RESEARCH ARTICLE

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Clinical assessment of *TGFB1* and *HP* Relative Gene Expression in the Peripheral Blood of Prostate Cancer Patients

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Abstract

Objective: This study aimed to assess the relative gene expression level of transforming growth factor-β1 (*TGFB1*) and haptoglobin (*HP*) in the peripheral blood of prostate cancer (PCa) patients and evaluate their diagnostic ability. **Methods:** A total of 125 participants were enrolled in the present study. Among them, 75 PCa patients, 25 benign prostatic hyperplasia (BPH) patients, and 25 healthy volunteers served as the control group. The relative *TGFB1* and *HP* gene expression level was quantified using real-time polymerase chain reaction. Further, free and total PSA levels were determined using electrochemiluminescence assays. **Results:** *TGFB1* was significantly over-expressed, whereas *HP* was significantly downregulated in the peripheral blood of PCa patients compared to BPH and control groups (p-value ranges from 0.034 to <0.001). Moreover, the high expression level of *TGFB1* was associated with an increased risk of PCa development with OR=1.412 (95%CI: 1.081-1.869, p= 0.012). *TGFB1* and *HP* relative expression levels had lower diagnostic performance to differentiate PCa from normal and BPH individuals compared to PSA, however, the combination of the tested parameters improved the diagnostic efficacy. **Conclusions:** *TGFB1* and *HP* relative expression have moderate diagnostic efficacy in discriminating patients with PCa from BPH and healthy subjects. Furthermore, over-expression of *TGFB1* may contribute to the pathogenesis of PCa.

Keywords: Biomarkers- Haptoglobin (*HP*)- Prostate cancer (PCa)- Prostate-specific antigen (PSA)

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Introduction

Prostate cancer (PCa) is a common male malignancy worldwide. It is the second leading cause of cancer-related mortality in men, accounting for 30,000 deaths annually in the United States [1, 2]. In Egypt, there is a growing concern about PCa, which is currently the fourth most prevalent tumor. This may be attributed to the fact that 7.2% of Egypt's male population in all aged, hence at risk for PCa incidence [3]. Further, the mortality rate from PCa in Egypt is higher than in the United States [4].

Prostate-specific antigen (PSA) has traditionally been the only biomarker used to detect PCa. Because elevated PSA levels in the blood may be driven by conditions such as benign prostatic hyperplasia (BPH) and prostatitis, none of the PSA cut-offs currently in use consistently identify patients with PCa and exclude patients without cancer. PCa incidence in patients with PSA levels below the accepted level of 4.0 ng/ml is similar to that of PCa in patients with PSA between 4.0-10.0 ng/ml [5, 6]. As a result, a transrectal ultrasound-guided prostate biopsy is critical for making a final diagnostic decision [7]. Therefore, more specific and sensitive non-invasive procedures for detecting PCa at an early stage are required. For that, novel biomarkers were explored, including prostate health index (PHI) as a simple blood test with higher specificity than free and total PSA. The PHI is calculated using the formula: (p2PSA/free PSA) × √PSA, which combines three forms of PSA (total, free, and a PSA isoform called p2PSA) into a single score that can be used to aid in clinical decision-making [8, 9]. Additionally, a serum 4-kallikrein panel composed of total and free PSA, intact PSA, and human kallikrein 2 combined with age may inform initial screening in men who have never undergone biopsy as well as in those with a prior negative biopsy [10]. Moreover, ghrelin-O-acyltransferase increased in urine and plasma samples from PCa patients [11].

Transforming growth factor-beta1 (TGF-β1) is a cytokine that belongs to the TGF-β superfamily, playing a crucial role in immune homeostasis and tolerance by inhibiting the proliferation and function of several components of the immune system. TGF-β signaling abnormalities underpin inflammatory diseases, promote tumor emergence, and play essential roles in immune suppression within tumor microenvironments [12]. TGF-β1 has a dual function in carcinogenesis where it

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acts as a tumor suppressor in the early stages whereas it promotes invasion and metastasis in the later stages [13-15]. Over-expression of TGF-β1 was found in tissue and plasma samples from patients with advanced stages of several solid tumors [16-20].

Haptoglobin (HP) is a late positive acute-phase reactant protein produced by the liver in response to inflammation. Lung, spleen, kidney, skin, and adipose tissue are also involved in *HP* formation. Usually, HP binds to free hemoglobin released from erythrocytes during intravascular hemolysis to form a complex that is promptly deleted [21-23]. However, HP is involved in autoimmune disorders, neurodegenerative diseases, and tumor angiogenesis [24, 25]. Furthermore, serum HP was found to be elevated in various cancer types, including buccal, lung, and colorectal cancer [26-28]. Worth mentioning that pro-haptoglobin, a precursor form of HP, stimulates endothelial angiogenesis, and that function is associated with TGF-β signaling via activin receptor-like kinase1 (AKL1)-Smad1/5 activation [29, 25].

Consequently, this study aimed to assess the relative gene expression levels of *TGFB1* and *HP* in the peripheral blood of PCa patients and to compare their diagnostic efficiency with PSA.

Materials and Methods

Subjects

Seventy-five patients with clinically, radiologically, and histologically proven PCa who did not receive any surgical or therapeutic intervention, as well as twenty-five BPH patients were enrolled from the medical oncology clinic, national cancer institute (NCI), Cairo University, Egypt, in the period between March 2018 and September 2020. Patients with pathologies other than adenocarcinoma or who received any treatment were excluded. In addition, 25 healthy volunteers were included as a normal control group.

Four milliliters of venous blood were collected from each subject and aseptically separated into two parts; the first was collected on EDTA-containing tubes for molecular analyses, and the other was collected on gel-containing tubes, left to clot, and then centrifuged for 5 min at 1000 xg to obtain sera for biochemical analyses.

Biochemical analyses

Elecsys® dual monoclonal antibody sandwich assays (Roche Diagnostics) based on electrochemiluminescence technology have been used to measure total and free PSA. The low detection limits for total and free PSA assays were 0.011 ng/ml and 0.018 ng/ml, respectively. The intra-assay coefficient of variation (CV) for total and free PSA assays was 2.62% and 1.38%, respectively whereas the inter-assay CV was 2.94% and 4.1%, respectively. The analyses were carried out on Cobas e 411 analyzers (Roche Diagnostics, In, USA). The PSA ratio was then calculated as free PSA/total PSA.

Molecular analyses

QIAamp RNA Blood Mini Kit (Cat# 52304, Qiagen) was used to extract total RNA from 1.5 ml of whole blood according to the manufacturer's instructions. RNA concentration and purity were determined spectrophotometrically at 260 and 280 nm, and its integrity was confirmed by electrophoresis on 1% of ethidium bromide-stained agarose gel.

Using the QuantiTect® Reverse Transcription Kit (Cat# 205311, Qiagen), 1 μg of total RNA was reverse transcribed to produce the first cDNA strand. QuantiTect® Primer Assays for *TGFB1* and *HP* (Cat# QT00000728 and QT00071449, respectively; by Qiagen) were used for the real-time polymerase chain reaction (PCR) reactions in a final volume of 25 μl with 2 μl of cDNA as a template, 2.5 μl of 10x QuantiTect® Primer Assay, 12.5 μl of 2x QuantiTect SYBR® green PCR master mix (Qiagen), and 8 μl DEPC-treated water. The PCR conditions were as follows: 15 min at 95°C for initial denaturation followed by 45 amplification cycles of denaturation at 95°C for 15 sec, annealing at 55°C for 30 sec, and extension at 72°C for 30 sec. MicroAmp® fast optical 96-well reaction plate with MicroAmp® optical adhesive film was used for PCR reactions (Applied Biosystems). The plate was loaded into the PCR system in Step One Real-Time device (Applied Biosystems, CA, USA). Each assay was performed in triplicate, and melting curve analyses of all real-time PCR reactions were performed and shown to produce a single DNA duplex. Quantitative calculations were determined using the $2^{-\Delta\Delta Ct}$ method [30]. The expression of mRNAs was normalized to the expression of β-actin (Cat# QT00095431) purchased from Qiagen (Hilden, Germany).

Statistical analysis

IBM SPSS advanced statistics version 25 was used for data analysis (IBM Corp, NY, US). The Shapiro-Wilk test was used to assess the way through which quantitative data were distributed. Normally distributed data were expressed as mean±SD while non-normally distributed data were expressed as median and interquartile range (25th and 75th percentile). The descriptive measurements for categorical data were provided as frequencies (percentages). The comparison of continuous variables between groups was performed using one-way ANOVA followed by Tukey's post hoc for multiple comparisons or Kruskal-Walli's test followed by Dunn's post hoc for multiple comparisons as appropriate. The Chi-square test was performed to compare the differences between categorical variables. To investigate the strength of the association of the circulating gene expression levels of *TGFB1* and *HP* with the susceptibility to PCa, unconditional logistic regression analyses were performed. The strength of the association was measured by crude and adjusted odd ratio (OR) for age and their corresponding 95% confidence interval (CI). Receiver operating characteristic (ROC) curve analysis was used to establish the diagnostic value of the relative *TGFB1* and *HP* gene expression. All p-values were two-sided, and p-values ≤0.05 were considered significant.

Results

General characteristics of patient

The basic characteristics of the studied groups are shown in Table 1. Compared to the control group, BPH

and PCa patients were older (p<0.001) with considerably high serum concentrations of free PSA (p=0.019 and p<0.001, respectively). However, a significant increase in total PSA level and a significant decrease in the PSA ratio were observed in the PCa patient group only (p<0.001 and p=0.034, respectively). Although PCa patients were age-matched with BPH patients (p=0.554), they had significantly high levels of serum total and free PSA (p<0.001), accompanied by a significant decrease in the PSA ratio (p<0.001) in comparison with BPH patients. Almost half of PCa patients suffered from metastasized tumors, and more than 70% had a high Gleason score (≥7).

Concerning gene expression levels, *TGFB1* showed a significant up-regulation in peripheral blood of PCa patients compared to controls (with median expression value of 4.63 vs. 1.49, p<0.001) and BPH patients (with median expression value of 4.63 vs. 3.58, p=0.034), respectively. However, the relative expression level of *TGFB1* did not vary significantly between the control group and BPH patients (p=0.289) (Figure 1A). On the other hand, *HP* relative expression was significantly downregulated in BPH (with median expression value of 0.066 vs. 0.22, p=0.001) and PCa patients (with median expression value of 0.039 vs. 0.22, p<0.001) compared to controls. Moreover, *HP* was significantly down expressed in PCa patients compared to BPH patients (p=0.028) (Figure 1B).

Association of TGFB1 and HP relative expression with the studied parameters in PCa patients

PCa patients were divided into 2 sub-groups (high vs. low) according to the median value of the relative gene expression level. Both *TGFB1* and *HP* gene expression levels were not associated with any of the studied parameters (Table 2).

Relative TGFB1 and HP expression levels as risk factors

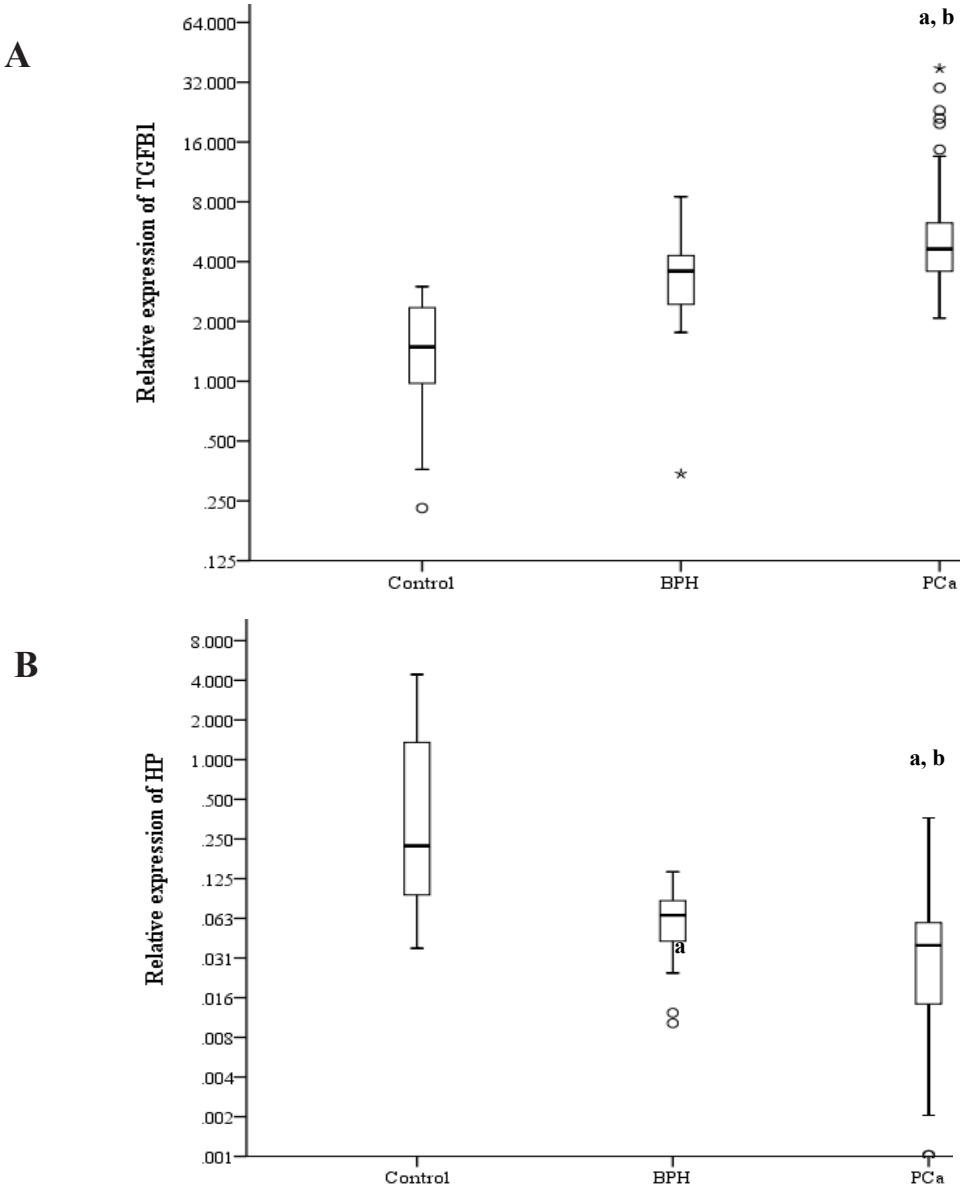


Figure 1. Relative Expression Level of (A) *TGFB1* and (B) *HP* in the Peripheral Blood of the Studied Groups. *TGFB1:* Transforming growth factor-beta1, *HP,* Haptoglobin; BPH, Benign prostate hyperplasia; PCa, Prostate cancer. In multiple comparisons, [a] p<0.05 vs. control and [b] p<0.05 vs. BPH. Box plot indicates median and interquartile range. Outliers between 1.5 and 3 box lengths are depicted by "○"and extreme values more than 3 box lengths are shown by "*".

Table 1. General Characteristics of Study Population

	Control (n=25)	BPH (n=25)	PCa (n=75)	p-value between groups
Age (years)	35.00±5.97	65.00 (60.50-69.50) [a]	68.18±7.21[a]	<0.001
Total PSA (ng/ml)	1.04 (0.75-1.56)	2.47 (1.24-6.80)	50.54 (11.00-115.00)[a, b]	<0.001
Free PSA (ng/ml)	0.27±0.09	0.69 (0.41-1.36)a	8.14 (1.83-17.37)[a, b]	<0.001
PSA ratio	0.28 (0.16-0.36)	0.30±0.10	0.17 (0.10-0.24)[a, b]	<0.001
Gleason score				
<7	-	-	20 (26.7)	
≥7	-	-	55 (73.3)	
Tumor's localization				
Localized	-	-	43 (57.3)	
Metastatic	-	-	32 (42.7)	

Data are expressed as mean±SD for Gaussian data, median (interquartile range) for non-Gaussian data, and frequency (percentage) for categorical data. BPH: Benign prostate hyperplasia; PCa, Prostate cancer; PSA, Prostate-specific antigen; In multiple comparisons, [a] p<0.05 vs. control, and [b] p<0.05 vs. BPH.

for PCa

Table 3 shows the results of the binary logistic regression analyses performed to test the associations of *TGFB1* and *HP* relative expression with PCa. The results demonstrated that only the high expression of *TGFB1* was associated with an increased risk of PCa after adjusting for age as a potential confounder.

Efficacy of TGFB1 and HP relative expression as potential diagnostic biomarkers for PCa

Figure 2 illustrates the ROC curves of *TGFB1* and *HP* relative expression levels, total and free PSA concentrations, and the PSA ratio to discriminate PCa patients from healthy controls and BPH patients. Although total and free PSA had strong diagnostic values, both

Table 2. Association of *TGFB1* and *HP* Relative Expression Levels with the Studied Parameters in PCa Group

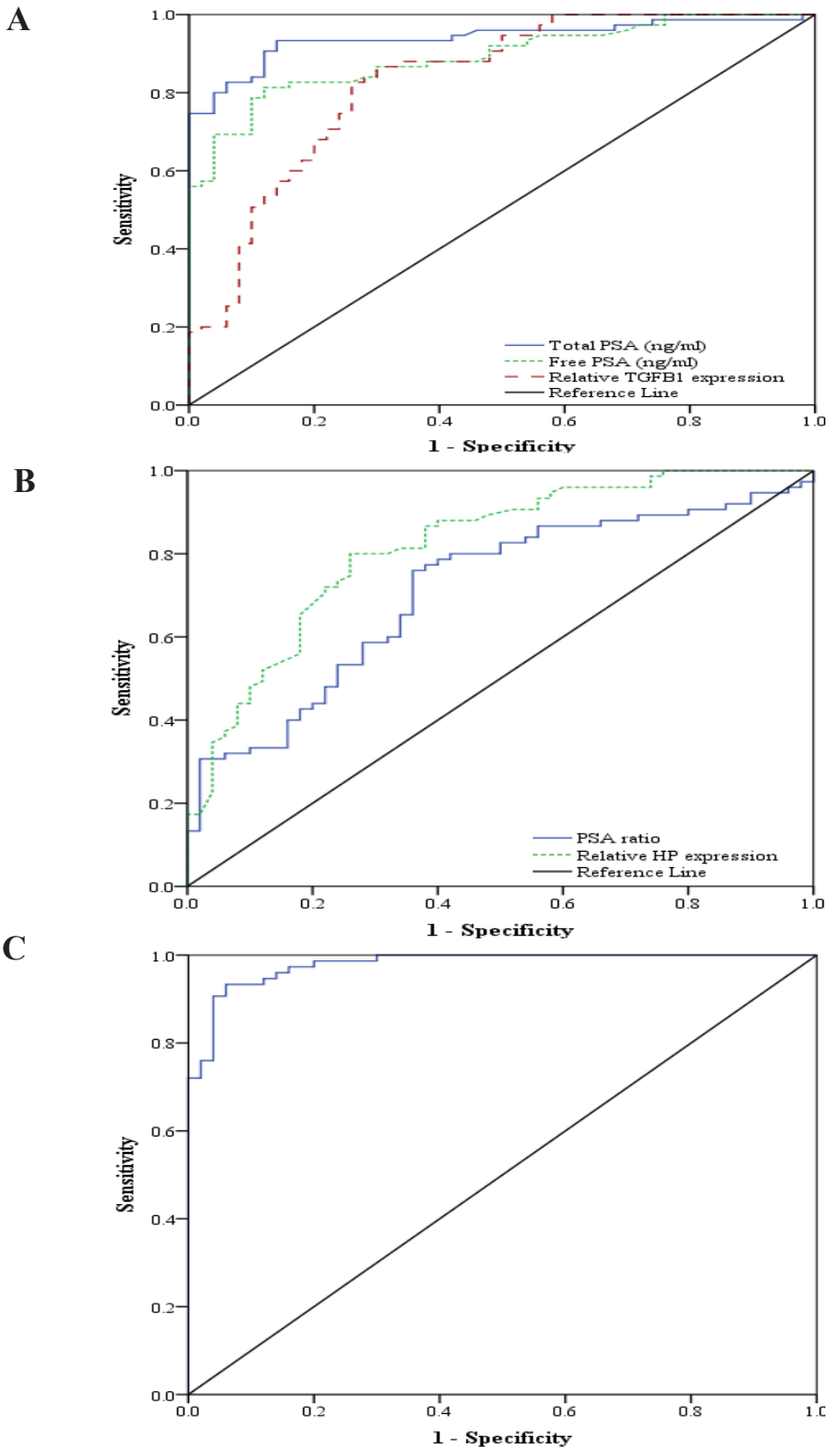
	TGFB1 expression			*HP* expression		
	High expression (n=38)	Low expression (n=37)	p-value	High expression (n=37)	Low expression (n=38)	p-value
Total PSA (ng/ml)						
<4	4 (10.5)	1 (2.7)	0.154	2 (5.4)	3 (7.9)	0.149
4–10	8 (21.1)	4 (10.8)		6 (24.3)	3 (7.9)	
>10	26 (68.4)	32 (86.5)		29 (70.3)	32 (84.2)	
Free PSA (ng/ml)						
>0.9	30 (78.9)	32 (86.5)	0.389	32 (86.5)	30 (78.9)	0.389
<0.9	8 (21.1)	5 (13.5)		5 (13.5)	8 (21.1)	
PSA ratio						
>0.25	8 (21.1)	10 (27)	0.545	9 (24.3)	9 (23.7)	0.948
<0.25	30 (78.9)	27 (73)		28 (75.7)	29 (76.3)	
Gleason score	12 (31.6)	8 (21.6)				
<7	26 (68.4)	29 (78.4)	0.33	12 (32.4)	8 (21.1)	0.265
≥7				25 (67.6)	30 (78.9)	
Distant metastasis						
Present	18 (47.4)	14 (37.8)	0.404	13 (35.1)	19 (50)	0.193
Absent	20 (52.6)	23 (62.2)		24 (64.9)	19 (50)	

Data are expressed as frequency (percentage), PCa, Prostate cancer; PSA, Prostate-specific antigen; *TGFB1*, Transforming growth factor-beta1; HP, Haptoglobin.

Table 3. Logistic Regression Analysis of *TGFB1* and *HP* Relative Expression as Potential Risk Factors for Prostate Cancer

Variable	Crude OR (95% CI)	p-value	†Adjusted OR (95% CI)	p-value
TGFB1 relative expression	1.948 (1.447-2.623)	<0.001	1.412 (1.081-1.869)	0.012
HP relative expression	1e-6 (0-0.016)	0.005	0.0003 (0-4.307)	0.097

TGFB1, Transforming growth factor-beta1; *HP*, Haptoglobin; OR, Odd ratio; CI, Confidence interval; †, adjusted for age



	AUC	95%CI		*p*-value	Cut-off	Sens (%)	Spec. (%)	PPV (%)	NPV (%)	Accuracy (%)
		Lower bound	Upper bound							
TGFB1 relative expression	0.830	0.755	0.906	<0.001	≥3.30	82.70	74.00	82.67	72.55	79.20
HP relative expression	0.818	0.743	0.894	<0.001	≤0.062	80.00	74.00	82.19	71.15	77.60
Total PSA (ng/ml)	0.940	0.897	0.982	<0.001	≥5.420	93.30	86.00	90.91	89.58	90.40
Free PSA (ng/ml)	0.891	0.836	0.946	<0.001	≥1.108	82.70	84.00	88.57	76.36	83.20
PSA ratio	0.710	0.619	0.802	<0.001	≤0.245	76.00	64.00	76.00	64.00	71.20
Combined parameters	0.979	0.960	0.998	<0.001	-	93.30	94.00	95.89	90.38	93.60

Figure 2. Receiver Operating Characteristic (ROC) Curves of A) *TGFB1* expression level as well as total PSA and free PSA, B) *HP* expression level and PSA ratio, and C) Combination of all tested parameters for discriminating prostate cancer patients from healthy controls and benign prostatic hyperplasia patients. *TGFB1,* Transforming growth factor-beta1; *HP*, Haptoglobin; PSA, Prostate-specific antigen; AUC, Area under curve; CI, Confidence interval; Sens, Sensitivity; Spec, Specificity; PPV, Positive predictive value; NPV, Negative predictive value.

genes were found to have high diagnostic efficacy as well. The combination of the tested parameters improved the diagnostic efficacy, as evidenced by the increased area under curve.

Discussion

PCa is one of the most common non-cutaneous malignancies, asymptomatic in its earliest stages, and a major cause of cancer fatality in men [31, 32]. Thus, it is important to detect and diagnose PCa early in its course. Therefore, this study aimed to assess the relative expression of *TGFB1* and *HP* in the peripheral blood of PCa patients and to evaluate their diagnostic performance as non-invasive biomarkers for PCa.

In the current work, the relative *TGFB1* gene expression was up-regulated in the peripheral blood of PCa patients compared to controls and BPH patients in line with the results of Faria et al. [33], who reported a significant increase in the relative *TGFB1* gene expression in PCa patients compared to control. However, the authors did not find a significant difference between BPH and PCa groups. In contrast, Soulitzis et al. [34] found a down-regulation in the tissue *TGFB1* expression of PCa and BPH patients. Elevated TGF-β1 level in cancer can result from both increased cytokine expression by tumor cells and the recruitment of TGF-β1-producing cancer-associated cells, such as stromal fibroblasts, immature myeloid cells, dendritic cells, and tumor-associated macrophages, into the tumor microenvironment [35-38].

Despite its ability to inhibit cell proliferation, TGF-β1 is highly expressed within the tumor tissue or plasma of various cancers. Angiogenesis, tissue invasion, metastasis, and the epithelial-mesenchymal transition are among the specific aspects of tumor progression that are associated with elevated *TGF-β1* expression. Many studies have also linked elevated *TGF-β1* expression levels with advanced tumor stages and decreased patient survival [39-46]. This seems to concur with the results of the current work, which showed that high *TGFB1* expression was associated with an increased risk of developing PCa after adjusting for age as a potential confounder.

When considering HP, its expression levels vary according to the malignant state. HP mRNA was down-regulated in PCa [47-49] in line with the results of the current study that showed a low expression level of *HP* in the peripheral blood of PCa patients compared to BPH and control participants. On the other hand, Fujimura et al. [50] reported significantly high HP protein levels in sera of PCa patients compared to benign prostate disease or normal subjects. Similarly, tissue HP was overexpressed in colorectal, ovarian, and liver cancer [51, 28, 52]. This variance may be attributed to HP's distinct orientation of the Th1/Th2 response, which results in immunomodulating effects [47]. Further, HP may decrease because of oncogenic changes that promote tumor formation and frequently interfere with HP expression [48].

Although PSA is the golden standard biomarker for PCa diagnosis, several studies revealed that total PSA has high sensitivity but lacks specificity [53-55]. Furthermore, there is an argument about the diagnostic efficiency of the PSA ratio; some studies revealed that the PSA ratio enhances the sensitivity and specificity for PCa diagnosis than that of the total PSA [56, 57], while others suggested that the PSA ratio lacks the efficiency to diagnose PCa [58, 59]. This concurs with the results of the present study, which showed that the PSA ratio had the lowest diagnostic efficacy compared to total and free PSA levels.

Moreover, the diagnostic values of the circulating *TGFB1* and *HP* expression level to discriminate between PCa patients from BPH and healthy subjects were examined herein. The results showed that *TGFB1* and *HP* expression levels have superior diagnostic efficacy over the PSA ratio but are inferior to total and free PSA. However, the combination of the tested parameters improved the diagnostic efficacy, as evidenced by the increased area under curve.

In conclusion, this work sheds light on the circulating *TGFB1* and *HP* expression levels in PCa, showing a significant over-expression of the former and a significant down-regulation of the latter in PCa patients compared to BPH and healthy subjects. Moreover, the results pointed out that the high *TGFB1* expression level was associated with higher susceptibility to PCa. Thus, lowering the levels of *TGFB1* expression may provide novel approaches for PCa treatment; further studies are required to verify this hypothesis. Finally, *TGFB1* and *HP* expression levels appear to be good candidates as biomarkers in clinical practice to diagnose PCa, especially when combined with the traditional biomarkers (total PSA, free PSA, and PSA ratio).

Author Contribution Statement

Conception: Iman A Abdelgawad, Fatma F Abdel Hamid, and Ahmed F Soliman; (II) Provision of study materials or patients: Noha H Radwan, and Mohammed S Yahya; (III) Collection and assembly of data: Mohammed S Yahya, and Ahmed F Soliman; (IV) Preparation of the manuscript: Mohammed S Yahya, and Ahmed F Soliman; (V) Revision for important intellectual content: Iman A Abdelgawad, Fatma F Abdel Hamid, and Noha H Radwan; (VI) Final approval of manuscript: All authors

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Ethics approval and consent to participate

All participants provided informed written consent before the start of the study. This work was executed per the Helsinki Declaration for human experiments, and the study protocol was approved by the NCI institutional review board under an approval number of 201617030.4.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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