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Investigating the expression changes of genes involved in the biosynthesis of androgens in prostate adenocarcinoma and benign prostatic hyperplasia

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ABSTRACT: Prostate cancer (PCa) is the second most frequently diagnosed malignancy in men worldwide. Androgens are critical for normal prostate development and function, as well as prostate cancer initiation and progression. There are various enzymes in the path of androgen biosynthesis, each of which can be a potential target for designing new therapeutic approaches. Studies have shown that the expression profile of genes involved in the biosynthesis of androgens changes in prostate cancer In this study, we investigated the expression changes of 4 genes involved in the biosynthesis of androgens at both transcription and translation levels in 80 patients with prostate adenocarcinoma, 80 adjacent healthy samples and 80 benign hyperplasia (BPH) samples by RT-qPCR and Western Blot methods, which included AR, HSD3B1, HSD3B2, SRD5A2. PCa showed a significant increase in the expression of the gene AR compared with BPH and Ctrl tissues (p=0.0001), as well as significant increase in the expression of the gene SRD5A2 (p=0.0038), HSD3B1 (p=0.0011) and HSD3B2 (p=0.0469) in BPH samples compared with PCa and Ctrl tissues. The results showed the presence and expression of all enzymes in all groups. But as can be seen, the expression levels of SRD5A2 and HSD3B2 proteins in the BPH group are higher than those in the Ctrl and PCa groups. The results of this study showed that there are extensive changes in the expression profile of genes involved in the biosynthesis of androgens in prostate adenocarcinoma and benign hyperplasia, these results support the claim that genes and enzymes involved in the biosynthesis of androgens can be used as diagnostic and therapeutic targets.

Keywords: *Androgens, Benign Prostate Hyperplasia, Gene expression, Real-time PCR, Prostate Cancer*

INTRODUCTION

Prostate cancer (PCa) is the most frequently diagnosed malignancy among males in majority of economically developed countries, and is the second most common cancer in men worldwide [1]. Benign prostatic hyperplasia (BPH) is an enlargement of the prostate gland that is frequently found in aging men and contributes to lower urinary tract symptoms [2]. The development

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and progression of prostate cancer is critically dependent upon the actions of androgens on the androgen receptor (AR) and the subsequent reactions that occur as a result of this activation [3]. Androgens, particularly testosterone (T) and dihydrotestosterone (DHT), which serve as the major endogenous ligands of AR, are the key drivers for both the initiation and progression of prostate cancer [4]. In males, androgens synthesis occurs in the Leydig cells and zona reticular is layer of adrenal gland [5]. Biosynthesis of androgens begins with cholesterol as the source material and, following the enzyme family (HSD3B, CYP17, SRD5A) catalysed synthesis of several intermediate compounds, culminates in the production of testosterone and Dehydroepiandrosterone (DHEA), which is then released into the circulation [6]. Although testes are the major source of testosterone in normal men, the intratumoral synthesis of testosterone from weak adrenal androgens appears to be a substantial source of prostatic testosterone [7]. Androgen deprivation therapy (ADT) is universally accepted as first-line treatment of symptomatic prostate cancer. ADT can be achieved by: suppressing the secretion of testicular androgens. Inhibiting the action of circulating androgens at the level of their receptor using competing compounds is known as anti-androgens [8].

Several studies have shown that intraprostatic testosterone and Dihydrotestosterone (DHT) levels do not decline as markedly as serum levels after ADT [9]. Three putative synthetic pathways currently exist that might lead to increased levels of signaling androgens within the tumor that including the canonical pathway, backdoor pathway, and 5α-dione [10]. The steps from cholesterol to Dihydrotestosterone (DHT) in the canonical pathway are known to occur in the adrenal glands and the steps from cholesterol to testosterone are the same as the reactions that occur in the testes during canonical steroid biosynthesis [11]. Steroidogenic enzymes, such as CYP11A1, CYP17A1, HSD3B1, AKR1C3 and SRD5A, are essential to catalyzing the conversion of the initial substrate cholesterol into potent androgens that confers the prostate cancer progression [4]. Accumulating evidence indicates that many steroidogenic enzymes are upregulated in the progression set. After cleavage of the cholesterol side chain, the resulting pregnenolone is converted to DHEA by the activity of CYP17A1 enzyme. Due to the activity of AKR1C3 and CYP11A1, DHEA is first converted to Androstenediol and finally to testosterone due to the activity of the HSD3B enzyme family. Testosterone can be converted into a potent androgen called dihydroxytestosterone by the enzymatic activity of SRD3B [12]. Since the Androgens action in prostatic cells is mediated by AR, Genetic alterations in this receptor can affect the biosynthesis of androgens. AR amplification is the most frequent genetic alteration reported for castrate-resistant prostate cancers (CRPC) tumors, as observed in up to 50% of the cases [13-15]. AR amplification has been linked to AR overexpression in clinical samples and experimental systems and thereby to sensitizing tumor epithelial cells to low androgen levels [16]. CYP17A1 is a multifunctional, hydroxylase type enzyme of the cytochrome p450 family, expressed in the endoplasmic reticulum of testicular Leydig cells and the adrenal cortex [17]. Genetic variations that affect the enzyme encoded by the SRD5A2 gene, which converts testosterone to DHT, may be associated with the risk of developing prostate cancer or disease progression [18, 19]. The genes HSD3B1 and HSD3B2 are intimately involved in androgen metabolism and cell proliferation in the prostate. Each shows intraspecific polymorphism and variation among racial-ethnic groups that is associated with the risk of prostate cancer [20]. As regards, significant correlations were revealed among BPH and PCa. BPH could lead to escalating risks of PCa. Meanwhile people with a history of BPH might be more vulnerable to PCa [21]. Considering the important role of the androgen biosynthesis pathway in the formation of prostate cancer and benign prostatic hyperplasia, in this study, we investigated the changes in the expression of the genes controlling this pathway at two levels mRNA and Protein. For this purpose, we investigated the expression of these genes in three groups of prostate cancer malignant tissue, adjacent healthy tissue and benign hyperplasia tissue sample.

MATERIALS AND METHODS

Clinical tissue sample

The formalin-fixed paraffin-embedded (FFPE) pros-

tate tissue samples were obtained from 240 patients including 80 prostate adenocarcinoma 80 normal adjacent tissues and 80 benign prostate hyperplasia. All samples were provided by the Khatam-al-Anbya Hospital, Tehran, Iran between 2020-2021. All patients gave their informed consent, In addition, the pathobiological information of each patient was received through the hospital and the study was approved by the Ethics Committee of Islamic Azad University, Tabriz Branch (IR.IAU.TABRIZ.REC.1400.178).

RNA Isolation and cDNA synthesis

RNA extraction from FFPE section was performed as previously described(22), Briefly, under appropriate conditions, tissue samples were deparaffined by xylenen then protein digestion was performed by proteinase K and its buffer (Tris-HCl 100mM + NaCl 200mM + EDTA 2mM + SDS1%) after that 1ml Trizol solution added to samples, Continue the steps according to manufacturer's instructions. RNA samples were quantified spectrophotometrically and loaded onto 1.5% agarose gel to visualize the degree of RNA integrity. The reverse transcription was carried out with Thermo Scientific RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, cat. K1622) in according to the manufacturer's instructions.

Real-time PCR

Expression levels of the genes were measured by real-time PCR using the RealQ Plus 2x Master Mix Green (Ampliqon, Denmark). Real-time PCR was carried out on a RotorGene Q real-time PCR system (Qiagen, Germany). Signals were normalized to the "housekeeping" gene GAPDH as an endogenous internal control. Gene-specific oligonucleotide primers were used for HSD3B1, HSD3B2, SRD5A2 and AR (Table 1). The optimal annealing temperature for each primer set was determined prior to the analysis of experimental samples. Triplicate real-time PCR reactions were run for each sample. Averaged Ct values for each reaction were normalized to GAPDH values. Finally, relative gene expression was calculated using the 2^-ΔΔct method.

Protein Extraction and Western Blotting

Protein extraction from the FFPE section was carried out as previously described (23). Briefly, Deparaffinization process was done by heating, then homogenization of the deparaffinized block in lysis buffer. Cell lysate were separated in 10% SDS-PAGE. Proteins were electro-blotted onto polyvinylidene fluoride (PVDF) membranes and blocked by incubation with TBST (20mM Tris (pH=7.6), 150mM NaCl and

Table 1. Clinicopathological characteristics of the studied population and the relationship of these data with the incidence of malignancy.

Clinical parameter	Benign prostatic hyperplasia	Prostatic Adenocarcinoma	P. Value
Age (mean±SD)	61.8±7.2	68.3±5.9	0.36
Pathology stage	Negative for malignancy	Stage 2: 27 (67.5 %) Stage 3: 13 (32.5 %)	-
Gleason Score ND 3+3=6 3+4=7 5+5=10	75 (93.75%) 5 (6.25%) - -	- 58 (72.5%) 13 (16.25%) 9 (11.25%)	-
PSA range 10> 10≤	17 (21.25%) 63 (78.75%)	13 (16.25%) 67 (83.75%)	0.08
Testosterone (ng/ml, mean±SD)	473.8±96.2	514.6±102.7	0.06
Prostate Volume (g, mean±SD)	44.6±8.3	39.4±12.9	0.14

Table 2. Primers sequences used in RT-qPCR.

Gene	Sequence	Tm	ID	Ac	Product
AR	5'- CAACTCCTTCAGCAACAGCA-3' 5'- CCGACACTGCCTTACACAAC-3'	59	367	NM_000044.6	160
SRD5A2	5'- CTGGGACGGTACTTCTGGG-3' 5'- CTCCCTCGATTGAGCAGTGAG-3'	60	6716	NM_000348.4	79
HSD3B1	5'- CACATGGCCCGCTCCATAC-3' 5'- GTGCCGCCGTTTTTCAGATTC-3'	60	3283	NM_000862.3	90
HSD3B2	5'- CTTGTGCGTTAAGACCCACAT-3' 5'- GGGTTGACTGTAGAGAACTTTCC-3'	60	3284	NM_000198.4	124
GAPDH	5'-GTCTCCTCTGACTTCAACAGCG-3' 5'-ACCACCCTGTTGCTGTAGCCAA-3'	60	2597	NM_002046.7	131

0.2% Tween-20) containing 5% non-fat dry milk under appropriate conditions for 1hr at room temperature. After blocking, membranes were washed in TBST three times for 5 min and then probed with the mAbs overnight at 4°C. Subsequently, the membranes were washed with TBST and incubated for 1hr at room temperature with rabbit-anti mouse-HRP in TBST containing 5% non-fat dry milk. Finally, membranes were washed three times, followed by imaging with Geldoc. Results analyzed by Image J software (Ver. 1.8.0-172).

Statistical Analysis

Statistical analyses were performed using Prism version 5.0 (GraphPad Software, San Diego, CA). One-way analyses of variance (ANOVA) were used to compare differences regarding quantitative gene expression. Fisher's exact tests or chi square were used for association analyses. Data are presented as the mean standard error, where $P < 0.05$ was considered statistically significant.

RESULTS

On histology of prostate biopsy specimens, Serum testosterone levels were higher in the PCa (514.6±102.7 ng/ml) than in the BPH group (473.8±96.2ng/ml) but this difference was not statistically significant ($p = 0.06$). Serum testosterone also did not differ significantly with Gleason score in patients with prostate cancer. Serum PSA values were significantly higher in men with prostate cancer and BPH than normal range (4.5 ng/ml≥ (60-69 years old)), but did not differ significantly between PCa and BPH group. Serum testosterone levels of patients with prostate cancer did not significantly differ from those of patients with BPH and were not related to grade in prostate cancer patients. In order to investigate the expression of genes involved in the biosynthesis of androgens and androgen receptor, candidate genes were evaluated at the mRNA level by qRT-PCR method between patients with prostate adenocarcinoma, adjacent healthy tissue (in the same patients) and patients with benign prostatic hyperplasia. Since the Androgens action in prostatic cells is mediated by AR. We measured AR gene expression in prostate tissues. ANOVA test was used to check the significance of gene expression changes between three groups: Comparison of the gene expression profiles of BPH and PCa showed a significant increase in the expression of the gene AR compared with BPH and Ctrl tissues ($p=0.0001$), as well as significant increase in the expression of the gene SRD5A2 ($p=0.0038$), HSD3B1 ($p=0.0011$) and HSD3B2 ($p=0.0469$) in BPH samples compared with PCa and Ctrl tissues (Fig. 2-A). Comparison between two groups PCa vs. BPH showed significant increase in AR gene ($p=0.0007$) and decrease in SRD5A2 ($p=0.0009$). Comparison between two groups PCa vs. Ctrl showed significant increase in AR gene ($p=0.0001$) and HSD3B1. Comparison between two groups BPH vs. Ctrl showed significant increase in HSD3B1 gene ($p=0.0008$) and

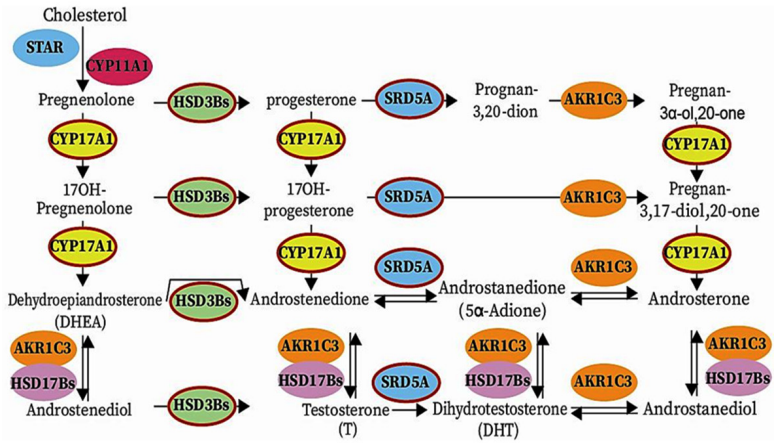

Fig. 1. Biosynthesis of androgens Pathway from primary precursor of cholesterol; As can be seen, various enzymes are involved in the process of converting cholesterol to testosterone and dihydrotestosterone, among which CYP17A1, SRD5A, HSD3B family enzymes play a role in different stages. CYP17A1 enzyme is involved in converting progesterone to 17OH progesterone and converting 17OH progesterone to Dehydroepiandrosterone (DHEA) and androstenedione. HSD3B enzyme is involved in converting Androstenediol to Testosterone and SRD5A converted Testosterone to Dihydrotestosterone. It is expected that the change in the expression of each of these enzymes can be effective in the process of producing androgens

HSD3B2 gene (p=0.0138). Western blot analysis was performed to investigate changes in protein expression (enzymes involved in the biosynthesis of androgens). The results showed the presence and expression of all

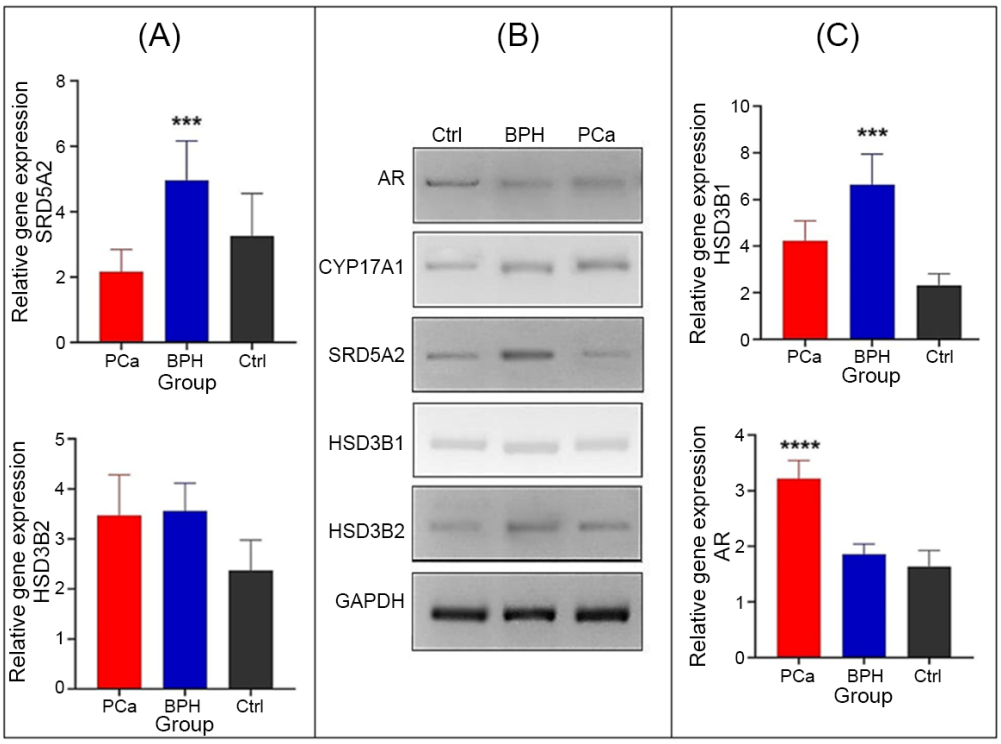

Fig. 2. Alterations in the expression of genes involved in the biosynthesis of androgens in the three groups of PCa, BPH and control. As can be seen, AR gene expression in the PCa group has increased significantly compared to the other two groups (****), HSD3B1 and SRD5A2 gene expression has also significantly increased in the BPH group compared to the other two groups (***). Fig. (2B). western blot results show that all proteins are expressed in all groups, but SRD5A2 protein expression was higher in BPH group compared to other two groups. Fig. (2C). shows the expression changes of 4 genes at the RNA level in all groups in one view.

enzymes in all groups. But as can be seen, the expression levels of SRD5A2 and HSD3B2 proteins in the BPH group are higher than those in the Ctrl and PCa groups. Although AR gene expression (mRNA) was high in the PCa group, the results of western bloting analysis showed that it has a low expression compared to the Ctrl and BPH groups (Fig. 2-B).

DISCUSSION

Huggins and Hodges showed that marked reductions in serum testosterone levels by castration or high-dose estrogen therapy resulted in the regression of metastatic prostate cancer. This work and subsequent studies led to the hypothesis that higher testosterone levels cause enhanced growth of prostate cancer and resulted in the use of androgen ablation therapy for the management of prostate cancer [24]. Androgens play an essential role in the development and maintenance of normal male physiology [25]. For almost 70 years, suppression of circulating androgen levels via surgical or medical castration has been the cornerstone of the management of patients with advanced-stage disease [26]. Evidence that has accumulated over the past decade clearly indicates that this castration- resistant growth is driven, to a large extent, by continued AR signaling. Data from a number of studies have indicated that, although castration leads to very low levels of circulating testosterone, androgens can still be isolated from prostate cancer tissues at clinically relevant concentrations [27-29].

Today, it is known that prostate cancer cells have the ability to intratumoral biosynthesize androgens; this ability has made cancer cells resistant to castration and androgen deprivation therapy treatments. In this study, we investigated the expression changes of genes involved in the intratumoral biosynthesis of androgens in three groups: tumor tissue, adjacent healthy tissue and benign hyperplasia tissue. The candidate genes of this study include: SRD5A2, HSD3B1 and HSD3B2. Since androgens exert their effect on cancer cells through the androgen receptor, we also investigate the expression alteration of this gene in this study. Results showed in PCa group a significant increase in the expression of the gene AR compared with BPH and Ctrl tissues ($p=0.0001$), as well as significant increase in the expression of the gene SRD5A2 ($p=0.0038$), HSD3B1 ($p=0.0011$) and HSD3B2 ($p=0.0469$) in BPH samples compared with PCa and Ctrl tissues. Increased AR expression is consistently seen in tumor epithelial cells during development of CRPC. Overexpression of the AR in tumor epithelial cells could be a result of AR gene amplification, but is probably also an instant response to castration as androgens normally suppress AR transcription in prostate epithelial cells. Increased expression of AR can be considered as a survival mechanism in cancer cells, increased expression of this gene in prostate cancer has been reported in various studies [30-32]. The 2-5a-reductase plays a crucial role in male sex differentiation by converting testosterone into 5 DHT in the peripheral target tissues. Type 2-5a-reductase (SRD5A2) is the major form expressed in normal prostate. However, work from several groups indicates that the type 1 5a-reductase (SRD5A1) is increased in primary PCa based on transcript and protein levels; In contrast, levels of SRD5A2 in PCa are not increased and were decreased in some studies [33]. In the Intratumoral androgens biosynthesis, the SRD5A2 enzyme catalyzes the conversion of testosterone to DHT. In this study, we observed a decrease in the expression of this gene in the cancer group compared to healthy tissue and benign hyperplasia tissue. In a recent study, Lundgren et al reported that high DHT levels were positively correlated to a lower risk for prostate cancer death in the entire cohort [34]. DHT plays an important role in the development of BPH that is, pathologic prostate enlargement caused by an increase in cell number arising from both cellular hyperplasia and reduced apoptosis. In a study, Khvostova et al compared the expression of gene SRD5A2 in three groups of prostate cancer, benign hyperplasia and healthy samples and reported that the expression of SRD gene in the prostate cancer group was reduced compared to the healthy group and benign hyperplasia [35]. These results in other studies reported too [36]. Another enzyme that is involved in prostate intratumoral biosynthesis is family HSD3B, in peripheral tissues, including the prostate, 3β-HSD1 predominates, whereas 3β-HSD2 is expressed preferentially in the adrenal glands and gonads [37]. In this study, we reported a significant increase in the expres-

sion of HSD3B1 gene in the BPH group, in another study conducted by us in a smaller number of samples, the same results were repeated. These results are consistent with the results reported by Khvostova et al [35].

In this study, we also evaluated the presence of proteins related to these genes by western blot method. The presence of all enzymes was observed in all groups, and the expression level of SRD5A2 protein was significantly observed in the hyperplasia sample compared to the other two groups. When comparing gene expression profiles in benign and malignant tumors, we demonstrated significant increases in the expression of the genes HSD3B1 and SRD5A2 in benign tissues compared to malignant tissues. This finding is consistent with an earlier published study from Elo et al., in which they reported significantly higher expression of SRD5A2 mRNA in BPH tissue compared with carcinoma specimens, as revealed by Northern blotting. The lower SRD5A2 mRNA level in cancerous tissues could be due to the relative lack of stromal cells [38].

DECLARATION OF INTEREST

The authors declare that they have no conflicts of interest that could be perceived as prejudicing the impartiality of this study.

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