in patients with PSA level  $\geq 4$  might be due

to prostatitis or urinary tract infections, in which its concentration has been dramatically increased. The inhibition values gradually increased according to different stages and grades of PC indicating that more antibodies are produced as the PC progresses.

To have better insight into the recognition of  $16\alpha$ -OHE<sub>1</sub>-ER complex by DPC antibodies, we determined the affinity of antibodies by quantitative precipitin titration. The affinity constant was highest for DPC patients that further demonstrates high recognition of this complex by these antibodies. The high binding of this complex by DPC antibodies indicates possible participation of  $16\alpha$ -OHE<sub>1</sub>-ER complex in PC. Estrogen metabolites (including  $16\alpha$ -OHE<sub>1</sub>) are present in tissues, bile, urine, and blood,<sup>29</sup> and ER had been expressed in the prostate, as a result, complexes have been formed and PC antibodies showed high binding. Therefore, it could be possible that the  $16\alpha$ -OHE<sub>1</sub>-ER complex might be one of the important factors toward the generation of antibodies in PC. The induced antibodies showed cross-reactivity towards other antigens. In clinical practice, these patients must be screened for the presence of  $16\alpha$ -OHE<sub>1</sub>-ER complex by more advanced monoclonal antibodies against this antigen. Patients should also be screened with the antibodies against this antigen that might help in the early detection of PC. The cross-reactivity of these molecules is also screened in these patients. Depression is common among PC patients, therefore, DPC patients must be screened with these two parameters to know some better outcomes.

### CONCLUSIONS

In conclusion, the proposed mechanism for PC includes the generation of antibodies against  $16\alpha$ -OHE<sub>1</sub>-ER through the formation of the  $16\alpha$ -OHE<sub>1</sub>-ER complex.  $16\alpha$ -OHE<sub>1</sub> and ER come in contact with each other to form  $16\alpha$ -OHE<sub>1</sub>-ER complex in prostate tissues. Furthermore, DPC patients showed the highest recognition to  $16\alpha$ -OHE<sub>1</sub>-ER indicating that depression somehow triggers the

production of PC antibodies against this complex. The formation of complex induces its immunogenicity leading to the induction and elevated levels of PC antibodies (Figure 4). Depression augments the production of antibodies through the generation of pro-inflammatory conditions in these patients.

### CONFLICT OF INTEREST

The authors declare no competing interests.

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