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Original Research

16α-hydroxyestrone and its receptor complex: high affinity antigen for antibodies from prostate cancer patients

Wahid Ali Khan[1,*], Mohd. Wajid Ali Khan[2,3]

[1]Department of Clinical Biochemistry, College of Medicine, King Khalid University, 960 Abha, Kingdom of Saudi Arabia
[2]Department of Chemistry, College of Science, University of Hail, 2440 Hail, Kingdom of Saudi Arabia
[3]Molecular Diagnostic and Personalised Therapeutics Unit, University of Hail, 2440 Hail, Kingdom of Saudi Arabia

***Correspondence:** wahidalikhan@rediffmail.com (Wahid Ali Khan)

Abstract

Background and objectives: Elevated levels of 16α-hydroxyestrone (16α-OHE$_1$) have been linked to increased risk of prostate cancer (PC) and estrogen receptor (ER) had been expressed in prostate tissue but the combined effect of 16α-OHE$_1$ and ER (α) is lacking. We investigated the binding specificity of antibodies from PC with 16α-OHE$_1$-ER complex in the sera from PC patients.

Materials and methods: The antibodies in the serum from 60 PC patients and 40 control subjects were evaluated from ELISA (direct binding and competition) and quantitative precipitin titration. Competition ELISA was also used to estimates 16α-OHE$_1$ concentration and 2-hydroxyestrone (2-OHE$_1$)/16α-OHE$_1$ ratio in PC patients.

Results: Antibodies from PC patients demonstrate high binding to 16α-OHE$_1$-ER in comparison to ER ($p < 0.05$) or 16α-OHE$_1$ ($p < 0.001$). The relative affinity of PC IgG was found to be high for 16α-OHE$_1$-ER (1.19×10^{-7} M) as compared to ER (1.45×10^{-6} M) or 16α-OHE$_1$ (1.13×10^{-6} M).

Conclusion: High affinity of PC IgG with 16α-OHE$_1$-ER might explain the possible antigenic role and 16α-OHE$_1$-ER acted as high affinity antigen for antibodies from PC. The interaction between 16α-OHE$_1$ and ER makes a complex in the prostate tissues and this may generate antibodies against this complex in the cancer patients.

Keywords

Prostate cancer; 16α-hydroxyestrone; Estrogen receptor; ELISA; Antibodies

1. Introduction

Prostate cancer is the second most common cancer in males worldwide [1]. Although, the growth and development of prostate gland can be controlled by androgen but estrogen can also play an important role in its development and carcinogenesis [2, 3]. Not only the estrogen but its metabolites such as 16α-hydroxyestrone (16α-OHE$_1$) and 2-hydroxyestrone (2-OHE$_1$) can also be related with PC [4]. These are predominant metabolites that are produced during oxidative metabolism of estrogen [5]. 16α-OHE$_1$ is an active estrogen metabolite that is bound to its receptor with high affinity and can function as estrogen agonist to induce various responses [6]. In contrast, 2-OHE$_1$ has weak estrogen activity and inhibits angiogenesis [7]. Their ratio (i.e., 2-OHE$_1$/16α-OHE$_1$) has been more related to breast cancer [8] than PC. In prostate cancer, patients with high urinary 2-OHE$_1$/16α-OHE$_1$ ratio had a 40% non-significant reduction in the risk of PC, with a condition in which prostate-specific antigen (PSA) concentration higher than 4 ng/mL, was excluded from control subjects [9]. PSA levels were closely linked to prostate size and larger prostate gland was also related with estrogen levels [10]. Later study on the PC showed that there is no difference in the concentration

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of estrogen metabolites rather DNA adducts formation by estrogen, were found to be more active in PC patients than control subjects [11]. Recently, to probe the role of estrogen in PC, 15 urinary estrogen metabolites were determined in different PC patients and it was found a modest difference in the estrogen metabolites concentration between the cancer patients and control subjects [12]. 4-hydroxyestrone (4-OHE_1) ranked higher in abundance among cases than control groups. Concentration of estriol (E_3), estrone (E_1), 16-ketoestradiol (16-kE2), 2-hydroxyestrone (2-OHE_1) and estradiol (E_2) were the highest among all groups, about 60–70% of the total urinary metabolites and E_3 was the dominant estrogen in all study groups. On the other hand, 4-methoxyestradiol (4-$MeOE_2$) was least abundant metabolites detected in the urine of all the groups. In another study, same urinary estrogen metabolites and their ratio were determined in PC patients. This study showed that oxidative metabolism of estrogen favoring 2-hydroxylation over 16α-hydroxylation, was associated with reduced risk of PC [13]. Previous studies from the lab also showed an important role of catechol estrogen modified DNA in the etiopathogenesis of PC [14]. Recent study showed that 16α-hydroxyestrone is responsible for causing breast cancer [15]. Infect, these estrogen metabolites play an important role in cancer as well as in autoimmune diseases [15–26].

The importance of estrogen in PC can be explained by two important facts. One is the presence of estrogen receptor (ER) in the prostate tissues in these patients [27] and the other is the response to estrogen therapy by the PC patients [28]. The effect of estrogen is mediated by two receptors: ERα and ERβ. These two receptors are expressed in prostate tissues. ERβ is the most prevalent and mainly expressed in basal-epithelial cells of prostate while ERα is mainly confined to stromal cells occasionally found in basal-epithelial [29]. Although, PC specimen express both ERα and ERβ but their concentration remains unknown at different stages of the cancer [30]. The expression and functions of both the receptors mediate the development and growth of PC. The loss of ERβ is linked to the development from normal tissues to PC, whereas, those cancers retaining their expression might have a chance for recurrence [31]. In addition, expression of isoform of ERβ (ERβ2 and ERβ5) showed a prognostic biomarker in PC patients [32]. Estrogen plays important role in prostate carcinogenesis [33]. Circulating levels of estradiol (estrone) were slightly higher in African American men than in Caucasian men, whereas, these males have twice the prostate cancer risk of Caucasian [34]. Therefore, increased circulating estrogen might increase prostate cancer risk. Epidemiological data from various source showed mixed results. One study showed an association of increased plasma estrogen with an elevated risk of prostate cancer [35] and another corelating risk with elevated estrogens [36]. While other studies showed opposite finding i.e., increasing prostate cancer risk associated with decreasing levels of estradiol [37]. Other results showed that estrogen metabolic pathway favoring 2-hydroxylation over 16α-hydroxylation might reduce the risk of clinically evident prostate cancer [38]. The study also confirmed an association between the risk of PC and higher urinary levels of 16α-OHE_1 and protective effect of higher 2-OHE_1 to 16α-OHE_1 ratio [39].

Estrone and estradiol are oxidatively metabolized via two major pathways: formation of catechol estrogen and 16α-hydroxylation. The catechol estrogens are 2-hydroxy and 4-hydroxyestrogens that are further inactivated in liver by conjugation reaction such as glucuronidation and sulfation. The other pathway of inactivation occurs in extra hepatic tissues by O-methylation catalyzed by catechol-o-methyl transferase [40].

There are significant evidences that showed that estrogen metabolites play an important role in PC. One study showed a negative relation between the risk of PC and elevated ratio of 2-OHE_1/16α-OHE_1 and positive relation between PC and 16α-OHE_1 [9]. Most of the studies on PC either explain the role of urinary estrogen metabolites in PC or expression of ER in PC but none of them could explain the combined effects of estrogen metabolite and its receptor on PC. To test this important hypothesis, we investigate antibodies from PC to 16α-OHE_1-ER because estrogen metabolite directly involved in PC and ER had been expressed in prostate tissues. This gives us opportunity to screen PC patient's sera with 16α-OHE_1-ER to probe their role. Furthermore, antibodies induced against this complex can also be used as an immunological probe for the determination of 16α-OHE_1 concentration and 2-OHE_1/16α-OHE_1 ratio in the PC patients.

2. Material and methods

2.1 Prostate cancer patients and controls

We have recruited 60 PC patients and their blood samples were taken to access antibodies against 16α-OHE_1-ER complex. Their ages range from 45 to 87 years (65 $\pm$ 7.1), who underwent a prostate biopsy. Experienced pathologist evaluated biopsy samples through routine histology diagnosis. We have taken prostate cancer patients more of stages III and IV as compared to I and II. Gleason score of 8 for almost all of the samples were taken. We have chosen different stages of the disease for PC patients. The controls (n = 40) were the males, age-matched, normal individuals, who were free from PC, coming to the hospital for routine checkup or blood donor or hospital staff. Baseline characteristics of selected patients and controls were given in Table 1. Spot urine samples from 35 patients and 30 control subjects were also taken for the estimation of estrogen metabolites and their ratio. All serum samples (patients and controls) were heated at 56 °C for 30 min to deactivate complement protein and then stored at –20 °C with sodium azide (0.1%) as preservatives. Prior consent from all the subjects was taken and finally, this study was approved by the Institutional Ethical Review Board before subject enrolment (No.: 1/53/39).

2.2 16α-OHE_1-ER complex formation

16α-OHE_1-ER complex was formed as described previously [15]. Briefly, 16α-hydroxyestrone (16α-OHE_1) with a concentration of 1–10 mM was incubated with ER (1 mg) in

TABLE 1. Baseline characteristics of subjects and estimation of 16α-OHE$_1$ and 2-OHE$_1$/16α-OHE$_1$ ratio in prostate cancer patients.

Characteristics	Prostate cancer (n = 60)	Controls (n = 40)
Age (years)	65 $\pm$ 7.1	62 $\pm$ 8.3
BMI (Kg/m^2)		
$<$25	15 (25%)	9 (22.5%)
25–29.9	36 (60%)	25 (62.5%)
$\geq$30	9 (15%)	6 (15%)
Smoking status		
Never	24 (40%)	17 (42.5%)
Past	15 (25%)	12 (30%)
Current	21 (35%)	11 (27.5%)
Employed		
Yes	35 (58.3%)	27 (67.5%)
No	14 (23.3%)	8 (20%)
Retired	11 (18.3%)	5 (12.5%)
Family history of prostate cancer		
Yes	21 (35%)	8 (20%)
No	39 (65%)	32 (80%)
Race or ethnic group		
White/Caucasian	37 (61.7%)	22 (55%)
Black/African	10 (16.7%)	8 (20%)
Asian	8 (13.3%)	6 (15%)
Others	5 (8.3%)	4 (10%)
16α-OHE$_1$ estimation in urine (n = 35) by$^{\#}$		
Anti-16α-OHE$_1$-ER antibodies	5.2 ng/μg creatinine^{c}	5.2 ng/μg creatinine^{a}
Human 16α-hydroxyestrone ELISA Kit	5 ng/μg creatinine	-
2-OHE$_1$/16α-OHE$_1$ ratio*	1.65	-
2-OHE$_1$/16α-OHE$_1$ ratio$^{€}$	1.61	-

$^{\#}$The amount of 16α-OHE$_1$ level was measured by ELISA and the values are corrected with creatinine. 2-OHE$_1$/16α-OHE$_1$; *Ratio estimated by anti-16α-OHE$_1$-ER antibodies; $^{€}$Ratio estimated by commercially available kit.

^{a}n = 30. ^{c}Correlation coefficient r = 0.94 ($p < 0.001$).

potassium phosphate buffer (0.1 M, pH 6) and 1 μM sodium cyanoborohydride was mixed. The reaction mixture was kept for 48 h at 37 °C with shaking. 16α-OHE$_1$ was dissolved in ethonal 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 16α-OHE$_1$.

2.3 Antibodies against 16α-OHE$_1$-ER complex

Antibodies against 16α-OHE$_1$-ER were induced in experimental animals (female rabbits, n = 8) as mention previously [16]. We also induced antibodies against 16α-OHE$_1$ and ER to checked their immunogenicity, whether they alone have any effects on the induction of antibodies or not. Briefly, 16α-OHE$_1$-ER (50 μg) was mixed with equal volume of complete Freund's adjuvant and the mixture injected intramuscularly in the experimental animals. Later doses were given with incomplete Freund's adjuvant. Each rabbit was given 8 injections (weekly) with a total of 400 μg of all antigens. Pre-immune sera served as negative control and were taken prior to the immunization.

2.4 Purification of antibodies against 16α-OHE$_1$-ER complex in prostate cancer

Immunoglobulin G was isolated and purified from the sera of PC patients on a Protein A-Agarose column as described previously [41]. The purity and homogeneity of the purified IgG was checked on 7.5% PAGE. The concentration of immunoglobulin G was evaluated by taken the formula 1.40 OD$_{280}$ = 1.0 mg/mL.

2.5 ELISA

Antibody screening was done in PC or immunized sera by direct binding ELISA as mention earlier [25]. Competition ELISA was also used for specific binding of PC/immunized antibodies to 16α-OHE$_1$-ER complex [25]. Briefly, this complex (100 μL, 2.5 μg/mL) was coated onto microtiter plate for 2 h at 25 °C and later for 24 h at 4 °C. This plate was washed with TBS-T and unoccupied sites were blocked with 100 μL of BSA (1.5%). Immune complexes were prepared by incubating 100 μL of PC/immunized sera (1 : 100 dilution) with increasing concentration of 16α-OHE$_1$-ER complex (or 16α-OHE$_1$ or ER) at 37 °C for 2 h and 4 °C, overnight. 100 μL of immune complex was incubated in each well and anti-human IgG-alkaline phosphatase conjugate was finally added,

followed by addition of p-nitrophenyl phosphate as substrate to developed the reaction. The absorbance was taken at 410 nm on to a microplate reader and data was present as percent inhibition. For 16α-OHE$_1$, we used the Human 16α-hydroxyestrone ELISA Kit (Glory Science Co. Lt, Shirley, NY, USA) and for 2-/16α-OHE$_1$ ratio, the Estramet 2-hydroxyestrogen/16α-OHE$_1$ ELISA Kit (CD Diagnostics, Claymont, DE, USA) was used.

2.6 Quantitation and formation of immune complexes from prostate cancer patients

Quantitation and formation of immune complexes were done as mention previously [26]. Briefly, PC IgG (100 μg) was incubated with increasing amount (0–40 μg) of various antigens (16α-OHE$_1$-ER, ER and 16α-OHE$_1$) 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 control that were 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 protein bound in immune complex were determined by colorimetric methods [42]. The affinity constant was calculated by determining affinity using Langmuir plot [43].

2.7 Statistical analysis

Statistical significance was determined using the student's *t*-test (SPSS Statistic 22, IBM, Armonk, NY, USA) and normality test was applied. A *p*-value of $p < 0.05$ was taken as statistical significance.

3. Results

3.1 Characterization of 16α-OHE$_1$-ER complex

Incubation of 16α-OHE$_1$ with ER resulted in the formation of high molecular weight complex that showed less mobility on the SDS-PAGE relative to ER [44]. Molecular weight of newly synthesized complex is closed to 68 kDa. UV absorption spectra revealed that 16α-OHE$_1$-ER demonstrated high absorbance, which was about 38.3% UV hyperchromicity compared to ER at 280 nm (Table 2, Ref. [15]).

TABLE 2. Characterization of 16α-OHE$_1$-ER$_1$ adducts and control.

Parameters	16α-OHE$_1$-ER$_1$ Complex	ER
Hyperchromacity at 280 nm (%)	38.3%	-
Molecular Weight (kDa)	≈68	=68
Band on SDS-PAGE	Thick	Sharp
Mobility on SDS-PAGE	Less	More

Adapted from [15]. The experiment was done by incubating 16α-OHE$_1$-ER and ER in 0.1 M potassium phosphate, pH 6.0, containing 1 μmol of sodium cyanoborohydride and 0.1% ethanol at 37 °C for 48 h. Hyperchromacity of 16α-OHE$_1$-ER was calculated by measuring OD of 16α-OHE$_1$-ER as compared to ER.

3.2 Antibodies against 16α-OHE$_1$-ER complexes in the sera of prostate cancer patients

Serum samples collected from 60 patients and 40 control subjects, were tested for the presence of antibodies against 16α-OHE$_1$-ER, ER and 16α-OHE$_1$ by direct binding ELISA. Nearly all the selected sera demonstrate high binding to 16α-OHE$_1$-ER in comparison to ER or 16α-OHE$_1$ ($p < 0.05$ or $p < 0.001$). Normal human sera showed no appreciable binding to either of the antigens (Fig. 1). Binding specificity was also checked with ER and 16α-OHE$_1$ and it was found that their binding is less as compared to 16α-OHE$_1$-ER. 16α-OHE$_1$ did not showed any binding with either antibodies from PC or normal subjects. In all our experiments we have chosen ERα because this isoform showed better results compared to ERβ. Competition ELISA was further used to detect binding specificities of antibodies from PC to 16α-OHE$_1$-ER, ER and 16α-OHE$_1$. 16α-OHE$_1$-ER showed an inhibition to about 59.8% ± 7.3% (37.3%–81.9%) in the antibody activity.

ER demonstrates less inhibition, that was about 42.3% ± 5.3% (15.5%–65.3%) and 16α-OHE$_1$ showed no appreciable inhibition to antibodies from PC (12.4% ± 3.9%) (Fig. 2a). The antibodies from PC were isolated and purified by affinity chromatography on Protein A-Agarose column (Sigma, St. Louis, MO, USA). Purity of the isolated IgG from PC patients was checked by running SDS-PAGE and it was found to be a single homogenous band on the gel (Fig. 3).

In competition binding assay, 16α-OHE$_1$-ER shown an inhibition of about 69.3% ± 10.3% (41.8%–85.3%) in the antibody activity, while for ER, it was about 46.3% ± 3.2% (18.1%–69.8%). The inhibition of PC IgG was also evaluated with 16α-OHE$_1$ and it was found to about 15.9% ± 3.9% (Fig. 2b).

Binding specificities of antibodies from PC were also checked according to various clinical characteristics, they had during the study. Accordingly, we divided them in eight groups based on what clinical characteristics they had during the course of the study. Whether the cancer patients are ER positive or not? PSA less than or greater than 4, 12-OHE$_1$/16α-OHE$_1$ ratio and BMI. Among all, cancer patients who were ER positive showed the highest inhibition (76.3% ± 8.9%), followed by patients with smoking (75.8% ± 5.4%), low 12-OHE$_1$/16α-OHE$_1$ ratio (72.4% ± 7.3%) and PSA level ≥4 (71.3% ± 7.8%) (Table 3). Inhibition values according to BMI showed that obese and overweight patients have high inhibition values (75.3% ± 8.3% and 72.3% ± 9.8%) and depend on this parameter. While for other groups such as ER negative, diabetes, hypertension and herbal medication, PSA <4 and high 12-OHE$_1$/16α-OHE$_1$ ratio have no major effects on the inhibition values (Table 3).

3.3 Affinity of antibodies against 16α-OHE$_1$-ER in prostate cancer patients

The antigen-antibody interaction was further characterized by estimating affinity constant. In this technique, varying amounts of different antigens (16α-OHE$_1$-ER, ER and 16α-OHE$_1$) were treated with constant amount of PC IgG (100

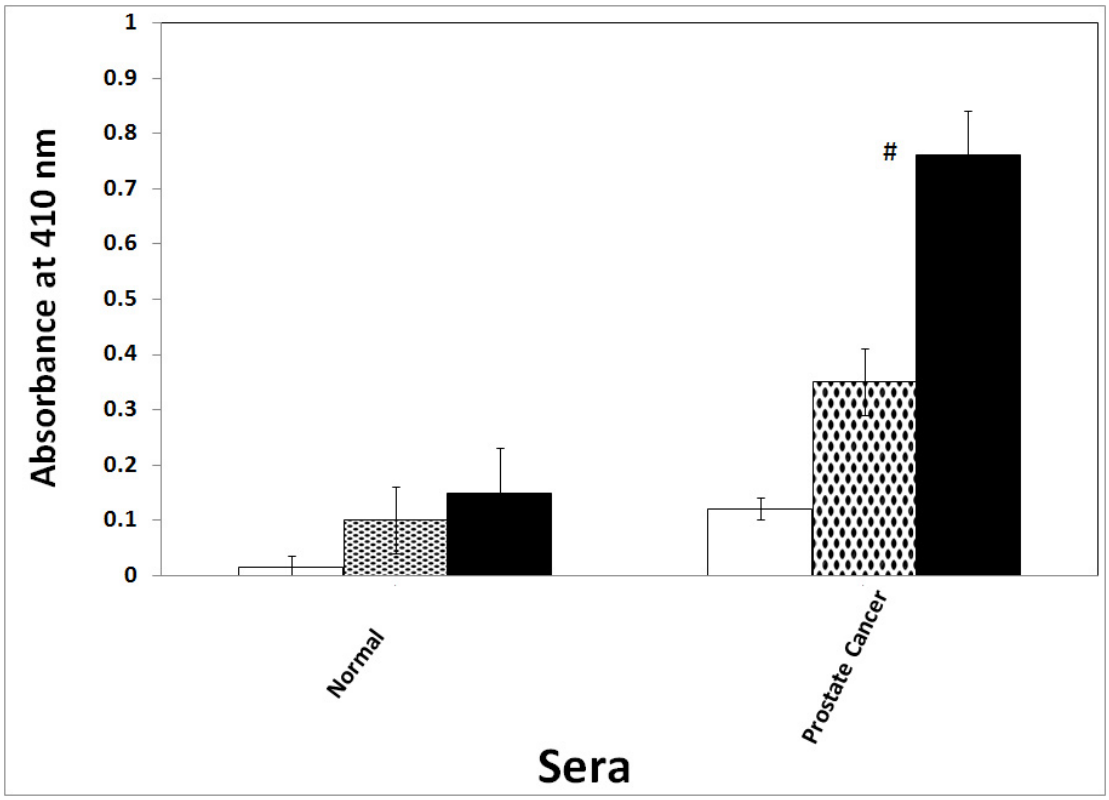

FIG. 1. Direct binding ELISA of controls and prostate cancer (PC) patients. Direct binding enzyme-linked immunosorbent assay of control (n = 40) and PC antibodies (n = 60) to 16α-OHE$_1$-ER (■), ER (▒) and 16α-OHE$_1$ (□). Microtitre plates were coated with 100 μL of respective antigen (2.5 μg/mL). The reaction was developed with p-nitrophenyl phosphate as the substrate and the absorbance was recorded at 410 nmas described in "Materials and Methods". Each histogram represents the mean $\pm$ SD. $^{\#}p < 0.001$, $p < 0.001$, significantly higher binding than normal sera and 16α-OHE$_1$ in PC; $p < 0.05$ significantly higher binding than ER in PC.

μg, n = 8). Normal human IgG was a negative control that was also treated with the same conditions. The data showed that about 24 μg of 16α-OHE$_1$-ER complexes was bound to about 73 μg of PC IgG. With ER, a maximum of 32 μg of ER was bound to about 61 μg of cancer IgG. Similarly, with 16α-OHE$_1$, a maximum of 35 μg of 16α-OHE$_1$ was bound to about 59 μg of PC IgG. Langmuir plot was used to evaluates the apparent association constant (Fig. 4). The affinity constant of prostate cancer IgG was found to be of the order of 1.19×10^{-7} M, 1.45×10^{-6} M and 1.13×10^{-6} M for 16α-OHE$_1$-ER, ER and 16α-OHE$_1$, respectively. Affinity of PC IgG from the patients was found to highest for 16α-OHE$_1$-ER in comparison to ER or 16α-OHE$_1$.

3.4 Induced antibodies against 16α-OHE$_1$-ER and their characterization

The antigencity of 16α-OHE$_1$-ER with their suitable controls were induced in experimental animals (female rabbits). The 16α-OHE$_1$-ER was found to be highly immunogenic ($\geq$1 : 25600) triggering high titer antibodies [44]. Pre-immune sera did not show any binding to 16α-OHE$_1$-ER and served as negative control. The titer shown by ER and 16α-OHE$_1$ was low incomparison to 16α-OHE$_1$-ER. In competition ELISA, induced antibodies in the serum showed an inhibition of about 75.3% in the antibody activity with 16α-OHE$_1$-ER as an inhibitor at 20 μg/mL and 50% inhibition was achieved at 7.7 μg/mL (Fig. 5a).

For ER and 16α-OHE$_1$, the inhibition values were found to be 71.8% and 64.3%, respectively and 50% inhibition was achieved at 13.8 μg/mL and 17.3 μg/mL. The induced IgG was isolated and purified on protein A-Agarose column and their cross-reactivity was also checked. The inhibition value for 16α-OHE$_1$-ER with induced IgG was found to be 95%. While for ER and 16α-OHE$_1$, it was found to be 91% and 84.3% (Fig. 5a). Immunocross-reactivity of anti-16α-OHE$_1$-ER antibodies was also checked with 16α-OHE$_1$-ER, ER, 16α-OHE$_1$, 2-OHE$_1$, progesterone receptor (PR), 4-OHE$_1$, just to rule out whether anti-16α-OHE$_1$-ER antibodies shared common epitopes on these antigens. The anti-16α-OHE$_1$-ER antibodies recognized its own antigen (i.e., 16α-OHE$_1$-ER) in addition to the cross-reactivity shown with 16α-OHE$_1$ (Fig. 5b).

Similar is the case for anti-16α-OHE$_1$ antibodies in which these antibodies showed binding with 16α-OHE$_1$-ER. As anti-16α-OHE$_1$-ER antibodies showed cross-reactivity with 16α-OHE$_1$, so these antibodies can be used as probe for the estimation of 16α-OHE$_1$ in the urine of PC patients. The mean value of 16α-hydroxyestrone (16α-OHE$_1$) was 5.2 ng/μg creatinine, as estimated by anti-16α-OHE$_1$-ER antibodies, which is comparable to the value obtained by using a commercially available kit (5.0 ng/μg creatinine) (Table 1). In healthy controls (n = 30), the mean value of

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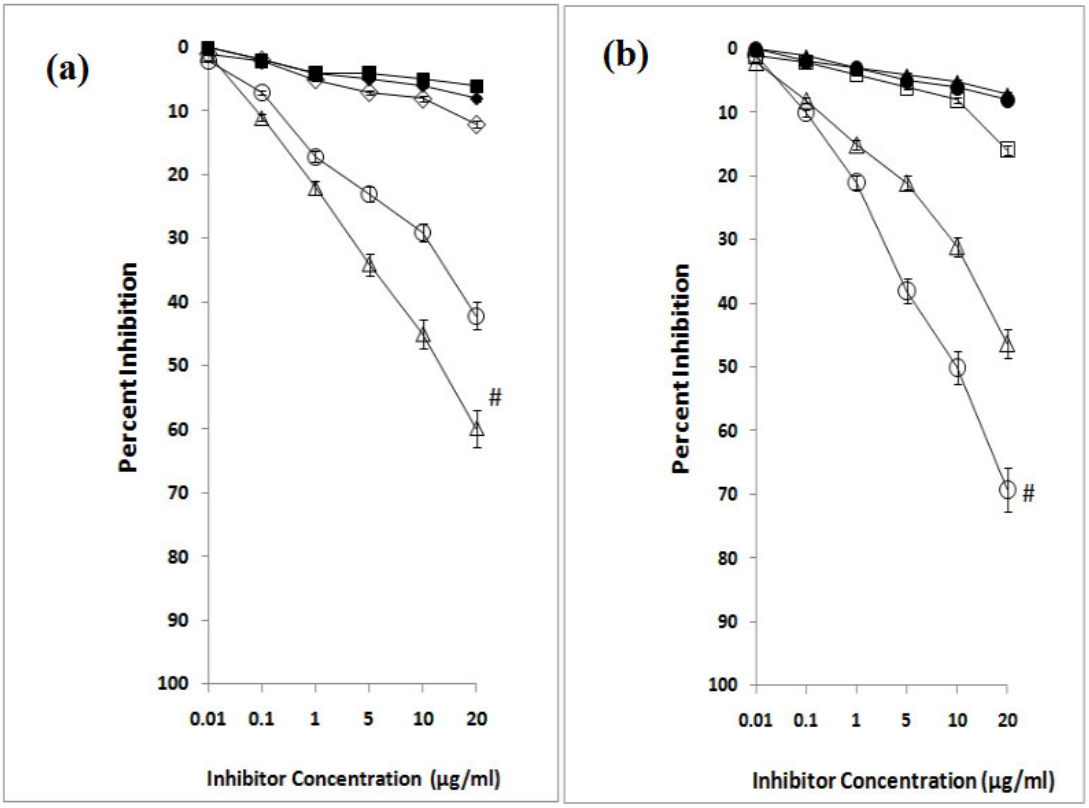

FIG. 2. Inhibition ELISA of antibodies in PC and control groups. (a) Inhibition ELISA of anti-(16α-OHE$_1$-ER, ER, 16α-OHE$_1$) PC (-Δ-, -○-, -◇-) and normal (-■-, -♦-) sera with 16α-OHE$_1$-ER, ER, 16α-OHE$_1$. Microtitre plates were coated with respective antigens (2.5 μg/mL). Note: Inhibition values for normal sera with 16α-OHE$_1$ were negligible and are not shown. #Significantly higher inhibition than ER ($p < 0.05$) and 16α-OHE$_1$ ($p < 0.001$). (b) Inhibition of PC 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). Inhibition values for normal IgG with 16α-OHE$_1$ were negligible and are not shown. #Significantly higher inhibition than ER ($p < 0.05$) and 16α-OHE$_1$ ($p < 0.001$).

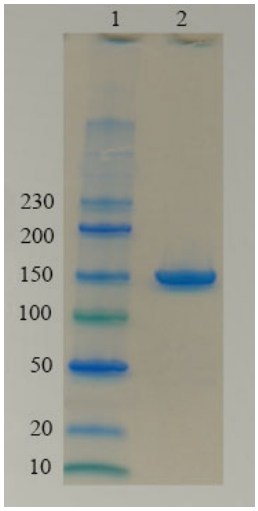

FIG. 3. SDS-PAGE of purified IgG on 7.5% polyacrylamide gel (Lane: 1. Protein Marker (kDa), 2. Purified IgG).

16α-OHE$_1$ was found to be 4.4 ng/μg creatinine. While, the 2-HE$_1$/16α-OHE$_1$ ratio for the prostate cancer was found to be 1.65.

4. Discussion

Estrogen (and its metabolites) can function as potential agent in the progression and development of PC [45]. They play a causative role in PC but the exact mechanism remains unknown. The potential mechanism that can explain the role of estrogen in PC includes epigenetic modification and estrogenic imprinting hyperprolectinemia, direct genotoxicity, inflammation and receptor-mediated actions. Although, estrogen can be used as potential hormonal therapy in PC but it can also cause this cancer [46]. Estrogen mediated its effect through the binding to its receptor (ERα and ERβ) in the cells. Both the receptors for estrogen are expressed in normal prostate. ERα is expressed in the stromal cells and ERβ is found in the basal cells of prostate. ERβ has tumor suppression role in which its expression is decreased leading to methylation of CpG dinucleotide in the gene [47]. Moreover, polymorphism in codon 10 of ERα is a risk factor for PC [48]. Estrogen act as causative factor not only through their receptors but also through their role as genotoxic agent [49]. Estrogen can be oxidized to active catechol-estrogen metabolites by P450-mediated hydroxylation [50]. These metabolites lead to the generation of ROS that can damaged DNA and make DNA adducts [49]. Once DNA get damaged it alters its antigenicity leading to the generation of autoanti-

TABLE 3. Clinical characteristics and immunological data of different prostate cancer patients.

Prostate cancer patients (n = 60)	Maximum percent (%) inhibition at 20 μg/mL		
	16α-OHE$_1$-ER^{a,*}	ER^b	16α-OHE$_1$^{c}
Overall	69.3 $\pm$ 10.3	46.4 $\pm$ 3.2	15.9 $\pm$ 3.9
Estrogen receptor (ER)			
Positive (n = 35)	76.3 $\pm$ 8.9	52.3 $\pm$ 4.5	14.9 $\pm$ 4.1
Negative (n = 25)	67.9 $\pm$ 11.3	45.8 $\pm$ 3.1	11.2 $\pm$ 3.1
Smoking at baseline			
Current/Past (n = 36)	75.8 $\pm$ 5.4	45.1 $\pm$ 8.1	13.5 $\pm$ 9.1
Never (n = 24)	68.3 $\pm$ 11.8	43.4 $\pm$ 3.5	11.2 $\pm$ 8.1
Diabetes medications (n = 22)	67.3 $\pm$ 8.4	45.8 $\pm$ 4.1	14.3 $\pm$ 3.1
Hypertension medications (n = 25)	68.5 $\pm$ 3.8	43.4 $\pm$ 5.7	13.8 $\pm$ 3.4
Herbal medications (n = 10)	67.3 $\pm$ 4.9	44.3 $\pm$ 8.1	12.3 $\pm$ 6.2
PSA (ng/mL)			
$<$4 (n = 15)	69.3 $\pm$ 8.9	45.3 $\pm$ 8.4	11.5 $\pm$ 4.1
$\geq$4 (n = 45)	71.3 $\pm$ 7.8	48.4 $\pm$ 4.3	15.8 $\pm$ 3.9
2-OHE$_1$/16α-OHE$_1$ ratio			
High (n = 28)	67.2 $\pm$ 11.4	47.4 $\pm$ 3.1	11.4 $\pm$ 3.9
Low (n = 32)	72.4 $\pm$ 7.3	48.5 $\pm$ 5.4	12.1 $\pm$ 8.9
BMI (Kg/m^2)			
$<$25 (n = 15)	68.3 $\pm$ 9.4	45.8 $\pm$ 3.7	13.3 $\pm$ 1.3
25–29.9 (n = 36)	72.3 $\pm$ 9.8	44.3 $\pm$ 8.1	14.4 $\pm$ 2.9
$>$30 (n = 9)	75.3 $\pm$ 8.3	43 $\pm$ 4.2	16.9 $\pm$ 4.5
NH IgG (n = 25)	8.2 $\pm$ 2.6	7.9 $\pm$ 3.1	5.4 $\pm$ 1.9

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

NH IgG, normal human IgG.

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

^{a}16α-OHE$_1$-ER as inhibitor, ^{b}ER as inhibitor, ^{c}16α-OHE$_1$ as inhibitor.

bodies in autoimmune diseases [15–26, 44]. P450-mediated hydroxylation also produced 16α-hydroxyestrone metabolites that exert its effect through binding to its receptor [51]. Elevated levels of urinary 16α-OHE$_1$ were associated with increased risk of prostate cancer [9] and ER had been expressed in prostate, so there might be a good opportunity to know the combed effect of 16α-OHE$_1$ and ER on PC. To study this important phenomenon, the binding affinity of the antibodies from PC with 16α-OHE$_1$-ER was measured to check whether this complex (16α-OHE$_1$-ER) has any affinity with antibodies from PC. The binding specificity of antibodies from sera of 60 PC patients and 40 controls to 16α-OHE$_1$-ER was checked with direct binding and inhibition ELISA. This complex showed high binding with almost all the chosen sera compared to controls ($p < 0.001$). The combination of 16α-OHE$_1$ and ER exposed specific groups/molecules that can function as good epitopes for the prostate cancer IgGs. These results showed that 16α-OHE$_1$-ER can acts as better inhibitor showing a substantial difference in the binding of 16α-OHE$_1$-ER over ER ($p < 0.05$) or 16α-OHE$_1$ ($p < 0.001$). This data is similar to our previous studies from the lab that showed high binding of 16α-OHE$_1$ and ER adduct by breast cancer IgGs [44]. Various therapeutic approaches targeting the use of monoclonal antibodies (Mabs) to prostate specific antigen in PC had been taken into consideration [52]. These approaches include early detection of PC with the use of monoclonal antibodies with hormone and chemotherapy [53]. These Mabs in PC include anti-human epidermal growth factor receptor-2 (HER2) Mabtrastuzumab, anti-epidermal growth factor receptor (EGFR) Mabscetuximab and panituzumab and the anti-vascular endothelial growth factor (VEGF) Mabbevacizumab [54]. In animal model, anti-IL-20 monoclonal antibodies suppress PC growth and therefore, can be a novel target for the treatment of PC [55]. The presence or high level of serum antibodies against genitouring pathogens was not associated with PC [55].

The specificities of antibodies from PC were also tested according to various clinical characteristics in PC patients. Among them, those PC patients who expressed ER showed the highest inhibition followed by those cancer patients who had history of smoking, low 2-OHE$_1$/16α-OHE$_1$ ratio and PSA level $\geq$4. As mention already, ERα and ERβ are expressed in prostate tissues and they are present in prostate during carcinogenesis [27]. Such binding might be observed because these patients already had antibodies against ER, which in combination of 16α-OHE$_1$, generate more immunological response. Cigarette smoking may increase the risk of PC by effecting circulatory hormone or through exposure to various carcinogens [56]. It might be possible that smoking increases the concentration of 16α-OHE$_1$ in these cancer patients that come in contact with ER, makes a complex and thus generate antibodies against this complex and showed high binding. As far as the high binding of patients with low 2-OHE$_1$/16α-OHE$_1$ ratio is concern, low

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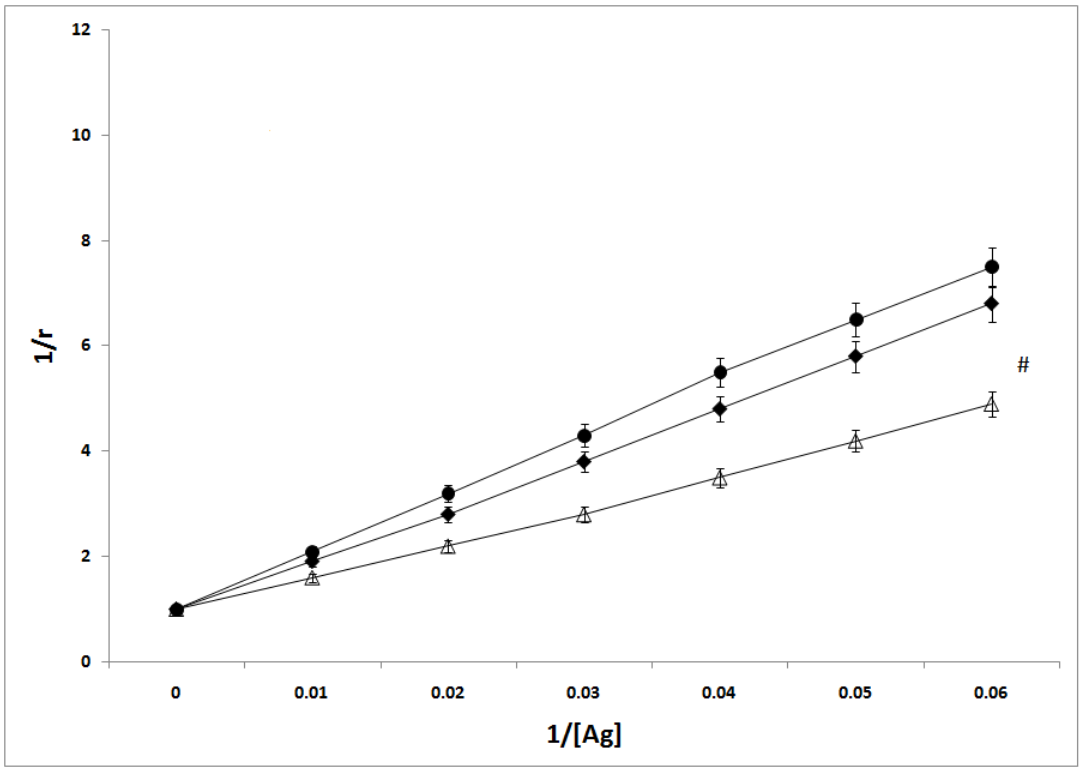

FIG. 4. Determination of apparent association constant by Langmuir plot. Antigens were 16α-OHE$_1$-ER (-Δ-), ER (-$\blacklozenge$-) and 16α-OHE$_1$ (-$\bullet$-). Immune complexes were prepared by incubating 100 μg of IgG with varying amounts of different antigens (0–100 μg) in an assay volume of 400 μL for 2 h at room temperature and overnight at 4 °C. The binding data were analyzed for antibody affinity as described in "Materials and Methods". #Significantly higher binding than ER ($p < 0.05$) and 16α-OHE$_1$ ($p < 0.001$).

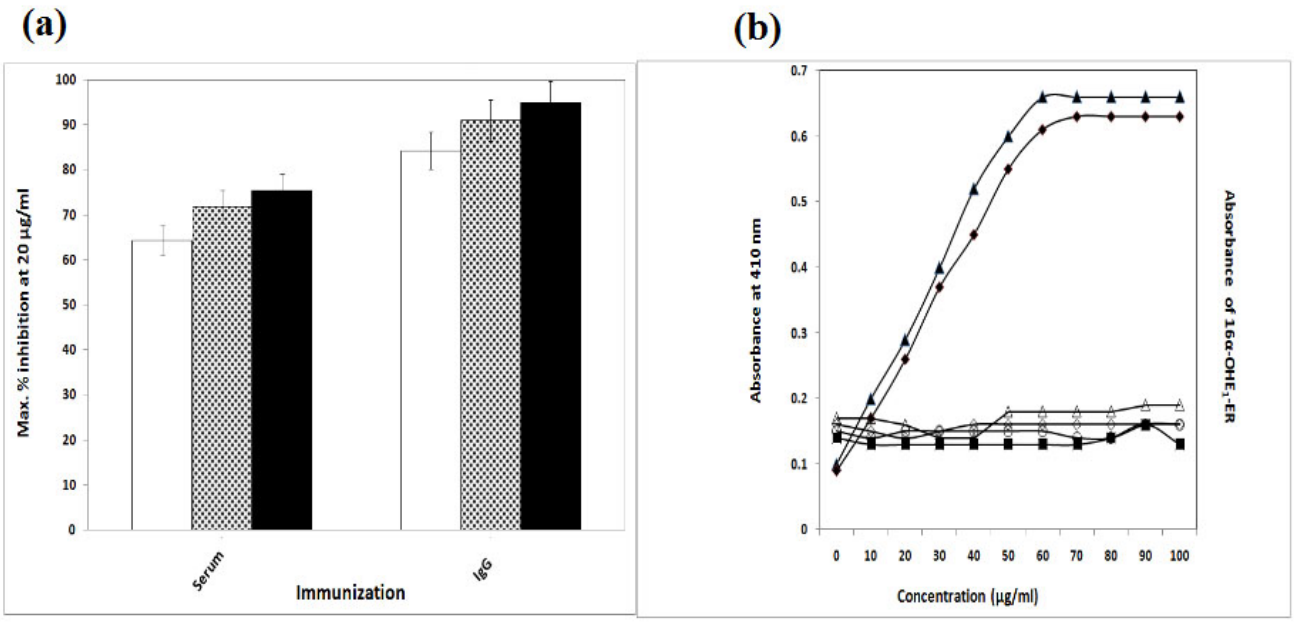

FIG. 5. Inhibition ELISA and immunecross-reactivity of immune IgG against 16α-OHE$_1$-ER. (a) Inhibition ELISA of anti-(16α-OHE$_1$-ER, ER, 16α-OHE$_1$) immune sera (■, ▒, □) and anti-(16α-OHE$_1$-ER, ER, 16α-OHE$_1$) IgG binding to 16α-OHE$_1$-ER (■), ER (▒), 16α-OHE$_1$ (□). Inhibition values for pre-immune sera and IgG with 16α-OHE$_1$-ER, ER, 16α-OHE$_1$, were negligible and are not shown. Microtire plates were coated with respective antigens (2.5 μg/mL). (b) Immunecross-reactivity of immunized IgG against 16α-OHE$_1$-ER with similar molecules. Estimation of immune cross-reactivity of anti-16α-OHE$_1$-ER antibodies against 16α-OHE$_1$-ER (-▲-), ER (-Δ-), 16α-OHE$_1$(-$\blacklozenge$-), 2-OHE$_1$(-○-), progesterone receptor (PR) ($\lozenge$), 4-OHE$_1$ (-■-).

ratio means high concentration of 16α-OHE$_1$, which is an active estrogen metabolite and elevated urinary level have been associated with increased risk for PC [9]. Estrogen metabolites such as 16α-OHE$_1$ involved in the release of

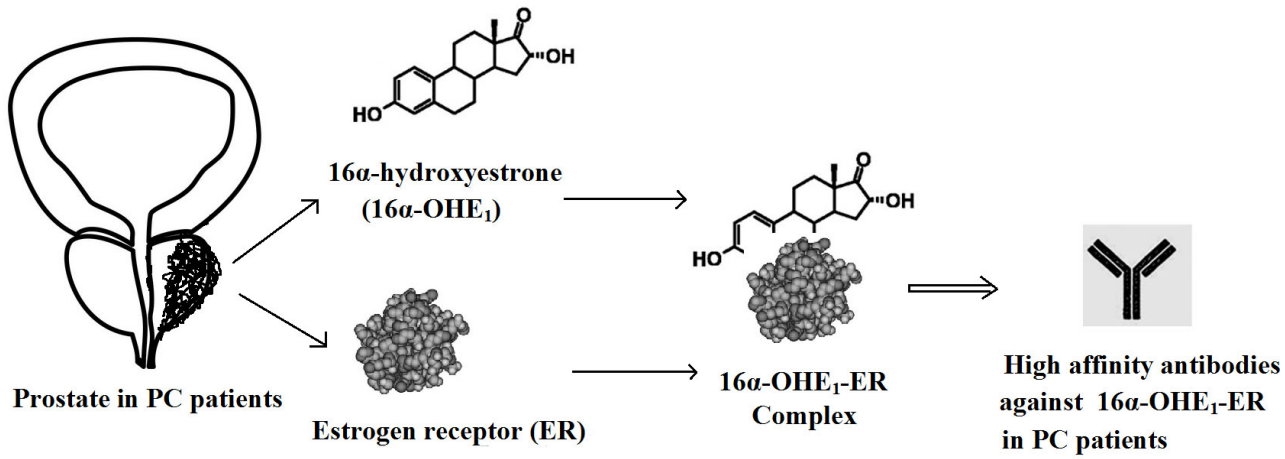

FIG. 6. The proposed mechanism for the production of high affinity antibodies in prostate cancer (PC) patients.

inflammatory mediators from the human amnion-derived cells [57] and somehow linked to inflammation. Again, high binding is due to the autoantibodies produced during inflammatory conditions. High binding in patients with PSA level $\geq$4 might be due to prostatitis or urinary tract infections, in which its concentration has been dramatically increased. The inhibition values gradually increased according to different stages and grades of PC indicating that more antibodies are produced as the PC progresses [58]. In addition, depression augments the production of antibodies against 16α-OHE$_1$-ER complex in prostate cancer patients [58]. Depression increased the production of antibodies through the generation of pro-inflammatory conditions in these patients.

To further confirmed the recognition of 16α-OHE$_1$-ER complex by antibodies from PC, we determined the affinity of antibodies by quantitative precipitin titration. The affinity constant of the order of 10^{-7} M clearly demonstrates high recognition of this complex by PC antibodies. The high recognition of 16α-OHE$_1$-ER complex by PC IgGs indicates possible participation of 16α-OHE$_1$-ER complex in prostate cancer pathogenesis. Studies have shown that estrogen metabolites (including 16α-OHE$_1$) are present in tissues, bile, urine and blood [7]. The production of antibodies in prostate cancer might arise as a result of formation of 16α-OHE$_1$-ER complex. Therefore, it could be possible that 16α-OHE$_1$-ER complex might be one of the important factors toward the generation of antibodies in prostate cancer. The induced antibodies showed cross-reactivity towards other antigens (i.e., anti-16α-OHE$_1$-ER with 16α-OHE$_1$ and anti-16α-OHE$_1$ antibodies with 16α-OHE$_1$-ER). Because anti-16α-OHE$_1$-ER antibodies showed cross-reactivity with 16α-OHE$_1$, these antibodies can be used as immunochemical probe for the estimation of 16α-OHE$_1$ in PC patients. These antibodies (i.e., anti-16α-OHE$_1$-ER antibodies) were also used to determined 2-OHE$_1$/16α-OHE$_1$ ratio in PC patients.

The proposed mechanism for the generation of antibodies in PC includes the production of antibodies against 16α-OHE$_1$-ER through the formation of 16α-OHE$_1$-ER complex. 16α-OHE$_1$ and ER bind to each other to formed 16α-OHE$_1$-ER complex in prostate tissues. Formation of complex modified its immunogenicity leading to the generation and elevated levels of PC antibodies (Fig. 6). We strongly recommended to use other techniques like western blotting and immunohistochemistry of the cancer patient's samples to confirmed their systemic level and in the tissue. This would help us to know their levels in the serum to compare with in the tissues of the cancer patients.

5. Conclusions

In conclusion, our data clearly demonstrates the possible antigenic role of 16α-OHE$_1$-ER in the generation of antibodies in PC patients. Results imply that combination of 16α-OHE$_1$ and ER generates discriminating epitopes, which were highly recognized by PC IgG. Anti-16α-OHE$_1$-ER antibodies shown to represent an alternate immunological probe for the estimation of 16α-OHE$_1$ and 2-OHE$_1$/16α-OHE$_1$ ratio in the urine of different PC patients, although we recommended to generate monoclonal antibodies-based probe for more specificity and accuracy.

Abbreviations

16α-OHE$_1$, 16α-hydroxyestrone; PC, prostate cancer; ER, estrogen receptor; 2-OHE$_1$, 2-hydroxyestrone; 16α-OHE$_1$-ER, 16α-hydroxyestrone-estrogen receptor; ELISA, enzyme linked immunosorbent assay; PSA, prostate-specific antigen; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Author contributions

WAK conceived, designed, performed the experiments and wrote the paper; MWAK performed the experiments and analyzed the data.

Ethics approval and consent to participate

Prior consent from all the subjects was taken and finally, this study was approved by the Institutional Ethical Review Board (Deanship of scientific Research, KKU, 1/53/39).

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Conflict of interest

The authors declare no conflict of interest.

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