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Prostate Cancer: From Genomics to the Whole Body and Beyond

Lead Guest Editor: Fabio Grizzi
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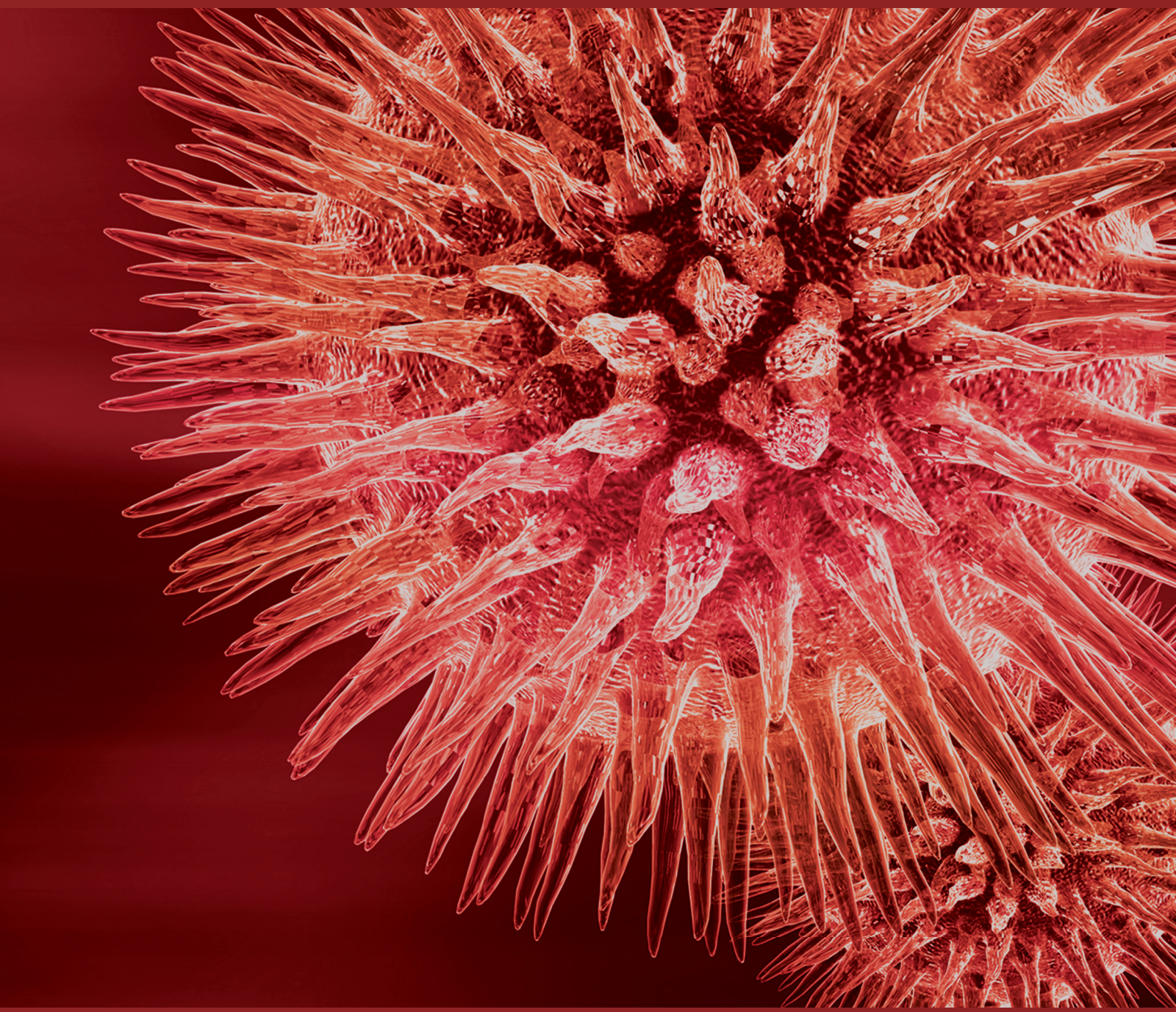

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Editorial

Prostate Cancer: From Genomics to the Whole Body and Beyond

Fabio Grizzi,[1] Gianluigi Taverna,[2,3] Richard J. Cote,[4,5,6] and Giorgio Guazzoni[2]

[1]*Department of Immunology and Inflammation, Humanitas Clinical and Research Center, Rozzano, 20089 Milan, Italy*
[2]*Department of Urology, Humanitas Clinical and Research Center, Rozzano, 20089 Milan, Italy*
[3]*Department of Urology, Humanitas Mater Domini, Castellanza, 21053 Varese, Italy*
[4]*Department of Pathology and Laboratory Medicine, University of Miami Miller School of Medicine, Miami, FL 33136, USA*
[5]*Sylvester Comprehensive Cancer Center, University of Miami Miller School of Medicine, Miami, FL 33136, USA*
[6]*Department of Biochemistry, University of Miami Miller School of Medicine, Miami, FL 33136, USA*

Correspondence should be addressed to Fabio Grizzi; fabio.grizzi@humanitasresearch.it

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Prostate cancer represents the second most common cancer in men globally [1]. Prostate, lung and bronchus, and colorectal cancers account for 44% of all cases in men, with prostate cancer alone accounting for 1 in 5 new diagnoses. Despite rapid advances in the fields of molecular and cell biology, how neoplastic prostate cells progress through carcinogenesis remains widely debated.

Prostate cancer is recognized as a complex and multifactorial dynamical disease that is discontinuous in *space* and *time*, but advances through qualitatively different states. It is known that prostate cancer is characterized by a high degree of pathological and genetic heterogeneity compared to other human cancers. Recently, several studies have investigated the molecular basis of primary prostate cancer and have identified recurrent genomic alterations, including mutations, DNA copy-number changes, gene rearrangements, and gene fusions [2–4]. Heterogeneous genomic aberrations may lead to prostate cancer onset, disease progression, and metastatic potential. This heterogeneity may also contribute to the variable drug responses observed among affected patients. Serum Prostate-Specific Antigen (PSA) is, currently, the most important biomarker for the detection, follow-up, and therapeutic monitoring of prostate cancer. PSA based screening for prostate cancer has had an important impact on the epidemiology of the disease. Its use has been associated with a significant reduction in prostate cancer mortality, but has also resulted in the overdiagnosis and overtreatment of indolent prostate cancer, exposing many men to treatments without benefits [5]. Its low specificity and sensitivity are mainly attributable to the fact that serum PSA may also be increased in benign conditions, such as benign prostatic hyperplasia and chronic prostatitis. Additionally, serum PSA levels are affected by biologic variability that may be related to differences in androgen levels or prostate manipulation and may have distinct racial variation [6]. Ludwig et al. recently reported that men with an undetectable serum PSA 20 years after radical prostatectomy had a very low rate of recurrence and no deaths due to prostate cancer, suggesting that 20 years is a reasonable time to discontinue PSA testing [7]. Given that an elevated PSA can be difficult to interpret, in the last decade proteomic and genomic technologies have been applied as powerful ways to uncover biomarkers of detection, prognosis, and prediction and to improve the understanding of prostate cancer biology. Several investigators have proposed alternative biomarkers that include the [−2]proPSA isoform [also called prostate health index (PHI)], the 4K score, a combination of four kallikrein proteins, and immunological or genomic biomarkers. It has been proposed that biomarkers for prostate cancer may be roughly classified in five categories based on their origin: genome, epigenome, transcriptome, proteome, or metabolome. Circulating tumor cells (CTCs) have been detected in different epithelial cancer types and have emerged as promising prognostic biomarkers [8]. Furthermore, the discovery of microRNAs (miRNAs) has led to investigating this class of small noncoding RNAs as new biomarkers for prostate cancer detection and prognosis. However, due to the small quantity of these molecules and the lack of standard strategies for normalization and validation

as well as the high degree of inconsistency among studies, the discovery of such biomarkers is still challenging. The study of prostate cancer metabolism represents another topic of great interest to understand the mechanisms underlying the development and progression of prostate cancer [9]. These metabolic features are of clinical interest as they present a variety of potential therapeutic targets.

Alternative screening strategies have also been proposed. Actually, nearly 90% of prostate cancers are clinically localized at the time of their diagnosis. The clinical behavior of localized prostate cancer is, however, highly variable. Some men will have aggressive cancer leading to metastasis and death from the disease while others will have indolent cancers that are cured with initial therapy or may be safely observed. Multiple risk stratification systems have been developed, combining the best currently available clinical and pathological parameters that include the digital rectal examinations, serum PSA levels histological Gleason score, and clinical and pathological staging; however, these tools still do not adequately predict outcome. Today, the diagnosis of prostate cancer remains based primarily on the microscopic observation of prostate tissue sampled throughout needle biopsy. Conventionally, a systematic prostate biopsy is performed using transrectal ultrasound to obtain 10 to 12 tissue cores. Even though systematic prostate biopsy represents the standard strategy, this approach misses 21% to 28% of prostate cancers and undergrads 14% to 17% [10–12]. Although pathologic grading and staging is one of the strongest predictors of prostate cancer outcome, recent changes to Gleason score assignment have improved the risk stratification and reproducibility of grading. There is great potential, however, for further improvement/optimization based on specific histological features that are not currently accounted for by the Gleason scoring systems and by additional quantitative analysis. Even more sophisticated and precise imaging tools also have been introduced to enhance diagnostic performance. Multiparametric magnetic resonance imaging/ultrasound fusion biopsy has been reported as a tool able to improve detection of high-grade cancers when compared to systematic biopsy. Furthermore, it has been shown that Positron Emission Tomography/Computed Tomography (PET/CT) and whole body magnetic resonance imaging (MRI) scans have the potential to improve detection and to assess response to treatment of all states of advanced prostate cancer. However, standardization of acquisition, interpretation, and reporting of whole body (WB) MRI and PET/CT scans is required to assess the performance of these techniques in clinical trials of treatment approaches in advanced prostate cancer.

Cancer research has generated an intricate "body of knowledge" showing that prostate cancer is a disease that involves dynamic changes in the genome. Further risk stratification using molecular features could potentially help distinguish indolent from aggressive prostate cancer. Further studies are also needed to investigate the potential predictive value of this procedure to identify prostate cancer. Additionally, circulating tumor cells and cell-free circulating tumor DNA in the blood have emerged as potential promising tumor avatars. microRNAs and the study of the prostate cancer metabolism are further attractive areas of research. Recently, it has been demonstrated that a trained canine olfactory system can detect prostate cancer specific volatile organic compounds (VOCs) in urine samples with high estimated sensitivity and specificity [13]. This approach might have the potential to offer a noninvasive alternative to PSA sampling and prostate biopsy for detecting prostate cancer. In addition, the results suggest that prostate cancer specific VOCs might depend on a metabolic process of the tumor.

It is now ascertained that prostate cancer is governed by a "multiscale causality." This not only recognizes multiple processes and controls acting at multiple scales, that is, from the gene level [2] to that of organism with neurobiological and psychological evidences [14, 15], but, unlike a strict reductionist approach, may also recognize the fact that relevant "first principles" may reside at scales other than the smallest microscales. In other words, the observed phenomenon at each level of biological organization, that is, scale, has structural and behavioral proprieties that do not exist at lower or higher organizational levels. In addition, although each of the spatial scales may have multiple temporal scales, biological process that takes place at a lower scale generally happens much faster than those at a higher scale. It is now clear that "prostate cancer" admits many descriptions (ways of looking at the system), each of which is only partially true. Each way of looking at a "cancer system" requires its own description, its own mode of analysis, and its own breakdown into different parts.

It is now clear that observing the prostate cancer as a dynamical disease will reveal more about its underlying complex behavioral features. This way of thinking may further help to clarify concepts, interpret new and old experimental data, indicate alternative experiments, and categorize the acquired knowledge on prostate cancer and its precursor lesions. It is encouraging that medicine, biology, psychology, and mathematics continue to contribute together towards a comprehensive understanding of prostate cancer complexity.

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Fabio Grizzi
Gianluigi Taverna
Richard J. Cote
Giorgio Guazzoni

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Review Article

Establishment and Application of Prostate Cancer Circulating Tumor Cells in the Era of Precision Medicine

Yoon Seok Suh, Jae Young Joung, Sung Han Kim, Ho Kyung Seo, Jinsoo Chung, and Kang Hyun Lee

Center for Prostate Cancer, Hospital, National Cancer Center, Goyang, Gyeonggi-do, Republic of Korea

Correspondence should be addressed to Kang Hyun Lee; uroonco@ncc.re.kr

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Prostate cancer (PC) is the second most common cancer in men and is the fifth leading cause of cancer-related deaths worldwide. Additionally, there is concern for overdiagnosis and overtreatment of PC. Thus, selection of an appropriate candidate for active surveillance as well as more accurate and less invasive tools for monitoring advanced PC is required. Circulating tumor cells (CTCs) have emerged as a liquid biopsy tool; there have been several reports on its role, technologies, and applications to various cancers, including PC. Liquid biopsy using CTCs has been gaining attention as a minimal invasive tool for investigation of biomarkers and for prognosis and assessment of response to therapies in patients with PC. Because of the lower invasiveness of liquid biopsy using CTCs, it can be performed more frequently; accordingly, personalized disease status can be successively determined at serial time points. CTC analysis enables detection of genomic alterations, which is drug-targetable, and it is a potential tool for monitoring response to therapeutic agents in patients with PC. This review focuses on the characteristics, technologies for analysis, and advantages and disadvantages of CTCs as a liquid biopsy tool and their application in PC. Finally, we propose future directions of CTCs.

1. Introduction

Prostate cancer (PC) is the second most common cancer in men and is the fifth leading cause of cancer-related deaths worldwide [1]. In the United States, PC is the second leading cause of cancer-specific deaths in men, and approximately 26,120 PC-related deaths were recorded in 2016 [2]. Although PC has a high prevalence among cancers as stated above, there is also a concern about overdiagnosis and overtreatment of PC. With an emerging concept of indolent PC [3], active surveillance, prescribed in several guidelines for active surveillance eligibility, of localized PC with low malignant potential is gaining wide acceptance [4–17]. However, after using the current method of prostate biopsy for diagnosing PC, there is a possibility of significant upgrade of PC [18]. Additionally, the patient undergoing active surveillance needs to undergo repeated prostate biopsies to determine disease progression. However, prostate biopsy by transrectal or transperineal approach is an invasive procedure and occasionally associated with morbidity, such as hematuria, hematochezia, acute urinary retention, urinary tract infection, and bacteremia [19]. Thus, to select an appropriate candidate for active surveillance, more accurate and less invasive tools for monitoring PC are required.

On the contrary, PC at an advanced stage is a lethal disease; however, several treatment strategies are available for locally advanced or metastatic PC, and overall survival (OS) in patients with even metastatic castration-resistant PC (CRPC) has been improved with the development of several promising drugs [20]. Quantitation of therapeutic response in metastatic CRPC is difficult due to lack of tools [21]. In a recent meta-analysis [22], circulating tumor cells (CTCs) positively indicated poor prognosis and CTC counts were a potential independent prognostic factor of survival rate in patients with CRPC. Hence, early detection of drug response to specific therapeutics and recurrence using CTCs would result in enhancing treatment outcome and reducing socioeconomic burden in treating PC.

Liquid biopsy using CTCs is gaining attention as a minimal invasive tool for investigation of biomarkers. Because of

less morbidity of liquid biopsy using CTCs, it can be performed more frequently; accordingly, personalized disease status can be successively achieved at serial time points [23]. This is advantageous during treatment period for measuring tumor burden and early detection of recurrence or resistance [24]. Changes in CTC counts during systemic therapy can be used as a tool for monitoring treatment response [25].

Liquid biopsy using CTCs can aid in selection of an appropriate candidate for active surveillance and monitoring response to active surveillance or therapies and recurrence. Investigation of CTCs to discover new, more effective, and less invasive biomarkers is expected to contribute to reducing morbidity and costs associated with PC, the prevalence of which is increasing. In this review, we discussed the technology, advantages and disadvantages, applications, and the future direction of CTCs in PC.

2. Circulating Tumor Cells

Historically, CTCs were observed for the first time by Thomas Ashworth in the blood of a cadaver with metastatic cancer [26]. CTCs in the peripheral blood are originated from the primary tumor or metastatic foci and are circulated along the blood vessels and spread throughout the body [27]. They are involved in cancer metastasis, and protein expression and localization of CTCs at the cellular level were highly heterogeneous, which reflects the primary tumor and metastatic site [28]. CTCs have been detected in several malignancies, such as those of the prostate, breast, colon, lung, kidney, and bladder [29–37].

In the urological field, especially, elevation of CTC level in the peripheral blood was noted in patients with advanced renal cell carcinoma (RCC) and was associated with an aggressive phenotype [38]. CTC level in the peripheral blood was correlated with lymph node involvement and metastasis. CTC enumeration in the peripheral blood and expression of vimentin in CTCs were correlated with RCC progression [33]. CTCs were also detected in patients with metastatic bladder cancer [34, 35]. CTC levels were higher in the peripheral blood of patients with bladder cancer than in the control group. Quantification of CTCs using the expression of folate receptor α showed 82% sensitivity and 62% specificity in case of bladder cancer detection [36]. Enumeration of CTCs in the peripheral blood was an early predictor of bladder cancer recurrence and overall cancer-specific mortality [37].

There are several types of CTCs. Traditional CTCs were confirmed with a viability test, nuclear localization of cytokeratins, and an absence of CD45, which implies epithelial and nonhematopoietic origins, respectively [39]. They are larger cells with a subcellular feature or irregular morphology. Cytokeratin negative (CK−) CTCs or cancer stem cells undergo epithelial-mesenchymal transition (EMT) [40]. CK− CTCs are considered to have genomic profile or gene expression specific to cancer and morphology similar to a cancer cell and are prone to resistance and metastasis. Nuclear fragmentation or cytoplasmic bleb associated with apoptosis of CTCs was identified with Epic Sciences technology [40]. Efficacy of therapy can be checked by serial measurement of the ratio of traditional CTCs to apoptotic CTCs. Small CTCs are CD45− and CK+, and their morphology is similar to that of white blood cells [41]. CTC cluster is a bounded form of two or more CTCs [42, 43]. The CTC cluster is thought to contain CK− or small CTCs. Aceto et al. reported that this cluster is related to increased risk of metastasis and poor prognosis [42].

Small CTCs are related to differentiation into small cell carcinomas and progressive disease, which need a different treatment strategy.

Despite therapeutic and prognostic role of CTCs, detecting CTCs is difficult because they are present in small numbers [44]. The detection frequency of CTCs is 1–10 CTCs/mL of whole blood from patients with metastatic cancer [45]. Thus, fine and precise technologies with high sensitivity and specificity are required to detect various CTC subtypes in patients with numerous cancer types [40, 46].

3. Technologies Based on CTC Analysis

Analysis of CTCs includes their isolation and enrichment, detection, enumeration, and molecular characterization [47]. To isolate and enrich CTCs using filtration devices, physical features, such as size, electrical charge, and density, are considered [48].

The CellSearch® system (Veridex, Raritan, NJ, USA) was approved by the Food and Drug Administration (FDA) for monitoring patients with metastatic PC [49]. Currently, this is the only test for detecting CTCs that has received approval from the FDA. Briefly, 7.5 mL peripheral blood is sampled in an EDTA tube, centrifuged, and placed in the preparation system that enriches the tumor cells immunomagnetically with magnet and ferrofluid nanoparticles. CTCs of epithelial origin can be counted with a nuclear stain and a fluorescent antibody conjugate against EpCAM+; CD45−; and cytokeratins 8, 18+, and/or 19+ in the peripheral blood [43, 50]. For becoming CTCs, cells should possess a nucleus with diameter larger than 5 μm and should be negative for the CD45 marker and positive for CK. If five or more CTCs are found, the result is considered positive. This test has a detection limit of 1 CTC/7.5 mL whole blood with 93% recovery capacity.

Although it is the only method approved by the FDA, there are several limitations. Specific equipment—the CellTracks AutoPrep and the CellTracks Analyzer II unit (Veridex LLC, Raritan, NJ, USA)—is required to perform the test. Considering the sensitivity and specificity, CTCs are not yet utilized practically; therefore, analyzing content in CTC, such as that of miRNAs, to detect cancer biomarkers is gaining interest [51]. Using antibodies that can recognize tumor marker may be biased due to requirement for enough expression of protein on the cell surface [52]. Some tumors do not express EpCAM and CKs, which can be downregulated during EMT [52].

Maintrac method employs microscopy for identifying CTCs [53]. For analysis, cells are prepared with single centrifugation and erythrocyte lysis. The processes of purifying or enriching cells can be omitted with this method. Instead, cells are identified among the mixture of blood components. EpCAM antibody is used for identification of cells. With this method, live EpCAM+ propidium-excluding cells can

be counted as cancer cells. The suspension is analyzed by fluorescence microscopy, and the events are automatically counted. Previous studies demonstrated that adding CK- or CD45- specific antibodies does not have any merit [54, 55]. Maintrac uses the dynamics of cell count, and varying tumor cell number is an indicator of cancer activity. This method was used for determining the outcome of chemotherapy and for monitoring the response to hormone therapy [54, 56–58]. Additionally, early detection of cancer recurrence was verified with this method [59, 60].

However EpCAM+ cells can be detected in the peripheral blood when inflammation disease or skin burns are present [61]. Thus, diagnosis of cancer by EpCAM+ cells is not appropriate.

CTCs can be separated based on antigen-antibody interactions. Antibodies against tumor specific biomarkers, such as PSA, Her2, and EpCAM, are used. Magnetic-activated cell sorting (MACS) is a commonly used separation method based on magnetic nanoparticles. Microfluidic separation and immunomagnetic assay were used in previous studies [62–66]. Viruses with oncolytic features were used for detecting CTCs [67]. In other studies, for better control of the magnetic field, magnetic structure with a microscale was implemented [68–70].

A filtering-based method considering cell size was employed to capture CTCs [71]. The ScreenCell method captures CTCs with an isolation device using the drawn peripheral blood for 4 hours [72]. It allows isolation of CTCs by a filtration-based device using the whole blood.

Previous studies reported detection of CTCs by GILUPI GmbH *in vivo* [73, 74]. By inserting a metal wire that is coated with an antibody into a peripheral vein for 30 minutes, CTCs bind to the antibodies. After isolating CTCs, several methods, including immunofluorescence staining and molecular genetics, could be employed [67, 75]. Analyzing higher blood volume for detecting CTCs is an advantage of this method. Viatar CTC Solutions developed a method based on therapeutic oncopheresis that uses a mechanical filtration system for dialysis of CTCs for 4 hours [76].

CTC detection can be performed using RNA- or DNA-based technology with improved sensitivity. AdnaTest kit (Qiagen, Hannover, GE) uses simultaneous amplification and detection of multiple transcripts of circulating RNA or DNA to detect CTCs [77]. This method utilizes multiplex reverse transcription polymerase chain reaction (RT-PCR). In addition, other methods, such as CTC filters, acoustic-based separation, and microscopy, could be used to detect CTCs [78–80].

Methods for CTC isolation should allow their identification, enumeration, and characterization. ViewRNA ISH Cell Assays is a method that enables multiplex, single-molecule detection of specific RNA target with in situ hybridization technology [81]. Proprietary probe design allows high sensitivity and specificity using background suppression and branched DNA signal amplification.

After removal of the primary tumor, biopsy of the tumor by tissue typing is not possible [82]. In this case, tissue sections from the primary tumor can be used for typing, and CTC characterization can be performed to identify the tumor phenotypes. FISH assays were used on CTCs and identification of Her2, IGF-1R, Bcl-2, AR status, ERG, and PTEN was performed [83–86].

4. Advantages and Disadvantages of CTCs as a Liquid Biopsy Tool

Tissue biopsy is invasive, unsuitable to be performed repeatedly, and unpredictive for understanding effectiveness of treatment, disease progression, and metastasis risk [87]. CTCs can render ongoing information of metastasis reflecting the patient's disease status [83]. CTC detecting method using the peripheral blood is feasible and safe to be performed. Additionally, successive and repeated sampling is available. These characteristics of liquid biopsy allow monitoring the disease status, including progression and response to therapies.

Despite the aforementioned merits of methods for CTC detection, some demerits should be overcome before adopting it practically; several clinical, technical, and biological problems need to be solved. Because tumor is characteristically heterogeneous, revealing the source of CTCs is the most important to understand its clinical usefulness.

Since CTCs are present at low levels in the whole blood, assays with higher sensitivity are required. Droplet digital PCR (ddPCR) is a promising method, but sufficient blood is needed for appropriate analysis. Another hurdle is measuring the changes in CTC counts that might be minute. These demerits should be addressed to consider CTCs as a monitoring marker for cancer.

Since CTCs are extremely rare in the blood, sensitivity and specificity are not enough for accurate detection of CTCs, and enumeration has not been accepted as a method for tumor staging, CTCs cannot be used routinely in clinical practice yet. Despite the presence of various methods for CTC detection, none of them are established to be clinically applicable due to narrow detection spectrum, low purity, and loss of CTCs [116]. Additionally, methods for CTC detection are usually accompanied by complicated processes, long time for CTC detection, and significant costs.

5. Application of CTCs in PC

Studies that reported CTC detection in patients with PC are summarized in Table 1. Some studies demonstrated the association of CTCs with biochemical recurrence after radical prostatectomy. In the study with 250 high-risk patients with PC, presence of prostate stem cell antigen (PSCA) mRNA in the peripheral blood was reported to be a significant predictor of biochemical recurrence after radical prostatectomy (HR, 4.549; 95% CI, 1.685–12.279) [93]. Joung et al. reported that prostate specific membrane antigen (PSMA) mRNA in the peripheral blood was a predictor of biochemical recurrence after radical prostatectomy [95]. Nested RT-PCR assay detecting PSMA mRNA-bearing cells in the peripheral blood was employed to detect CTCs. PSMA mRNA (HR, 3.697; 95% CI, 1.285–10.634; $P = 0.015$) was an independent predictor of biochemical recurrence.

Table 1: Studies that reported detection of circulating tumor cells in patients with prostate cancer.

Study	Number ofpatients	CTCs enrichment method	Markers	Stage of disease	Treatment
Danila et al. [88] (2007)	120	CellSearch	CD45− CK+	CRPC	Chemotherapy
de Bono et al. [89] (2008)	231	CellSearch	CD45− CK+	CRPC	Chemotherapy
Goodman Jr. et al. [90] (2009)	100	CellSearch	CD45− CK+	CRPC	Chemotherapy
Scher et al. [91] (2009)	156	CellSearch	CD45− CK+	CRPC	Surgery/chemotherapy
Olmos et al. [92] (2009)	119	CellSearch	CD45− CK+	CRPC	Chemotherapy
Joung et al. [93] (2010)	103	Nested RT-PCR	PSCA-mRNA+	High-risk PC	Radical prostatectomy
Coumans et al. [94] (2010)	179	CellSearch	EpCAM+ CD45− CK+	CRPC	Chemotherapy
Joung et al. [95] (2010)	134	Nested RT-PCR	PSMA mRNA+	Localized PC	Radical prostatectomy
Strijobs et al. [96] (2010)	154	CellSearch	CD45− CK+	CRPC	Docetaxel based chemotherapy
Danila et al. [97] (2011)	48	CellSearch	CD45− CK+	CRPC	Abiraterone
Scher et al. [98] (2013)	144	CellSearch	CD45− CK+	CRPC	Cabozantinib
Thalgott et al. [99] (2013)	55	CellSearch	CD45− CK+	CRPC	Docetaxel based chemotherapy
Okegawa et al. [100] (2014)	57	CellSearch	CD45− CK+	CRPC	Docetaxel based chemotherapy
Danila et al. [101] (2014)	97	RT-PCR assay	Not reported	CRPC	Not reported
Goldkorn et al. [102] (2014)	263	CellSearch	CD45− CK+	CRPC	Docetaxel based chemotherapy
Antonarakis et al. [103] (2014)	62	AdnaTest	PSA+ PSMA+or EGFR+	CRPC	Enzalutamide and abiraterone
Chang et al. [104] (2015)	70	CellSearch	Not reported	CRPC	Docetaxel based chemotherapy
Scher et al. [105] (2015)	711	CellSearch	CD45− CK+	CRPC	Abiraterone or prednisone
Fleisher et al. [106] (2015)	258	CellSearch	EpCAM+ CD45− CK+	CRPC	Enzalutamide
Thalgott et al. [107] (2015)	33	CellSearch	EpCAM+ CK+ nucleic acid+	CRPC	Docetaxel based chemotherapy
Lorente et al. [108] (2015)	439	CellSearch	CD45− CK+	CRPC	Abiraterone orchemotherapy
Crespo et al. [109] (2015)	48	CellSearch	CD45− CK+	CRPC	Enzalutamide and abiraterone
Onstenk et al. [110] (2015)	29	CellSearch	CD45− CK+	CRPC	Cabazitaxel based chemotherapy
Antonarakis et al. [111] (2015)	37	AdnaTest	PSA+ PSMA+ or EGFR+	CRPC	Chemotherapy
Bitting et al. [112] (2015)	89	CellSearch	CD45− CK+	CRPC	Chemotherapy
Lorente et al. [113] (2016)	486	CellSearch	CD45− CK+	CRPC	Abiraterone plus prednisone or prednisone
Vogelzang et al. [114] (2017)	208	CellSearch	CD45− CK+	CRPC	Docetaxel based chemotherapy
Tsumura et al. [115] (2017)	59	CellSearch	DAPI+ CK+ CD45−	Nonmetastatic PC	Brachytherapy

CTC: circulating tumor cell; CK: cytokeratins; CRPC: castrate-resistant prostate cancer; EpCAM: epithelial cell adhesion molecule; RT-PCR: real-time polymerase chain reaction; PSCA: prostate stem cell antigen; PC: prostate cancer; PSMA: prostate specific membrane antigen; EGFR: epidermal growth factor receptor.

CTCs are often found in patients with mCRPC and it showed a prognostic significance [102]. de Bono et al. reported that elevated level of CTCs was a prognostic factor in patients with mCRPC [89]. Unfavorable group that had 5 or >5 CTCs/7.5 mL peripheral blood showed significantly shorter OS (median OS 11.5 versus 21.5 months) than that showed by favorable group (≤5 CTCs/7.5 mL peripheral blood). In this study, level of CTCs was a stronger predictor of OS than PSA was.

In a phase III clinical study, CTC level elevation after three cycles of docetaxel with lenalidomide chemotherapy in patients with mCRPC predicted poor survival [114]. Scher et al. reported in their phase III trial that level of lactate dehydrogenase and number of CTCs in the whole blood were predictors of OS in patients with mCRPC that had been treated with abiraterone acetate and docetaxel [105]. Two-year survival of patients with ≥5 CTCs/7.5 mL was 2%, while that of patients with <5 CTCs/7.5 mL was 46% (CTCs were counted at 12 weeks). Lorente et al. demonstrated that decline in CTC count by 30% after treatment from an initial count ≥5 cells/7.5 mL is independently associated with CRPC OS following chemotherapy and abiraterone treatment [113].

CTCs have emerged as a biomarker in mCRPC that guides therapeutic decisions. For evaluating predictor of resistance to treatments, molecular alterations in androgen receptor (AR) on CTCs were investigated. Antonarakis et al. demonstrated an association of detection of AR splice variant-7 (AR-V7) in CTCs and resistance to AR-targeting treatments in patients with mCRPC [103]. Recently, a real-time CTC-based assay of nuclear AR expression in CTCs of patients with CRPC was developed using CellSearch System [109].

Recently, no significant correlation between detection of AR-V7 positive CTCs and primary resistance to taxane chemotherapy was demonstrated in patients with mCRPC [111, 117]. Thalgott et al. reported that categorical status of CTC count, which was assessed after a cycle of taxane chemotherapy, was a predictor for progression-free survival and OS in patients with mCRPC [107]. This implies that categorical CTC count can act as a predictor of treatment response to taxane chemotherapy. However, AR-V7 status was not a predictor of response to cabazitaxel [110, 118].

6. Future Directions of CTCs

Cancer biomarker development using CTCs is a promising and rapidly expanding field. The emergence of ddPCR and next-generation sequencing (NGS) enabled improved detection rate and minimized time and expenses. Gulati et al. reported biomarker panel with an improved sensitivity and specificity compared to those of previous single markers [119]. However, most of the other markers are still experimental; accordingly establishment of these markers is required in the future. For improving accuracy of CTCs as a biomarker for cancer activity, usage of different molecular alteration levels, such as combining proteomics, genomics, and transcriptomics, would be beneficial [120, 121].

The analysis technique of CTCs has to be innovated with high selectivity and sensitivity that can be utilized for CTC purification, downstream CTC characterization, and retaining viable CTCs for ex vivo expansion [122]. Various CTC detection methods may count different subclasses of CTCs, and the best CTCs, which are clinically relevant, have not been established. Thus, the best analysis methods should be investigated for use as a biomarker of cancer and monitoring response to therapies.

7. Conclusion

In the era of precision medicine, treatment of PC will be tailored based on behavior of PC in each patient. Liquid biopsy using CTCs is a promising tool as a marker for prognosis and assessment of response to therapies in patients with PC. CTC analysis has a minimal invasive nature; accordingly, it is a suitable follow-up of biopsy, when serial PC behavior is required. CTC analysis enables detection of genomic alterations, which is drug-targetable, and it is a potential tool for monitoring response to therapeutic agents in patients with PC.

The utility of CTCs in patients with CRPC and localized PC after radical prostatectomy has been reported. Identification of gene expression, such as AR expression variants in CTCs, can be used as promising marker for selection of therapeutics in patients with mCRPC. However, application of CTCs in candidate for active surveillance is not well evaluated; accordingly, development of CTC marker that is specific in patients eligible to current active surveillance criteria is required for reflecting the increasing trend of active surveillance.

Future research on CTCs should be focused on developing a marker for CTCs with improved sensitivity and specificity, prior to their application to the detection of PC. The ideal marker for PC should be expressed on most of the CTCs, but not on other cells in the blood, and its expression should be maintained throughout the course of PC. The effort for using different molecular alteration levels has to be made for improving accuracy of CTCs as well. Finally, the technique used for CTC analysis should be improved to obtain high selectivity and sensitivity for the application of CTCs in PC.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Yoon Seok Suh and Jae Young Joung contributed equally to this work.

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Research Article

Pretreatment Serum Cystatin C Levels Predict Renal Function, but Not Tumor Characteristics, in Patients with Prostate Neoplasia

Feilong Yang, Dawei Li, Yu Di, Yongzhen Zhang, Yuanwei Zang, Juchao Ren, Lei Yan, Zunlin Zhou, Hainan Liu, and Zhonghua Xu

Department of Urology, Qilu Hospital of Shandong University, Jinan 250012, China

Correspondence should be addressed to Dawei Li; lidaweimd@aliyun.com

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To evaluate the role of Cystatin C (Cys-C) in tumorigenesis and progression of prostate cancer (PCa), we retrospectively collected the clinical information from the records of 492 benign prostatic hyperplasia (BPH), 48 prostatic intraepithelial neoplasia (PIN), and 173 PCa patients, whose disease was newly diagnosed and histologically confirmed. Pretreatment serum Cys-C levels were compared across the various groups and then analyzed to identify relationships, if any, with clinical and pathological characteristics of the PCa patient group. There were no significant differences in serum Cys-C levels among the three groups ($P > 0.05$). In PCa patients with normal SCr levels, patient age was correlated with serum Cys-C level ($P \leq 0.001$) but did not correlate with alkaline phosphatase (AKP), lactate dehydrogenase (LDH), prostate specific antigen (PSA), Gleason score, or bone metastasis status ($P > 0.05$). Age and SCr contributed in part to the variations in serum Cys-C levels of PCa patients ($r = 0.356$, $P \leq 0.001$; $r = 0.520$, $P \leq 0.001$). In conclusion, serum Cys-C levels predict renal function in patients with prostate neoplasia, but were not a biomarker for the development of prostate neoplasia, and were not correlated with the clinicopathological characteristics of PCa.

1. Introduction

Prostate cancer (PCa) develops in the unique gland of the male reproductive system, where it becomes a detriment to men's health. In 2015, PCa was ranked the second most frequently diagnosed cancer in males worldwide and the fifth leading cause of cancer deaths in the world [1]. In the United States, it was estimated that 241,740 new cases developed in 2012, making it the most frequently diagnosed cancer type therein [2]. In addition, 28,170 deaths were attributed to PCa, accounting for more than ten percent of cancer deaths in men [2]. The incidence of PCa varies widely worldwide. PCa is least common in South and East Asia and most common in United States, with moderate incidences in Europe. Moreover, in China, it was estimated that the incidence of prostate cancer was ranked sixth and the mortality of prostate cancer was ranked seventh in men [3]. Though widely studied, the precise mechanism of prostate cancer has not yet been fully clarified and further investigation is needed.

Cystatin C (Cys-C), encoded by the CST3 gene, belongs to the type two cystatin superfamily and has been extensively studied since it was first described in 1961 [4, 5]. CST3 is located on the short arm of chromosome 20, spans 7.3 kb [6], contains four exons, encodes a 120-amino acid active cysteine proteinase inhibitor [7], and shares several features with housekeeping genes [6]. Cys-C is ubiquitously expressed in nucleated cells [8, 9] in tissues such as the testis, epididymis, seminal vesicle, and prostate [10] and is then secreted into various human fluids to inhibit the activity of cysteine proteases such as papain and cathepsins B, H, K, and L [11]. Moreover, Cys-C is considered to function as a p53-inducible tumor suppressor and apoptotic mediator that negatively regulates cathepsin L activity during carcinogenesis [9]. Therefore, Cys-C is believed to play a critical role in the tumor suppressive function of p53 [9], as well as in extracellular, protein homeostasis. An imbalance between Cys-C and cysteine proteinases has been observed in the pathogenesis of a broad spectrum of diseases [12, 13],

including cancer [14–17]. However, the diagnostic role of Cys-C in cancers, such as renal cell carcinoma [18] and pancreatic tumors [18], has been dismissed. Recent studies by Wegiel et al. and Jiborn et al. indicated that Cys-C was downregulated in PCa specimens [19, 20]. Cys-C was also found to modulate the invasion of PCa cells by means of the androgen receptor and MAPK/Erk2 pathways [19]. Aberrant expression of Cys-C is associated with neuroendocrine differentiation in PCa [20]. Studies from another group also revealed that serum Cys-C levels may distinguish PCa patients from BPH patients and functioned as an indicator for the treatment of metastatic PCa with zoledronic acid in a small patient group [15, 21]. Taken together, published studies have reported both positive [15, 21, 22] and negative [23] effects of serum Cys-C levels on predicting malignancies. Thus, the feasibility of using serum Cys-C levels in cancer detection remains controversial.

We evaluated the diagnostic significance of circulating Cys-C levels in patients with prostate neoplasia, including BPH, PIN, and PCa. We also explored the relationship between serum Cys-C levels and clinicopathological characteristics of PCa patients.

2. Materials and Methods

2.1. Patient Population. The study was reviewed and approved by the Ethics Committee at Qilu Hospital of Shandong University and the Approval Number is KYLL-2015(KS)-156. We retrospectively collected clinical and pathological information from the records of inpatients that were newly diagnosed with prostate neoplasia and treated at the Department of Urology, Qilu Hospital of Shandong University between February 2010 and September 2013. Histologic confirmation of BPH, PIN, or PCa was obtained for all patients. None of the patients received preoperative hormonal therapy or radiotherapy. Patients with clinical characteristics such as (a) coexistence of prostate neoplasia and malignancy of other tissues or organs, (b) histologically diagnosed PCa but not adenocarcinoma, or (c) inadequate clinical information were excluded.

2.2. Sample Test and Data Collection. After an overnight fast, 5 mL of venous blood was obtained from patients with prostate neoplasia and assayed immediately before clinical treatment. Blood samples were deposited into test tubes containing a clot activator and gel, allowed to clot at room temperature, and subsequently centrifuged at 2000 rpm for 10 min. Serum was then collected to determine the concentration of Cys-C and other biochemical markers. The circulating Cys-C levels were tested with immunoturbidimetric method using a Roche Cobas 8000 analyzer with reagents purchased from BioSino Bio-Technology & Science Inc., Beijing, China. The levels of SCr, AKP, and LDH were determined using a Roche Cobas 8000 analyzer with reagents purchased from Roche. PSA was quantified using a Roche Cobas 601 analyzer, also with Roche reagents. The tests were completed according to the manufacturers' instructions. Clinical data, including age, SCr, Cys-C, PSA, AKP, LDH, Gleason score, and ECT (Bone Imaging), were retrieved from patient files. We first compared pretreatment serum Cys-C levels among all patients in the three groups. Subsequently, subclass analyses were conducted to exclude the possible effects of renal function on the pretreatment levels of Cys-C. Furthermore, we performed statistical analyses to compare the BPH and PIN groups. We also analyzed the association of serum Cys-C levels with clinical characteristics of PCa patients. Linear correlations among age, SCr, and Cys-C were also evaluated.

2.3. Statistical Analysis. The normal distribution of quantitative data in the various groups was assessed by the Kolmogorov-Smirnov test. Normally distributed data were expressed as the mean ± standard deviation (SD), while the median (range) was reported for data not following a Gaussian distribution. Statistical analyses were accordingly performed using the parametric Student's t-test, one-way ANOVA, or the nonparametric Mann–Whitney U test and Kruskal-Wallis H test. Qualitative data were reported as numbers and percentages, and the Pearson χ^2 test was used to compare differences among various groups. Pearson correlation coefficients were calculated to examine the associations among age, SCr, and Cys-C. Data were analyzed and processed using the Statistical Package for Social Sciences version 16.0 (SPSS 16.0, SPSS Inc., Chicago, IL, USA). All probabilities (P) were two-tailed and P values less than 0.05 were considered statistically significant.

3. Results

3.1. Characteristics of Study Population. The study group consisted of patients with prostate neoplasia consecutively presenting at the Department of Urology, Qilu Hospital of Shandong University. In total, 492 BPH, 48 PIN, and 173 PCa patients conforming to inclusive criteria were eligible for inclusion in the final study. The ages of eligible BPH, PIN, and PCa patients were 70.50 ± 7.55, 70.35 ± 7.95, and 70.88 ± 8.01 years, respectively ($P = 0.775$). All patients were further grouped according to their levels of SCr ($P = 0.916$). Clinical characteristics of enrolled patients are presented in Table 1.

3.2. Serum Cys-C Levels in Patients with Prostate Neoplasia. The levels of serum Cys-C were 1.04 (0.59–4.02), 1.02 (0.59–2.43), and 1.03 (0.59–3.08) mg/L in the BPH, PIN, and PCa groups, respectively ($P = 0.765$) (Figure 1(a)). Patients were then further grouped and analyzed according to their levels of SCr (less or greater than 115 μmol/L) ($P = 0.916$) (Table 1). There were insignificant associations with serum Cys-C levels among patients with SCr levels less than 115 μmol/L ($P = 0.769$) (Figure 1(b)) or greater than 115 μmol/L ($P = 0.609$) (Figure 1(c)). Levels of serum Cys-C were higher in BPH ($P < 0.001$) and PCa ($P \leq 0.001$) patients with SCr levels greater than 115 μmol/L (Figures 1(d) and 1(e)) than those in the PIN group. Moreover, the levels of serum Cys-C in PIN patients with SCr levels greater than 115 μmol/L were similar to those in patients with SCr levels less than 115 μmol/L ($P = 0.126$) (Figure 1(f)).

3.3. Association of Serum Cys-C Levels with Clinical Characteristics of PCa. Considering the effect of SCr on levels of serum

Table 1: Clinical characteristics of patients with prostate neoplasia.

Characteristics	BPH	PIN	PCa	*P* value
Patients (*n*)	492	48	173	
Age (y)	70.50 ± 7.55	70.35 ± 7.95	70.88 ± 8.01	0.775[a]
Cys-C (mg/L)	1.04 (0.59–4.02)	1.02 (0.59–2.43)	1.03 (0.59–3.08)	0.765[b]
SCr (*n*, %)				
≤115 μmol/L	455 (92.5)	44 (91.7)	159 (91.9)	0.916[c]
>115 μmol/L	37 (7.5)	4 (8.3)	14 (8.1)	

BPH: benign prostatic hyperplasia; PIN: prostate intraepithelial neoplasia; PCa: prostate cancer; Cys-C: cystatin C; SCr: serum creatinine; P^a: one-way ANOVA test; P^b: Kruskal-Wallis H test; P^c: Pearson χ^2 test.

Table 2: The level of serum Cys-C in PCa patients with normal SCr.

Characteristics	*n* (%)	Mean ± SD	*P* value
Patients (n)	159 (100)	1.04 ± 0.22	
Age			0.000[a*]
≤70 y	73 (45.91)	0.96 ± 0.17	
>70 y	86 (54.09)	1.10 ± 0.24	
AKP			0.133[*]
>125 U/L	20 (12.58)	0.97 ± 0.21	
≤125 U/L	139 (87.42)	1.05 ± 0.22	
LDH			0.368[*]
>230 U/L	24 (15.09)	1.07 ± 0.26	
≤230 U/L	135 (84.91)	1.03 ± 0.21	
PSA			0.471[*]
>5 ng/mL	147 (92.45)	1.04 ± 0.21	
≤5 ng/mL	12 (7.55)	0.99 ± 0.30	
Gleason score			0.574[b*]
≤5	4 (2.52)	1.03 ± 0.21	
6	22 (13.84)		
7	65 (40.88)		
8	40 (25.16)	1.05 ± 0.23	
9	21 (13.21)		
10	1 (0.63)		
Missing information	6 (3.77)	—	
ECT (bone metastasis)			0.432[c*]
Yes	27 (16.98)	1.06 ± 0.21	
No	55 (34.59)	1.02 ± 0.12	
Possible	26 (16.35)	—	
Unknown	51 (32.08)	—	

SCr: serum creatinine; PCa: prostate cancer; Cys-C: cystatin C; $P^a < 0.05$; P^b: Gleason score ≤ 7 versus Gleason score > 7; P^c: bone metastasis versus non-bone metastasis; P^*: Student's t-test.

Cys-C, the associations between serum Cys-C levels and clinical characteristics of 159 PCa patients with normal SCr (less than 115 μmol/L) were further evaluated. PCa patients were stratified accordingly, and these data are presented in Table 2. We found that the levels of serum Cys-C in older PCa patients (more than 70 years) were higher than in younger patients ($P \leq 0.001$) (Figure 2(a)). Moreover, there were insignificant associations between the levels of serum Cys-C and clinical characteristics, such as AKP, LDH, PSA, Gleason score, and bone metastasis status (all $P > 0.05$) (Figures 2(b)–2(f)).

3.4. Levels of Serum Cys-C Correlate with SCr and Age in PCa Patients. Using linear correlation analyses, there were positive correlations between circulating Cys-C levels, age ($r = 0.356$, $P \leq 0.001$) (Figure 3(a)), and SCr ($r = 0.167$, $P = 0.036$) (Figure 3(b)) in PCa patients. In addition, the SCr levels of PCa patients correlated with their pretreatment levels of serum Cys-C ($r = 0.520$, $P \leq 0.001$) (Figure 3(c)).

4. Discussion

Cys-C is a cationic, nonglycosylated protein with a molecular mass of 13 kDa. It is ubiquitously expressed in all nucleated

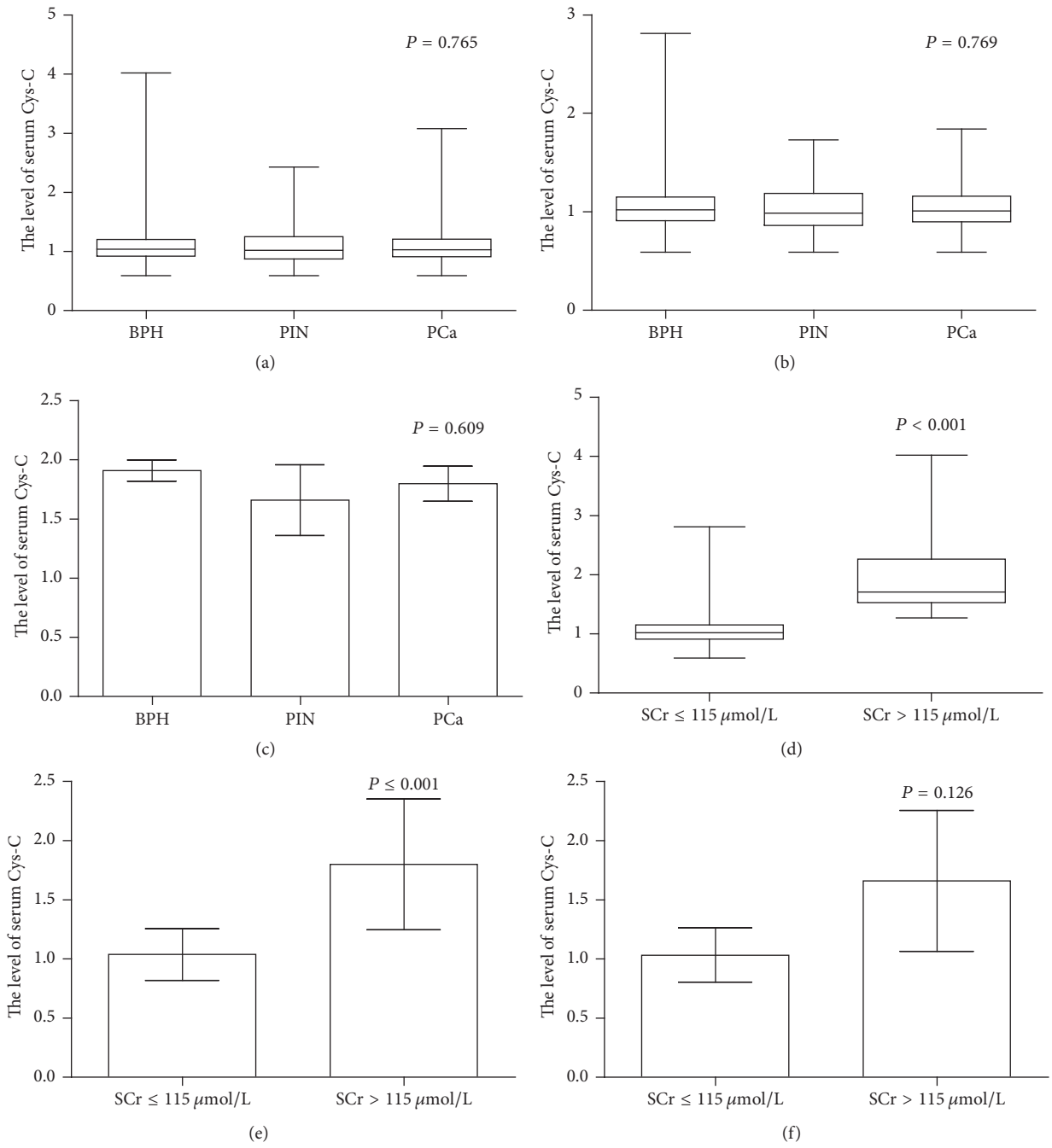

Figure 1: The comparisons of serum Cys-C in various groups of patients with prostate neoplasia. The comparison of serum Cys-C in total patients with prostate neoplasia ($P = 0.765$) (a); in patients with normal SCr ($P = 0.769$) (b); in patients with high SCr ($P = 0.609$) (c); in BPH patients ($P < 0.01$) (d); in PCa patients ($P \leq 0.001$) (e); in PIN patients ($P = 0.126$) (f). P: parametric Student's t-test.

cells [8], widely distributed in human biological fluids [9], freely filtered through renal glomeruli, and uniquely and almost completely reabsorbed and catabolized in the proximal tubules [18]. Therefore, its classic role as a sensitive marker for renal function has been extensively studied [24–27] and further confirmed in a meta-analysis [28]. In addition to its role in predicting kidney function, Cys-C is also a marker for inflammation [12], infection [13], tumorigenesis [16], prostate cancer pathological grade [20], malignant progression [14, 17], and several other processes [29, 30]. In the present study, we collected and analyzed clinical information to evaluate the diagnostic significance of circulating Cys-C in patients with prostate neoplasia and explored the relationship between serum Cys-C levels and clinicopathological characteristics of PCa patients. To our knowledge, this study is one of the first studies to focus on alterations circulating Cys-C concentrations in patients with PIN.

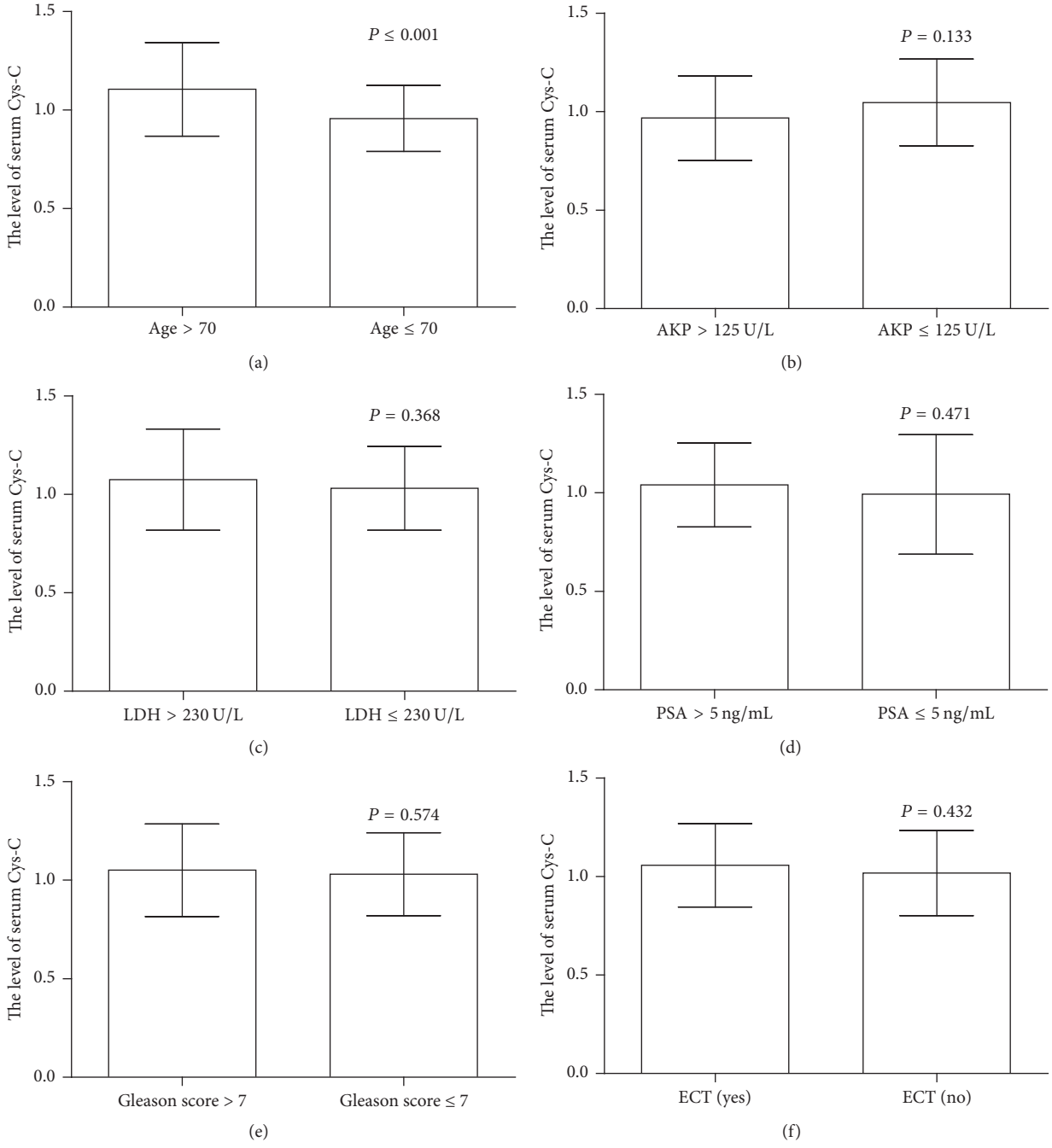

Figure 2: The comparisons of serum Cys-C in subgroup analyses of PCa patients with normal SCr. The subgroup comparison of serum Cys-C in patients with normal SCr based on patient age ($P \leq 0.001$) (a); AKP ($P = 0.133$) (b); LDH ($P = 0.368$) (c); PSA ($P = 0.471$) (d); Gleason score ($P = 0.574$) (e); the status of bone metastasis ($P = 0.432$) (f). P: parametric Student's t-test.

In the present study, there were no significant differences in the levels of serum Cys-C among all patients in the three groups ($P = 0.765$). Our result was in accordance with that from another study focusing on ovarian cancer, which excluded the role of serum Cys-C level as possible biomarker [23]. To exclude the impact of renal function on Cys-C levels, all patients were further grouped based on their SCr levels. Again, no differences in the levels of serum Cys-C were detected among the three prostate neoplasia groups in either the high SCr ($P = 0.609$) or normal SCr ($P = 0.769$) groups. However, a recent study found that the level of serum Cys-C could distinguish PCa from BPH patients [15]. The conflicting results between these two studies may be attributed to differences in the ages of the patient groups. In our study, patient age was normally distributed, and there were no significant differences among the three groups ($P = 0.775$). However, in the study by Tumminello et al. [15], PCa patients (72.4 ± 7.8 years) were much older than BPH patients

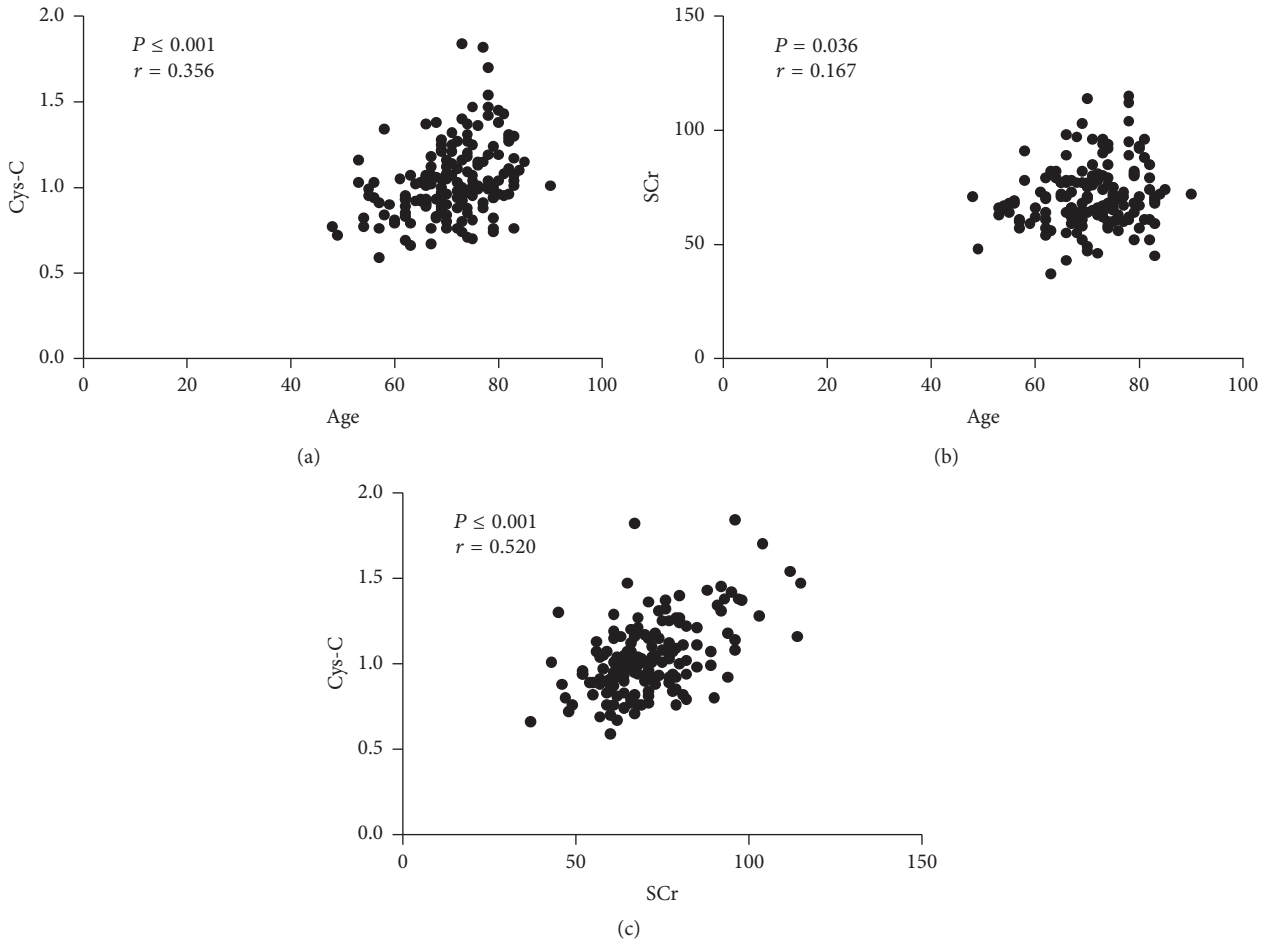

Figure 3: Correlations of serum Cys-C, SCr, and age in patients with PCa (normal SCr). Correlation of serum Cys-C and patient age (r = 0.356, $P \leq 0.001$) (a); correlation of SCr and patient age (r = 0.167, P = 0.036) (b); correlation of SCr and serum Cys-C (r = 0.520, $P \leq 0.001$) (c). P: statistical significance; r: correlation coefficient according to Pearson correlation test.

(62.8 ± 6.2 years). Age-related reductions in the glomerular filtration rate (GFR) [31, 32] lead to age-dependent increases in the concentrations of serum Cys-C [16]. Still, SCr affected the levels of serum Cys-C in both the BPH (P = 0.001) and PCa ($P \leq 0.001$) groups. However, SCr did not affect the level of serum Cys-C in the PIN group (P = 0.126). These results could be explained by the small number of PIN patients with SCr levels greater than 115 μmol/L (n = 4). However, the mean serum Cys-C level in PIN patients with high SCr (1.66 ± 0.60 μmol/L) was higher than that in PIN patients with normal SCr levels (1.03 ± 0.23 μmol/L).

We next investigated the possible relationship between circulating Cys-C and clinicopathological parameters in PCa patients with normal SCr. Unfortunately, when PCa patients were stratified according to levels of AKP, LDH, PSA, bone metastasis status, and Gleason score, no significant differences in serum Cys-C levels were found among the various groups (all $P > 0.05$). Our results were consistent with those of a previous study containing relatively few subjects [15]. However, we found that PCa patients older than seventy years of age had higher serum Cys-C levels than their younger counterparts (1.10 ± 0.24 versus 0.96 ± 0.17 mg/L, $P \leq 0.001$). As discussed above, older age may alter renal function as well as levels of serum Cys-C [33]. Next, the possible relationships among serum Cys-C, age, and SCr were tested using linear correlation analyses. As expected, serum Cys-C levels were positively correlated with patient age (r = 0.356, $P \leq 0.001$) and SCr (r = 0.520, $P \leq 0.001$). Moreover, we found that patient age was positively correlated with SCr (r = 0.167, P = 0.036).

Taken together, the value of serum Cys-C levels as a feasible predictor for PIN and PCa was limited for the following reasons. First, Cys-C is a housekeeping protein ubiquitously expressed in all nucleated cells and highly expressed in the male reproductive system. Unlike PSA, its expression was actually downregulated in prostate tumors and its circulating level may be affected by complex mechanisms. Second, prostate neoplasia was common in older males. Age-dependent reductions in GFR and declines in renal function would confound with changes in the levels of serum Cys-C [16]. Third, the male urethra traverses through the center of the prostate gland. Obstruction of the urethra caused by a prostate lesion may impair kidney function, which may impact levels of circulating Cys-C.

In conclusion, no statistically significant differences in the levels of serum Cys-C were found among the BPH, PIN, and PCa groups. Circulating Cys-C was not a potential marker for prostate tumorigenesis and was not a reliable predictor for clinicopathological characteristics of PCa patients. The increases in serum Cys-C levels in the elderly PCa group may be partly ascribed to age-dependent reductions in GFR.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Feilong Yang and Dawei Li contributed equally to the manuscript.

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Review Article

Genotyping the High Altitude Mestizo Ecuadorian Population Affected with Prostate Cancer

Andrés López-Cortés,[1] Alejandro Cabrera-Andrade,[1] Carolina Salazar-Ruales,[1] Ana Karina Zambrano,[1] Santiago Guerrero,[2] Patricia Guevara,[1] Paola E. Leone,[1] and César Paz-y-Miño[1]

[1]*Centro de Investigación Genética y Genómica, Facultad de Ciencias de la Salud Eugenio Espejo, Universidad Tecnológica Equinoccial, Avenue Mariscal Sucre, 170129 Quito, Ecuador*
[2]*Gene Regulation, Stem Cells and Cancer Programme, Centre for Genomic Regulation (CRG), The Barcelona Institute for Science and Technology, Universitat Pompeu Fabra (UPF), Dr. Aiguader 88 Street, 08003 Barcelona, Spain*

Correspondence should be addressed to Andrés López-Cortés; aalc84@gmail.com

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Prostate cancer (PC) is the second most commonly diagnosed type of cancer in males with 1,114,072 new cases in 2015. The MTHFR enzyme acts in the folate metabolism, which is essential in methylation and synthesis of nucleic acids. MTHFR C677T alters homocysteine levels and folate assimilation associated with DNA damage. Androgens play essential roles in prostate growth. The SRD5A2 enzyme metabolizes testosterone and the V89L polymorphism reduces in vivo SRD5A2 activity. The androgen receptor gene codes for a three-domain protein that contains two polymorphic trinucleotide repeats (CAG, GGC). Therefore, it is essential to know how PC risk is associated with clinical features and polymorphisms in high altitude Ecuadorian mestizo populations. We analyzed 480 healthy and 326 affected men from our three retrospective case-control studies. We found significant association between MTHFR C/T (odds ratio [OR] = 2.2; $P = 0.009$), MTHFR C/T+T/T (OR = 2.22; $P = 0.009$), and PC. The SRD5A2 A49T substitution was associated with higher pTNM stage (OR = 2.88; $P = 0.039$) and elevated Gleason grade (OR = 3.15; $P = 0.004$). Additionally, patients with ≤21 CAG repeats have an increased risk of developing PC (OR = 2.99; $P < 0.001$). In conclusion, genotype polymorphism studies are important to characterize genetic variations in high altitude mestizo populations.

1. Epidemiology of Prostate Cancer

Prostate cancer (PC) represents a significant health problem that involves the progressive accumulation of environmental, hormonal, genetic, and hereditary factors [1]. PC is the second most commonly diagnosed type of cancer in males, representing ~15% of all new cancer cases in 2015 (1,114,072 cases) [2]. Worldwide, the areas with a higher incidence of PC cases per 100,000 inhabitants are Oceania (101.9), North America (97.2), and Western Europe (94.9) [2–4]. In the Latin America region, PC was the most common malignancy diagnosed among males. The incidence of PC varied by 6-fold across this region during the period from 2003 to 2007. The highest age-standardized rates were observed in French Guyana (147.1) and Brazil (91.4) and the lowest ones were in Mexico (28.9) and Cuba (24.3). PC was one of the two leading causes of cancer deaths in males in Latin America, except in Chile, Argentina, Colombia, and El Salvador where it ranked third. Mortality rates varied by 4-fold, with the highest rates seen in Belize (28.9), Uruguay (21.8), and Cuba (24.1) and the lowest ones in Peru, Nicaragua, and El Salvador (rates between 6.8 and 9.7) [5]. In Ecuador 33% of all carcinoma diagnoses in males are prostate cancer, with an increase in the incidence from 23.7 per 100,000 inhabitants in 1985 to 54.4 in 2012 [2–4, 6]. Furthermore, the mortality rate associated with PC was 18.12 per 100,000 inhabitants in Ecuador in 2012 [2–4, 6, 7].

Table 1: Different NGS techniques used in prostate cancer studies.

Finding	NGS technology	Year	Reference
Deletion at chromosome 3p14 implicates FOXP1, RYBP, and SHQ1 as potential cooperative tumor suppressors	WES	2010	[21]
TMPRSS2-ERG, CTAGE5-KHDRBS3, and USP9Y-TTTY15 fusions, long noncoding RNAs (long ncRNAs), alternative splicing, and somatic mutations	RNA sequencing WGS	2011, 2012	[16, 18]
SPOP, FOXA1, and MED12 mutations	WES	2012	[20]
Variant at 8q24 and HOXB13	WGS	2012	[22]
Rearrangements, translocations, and deletions	WGS	2013	[23]
ADP-regulated signaling pathways-inhibitions of Wnt/B catenin signaling pathways	RNA sequencing	2014	[17]
Heterogeneity of AR gene expression, mutations, and splicing variants	Single-cell RNA seq	2015	[19]

2. Genomic Landscape in Prostate Cancer

Due to the fact that cancer rates are increasing every year, new technologies are being applied to detect, manage, and treat it according to the patient. Consequently, in the last years, next-generation sequencing (NGS) has emerged in order to provide a comprehensive characterization in cancer and other diseases [8]. Moreover, NGS technology allows for the identification of base substitution, insertion-deletion, copy number variance, and structural alteration with good sensitivity in cancer [9]. Some studies using NGS have been carried out, obtaining relevant information in breast cancer, lung cancer, ovarian cancer, colorectal cancer, and melanoma [10]. It is important to mention that there are available various NGS techniques that could be applied in cancer, specifically in prostate cancer.

2.1. Whole-Genome Sequencing (WGS). The WGS is the most complex technique based on the genomic DNA extraction from amplified and sequenced cancerous tissue giving, as a result, the somatic variations, such as mutations, insertions, and deletions, copy number variants, and rearrangements, among others. Nevertheless, the large amount of information that can be obtained with WGS, depending on the application (mutations, structural rearrangements, or others), needs time and a big coverage (100–200-fold coverage) that are translated into cost [11].

2.2. Whole-Exome Sequencing (WES). WES is based on the protein coding genes (exome) present in 1-2% of the human genome. It can give huge amounts of variation data of all coding regions of known genes. WES is cheaper than WGS because it analyzes a sequence of interest 70–100-fold coverage in order to identify the presence of mutations [12].

2.3. RNA Sequencing. RNA sequencing needs an additional step, which is the reverse transcription from cDNA, but it can give important data in cancer analysis, such as mutations, dysregulated genes, variants, and the level of expression. However, RNA sequencing experiments can be affected by technical effects in the sequencing steps and by contamination [13].

2.4. Single-Cell RNA Sequencing. The heterogeneity of cancer can be completely understood by single-cell RNA sequencing and it can be used as an instrument for clinical decisions. Besides, it identifies driver mutations, differentially expressed genes when it is compared with normal tissue, and drug resistance and it can also suggest other therapeutic alternatives [11]. The technique includes the separation of tumor cells by laser capture, cell sorting (FACS), or microfluidics, followed by cell isolation.

Furthermore, PC has been considered a challenge in diagnosis and prognosis because of the highly heterogeneous nature and the limited sample of tumor tissue. Consequently, merging traditionally diagnostic methods with NGS can diagnose PC with greater accuracy. There are plenty of publications regarding studies in PC that reported using NGS through whole-genome and whole-exome technology and RNA sequencing, among others. For instance, Robinson et al. (2015) reported that specific AR mutations can be linked to clinical phenotypes in order to determine the mutations that are responsible for resistance to therapy and the 40%–60% of the cases showed abnormalities of AR, ETS genes, TP53, and PTEN [14]. Another study is the one published by Lohr et al. (2014) who characterized circulating tumor cells by WES, and the variants were in concordance with the tumor biopsies; the used methodology gave an alternative because of the type of sample even though both techniques must be combined in order to reduce false-positives and reveal new mutations [15]. Another study is the one published by Berger et al. (2011), where they identified 3,866 putative somatic base mutations, mutated genes, and MAGI2 genomic rearrangements that are directed to improve the diagnostic and patient stratification by PI3 pathways [16]. There are other PC studies that include different NGS techniques in order to determine the following: signaling pathways, genomic alterations, repair defects, and gene merging (Table 1).

The main genetic alterations reported for prostate cancer are as follows: (a) merging: the main merging described is the one between TMPRSS2 and ETS family verified by various NGS techniques achieving the same result, constituting a powerful tool for diagnostic PC [16–18]; (b) mutations: P53 mutation is linked in most tumor types including hereditary.

It is involved in the response of cellular stress, cell survival, apoptosis, DNA repair, or change in the metabolism [11]; (c) pathways deregulations: there are studies that reported proliferative pathways that may promote cancer proliferation in the prostate. The AR abnormality is the most mentioned one in bibliography because it can modulate NCOA2, NCOR2, and regulatory elements like FOXA1 [16, 19–21]. Another signaling that can be found in prostate cancer is implied in cell growth and proliferation and apoptosis-like PI3K and PTEN [9]; and (d) variants and rearrangement: one variant described was at the 8q24 locus being a prostate cancer risk variant [22]. Furthermore, CHD1 regulates the chromatin state and its rearrangements are associated with more copy number variants [23].

3. The Steroid 5α-Reductase Type II Gene (SRD5A2) and Prostate Cancer Risk

The association of polymorphisms in the steroid 5α-reductase type II (SRD5A2) gene with prostate cancer risk in the high altitude mestizo Ecuadorian population was studied in 2009 by Paz-y-Miño et al. [24]. The steroid 5α-reductase type II enzyme is responsible for metabolizing the main androgenic hormone called testosterone that helps in the growth and development of the prostate. Due to the activity of the type II enzyme, testosterone is irreversibly converted into dihydrotestosterone (DHT), which is the reduced and metabolically more active form [25]. DHT then binds to the androgen receptor for the transactivation of androgen-sensitive elements as well as those that control cell proliferation. The SRD5A2 gene encodes the steroid 5α-reductase type II enzyme whose enzymatic activity may vary in such a way that it influences the incidence of PC [26].

The SRD5A2 gene plays an essential role in the induction of androgenic stimulation in the prostate and is highly polymorphic [27]. Hsing et al. suggest that genetic variations of this gene such as A49T and V89L are commonly studied and related to this type of cancer [26]. The V89L polymorphism, a missense mutation that substitutes leucine for valine at codon 89, identified by Makridakis et al., was reported to reduce in vivo steroid 5α-reductase activity [28]. Also, they found this substitution to be more common among Asians and believed this may explain the low risk for prostate cancer within this population. Another common polymorphism that has been reported to vary noticeably across populations is A49T, which results in an alanine residue at codon 49 being replaced with threonine. This missense substitution may enhance the conversion of testosterone into dihydrotestosterone because of increased enzymatic activity in vitro and has been associated with a higher risk of advanced prostate cancer in African American and Hispanic men living in the United States [29]. A total of 258 individuals, including 114 individuals with a previous diagnosis of prostate cancer and 144 control men, were analyzed to determine the association of SRD5A2 gene polymorphisms with the pathological characteristics of the tumor and the risk of PC in the high altitude mestizo Ecuadorian population.

The affected group had a median age of 70 years. 58% of the individuals were between 46 and 93 years old and 16% were older than 80 years. The pathological features of prostate tumors were quite variable, and the pathological stage of the tumor in most patients was moderate while 27% of the cases were diagnosed with advanced cancer. The 65% of the cases had serum prostate specific antigen levels greater than 10 ng/mL. The 23% of the individuals presented seminal vesicle invasion and 14% of the cases had positive surgical margins. The sixth level of Gleason grade is characterized because the cells are differentiated, in such a way that 59% of the cases have this score. There were significant differences between the tumor status and the presence of V89L polymorphism with the VV or VL genotype, where the LL genotype presented a highly significant reduction regarding the development of a high tumor stage (OR = 0.11, 95% CI = 0.04–0.27, and $P < 0.001$). While the higher pTNM stage (OR = 2.88, 95% CI = 1.15–7.21, and $P = 0.039$) and an elevated Gleason grade (OR = 3.15, 95% CI = 1.13–8.78, and $P = 0.043$) are associated with the A49T polymorphism.

Nam et al. and Li et al. showed that the V allele of the V89L polymorphism in the SRD5A2 gene was associated with PC risk because this V allele may encode for 5α-reductase variants with different activities, which are likely attributed to altered mRNA stability, which could alter the steroid 5α-reductase protein, leading to an increased cell division and, therefore, a higher likelihood of carcinogenesis [30, 31].

The genotype and allelic distribution of the V89L polymorphism in the Ecuadorian population support the hypothesis of the L allele having a protective status because of its suggested lower 5α-reductase enzymatic activity among men with the LL genotype [26, 28, 32, 33]. Other studies, however, have conversely found the LL genotype of the SRD5A2 V89L polymorphism to significantly increase the risk of PC [34–37]. Differences in genetic, dietary, and environmental factors among populations may explain the inconsistent results obtained in different studies. Our study also shows that men carrying the A49T variant have prostate tumors with a higher pathologic tumor-lymph node-metastasis (pTNM) stage (OR = 2.87; 95% CI = 1.15–7.21; $P = 0.039$) and a high Gleason grade (OR = 3.15; 95% CI = 1.13–8.78; $P = 0.044$). Our results agree with those of Jaffe et al., who reported the T allele to be linked to a greater frequency of extracapsular disease and a higher pTNM stage [38]. The genotype and allelic distribution are shown in Table 2, the OR test associated with polymorphisms is shown in Table 3, and the OR test associated with clinical data is shown in Table 4.

According to the 1000 Genomes' Project (Phase 3), the allele frequencies of the SRD5A2 V89L polymorphism are $G = 0.255$ and $C = 0.745$ in Colombians, $G = 0.406$ and $C = 0.594$ in Peruvians, $G = 0.233$ and $C = 0.766$ in Iberians, $G = 0.389$ and $C = 0.611$ in Japanese, $G = 0.387$ and $C = 0.613$ in Indians, $G = 0.524$ and $C = 0.476$ in Han Chinese, and $G = 0.320$ and $C = 0.680$ in the mestizo Ecuadorian population [39]. Also, the allele frequencies of the SRD5A2 A49T polymorphism are $C = 0.979$ and $T = 0.021$ in Colombians, $C = 0.982$ and $T = 0.018$ in Peruvians, $C = 0.981$ and $T = 0.019$ in Iberians, $C = 1.000$ and $T = 0.000$ in Japanese, $C = 1.000$ and $T = 0.000$ in Indians, $C = 1.000$ and $T = 0.000$ in Han Chinese, and $C = 0.550$ and $T = 0.450$ in the mestizo Ecuadorian population [39].

Table 2: Genotype distribution and allele frequency of polymorphisms in SRD5A2, MTHFR, and AR genes in high altitude Ecuadorian population with prostate cancer.

Gene	Polymorphism	Genotype	Genotypic frequency			Allele frequency		
			Control	Case	All	Control	Case	All
SRD5A2	A49T	A/A	0.347	0.289	0.322	0.674	0.553	0.627
		A/T	0.653	0.526	0.597			
		T/T	0.000	0.184	0.081	0.326	0.447	0.373
	V89L	V/V	0.153	0.404	0.264	0.469	0.684	0.564
		V/L	0.632	0.561	0.601			
		L/L	0.215	0.035	0.136	0.531	0.316	0.436
MTHFR	C677T	C/C	0.473	0.288	0.383	0.732	0.639	0.687
		C/T	0.518	0.702	0.607			
		T/T	0.009	0.010	0.009	0.268	0.361	0.313
AR	CAG repeats	≥22 CAGs	0.639	0.372	0.484			
		≤21 CAGs	0.361	0.628	0.516			
	GGC repeats	≥17 GGCs	0.389	0.281	0.329			
		≤16 CAGs	0.611	0.719	0.671			

Table 3: Association between genetic polymorphisms and prostate cancer risk among cases and controls.

Gene	Polymorphism	Genotype	Case versus control		
			OR	95% CI	*P* value
SRD5A2	A49T	A/A			Reference
		A/T	0.97	0.56–1.67	1
		T/T	0.40	0.31–0.52	0.000
		A/T + T/T	1.31	0.77–2.22	0.394
	V89L	V/V			Reference
		V/L	0.34	0.19–0.61	
		L/L	0.06	0.02–0.20	
		V/L + L/L	0.27	0.15–0.48	0.000
MTHFR	C677T	C/C			Reference
		C/T	2.22	1.26–3.92	0.008
		T/T	1.73	0.11–28.73	1
		C/T + T/T	2.21	1.26–3.89	0.008
AR	CAG repeats	≥22 CAGs			Reference
		≤21 CAGs	2.99	1.79–5.01	0.000

OR, odds ratio.

Table 4: Association of prostate cancer risk with genotype polymorphisms and clinical features.

Gene	Polymorphism	Genotype	Gleason score		OR	95% CI	*P* value	Tumor stage		OR	95% CI	*P* value
			2–6	7–10				T1-T2b	T2c-T4			
SRD5A2	A49T	A/A	20	6		Reference		17	10		Reference	
		A/T + T/T	36	34	3.15	1.13–8.78	0.044	26	44	2.88	1.15–7.21	0.039
	V89L	V/V	19	24		Reference		8	36		Reference	
		V/L + L/L	37	16	0.34	0.15–0.79	0.020	36	17	0.11	0.04–0.27	0.000
MTHFR	C677T	C/C	25	5		Reference		12	15		Reference	
		C/T + T/T	32	34	5.31	1.81–15.56	0.003	33	35	0.85	0.35–2.08	0.895
AR	CAG repeats	≥22 CAGs	19	8		Reference		19	8		Reference	
		≤21 CAGs	27	33	2.90	1.10–7.66	0.05	20	40	4.75	1.77–12.72	0.003

OR, odds ratio.

In conclusion, we found highly significant associations between two polymorphisms in the SRD5A2 gene. The V allele of the V89L polymorphism is associated with an increased risk, and the LL genotype has a protective role in the progression to a higher pTNM stage, while the TT homozygous genotype of the A49T polymorphism is associated with a higher pTNM stage and an elevated Gleason in the high altitude mestizo Ecuadorian population.

4. The Folate-Metabolizing MTHFR Gene and Prostate Cancer Risk

In 2013, López-Cortés et al. associated the folate-metabolizing genes with pathological characteristics of prostate cancer in the high altitude Ecuadorian mestizo population [40]. Regarding the folate cycle, the methylenetetrahydrofolate reductase (MTHFR), methionine synthase (MTR), and MTR reductase (MTRR) enzymes play an essential role in the folate metabolism [41], which is an important source for RNA and DNA synthesis and methylation [42]. The MTHFR enzyme controls a reaction of 5,10 methylenetetrahydrofolate to 5-methylenetetrahydrofolate [43–45], which is used as a methyl group donor for the remethylation of homocysteine to methionine, being a precursor of S-adenosylmethionine (SAM), essential in methylation of phospholipids, proteins, RNA, and DNA [41, 46, 47]. The remethylation of homocysteine to methionine is catalyzed by MTR in a reaction depending on vitamin B_{12} as an intermediary carrier of methyl group [48]. The MTR enzyme becomes inactive when the remethylation cofactor (vitamin B_{12}) is oxidized by the MTRR enzyme [46, 49]. The MTRR enzyme catalyzes the regeneration of methylcobalamin, MTR cofactor, keeping the MTR active [50]. On the other hand, 5,10-methylenetetrahydrofolate is used by the thymidylate synthase enzyme in the methylation of deoxyuridylate to *deoxythymidylate*, which is a source of thymidine required for DNA synthesis and repair [46].

Folate deficiency is associated with the rise in DNA rupture, chromosome damage, and formation of micronucleus in lymphocytes. These effects may depend on hereditary defects or be acquired in the absorption and metabolism of folic acid [51]. The MTHFR C677T single nucleotide polymorphism has been identified in the alteration of the levels of folates and homocysteine [52], triggering an alteration of the functioning of expressed proteins [46]. The presence of the allelic variants of the MTHFR C677T polymorphism causes reduced enzymatic activity, alteration in the homocysteine levels, and folate concentration in plasma [46, 50]. The MTHFR C677T variant has been associated with neural tube defects, cerebrovascular diseases [53, 54], coronary artery disease, venous thrombotic disease, and the rise in the risk of developing ovary cancer [55], esophagus cancer [56], gastric cancer [57], and prostate cancer [58]. On the contrary, a low risk of developing leukemia and colorectal cancer has been observed [59, 60]. A retrospective case-control study was conducted to establish the frequency of the C677T polymorphism in the MTHFR gene related to the folate metabolism, DNA synthesis and methylation, and their association with pathological characteristics in the high altitude mestizo Ecuadorian individuals with PC. Two hundred fourteen individuals altogether were analyzed. One hundred samples were taken from individuals diagnosed with PC after going through prostatectomy between 2004 and 2006. One the other hand, the control group was made up of 110 healthy men.

About the clinical-pathological parameters, 61% of the individuals affected by PC were aged between 63 and 79 years, whereas 16% were older than 79 years. Regarding pathological tumor stage, 30% of the cases were diagnosed with advanced cancer. Forty-one percent of the individuals had a Gleason score between 7 and 10 (poorly differentiated carcinoma). Concerning the PSA levels, 65% of the cases had a level higher than 10 ng/mL. Fourteen percent of the individuals had positive surgical margins, whereas 23% of the cases showed positive invasion of the seminal vesicle. The Gleason grading system for PC is the dominant method around the world in research and daily practice. A common practice has been to translate Gleason score of 2–4 carcinoma as well-differentiated, Gleason score of 5-6 as moderately differentiated, and Gleason scores of 7–10 as poorly differentiated [61]. Because there was no significant risk between the CT + TT genotypes and the Gleason score of 2–4 versus 5-6 (OR = 0.9; $P = 1$) and because there was significant risk between the Gleason score of 2–4 versus 7–10 (OR = 5.2, $P = 0.007$), an association between the Gleason grade and the MTHFR C677T polymorphism was shown.

The MTHFR gene has been widely studied, and it was observed that the T/T677 genotype reduces the risk of colorectal cancer, acute lymphocytic leukemia, and malignant lymphoma [59, 62, 63]. Hypermethylation plays an important role in ontogenesis, silencing expressions of CpG islands in regions that promote tumor suppressor genes [64]. On the contrary, Heijmans et al. [47], Sharp and Little [65], Robien and Ulrich [66], Shen et al. [67], Song et al. [56], and Miao et al. [57] have observed association between the MTHFR C677T variant and different types of cancer [55]. For this reason, there is a continuous debate on the effects of the MTHFR C677T polymorphism on PC [41]. In our study, we found that the MTHFR C/T genotype is significantly risky in affected individuals with an OR of 2.2 ($P = 0.008$), being a possible association between this polymorphism and PC; therefore, these results were similar to those provided by Marchal et al. [68] and Van Guelpen et al. [69]. The genotype and allelic distribution are shown in Table 2, the OR test associated with polymorphisms is shown in Table 3, and the OR test associated with clinical data is shown in Table 4.

According to the 1000 Genomes' Project (Phase 3), the allele frequencies of the MTHFR C677T polymorphism are $G = 0.457$ and $A = 0.543$ in Colombians, $G = 0.565$ and $A = 0.435$ in Peruvians, $G = 0.556$ and $A = 0.444$ in Iberians, $G = 0.620$ and $A = 0.379$ in Japanese, $G = 0.897$ and $C = 0.103$ in Indians, $G = 0.534$ and $A = 0.466$ in Han Chinese, and $G = 0.640$ and $A = 0.360$ in the mestizo Ecuadorian population [39].

In conclusion, the association between Gleason grade, MTHFR gene, and prostate cancer is an important contribution to understanding the different genetic behavior of cancer between the high altitude mestizo Ecuadorian population and populations worldwide.

5. The Androgen Receptor (AR) Gene and Prostate Cancer Risk

In 2016, Paz-Y-Miño et al. positively associated the androgen receptor CAG repeat length polymorphism with the risk of prostate cancer in the high altitude Ecuadorian mestizo and indigenous populations [70]. This study was performed to determine the association between CAG and GGC repeats and the risk of PC and histopathological characteristics of prostate tumors. The prostate cell cycle is mediated by the interactions of the androgen receptor (AR) gene, which is located on chromosome Xq12 and encodes a protein that has three major functional domains: the N-terminal domain (NTD), DNA-binding domain, and ligand-binding domain. The NTD, encoded by exon 1, regulates the transactivation of target genes and contains two polymorphic trinucleotide repeats: CAG and GGC, encoding polyglutamine and polyglycine, respectively [71]. The length of the CAG repeats correlates inversely with the AR transactivation function [72, 73]. Moreover, Hakimi et al. and Irvine et al. associated a low number of CAG repeats with an increased risk of PC [74–78]. Furthermore, the variants reported in the CAG and GGC repeats are highly polymorphic and associated with ethnic factors; thus, it may be important to determine their association with PC in different populations. Trinucleotide repeats are associated with human diseases and microsatellite instability [79]. The last one affects gene expression and protein function [80]. In addition to PC, the polymorphic CAG repeats have been associated with skin disorders [81, 82], breast cancer, polycystic ovary disease, Kennedy syndrome [83, 84], azoospermia, and oligospermia [85]. Furthermore, the effects of the repetition sequence GGC polyglycine have been associated with hypospadias and cryptorchidism [86, 87]; however, its role in transcription is unclear.

According to Kittles et al. the CAG and GGC repeats specifically vary depending on the ethnic group [88, 89]. The normal distribution of the CAG repeats is reported in a range of 6–39, with an average of 19-20 in African Americans, 21-22 in Caucasians, 22-23 in Asians, and 23 in Hispanics [90]. However and regarding South American countries, Brazil reported an average of 20.65 CAGs [91]. Moreover, Madjunkova et al. reported a mean repeat length of 21.5 CAGs in patients with PC from Macedonia [92]. Beilin et al. examined prostate adenocarcinomas, and the number of CAG repeats ranged from 12 to 30 and averaged 20, which was similar to that in a healthy Brazilian population [91]. Paz-y-Miño et al. reported that the repeat range in the mestizo control group was 16–30, with an average of 22, resembling Asians and Caucasians. The study was performed to determine the association between CAG and GGC repeats and the risk of PC and histopathological characteristics of prostate tumors. A total of 334 individuals were analyzed; 108 mestizo patients had a clinical diagnosis of prostate adenocarcinoma and 148 mestizo patients were healthy. Additionally, 78 healthy indigenous individuals were analyzed to determine the variety of trinucleotide repeats between different ethnic groups. The patient group presented with 12–30 repetitions with an average of 20. The most common numbers of CAG repeats were 21 and 22 (15.7 and 12%, resp.). The mestizo control group had 16–30 repetitions with an average of 22. The most common numbers of CAG repeats were 22 (25.7%) and 25 (12.8%). In the indigenous population, the repeat size ranged from 18 to 29 CAGs, determining the highest average of the three groups corresponding to 24 repetitions. Thus, the most common numbers of CAG repeats were 24 and 26, both with 24.4%. Statistically significant differences ($P < 0.001$) in the distribution of these trinucleotides were demonstrated ($\geq$22 CAGs versus $\leq$21 CAGs). In relation to tumor clinical characteristics, the presence of =21 CAGs showed significant association with tumor stage (OR, 4.75; 95% CI, 1.77–12.72; $P < 0.05$) and Gleason score (OR, 2.9; 95% CI, 1.1–7.66; $P = 0.03$) as in the ratio of risk of prostate cancer [70].

The ranges and averages of the CAG and GGC repeats in control mestizo population are similar to those of Asian and Caucasian-European populations. This may occur because the mestizo Ecuadorian population is considered a trihybrid, containing genes originated from America and descendants of Native Asians, Europeans, and Africans [93]. Other polymorphisms associated with PC in Ecuador also showed similar frequencies to Asians [24, 40]. Additionally, these results were consistent with the average in cases (19 CAGs) and controls (19-20 CAGs) in African Americans (17 CAGs) and in both cases and controls (21.95 CAGs) in Australians [94].

Finally, these results indicated that in mestizos the PC risk increased 2.99 times in males with $\leq$21 CAGs. By contrast, some studies did not identify an association with this repetition [43, 95, 96]. Several studies reported no association between the GGC repeat lengths, the PC risk, and pathological characteristics [34], stating that there were no significant differences between cases and controls [97]. Similarly, the risk of PC and the tumor characteristics did not differ in relation to the number of GGC repeats in this study [93].

6. Conclusions

Cancer research has evolved in parallel with cutting-edge technologies, leading to the development of a personalized genomic-based therapy. This tailored treatment not only takes into account the clinical aspects of each patient but also, and most importantly, the molecular characteristics of their tumors. Thus, to offer a precise anticancer therapy, personalized oncology identifies druggable cancer driver proteins based on their genomic alterations and differences between human populations. For instance, the 1000 Genomes' Project (Phase 3) demonstrates that the allele frequencies of the SRD5A2 V89L, SRD5A2 A49T, and MTHFR C677T genetic variants differ among the Latin American (Ecuador, Colombia, and Peru), Caucasian (Spain), and Asian (Japan, India, and China) populations. In conclusion, in order to implement successful pharmacogenomics tests at the hospitals in Ecuador, it is important to understand the genetic variability of the mestizo population. Likewise, genetic polymorphisms in the MTHFR, SRD5A2, and AR genes are associated with PC risk in high altitude mestizo Ecuadorian population.

Conflicts of Interest

The authors declare no conflicts of interest.

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Research Article

The Long-Term Effect of Radical Prostatectomy on Erectile Function, Urinary Continence, and Lower Urinary Tract Symptoms: A Comparison to Age-Matched Healthy Controls

Badereddin Mohamad Al-Ali,[1,2,3] Anton Ponholzer,[4] Herbert Augustin,[2] Stephan Madersbacher,[1] and Karl Pummer[2]

[1]*Department of Urology, Kaiser-Franz-Josef Spital, Vienna, Austria*
[2]*Department of Urology, Medical University of Graz, Graz, Austria*
[3]*Kazan Federal University, Kazan, Russia*
[4]*Krankenhaus der Barmherzigen Brüder, Department of Urology, Vienna, Austria*

Correspondence should be addressed to Badereddin Mohamad Al-Ali; bader1971@gmx.at

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Introduction. To analyze the impact of radical prostatectomy (RPE) on erectile function and lower urinary tract function in comparison to age-matched healthy men. *Materials and Methods*. Patients who underwent radical retropubic prostatectomy completed questionnaires containing the IIEF-5, the Bristol female LUTS questionnaire, and the International Prostate Symptom Score (IPSS). *Results*. Patients after RPE were included ($n = 363$). Age-matched healthy men ($n = 363$) were included. The mean IIEF-5 of patients aged 61–70 yrs after RPE was 10.4 ± 6.6 versus 18.8 ± 5.3 in the control cohort; the respective values for men aged 71–80 yrs after RPE were 7.2 ± 6.5 versus 13.6 ± 7.7 in the control cohort. Urinary incontinence after RPE was reported in 41.9% (61–70 years) and 37.7% (71–80) versus 7.5% and 15.1% in the control cohort. The mean IPSS of patients after RPE aged 61–70 yrs was 5.0 ± 4.4 versus 5.5 ± 4.9 in the control cohort; the respective values for men aged 71–80 yrs were 6.0 ± 4.9 versus 7.5 ± 5.7 in the healthy cohort. *Conclusions*. The negative effect of radical prostatectomy on erectile and urinary incontinence remains substantial. The physiologically declining erectile and lower urinary tract function with ageing reduces the difference between healthy men and those after surgery. Healthy men have a higher IPSS presumably due to the presence of bladder outlet obstruction.

1. Introduction

Besides the issue of overtreatment, the negative effect of active treatment of localized prostate cancer on lower urinary tract and erectile function is one of the major burdens regarding screening and treatment of prostate cancer [1, 2].

This negative effect of radical prostatectomy (RPE) on lower urinary tract function (erectile function, urinary incontinence) is well documented for two decades, although relevant discrepancies regarding the extent of this effect remain even after this long time period of clinical experience and research [3]. This is exemplified by the reported rates of erectile dysfunction after RPE [4]. Moreover, cohort studies generated by centres of excellence provide potency rates in the range of 90–95%; independent surveys and a recent meta-analysis of the placebo-arms of randomized controlled trials on penile rehabilitation suggest considerable lower rates between 20 and 30% [4, 5]. Similar discrepant data were reported for urinary incontinence [6, 7]. It remains debateable whether the robotic approach provides superior outcome since level I evidence is missing.

Given the life expectancy after RPE, the long-term effect of surgery on lower urinary tract function is of considerable interest and an important parameter for patients, surgeons, and also socioeconomic aspects. To analyze this long-term effect of surgery, the impact of age on lower urinary function has to be taken into consideration [8]. Moreover, to study the long-term effect of RPE on lower urinary tract function

two study designs are possible, that is, RCT in comparison to active surveillance/watchful waiting or a matched-pair and cross-sectional comparison to unoperated men. The latter approach was chosen in this study.

Men with a minimum follow-up of 5 yrs after nerve-sparing open retropubic RPE and no adjuvant therapy were compared to age-matched healthy men that were recruited via a voluntary health investigation. All patients completed the International Index of Erectile Function- (IIEF-) 5 [9], the Bristol female LUTS questionnaire [10], and the International Prostate Symptom Score (IPSS).

2. Materials and Methods

2.1. Study Design. A consecutive series of patients who underwent open uni- or bilateral nerve-sparing retropubic RPE were contacted by surface mail to complete a 10-page questionnaire. For the current study, only men with a minimum follow-up of 5 yrs after RPE and without any adjuvant therapy were eligible.

As a control cohort [11], age-matched men without previous prostate surgery or prostate specific medication (alpha-blockers, 5-alfa-reductase inhibitors, and anticholinergics) who underwent a voluntary health investigation were included. Moreover, the following parameters were routinely evaluated: (1) a clinical medical history, (2) documentation of all concurrent medical therapies, (3) physical examination with documentation of age, weight, height, body mass index, heart rate, blood pressure, echocardiogram, and spirometry, (4) sociodemographic parameters including marital status, cigarette smoking, alcohol consumption, level of education, and physical activity, (5) stress factors, (6) urinalysis, and (7) a blood laboratory evaluation of a total of 14 parameters including kidney and liver function tests, red and white cell counts, low and high density lipoprotein, cholesterol, and glucose. The control group was extracted from this database to match the RPE group regarding age. Institutional board approval was obtained.

2.2. Questionnaires. Besides various disease-specific aspects (PSA at diagnosis, histology of the RPE-specimen, PSA-relapse, adjuvant therapy, etc.) the questionnaire contained the IIEF-5, the IPSS, and the Bristol female LUTS questionnaire.

2.3. Statistical Analysis. All statistical analyses were conducted using Statistical Package for Social Sciences, version 10.0.7 (SPSS Inc., Chicago, IL) and Primer of Biostatistics, Version 5.0 (McGraw-Hill, 2002). All hypotheses testing was 2-sided with $p < 0.05$ considered to be significant.

3. Results

3.1. Characteristics of the Study Population. A total of 363 men with a mean age of 71 yrs (range: 61–80 yrs) and a mean follow-up of 7.1 yrs after nerve-sparing RPE (range: 5–13 yrs) entered the study. Tumour characteristics were as follows: PSA 8.5 ± 5.5 ng/mL (0.3–56 ng/mL), pT2, 67%, pT3, 33%, positive surgical margin, 22.9%, Gleason score 6, 48%, Gleason score 7, 41%, and Gleason scores 8–10, 11%. To assess the impact of age, patients were grouped into two age cohorts 60–70 yrs ($n = 176$; 66±2.9 yrs) and 71–80 yrs (75±2.9 yrs; $n = 187$). These patients were compared to 363 age-matched men who underwent a health investigation (60–70 yrs; 64 ± 2.9 yrs; $n = 257$; 71–80 yrs; 74 ± 2.8 yrs; $n = 106$).

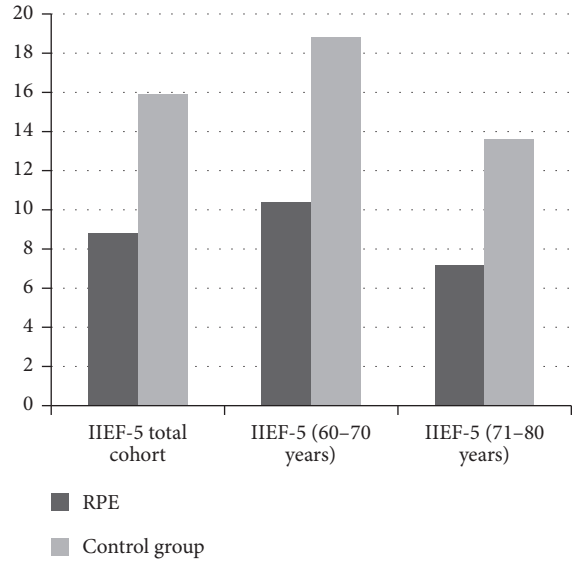

Figure 1: IIEF-5 scores in the RPE and the control group. The differences between the RPE and control cohort were significant for all three groups ($p < 0.001$).

3.2. Erectile Function. The mean IIEF-5 of the RPE cohort was 8.8±6.5 compared to 15.9±6.5 in the control group (Figure 1). In patients aged 61–70 yrs after RPE the IIEF-5 was 10.4 ± 6.6 as compared to 18.8 ± 5.3 in the healthy age-matched cohort (Figure 1). The respective values for men aged 71–80 yrs after RPE were 7.2 ± 6.5 versus 13.6 ± 7.7 in the healthy cohort (Figure 1). Moderate to severe ED (IIEF-5 <18) was present in 81% after RPE in both age groups as compared to 17.2% (61–70 yrs) and 37% in those aged 71–80 yrs in the healthy cohort (Figure 2). The risk for moderate/severe ED following RPE compared to healthy men declined from being 4.7-fold in younger age group to being 2.2-fold in the higher age group (Figure 2).

3.3. Urinary Incontinence. The overall prevalence of UI (definition: any involuntary loss during the past 4 weeks) was 39.9% for men after RPE as compared to 11.3% of the healthy cohort (Figure 3). In the 60–70 yrs cohort the prevalence of UI was 41.9% (RPE) and 7.5% (healthy men) and in those aged 71–80 yrs 37.7% (RPE) and 15.1% (healthy men), respectively (Figure 3). The following percentages refer to the number of incontinent patients in each group. Rare episodes of urinary incontinence (once per week or less frequent) were reported after RPE by 54.5% (60–70 yrs) and 36% (71–80 yrs) and in the control group by 27.3% and 18.2%. The respective percentages for more frequent urinary incontinence episodes (≥1/week)

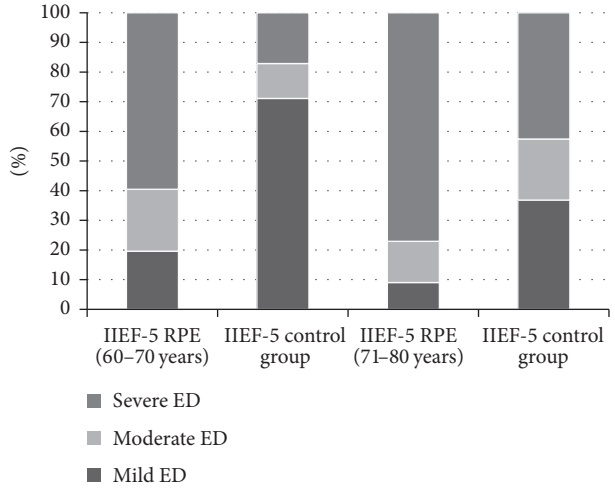

Figure 2: Distribution of mild/moderate/severe erectile dysfunction in the RPE and the control group. The differences between the RPE and control cohort were significant for all three groups ($p < 0.001$).

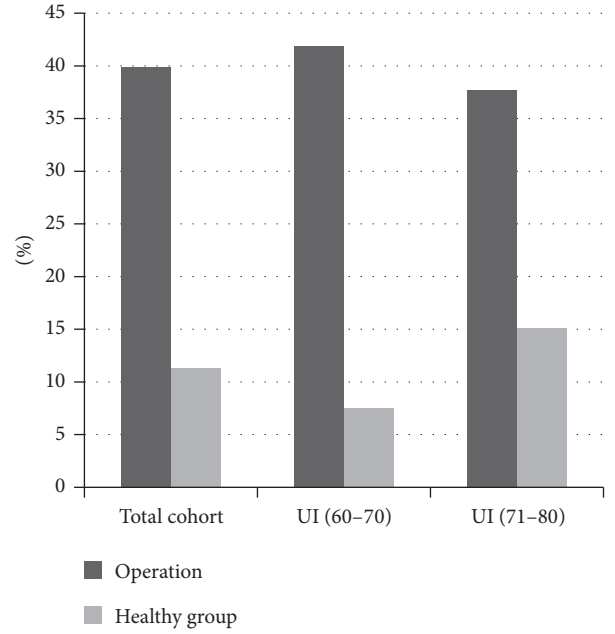

Figure 3: Prevalence of UI in the RPE and the control group. The differences between the RPE and control cohort were significant for all three groups ($p < 0.001$).

were 37.7%, 48.0%, 54.6%, and 45.5%, respectively. Any degree of quality-of-life impairment due to urinary incontinence after RPE was reported by 70.1% (60–70 yrs) and 66.1% (71–80 yrs) and by 81.8% (60–70 yrs) and by 83.3% (71–80 yrs) in the control cohort.

3.4. Lower Urinary Tract Symptoms. The mean IPSS was higher in the unoperated group (6.5±5.3) as compared to men after RPE (5.5±4.6) (Figure 4). In the younger age cohort, the IPSS was identical in men who were operated and unoperated

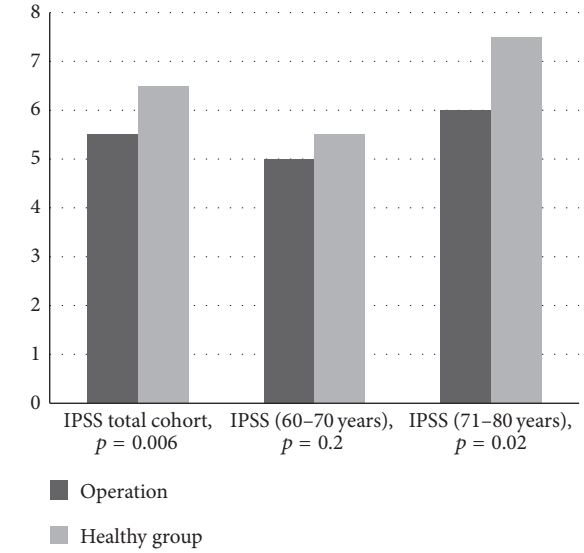

Figure 4: IPSS in the RPE and control group. The differences in the total ($p = 0.002$) and the 71–80 yrs ($p = 0.02$) group were significant.

on (5.0±4.4 versus 5.2±4.9). In the older age cohort men after surgery had a significantly lower IPSS 6.0 ± 4.9 as compared to unoperated men 7.5 ± 5.7 (Figure 4). Patients in the healthy cohort had higher IPSS scores (IPSS > 8), with a trend towards moderate and severe lower urinary tract symptoms in comparison to surgery cohort. In individuals aged 71–80 yrs, the percentage of men with moderate/severe LUTS increased from 30.8% after surgery to 38.8% in the healthy group.

4. Discussion

The aim of our study was to compare the long-term negative effect of RPE on lower urinary tract function to age-matched men participating in a health survey. A comparison to unoperated men might provide a better estimation of the potential long-term negative effect of surgery on lower urinary tract function than a longitudinal cohort study and could be of value in counselling the patient before surgery. To avoid any bias of secondary treatments, only men without adjuvant treatment after RPE were eligible. Moreover, our study had a mean follow-up of >7 yrs after RPE, which is the longest follow-up on this issue. We concentrated on three aspects, that is, erectile function (IIEF-5), urinary incontinence (Bristol LUTS questionnaire), and lower urinary tract symptoms (IPSS).

Reported potency rates after RPE differ substantially [4, 5]. A systematic review by Ficarra et al. [4] showed potency rates 12 months postoperatively from 10 to 73%, 42% to 76%, and 70 to 80% following retropubic, laparoscopic, and robot assisted RPE. Barry et al. [12] investigated 220 patients after open and 406 after robotic RPE. In this cohort patients were at least 66 yrs or older at the time of surgery with only 2.9% after open and 2.3% after robotic surgery reported to have no sexual problems [11]. In a recent meta-analysis of control arms

on penile rehabilitation after nerve-sparing RPE Schauer et al. showed that the rate of undisturbed erectile function is in the range of 20–25% in most studies and that these rates have not improved over the past 17 yrs [5]. In our study 61–70 yrs men after RPE had a 4.7-fold higher rate of moderate to severe ED; in the 71–80 yrs cohort this rate declined to 2.2. The only study available with a similar design was reported by Deliveliotis et al. [13] who studied 105 patients after RPE and 80 unoperated control patients recruited in the urological outpatient clinics (follow-up 2 years) [13]. Participants completed various questionnaires, none (with the exception of the AUA-symptoms score) of which was used by us (the validated IIEF-5 was not available then). In this series, erectile function decreased after RPE significantly with only 24.8% of patients having a firm erection compared to 72.8% of the control population [12]. These findings are in agreement with our study.

Similar to erectile dysfunction, the incidence of UI after RPE ranges in the literature between 2.5% and 87% [14]. While some centres of excellence report on continence rates beyond the 90% mark, other sources (e.g., Medicare data) suggest higher incontinence rates [12, 14]. This wide variation is further attributed to definition of UI, methods used for assessing the return of continence, time of reporting after surgery, and patient selection [12, 14]. Herein we used the rather strict definition of the Bristol female LUTS questionnaire, that is, any involuntary loss during the past 4 weeks [10]. By using this definition, already 7.9% of healthy men aged 60–70 yrs and 13.5% in the 71–80 yrs cohort have to be rated as incontinent. In the 60–70 yrs age group, men after RPE had a 5.5-fold higher risk for UI than healthy men; this excess risk declined to be 3-fold in the age group 71–80 yrs. This decline was not due to the lower UI-rates in the surgery group yet to the higher UI in the advanced age control group (13.1%).

The impact of RPE in LUTS was studied in several trials. Schwartz and Lepor performed a prospective study of 104 patients who underwent open radical prostatectomy and reported on the impact after 12 months [15]. In men with moderate/severe LUTS prior surgery, the total AUA symptom score, the symptom problem, and quality-of-life score decreased by 51% (−6.4), 57% (−4.2), and 25% (−0.7) 12 months after RPE [15]. Except for nocturia, all parameters of the AUA symptom score improved significantly [15]. In men with no/mild LUTS no significant changes were observed [15]. Matsubara et al. studied 117 patients after perineal radical prostatectomy and observed a decline of the IPSS in men with moderate/severe LUTS [16]. Wang et al. studied100 consecutive patients after robotic radical prostatectomy with a follow-up of 12 months [17]. The IPSS improved substantially from 14.1 before surgery to 2.9 after 12 months and the IPSS-Ql from 3.4 to 1.6, respectively [17]. Moreover, patients with no/mild LUTS experienced no relevant improvements [17]. Slova and Lepor prospectively followed 453 men for up to 48 months after surgery [18]. The AUA symptom score declined from 6.9 to 5.8 after 4 years, the corresponding numbers for men with an AUA symptom score <8 prior surgery were 3.2 to 4.9; for men with an AUA symptom score >8 the score declined from 13.6 to 7.3 [18]. Storage and voiding symptoms revealed similar patterns [18]. In our cross-sectional study men who were operated on had a lower IPSS as age-matched unoperated men. This underlines the altered natural history of the lower urinary tract in the absence of infravesical obstruction due to the removal of the prostate.

Donovan et al. recently published a series of patient reported outcomes including lower urinary tract and erectile function based on the ProtecT trial [19]. A total of 1643 men were randomized to active monitoring, surgery, or radiotherapy and followed for up to 6 years with several quality-of-life measures [19]. After 6 years (hence a follow-up comparable to our series), the rate of UI and erectile function was—as expected—higher after RPE as compared to active monitoring [19]. The absolute difference between surgery and conservative management, however, was considerably lower in the ProtecT trial as compared to our series [19]. One potential explanation for this discrepancy might be the fact that active surveillance has a negative impact on lower urinary tract function.

5. Conclusions

The long-term negative effect of radical prostatectomy on erectile and urinary incontinence remains substantial. The physiologically declining erectile and lower urinary tract function with ageing reduces the difference between unoperated men and those after surgery with advancing age. Unoperated men have a higher IPSS than those after RPE presumable due to the presence of bladder outlet obstruction.

Competing Interests

None of the contributing authors have any conflict of interests, including specific financial interests and relationships and affiliations relevant to the subject matter or materials discussed in the manuscript.

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Research Article

ZFP91: A Noncanonical NF-κB Signaling Pathway Regulator with Oncogenic Properties Is Overexpressed in Prostate Cancer

Lukasz Paschke, Karol Jopek, Marta Szyszka, Marianna Tyczewska, Agnieszka Ziolkowska, Marcin Rucinski, and Ludwik K. Malendowicz

Department of Histology and Embryology, Poznan University of Medical Sciences, 6 Swiecicki St., 60-781 Poznan, Poland

Correspondence should be addressed to Lukasz Paschke; paschkelukasz@gmail.com

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Novel molecular targets are being searched to aid in prostate cancer diagnosis and therapy. Recently, ZFP91 zinc finger protein has been found to be upregulated in prostate cancer cell lines. It is a potentially important oncogenic protein; however only limited data regarding its biological function and expression patterns are available. To date, ZFP91 has been shown to be a key factor in activation of noncanonical NF-κB signaling pathway as well as to be involved in HIF-1α signaling in cancer cells. The present study aimed to characterize *ZFP91* expression in prostate cancer specimens. Furthermore, since our earlier reports showed discrepancies between *ZFP91* mRNA and protein levels, we studied this interrelationship in LNCaP and PC-3 prostate cancer cell lines using siRNA mediated knockdown. QPCR analysis revealed marked upregulation of ZFP91 mRNA in the majority of prostate cancer specimens. Transfection of prostate cancer cells with *ZFP91* siRNA resulted in a 10-fold decrease in mRNA levels. On a protein level, however, no inhibitory effect was observed over the time of the cell culture. We conclude that *ZFP91* is overexpressed in prostate cancer and that potential accumulation of the ZFP91 protein in studied cells may be of importance in prostate cancer biology.

1. Introduction

Prostate cancer is worldwide the second most common cancer in men with over million of new cases diagnosed every year and is the fifth leading cause of death of cancer in men (WHO International Agency for Research on Cancer, http://www.iarc.fr/). With the advent of whole genome expression analyses and progress in molecular biology techniques several molecular pathways involved in prostate cancer pathogenesis have been discovered. Still novel targets are being searched to aid in diagnosis and treatment of this complex and heterogeneous disease [1]. In 2014 our team described changes in expression of little-studied ZFP91 zinc finger protein gene (*ZFP91*) in benign prostate hyperplasia (BPH) and in prostate cancer cell lines [2]. Recently found oncogenic properties of this gene prompted us to further study its significance in prostate cancer pathogenesis.

ZFP91 gene was discovered in 1995 by Saotome et al. [3]. In 2003, using cDNA microarray screening, Unoki et al. found its overexpression in mononuclear cells from patients with acute myelogenous leukemia (AML) and in several neoplastic blood cell lines [4]. Subsequently, in 2006 an interaction of ZFP91 with ARF tumor suppressor protein (cyclin-dependent kinase inhibitor 2A, isoform 4) was discovered. This protein serves important antioncogenic functions based on p53-dependent cell death or cell cycle arrest in response to oncogenes activation [5]. To date the most important findings regarding *ZFP91* gene functions come from works of Lee and Jin et al. This team patented concepts of ZFP91-based therapies and published a series of papers providing valuable insight into *ZFP91* role in human biology and cancer pathogenesis [6–9].

ZFP91 expression is positively regulated by agonists of the nuclear factor kappa B (NF-κB) signaling pathway through a promotor sequence located in *ZFP91* gene's 5′ upstream region. On the other hand, *ZFP91* overexpression results in increased NF-κB activity in a dose dependent manner. This effect is also dependent on presence of MAP3K14 protein, known also as NIK (NF-κB-inducing kinase). This is a key kinase in activation of noncanonical (alternative) NF-κB

signaling pathway [7, 10]. It has been shown that ZFP91 protein functions as an atypical E3 ubiquitin-protein ligase. Ubiquitination of NIK by ZFP91 leads to its stabilization and activation of the noncanonical NF-κB pathway and this pathway's target genes expression [7, 8]. What is important, available data imply oncogenic activity of the NIK and its overexpression has been associated with cancer pathogenesis in, for example, melanoma, pancreatic, breast, and lung cancer [11].

Oncogenic properties of *ZFP91* gene are not only limited to NIK stabilization and NF-κB signaling pathway activation. Most recently, it has been shown that *ZFP91* is overexpressed in human colon cancer and promotes this cancer progression. Through interaction with NF-κB/p65, ZFP91 protein upregulates hypoxia inducible factor-1α (HIF-1α). HIF-1α is a subunit of a key transcription factor responsible for cellular response to hypoxia and implicated on many levels in cancer pathogenesis and biology [12, 13]. With regard to prostate cancer, HIF-1α is overexpressed in actively growing prostate tissues: BPH and prostate cancer [14]. Under hypoxic conditions HIF-1α dependent signaling promotes epithelial to mesenchymal transition (EMT) in prostate cancer cells which is proven to play a role in cancer progression and invasiveness [15, 16].

In the current study, the *ZFP91* gene expression was examined in prostate cancer specimens and found to be markedly upregulated. To study further *ZFP91* expression in prostate cancer cells, LNCaP and PC-3 prostate cancer cell lines were transfected with *ZFP91* targeting siRNA. In the result a significant discrepancy between *ZFP91* mRNA level changes and protein levels in these cells was observed. This indicates that ZFP91 protein may be stabilized and accumulated in prostate cancer cells and this effect may be connected with oncogenic properties of *ZFP91*.

2. Materials and Methods

2.1. Prostate Cancer cDNA Samples. Prostate Cancer cDNA Array III (Origene, HPRT103) was utilized providing samples from 48 prostate cancer patients. Description of every sample includes relevant clinical information, full pathology report, and RNA quality data (data available at http://www.origene.com/qPCR/Tissue-qPCR-Arrays.aspx). In 9 cases tissue samples came from parts of prostate without pathological changes and in remaining 39 cases from parts of prostate with cancer. Each sample was evaluated for mRNA expression of *ZFP91* and of three reference genes: tubulin alpha 1b (*TUBA1B*), 5′-aminolevulinate synthase 1 (*ALAS1*), and β2-microglobulin (*B2M*). Out of these three, *TUBA1B* and *ALAS1* were selected using geNorm method as a reference to normalize data. Of note, selected genes were proven to be among the most stable and useful ones for normalization purposes in gene profiling studies of prostate tissues, both malignant and not [17].

2.2. Prostate Cancer Cell Lines. Prostate cancer cell lines, LNCaP and PC-3, were obtained from American Type Culture Collection (ATCC, Manassas, USA) and maintained in RPMI-1640 Medium (LNCaP) or F12K Medium (PC-3). Media were purchased from ATCC and supplemented with 10% fetal bovine serum. The cells were grown in 75 cm^2 flasks at 37°C in a humidified atmosphere of 5% CO_2. The culture medium was changed every 2 days. When cells reached approximately 80% confluence, they were either subcultured or harvested by 0.25% trypsin-EDTA. Harvested cells were frozen in −80°C for further analyses.

2.3. Transfection. LNCaP and PC-3 cells transfection conditions were optimized using siGLO Green Transfection Indicator (Dharmacon, GE Healthcare, Lafayette, USA) and Fluoview FV10i-LIV confocal microscope (Olympus, Melville, USA) for image acquisition. Cells were transfected with *ZFP91* siRNA (ON-TARGET*plus* SMARTpool, Dharmacon) or negative control siRNA (ON-TARGETplus Nontargeting control Pool, Dharmacon) or left untreated. DharmaFECT Transfection Reagents 2 and 3 (Dharmacon) were used as transfection agents. The procedure was performed on logarithmically growing LNCaP and PC-3 cells according to manufacturer's recommendations with several modifications of the procedure tested. Viability of cells was determined by microscopic evaluation and trypan blue exclusion test.

2.4. Total RNA and Protein Extraction. Total RNA and protein were extracted by means of NucleoSpin RNA/Protein and NucleoSpin RNA Clean-Up XS (Macherey-Nagel Ltd., Oensingen, Switzerland). RNA concentration and purity were determined spectrophotometrically (NanoDrop, Thermo Scientific, Waltham, USA). For each sample 1 μg of total RNA was reversely transcribed using MMLV reverse transcriptase kit (Novozym, Poznan, Poland) with Oligo dT (PE Biosystems, Warrington, UK) as primers. The reaction was performed at 42.8°C for 60 min (UNO II thermocycler, Biometra, Goettingen, Germany).

2.5. QPCR Analysis. Analyses were performed as described earlier [2, 18, 19]. Briefly, primers were designed by Primer 3 software (Whitehead Institute for Biomedical Research, Cambridge, USA) and purchased from the Laboratory of DNA Sequencing and Oligonucleotide Synthesis (Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland). Primers sequences are listed in Table 1. Real-time PCR was carried out in a LightCycler 2.0 thermocycler (Roche Diagnostics, Basel, Switzerland) with software version 4.05. SYBR Green detection system was used based on LightCycler FastStart DNA Master SYBR Green I mix (Roche). PCR reactions were carried out in 20 μL mixtures, containing 4 μL template cDNA, 0.2 μM of each gene specific primer, and 3.5 mM of Mg2+ ions. The real-time PCR program included a 10 min denaturation step to activate the Taq DNA polymerase, followed by a three-step amplification program: denaturation at 95.0°C for 9 s, annealing at 58.0°C for 5 s, and extension at 72.0°C for 5 s. Specificity of the reaction products was routinely checked by determination of melting points (0.1°C/s transition rate) and random sample separation in a 2.5% ethidium bromide/agarose gel (Figure 1). PCR efficiency was assessed by a serial dilution method. Briefly, products of PCR reactions were separated in a 2.5% agarose gel and specific bands were extracted using a DNA

Table 1: Oligonucleotide sequences of sense (S) and antisense (A) primers are shown for ZFP91 zinc finger protein (ZFP91), two primer pairs, tubulin alpha 1b (TUBA1B), 5′-aminolevulinate synthase 1 (ALAS1), and β2-microglobulin (B2M).

cDNA	GenBank accession number	Primer	Primer sequence (5′-3′)	Position	PCR product size (bp)
ZFP91	NM_053023	S	TGTCCTTGCCCATCCTCGCTA	1128–1148	190
		A	ACTCTTGAAGGCCCGAGCAC	1298–1317	
		S	GAAACCCCAAAGCCACGGAG	892–911	227
		A	CCTTCCATCTCACAACGGACA	1098–1118	
TUBA1B	NM_006082	S	TGGAACCCACAGTCATTGATGA	430–451	135
		A	TGATCTCCTTGCCAATGGTGTA	543–564	
ALAS1	NM_000688	S	AGACATAACATCTACGTGCAA	2031–2051	167
		A	GAATGAGGCTTCAGTTCCA	2179–2197	
B2M	NM_004048	S	CAGCCCAAGATAGTTAAGTG	385–404	262
		A	CCCTCCTAGAGCTACCTGT	628–646	

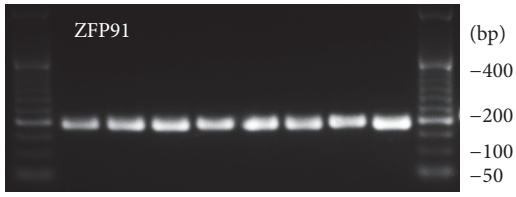

Figure 1: Ethidium bromide-stained 2.5% agarose gel showing random samples out of prostate cancer cDNA Array amplified with *ZFP91* primers. Note presence of reaction products with the expected size of 190 bp. As a DNA size standard O'RangeRuler 50 bp DNA Ladder (Fermentas) was used.

gel extraction kit (Millipore, Billerica, USA). The amount of extracted DNA was estimated spectrophotometrically. Extracted DNA was diluted (10-fold serial dilutions) in order to generate a standard curve for efficiency calculation (LightCycler software version 4.05.).

2.6. Western Blot Analysis. For each sample, 20 μg of protein was separated in a 4–20% gradient SDS-polyacrylamide electrophoretic gel and transferred onto a PVDF membrane. Transferred proteins were stained with Ponceau S. Membranes were incubated in a blocking buffer consisting of 5% nonfat dry milk in TBST for 1 h, followed by primary antibody incubation overnight at 4°C with rabbit anti-ZFP91 at 1 : 200 (sc-102172; Santa Cruz Biotechnology, Dallas, USA) or (to further validate the results) with rabbit anti-ZFP91 at 1 : 200 (ab30970; Abcam, Cambridge, UK) and with rabbit anti-β-Actin at 1 : 2000 (#5142; Cell Signaling Technology, Danvers, USA). Afterwards, membranes were thoroughly washed and incubated with an anti-rabbit HRP-linked antibody at 1 : 2000 (#7074; Cell Signaling Technology) for 1 h at room temperature. After washing, membranes were incubated with enhanced chemiluminescence (ECL Plus, Amersham, GE Healthcare) detection reagents (5 min, room temperature) and visualized on GelDoc-It Imaging System (UVP, Upland, USA) with use of VisionWorks LS Software. ECL DualVue Western Blotting Markers (GE Healthcare) served as a protein size standard. Band detection and quantification of band intensity were performed using TotalLab (Nonlinear dynamics, Newcastle upon Tyne, England).

2.7. Statistical Analysis. GraphPad Prism version 5.00 (GraphPad Software, San Diego, USA) was used to perform statistical analyses. Differences were considered significant at $p < 0.05$. For a more detailed description of particular experiment statistics, see description below each figure.

3. Results

3.1. ZFP91 mRNA Expression in Prostate Cancer Specimens and Its Correlation with Gleason Score. Samples with cDNA from 48 patients with pathologically confirmed prostate cancer were examined. Samples came from tissue fragments evaluated microscopically as normal prostate or prostate cancer. Using QPCR method *ZFP91* expression was analyzed in above-mentioned samples finding its significant overexpression in prostate cancer (Figure 2(a)). In some cases the difference was over 10-fold. On the other hand, the range of *ZFP91* expression in cancer samples was very wide and specimens with similar expression as in control group were also present.

In subsequent analysis, samples taken from prostate cancer fragments were divided according to the Gleason score (Figure 2(b)). No significant differences in *ZFP91* expression were noted between tissues with Gleason scores 7, 8, and 9. Samples with Gleason score 6 prostate cancer had *ZFP91* expression similar to control group, that is, significantly lower. However, this interesting result requires further studies due to limited number of samples in Gleason score 6 group.

3.2. ZFP91 siRNA Mediated Knockdown in LNCaP and PC-3 Prostate Cancer Cells. In order to optimize conditions for *ZFP91* gene knockdown in prostate cancer cells, LNCaP and PC-3 cell lines were firstly transfected with fluorescent indicator of transfection efficiency (siGLO Green Transfection Indicator, Dharmacon). After a series of experiments a protocol was established allowing successfully transfecting almost all

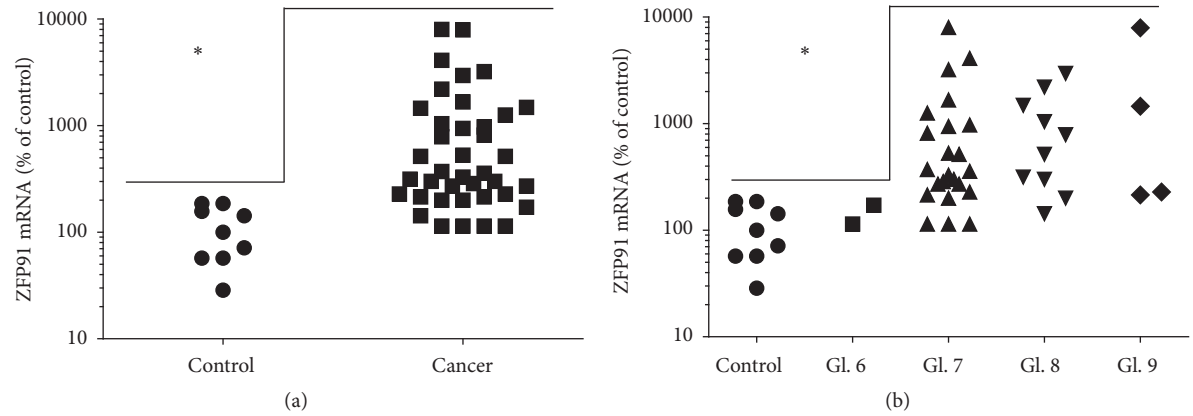

Figure 2: QPCR analysis of *ZFP91* gene expression in normal prostates (control $n = 9$) compared to prostate cancer specimens (Cancer $n = 39$) (a) and compared to prostate cancer specimens grouped according to Gleason score (Gl. 6 $n = 2$, Gl. 7 $n = 23$, Gl. 8 $n = 10$, and Gl. 9 $n = 4$) (b). Results are presented as a scatter plot and median expression in control group was assigned a value of 100. Statistical comparison by Mann-Whitney test (a) and by Kruskal-Wallis test followed by Dunn's test (b); $^{*}P < 0.05$.

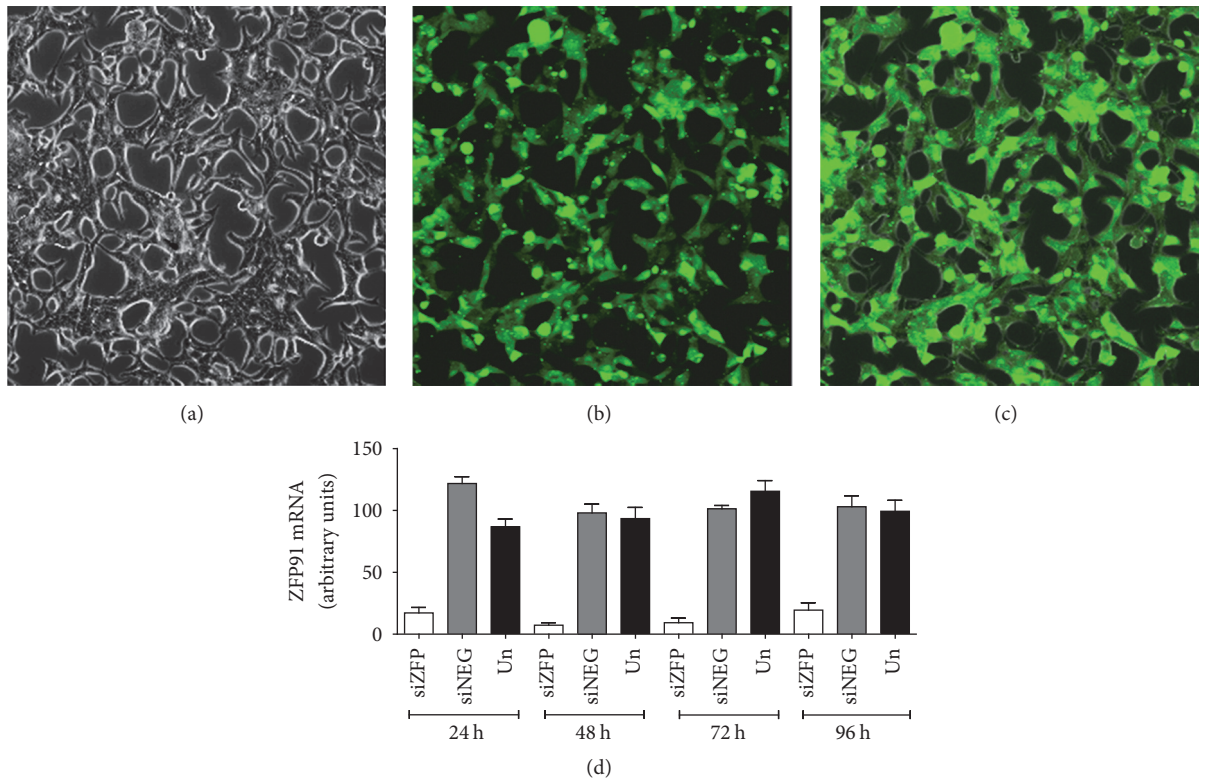

Figure 3: Transfection of LNCaP cell line with *ZFP91* siRNA. Top. cells transfected with siGLO Green Transfection Indicator showing good transfection efficiency. Representative cell images under bright field (a), fluorescence field with a FITC filter (b), and superimposed images (c). (d) QPCR analysis of *ZFP91* gene RNA interference. Cells transfected with *ZFP91* siRNA pool (siZFP), nontargeting siRNA control pool (siNEG), and untreated (Un). *ZFP91* mRNA levels at different time points were examined. Bars represent mean *ZFP91* expression ± SE; median value in untreated group was assigned a value of 100. Each experiment was performed in triplicate.

cells in a culture (a representative image for LNCaP cells is shown in Figure 3). In a next step, using a pool of predesigned *ZFP91* siRNA (ON-TARGET*plus* SMARTpool, Dharmacon) transfection conditions were further optimized in order to achieve maximal *ZFP91* knockdown while maintaining unaffected cell viability. *ZFP91* mRNA downregulation to the levels between 5 and 20 percent of control groups was achieved in LNCaP cells (Figure 3). Similar results were

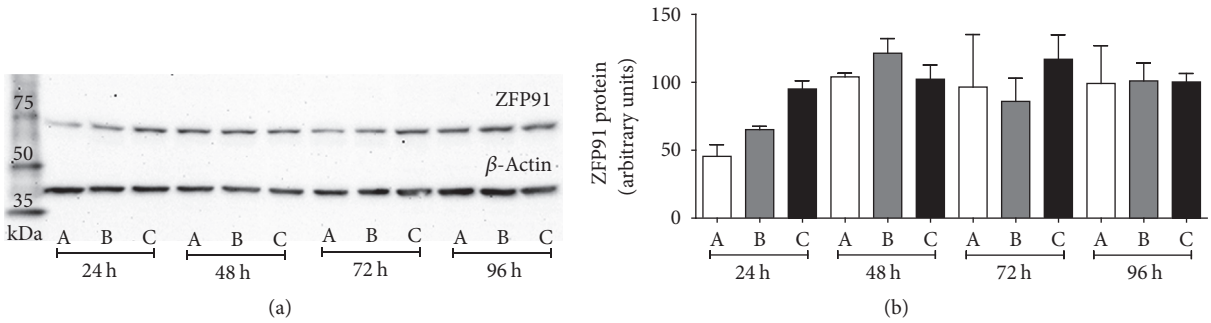

Figure 4: Representative experiment of ZFP91 protein immunoblotting in LNCaP cells transfected with *ZFP91* siRNA pool (A), nontargeting siRNA control pool (B), and untreated (C) at indicated time points. Bars represent protein expression relative to β-Actin levels. Median expression in untreated group was assigned a value of 100. Results are presented as means ± SE; each experiment was at least duplicated.

obtained in PC-3 cells (data not shown). What is important, the silencing effect of RNA interference was stable over at least 4 days of cell culture (Figure 3).

3.3. Discrepancy between ZFP91 mRNA and Protein Levels. Protein levels of ZFP91 were examined in prostate cancer cells treated with *ZFP91* siRNA. Despite marked *ZFP91* mRNA downregulation no effect on the protein level was observed. Figure 4 shows representative results of ZFP91 immunoblotting in LNCaP cells. Similar results were obtained for PC-3 cells (data not shown). What is important, this discrepancy between mRNA and protein levels was maintained over time of the cell culture. This finding was further validated by a set of experiments using, among others, different culture and transfection conditions and different ZFP91 primers and antibodies.

4. Discussion

The results of the present study show significantly elevated expression of *ZFP91* mRNA in prostate cancer samples compared with normal prostate, in some cases over 10-fold. These results are in accordance with an observation of Lee et al. of increased *ZFP91* mRNA staining in prostate cancer specimens using in situ hybridization method [6]. What is important, the hereby presented results come from a significant number of samples: 48, with full pathology reports available. As found in our previous work, similar *ZFP91* mRNA overexpression was not noted in prostate cancer cell lines, despite upregulated ZFP91 protein levels in these cells. This difference, although surprising, could be explained by genome differences between cancer cell lines and cancer specimens [20–23].

Analysis of *ZFP91* gene expression in prostate cancer samples subdivided according to Gleason score did not reveal significant differences between Gleason scores 7, 8, and 9. Lower *ZFP91* expression in samples with Gleason score 6 is an interesting result as it represents a low-risk prostate cancer and efforts are still being made to distinguish low- and high-risk disease [24]. However, the sample number in Gleason score 6 group is insufficient to draw any conclusions and this issue requires further study. Overall, no evidence suggests that observed *ZFP91* overexpression in prostate cancer is correlated with Gleason score or disease staging (data not shown). In this regard, it would be of interest to study potential differences between patients with high *ZFP91* expression in cancer samples and those with its expression unchanged. In the field of prostate cancer studies, genes whose expression is an independent risk factor of the disease course have been discovered [25]. Whether *ZFP91* gene expression could be such a factor remains to be answered. It is possible that it only accompanies other cellular processes (e.g., NF-κB pathway activation) and as such does not represent an independent risk factor. Abundance and complexity of factors influencing NF-κB pathway activation cause problems in selecting key compounds in the studied processes [26]. It would be important to test significance of the observed changes at the protein level, especially as marked discrepancies between mRNA and protein levels of *ZFP91* have been described. Marked overexpression of *ZFP91* in many samples of prostate cancer remains unclear and requires further investigation.

Studies on *ZFP91* gene's potential role in prostate cancer pathogenesis seem important not only due to its oncogenic properties. In recent years one of the thoroughly investigated molecular targets in prostate cancer therapy is factors involved in the NF-κB pathway activation. Its role as a key regulator of immune processes is well documented; still an increasing line of evidence implies its influence on cancer initiation and propagation. With regard to prostate cancer biology, particularly underlined are multidimensional relations between NF-κB pathway activation and activation of signaling pathways dependent on androgen receptor [27]. Interaction between androgen receptor and p52 factor (engaged in noncanonical NF-κB pathway activation) promotes prostate cancer cell growth, protects from apoptotic cell death, and influences expression of androgen receptor dependent genes [28–31]. Taking this into consideration, *ZFP91* may potentially influence androgen receptor signaling in prostate cancer cells and therefore affect the biology of prostate cancer.

With regard to NF-κB pathway function in prostate cancer cells, the influence of the canonical NF-κB pathway activation agonists such as TNF and IL-1β is relatively

well characterized. Less is known in this field regarding its noncanonical pathway. Functions of this additional and more specific NF-κB activation pathway are not thoroughly studied; however available data suggest its involvement in prostate cancer biology [10, 27]. In vitro stimulation of cancer cells with TNF and LIGHT (peptide activating both canonical and alternative NF-κB signaling pathway) stimulates *ZFP91* expression (in MCF-7 cells for TNF and HeLa cells for LIGHT) in a dose dependant manner [6, 8]. TNF influence on growth and function of prostate cancer cells in vitro has been well documented and, among other things, it is proven to induce apoptosis in LNCaP cells [32]. As for LIGHT, it enhances the proliferation rate of LNCaP cells [33]. Another agonist of both NF-κB signaling pathways, RANKL, stimulates migration of PC-3 cells and expression of genes associated with metastatic potential in these cells [34]. ZFP91 protein as a factor specifically regulating NF-κB noncanonical pathway activation [7, 8] may play an important role in above-mentioned relationships.

The present study is the first to our knowledge that directly tests the inhibition of *ZFP91* expression in prostate cancer cell lines using RNA interference. In the work of Unoki et al. *ZFP91* antisense oligonucleotides induced cell growth arrest and apoptosis in endometrial and colon cancer cell lines [4]. Lee and Jin et al. found that siRNA knockdown of *ZFP91* inhibited activity of HIF-1α and its dependent genes in colon cancer cell lines and induced apoptosis in breast cancer and stomach cancer cell lines [6, 9]. In our hands successful *ZFP91* mRNA knockdown was established in both LNCaP and PC-3 prostate cancer cell lines. On a protein level, however, no inhibitory effect was noted. Such phenomenon has been described in the literature and stems from only transient character of siRNA induced knockdown in cell culture [35, 36]. In cases of proteins with long turnover rate or proteins accumulated/stabilized in certain cell types siRNA mediated transfection does not result in phenotypic response. As found in our previous study, both LNCaP and PC-3 cell lines have elevated ZFP91 protein levels compared to normal prostate epithelial cells [2]. Taking this into consideration, together with a fact that ZFP91 protein knockdown was established in other cell lines, it may be hypothesized that in case of prostate cancer cells ZFP91 protein is accumulated or stabilized. Such posttranscriptional regulation of protein abundance has been described for other oncogenic proteins, for example, protein p53, where its accumulation involves enhanced translation of its mRNA and decreased proteolytic degradation [37]. It seems that in case of *ZFP91* expression no direct correlation exists between its mRNA and protein level, as described in our previous report and noted also in works of Lee and Jin et al. [2, 6]. Available data imply that *ZFP91* expression is subjected, at least partially, to a potent posttranscriptional regulatory mechanism. What is important, ZFP91 relative abundance in prostate cancer cells may play a role in this cancer biology.

A recent interesting study of Huang et al. presented effects of RNA-mediated knockdown of *ZFP91* pseudogene on biology of pancreatic cancer cells [38]. Such a silencing resulted in decreased pancreatic cancer cells proliferation rate, inhibition of their migratory ability, and a reversal, at least partial, of the epithelial to mesenchymal transition process in these cells. Long noncoding RNA (lncRNA) expressed by *ZFP91* pseudogene would be therefore another one in an emerging group of pseudogene lncRNAs involved in cellular development and cancer pathogenesis. Although no direct relation with *ZFP91* gene expression or function could be assigned to this, it is a relevant information as lncRNAs are capable of acting, for example, through generation of endogenous siRNAs and influencing mRNAs in the cell [39]. Whether results observed by Huang et al. are somewhat connected with interference in *ZFP91* expression requires further study.

In conclusion, recently discovered oncogenic properties of *ZFP91* should draw more attention to this subject and propel research regarding expression patterns and functions of this little-studied gene. The results presented in this study indicate that *ZFP91* is overexpressed in prostate cancer. A siRNA mediated knockdown of the *ZFP91* gene revealed a potential accumulation of the ZFP91 protein in prostate cancer cells. Taking into consideration known ZFP91 functions, this could play a role in NF-κB and HIF-1α signaling in prostate cancer. In order to explore the significance of *ZFP91* in prostate cancer biology further studies are definitely needed.

Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Diet, Lifestyles, Family History, and Prostate Cancer Incidence in an East Algerian Patient Group

Somia Lassed,[1,2] Cláudia M. Deus,[3] Nuno Lourenço,[4] Abderrezak Dahdouh,[5] Albert A. Rizvanov,[6] Paulo J. Oliveira,[3] and Djamila Zama[1]

[1] *Unité de recherche Valorisation des Ressources Naturelles, Molécules Bioactives et Analyses Physicochimiques et Biologiques (VARENBIOMOL), Université des Frères Mentouri, Constantine, 25000 Constantine, Algeria*
[2] *Laboratoire de Physiologie Animale, Département de Biologie Animale, Faculté des Sciences de la Nature et de la Vie, Université des Frères Mentouri, Constantine, 25000 Constantine, Algeria*
[3] *Center for Neuroscience and Cellular Biology (CNC), University of Coimbra, UC Biotech Building, Biocant Park, 3060-197 Cantanhede, Portugal*
[4] *Department of Informatics Engineering, Center for Informatics and Systems of University of Coimbra (CISUC), Polo II-Pinhal de Marrocos, 3030 Coimbra, Portugal*
[5] *Clinic of Urology-Nephrology and Kidney Transplant Daksi, 25000 Constantine, Algeria*
[6] *Institute of Fundamental Medicine and Biology, Kazan Federal University, Kazan, Russia*

Correspondence should be addressed to Djamila Zama; atrouzl@umc.edu.dz

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Prostate cancer (PC) is the fourth most common cancer in men and the sixth leading cause of death in Algeria. To examine the relationship between lifestyle factors, including diet, and family history and PC risk, a case-control study was performed in an eastern Algerian population, comprising 90 patients with histologically confirmed PC and 190 controls. Data collection was carried out through a structured questionnaire and statistical analysis was performed to evaluate the different variables. The data showed that consumption of lamb and beef meat and high intake of animal fat and dairy products increased PC risk. Seven to thirteen vegetables servings per week and fourteen or more servings decreased PC risk by 62% and 96%, respectively. Seven to fourteen fruit servings per week decrease PC risk by 98%. Green tea consumption reduced the risk of PC but the results were statistically borderline. Increased risk was observed for individuals with family history of PC in first and in second degree. A positive strong association was also found for alcohol and smoking intake and a dose-response relationship existed for quantity and history of smoking. This study suggests that dietary habits, lifestyle factors, and family history have influence on the development of PC in Algerian population.

1. Introduction

After lung cancer, prostate cancer (PC) is the most common cancer among men, with nearly a million new cases diagnosed worldwide [1]. PC incidence varies more than 25-fold worldwide; the rates are highest in Australia/New Zealand, Northern America, and Western and Northern Europe (ASR 111.6, 97.2, 94.9 and 85.0 per 100,000, resp.) and relatively high in certain regions such as the Caribbean (79.8), Southern Africa (61.8), and South America (60.1), but it is low in Asian population with estimated rates of 10.5 and 4.5 in Eastern and South-Central Asia [2]. According to GLOBOCAN 2012 database, PC in Algeria was the fourth most common cancer in men with 8.8 cases per 100,000 and the sixth leading cause of death with 4.9 deaths per 100,000. The incidence of PC in Algeria is considered as low when compared with some western countries such as Norway and Sweden (129.7 and 119.0 cases diagnosed and approximately 18 deaths per 100,000, resp.), but it is still high compared to many Asian countries especially China, Korea, and Bhutan (5.3, 3.2, and 1.2 cases diagnosed and 2.5, 1.3, and 0.7 deaths per 100,000, resp.) [2]. Numerous epidemiological studies showed that

the incidence of PC in Asian countries is low compared to the West. However, this incidence rapidly increases in Asian immigrants that have assimilated western diet and way of living, replacing soy, tea, fish, fruits, and vegetables consumption with red meat and fat-rich food [3]. Also, a study conducted in Western Australia described that the risk of PC and in particular aggressive PC increases significantly in about 80% of men who consume a western style diet [4]. The difference in PC rates may result in possible dietary, lifestyle, and environmental links to the development and progression of PC [5, 6]. Many epidemiological and case-control studies disclosed that some dietary factors such as animal fat [7], dairy products [8], and red meat [9] may increase the risk of PC [7–9], whereas intake of vegetables and fruit [10], fish [11], and green tea [12] appears to be potentially protective [10–12]. The aim of this study was to examine the relationship between dietary habits, family history of PC, alcohol, and smoking and PC risk in an East Algerian population.

2. Materials and Methods

2.1. Data Collection. The present study was approved by the Ethics Committee of the EHS Daksi, which certified that the data collection was performed at the Department of Urology and Renal Transplant without any risk for patients. Data were derived from a case-control study of PC, performed between 2011 and 2013 in the east of Algeria. From 100 cases of histologically confirmed PC, followed at the service of urology and at the emergency department in Clinic of Urology-Nephrology and Kidney Transplant Daksi, Constantine, one patient refused to participate and 9 were too ill to be interviewed. The cohort investigated consisted of 90 patients with a median age of 68.87 ± 0.73, ranging from 50 to 88 years. The total Gleason score, serum PSA level at diagnosis, and primary treatment taken for each case were obtained from the medical record. Controls were men, residing in the same geographical area of cases without either PC or prostate diseases. Those with other malignancies were also excluded. Among 200 men selected, 10 refused to participate for different reasons. The final control group consisted of 190 men with a median age of 67.13 ± 0.72, ranging from 50 to 88 years. Sixty-one men were treated at the hospital for some urologic diseases (35 had kidney stone, 25 had urinary infection, and 10 had other diseases), 14 were men from the hospital staff invited to participate, and 115 were men selected in the blood sample collection room of the Laboratory of Biochemistry of Establishment Public Hospital of Chelghoum Laid city.

Data collection was carried out through an interview, using structured questionnaire, including information on sociodemographic characteristics, dietary habits, family history of PC, smoking, and alcohol consumption (see Supplementary Material available online at http://dx.doi.org/10.1155/2016/5730569). All the interviews were filled face-to-face with case and control individuals. Questionnaire on dietary habits included questions about the frequency of intake of different food items during the past years. The first item was about the number of meals per day, followed by questions about the consumption of fish and red meat (lamb and beef), by using the question "how many times per week or month," and consumption of animal fat (from red meat) or dairy products (milk, cheese, and yogurt) by using the expressions "never, small, medium or great part." In the latter, "small or medium part" of dairy products meant one cup of milk or/and some other product per day, and "great part" meant more than 2 cups of milk per day or/and a large amount of other product. Another question was related to total fruit and vegetables consumption by using the question "how many times per week. The last food-related item is regarded as green tea consumption by using the question "how many cups per day." Participants were also asked about alcohol and smoking employing the terms "non-users," "former users," and "current users." Former users were defined as those who had consumed alcoholic beverages or tobacco earlier in their life but not during the last years and current users were considered as those who actually still consume alcoholic beverages or tobacco. The total tobacco consumption was determined using pack-years unity. The original questionnaire is presented as supplementary material. All the participants were asked about their dietary habits 10 years prior to the study because the clinical PC has a long latency phase, during which the disease is histologically present and the remote dietary intake may be more important than recent dietary intake when predicting the risk of PC [13].

2.2. Statistical Analyses. The data from 90 PC cases and 190 controls were analyzed using Graph Pad Prism version 7. Firstly we started by analyzing if the samples follow a normal distribution. We relied on the Shapiro-Wilk test at a significance level of 0.05. The results showed that the data did not follow a normal distribution so all the tests from here on will assume nothing about the distribution of the data; that is, we will rely on nonparametric tests to analyze the data. To check whether there were statistically significant differences between the control and cases groups, we used the Mann–Whitney test for continuous variables, such as PSA values.

The remainder of the variables (different types of food consumption, family history of PC, smoking habits, and alcoholic consumption) were grouped into categories, corresponding to the different possible answers. The individual frequency of the answers was measured and stored in a contingencies table. All tables were analyzed using the chi-square test. To compute the confidence intervals of the odds ratio (OR) we used on the Woolf logit method. p_{trend} values were determined using the chi-square test for trend.

3. Results

The Gleason score at diagnosis was between 5 and 7 in 38% of cases and it was between 8 and 10 in 62% of cases. 12% of cases were oriented toward prostatectomy as initial treatment and 88% were oriented to start hormone therapy. There were no statistical significant differences in age between cases and controls ($p = 0.101$). Moreover, the nonparametric test Kolmogorov-Smirnov showed that the ages of the two groups (control and cases) were taken from the same distribution. The median prostate-specific antigen (PSA) level was higher

Table 1: Prostate cancer cases and controls characterization.

	Cases, n (%)	Controls, n (%)	p value[b]
No. of subjects	90	190	
Mean age ± SD	68.87 ± 0.73[a]	67.13 ± 0.72[a]	0.1012
Mean PSA ± SD (ng/mL)	122 ± 22.04	1.71 ± 0.10	<0.0001****
Gleason score			
5–7	34 (38)	—	—
8–10	56 (62)	—	—
Primary treatment			
Prostatectomy	11 (12)	—	—
Hormone therapy	79 (88)	—	—
City of living			
Constantine	34 (38)	60 (32)	—
Mila	16 (18)	43 (23)	—
Guelma	4 (4)	10 (5)	—
Setif	5 (6)	12 (6)	—
Oum El Bouaghi	10 (11)	15 (8)	—
Jijel	8 (9)	19 (10)	—
Skikda	6 (7)	14 (7)	—
Bejaia	2 (2)	5 (3)	—
Tebassa	3 (3)	8 (4)	—
M'Sila	2 (2)	4 (2)	—

[a]Values are expressed as mean ± SD.
[b]Based on Mann-Whitney; PSA: prostate-specific antigen; **** $p < 0.0001$.

in diseased individuals than in controls ($p < 0.0001$). All the participants were living in the same geographical area distributed by different cities in the east of Algeria (Table 1).

Table 2 shows the odds ratio and the relative risk for the relationship between the intake of different food items and PC. When comparing men who ate more than three meals per day to men who consumed only two to three meals per day, no statistically significant associations were observed between numbers of meals consumed per day and PC risk. Also, no statistical association was found between fish consumption and PC, when comparing men who ate fish more than three times per month to those who had fish in their meals less than 2 times per month. A significant relationship between red meat consumption and PC risk was observed. A strong positive association ($p < 0.0001$) between lamb consumption and PC was found. The odds ratio and the relative risk increased significantly with the increased lamb meat meals consumed per month. Compared to those who never or rarely consumed lamb, they were, respectively, 3.33 (1.74 to 6.37) and 2.46 (1.49 to 4.06), $p = 0.0002$, in those who consumed lamb between one to two times per month and were 4.99 (2.36 to 10.53) and 3.10 (1.84 to 5.24), $p < 0.0001$, in those who consumed lamb three times or more per month. A positive association was also found between beef consumption and PC ($p_{\text{trend}} = 0.013$), but it was only significant in men who ate beef three times or more per month. Concerning animal fat derived from red meat consumption, a strong positive correlation was found ($p_{\text{trend}} < 0.0001$). Increased risk was observed for men with high intake of animal fat compared to those who never eat animal fat or just normally consumed a small part relative to the total of food intake daily [OR 7.38 (95% CI 3.87 to 14.06) and RR 3.02 (95% CI 2.17 to 4.20); $p < 0.0001$]. The dairy product intake was also associated with PC risk. The risk increased significantly in men with high intake of dairy products compared to those who consumed just a small or medium part daily. Inverse strong association was observed between total fruit and total vegetables consumption and PC risk ($p_{\text{trend}} < 0.0001$). The odds ratio and the relative risk decreased significantly with increased fruit and vegetables consumed per week. The results obtained showed that PC is less common in men who consumed vegetables 7 to 13 times per week and much less common for those who intake vegetables 14 or more times per week and fruit 7 to 14 times per week. Results concerning green tea consumption showed that PC risk decreased with the number of cups of green tea consumed per day. However, these results were not significant ($p_{\text{trend}} = 0.07$), and OR and RR were, respectively, 0.64 (95% CI 0.36 to 1.15) and 0.74 (95% CI 0.48 to 1.12), $p = 0.1569$, for those who drank 1 to 3 cups per day, and they were 0.40 (95% CI 0.08 to 1.92) and 0.51 (95% CI 0.14 to 1.82), $p = 0.2025$, for individuals who had consumption of more than 3 cups per day.

The results in Table 3 indicate that a higher proportion of cases had a family history of PC (22%) compared to controls (4%). A statistically significant association between family history of PC in first degree (father or brother) and PC risk was observed. Men with a family history of PC in second degree (grandfather or uncle) were also at higher risk of getting PC. A positive strong correlation was observed between PC incidence and alcohol consumption ($p < 0.0001$). The odds ratio and the relative risk for former users compared to nonusers were, respectively, 5.53 (95% CI 2.68 to 11.42) and 2.51 (95% CI 1.84 to 3.40). Also, smoking status showed a potent positive association between smoking and the risk of getting PC ($p_{\text{trend}} < 0.0001$). The odds ratio and relative risk for former smokers compared to nonsmokers were, respectively, 3.17 (95% CI 1.76 to 5.69) and 2.27 (95% CI 1.47 to 3.50) and, for current smokers, the values were, respectively, 4.05 (95% CI 1.84 to 8.89) and 2.60 (95% CI 1.57 to 4.31). According to total tobacco consumption, we found a dose-response relation to PC risk which was 3.56-fold higher in men who consumed more than forty pack-years (more than 292 kg of tobacco) in their life (Table 3).

4. Discussion

In this study, controls and PC-positive patients were collected in the same period and interviewed face-to-face using a structured questionnaire to decrease the probability of bias. Cases were interviewed only after the confirmation of their disease and controls were selected carefully and matched to disease cases based on age and region of residence. The use of past 10 years' dietary history is justified by the fact that PC has a long latency period (10 to 15 years) [13] and because dietary changes may have occurred after diagnosis of the disease, thus not reflecting the typical diet consumed before the onset of PC. Still, since this is a case-control study we cannot exclude a certain degree of recall bias [14].

Table 2: Association between the amount and kind of different food items and the risk of prostate cancer.

	Cases, *n* (%)	Controls, *n* (%)	Odds ratio[a]	95% CI[a]	Relative risk[a]	95% CI[a]	*p* value[a]
Number of meals/day							
2 to 3 meals	70 (78)	158 (83)	1				
>3 meals	20 (22)	32 (17)	1.41	0.75–2.63	1.25	0.84–1.86	0.3238
Fish, time/month							
0–2	55 (61)	118 (62)	1				
≥3	35 (39)	72 (38)	1.04	0.62–1.74	1.03	0.72–1.45	0.8957
Red meat, time/month							
Lamb							
0	17 (19)	95 (50)	1				
1-2	40 (44)	67 (35)	3.33	1.74–6.37	2.46	1.49–4.06	0.0002***
≥3	25 (28)	28 (15)	4.99	2.36–10.53	3.10	1.84–5.24	<0.0001****
p_{trend}[b]			<0.0001****				
Beef							
0	16 (18)	57 (30)	1				
1-2	39 (43)	81 (43)	1.71	0.87–3.36	1.48	0.89–2.45	0.1394
≥3	35 (39)	52 (27)	2.39	1.19–4.83	1.83	1.11–3.03	0.0170*
p_{trend}[b]			0.013*				
Animal fat							
Never or small part[1]	37 (41)	127 (67)	1				
Medium part[1]	10 (11)	43 (23)	0.79	0.36–1.74	0.83	0.44–1.56	0.7020
Great part[1]	43 (48)	20 (10)	7.38	3.87–14.06	3.02	2.17–4.20	<0.0001****
p_{trend}[b]			<0.0001****				
Dairy product							
Small or medium part[2]	45 (50)	144 (76)	1				
Great part[3]	45 (50)	46 (24)	3.13	1.84–5.31	2.07	1.49–2.88	<0.0001****
Total fruit, time/week							
<2	17 (19)	4 (2)	1				
2–6	59 (66)	34 (18)	0.40	0.12–1.31	0.78	0.60–1.01	0.198
7–14	14 (15)	152 (80)	0.02	0.00–0.07	0.10	0.06–0.17	<0.0001****
p_{trend}[b]			<0.0001****				
Total vegetables, time/week							
<7	18 (20)	7 (4)	1				
7–13	60 (67)	61 (32)	0.38	0.14–0.98	0.68	0.50–0.93	0.048*
≥14	12 (13)	122 (64)	0.03	0.01–0.10	0.12	0.06–0.22	<0.0001****
p_{trend}[b]			<0.0001****				
Green tea consumption, cups/day							
<1	67 (75)	122 (64)	1				
1–3	21 (23)	59 (31)	0.64	0.36–1.15	0.74	0.48–1.12	0.1569
>3	2 (2)	9 (5)	0.40	0.08–1.92	0.51	0.14–1.82	0.2025
p_{trend}[b]			0.07				

95% CI = 95% confidence interval. [a]Determined using the chi-square test. [b]Determined using the chi-square test for trend; * $p < 0.05$, *** $p < 0.005$, and **** $p < 0.0001$.

[1]Relative to the total of daily food intake. [2]One cup of milk or/with some other product (cheese, yogurt) per day. [3]More than 2 cups of milk per day or/with a big quantity of other product (cheese, yogurt).

The number of meals taken per day, which can be an indicator of total energy intake, showed no association with the risk of PC in this study. Theoretically, high energy intake can play a relevant role in several neoplasms since it may stimulate the sympathetic nervous system and basal metabolism leading to increased IGF-1 release which increases cell proliferation automatically through the stimulation of mitosis [15]. Several case-control studies reported an association [16–18] or no association [19, 20] between increased PC risk and high energy intake.

Numerous epidemiological studies examined the relationship between fish consumption and PC. Although many suggested risk reduction associated with high intake, others are contradictory. A follow-up of 47,882 men during 12 years

Table 3: Association between family history of prostate cancer, alcohol consumption, and smoking habits and the risk of prostate cancer.

	Cases, *n* (%)	Controls, *n* (%)	Odds ratio[a]	95% CI[a]	Relative risk[a]	95% CI[a]	*p* value[a]
Family history of prostate cancer							
Absent	70 (78)	182 (96)	1				
In first degree	11 (12)	3 (1)	9.53	2.58–35.20	2.82	2.01–3.96	0.0002***
In second degree	9 (10)	5 (3)	4.68	1.51–14.45	2.31	1.49–3.58	0.0063**
p_{trend}[b]			<0.0001****				
Alcohol status							
Nonusers	64 (71)	177 (93)	1				
Former users	26 (29)	13 (7)	5.53	2.68–11.42	2.51	1.84–3.40	<0.0001****
Smoking status							
Nonsmokers	22 (24)	99 (52)	1				
Former smokers	50 (56)	71 (37)	3.17	1.76–5.69	2.27	1.47–3.50	0.0001***
Current smokers	18 (20)	20 (11)	4.05	1.84–8.89	2.60	1.57–4.31	0.0006***
p_{trend}[b]			<0.0001****				
Total tobacco consumption, unity of pack-years							
0	22 (24)	99 (52)	1				
1–9	9 (10)	20 (11)	2.02	0.81–5.04	1.70	0.88–3.30	0.1326
10–19	15 (17)	27 (14)	2.50	1.14–5.46	1.96	1.12–3.42	0.0311*
20–29	11 (12)	23 (12)	2.15	0.91–5.05	1.78	0.96–3.29	0.0964
30–39	9 (10)	8 (4)	5.06	1.75–14.59	2.91	1.62–5.23	0.0033**
≥40	24 (27)	13 (7)	8.30	3.66–18.83	3.56	2.28–5.57	<0.0001****
p_{trend}[b]			<0.0001****				

95% CI = 95% confidence interval. [a]Determined using the chi-square test. [b]Determined using the chi-square test for trend; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$, and $^{****}p < 0.0001$.

in the Health Professionals Study found that the consumption of more than three times per week of fish was associated with a reduced risk of PC. The strongest association was found for metastatic cancer (RR 0.56; 95% CI 0.37 to 0.86, compared with infrequent consumption, i.e., less than twice per month) [11]. Similarly, five or more servings of fish per week versus less than once was associated with a significant reduction in PC risk [21]. Also, a recent study suggested that prostate tissue ω-3 fatty acids, especially eicosapentaenoic acid (EPA), are protective against PC progression in men with low risk for that disease [22]. However a meta-analysis study provided no strong evidence of a protective association of fish consumption with PC incidence [23]. No association was observed in our study between fish consumption and PC, which may be explained by the low levels of fish consumption, compared to other studies cited [11, 21].

Our study showed a strong association between red meat and high animal fat consumption and PC. Several previous studies examined the relationship between red meat and PC risk with conflicting results. Some studies suggested a positive association [9, 24, 25], whereas others have found no association [26, 27]. Among studies which suggested a positive association, a case-control study found that eating red meat (beef, lamb, or pork) at least 5 times per week increased the risk of PC incidence by 2.5-fold [9], which was confirmed by further studies [24]. The increased risk may stem from triggering of carcinogenesis by high fat content, formation of carcinogenic heterocyclic amines (HCAs) during cooking at high temperature, carcinogenic N-nitroso compounds (NOCs), and the promotion of carcinogenesis by heme iron [25, 26], although infection by tumorigenic pathogens has been increasingly denoted as possible cause [28]. Animal fat was positively associated with PC risk in this study, in line with previous works [7, 29, 30] but not others [31, 32]. The prospective cohort study of Giovannucci et al. concluded that red meat and total fat consumption was directly related to the risk of advanced PC and the association of the latter was primarily due to animal fat. However, fat from dairy products (with the exception of butter) or fish was unrelated to the risk of PC [7]. Also in a prospective study, men in the lower tercile of saturated fat consumption compared to those in the upper tercile had three times the risk of dying from PC [29]. The link between dietary fat content and the development of PC is possibly related to the increase in sex hormone and IGF-1 concentration [33], alteration of cell membrane function, and modulation of metabolic processes [34] or to increased oxygen radicals generation [35].

Total dairy product intake was also positively associated with the risk of PC in this study. The relationship between PC and dairy products showed significant positive association [8, 36, 37], overall null association [38, 39], or an inverse association [40]. In the epidemiologic follow-up study, results were similar to ours, showing that men in the highest tercile for dairy food intake had a relative risk of 2.2 (95% CI 1.2 to 3.9; $p_{trend} = 0.05$) compared with men in the lowest tercile [8]. However, in the majority of other studies the relative risk was lower, 1.63 (95% CI 1.14 to 2.32) and $p_{trend} =$

0.01, in a population based prospective study performed with 43,435 Japanese men [36] and 1.26 (95% CI 1.04 to 1.51) and $p_{\text{trend}} = 0.03$ in a large prospective study from Finland [37]. Several potential mechanisms explain the relationship between dairy product intake and the increased PC risk. First, the high level of calcium or phosphate content in plasma may lower intracellular bioactive metabolite of vitamin D, 1,25 dihydroxycholecalciferol concentration, [41] which is described to have an antiproliferative effect [42]. Second, higher intake of milk and calcium has been associated with increased plasma levels of IGF-1 [43]. Third, estrogen content in milk has been suggested also as possible explanation [44].

Among all food items studied, our study showed that total vegetables and fruit consumption presented a statistically significant protective effect on PC. These results support that high consumption of vegetables and fruit are associated with decreased risk of PC and are in agreement with the findings of a case-control study performed in Montevideo [10]. Nevertheless the finding in this context remains contradictory. Some studies suggested that total fruit or vegetable consumption may not exert a protective role [24, 45]. However, in another study a large fruit intake was associated with increased risk of PC [46].

Green tea polyphenols, especially catechins, inhibit carcinogenesis by different mechanisms [47] and in the majority of epidemiological studies a significant decrease in the risk of PC incidence was observed with increasing intake of green tea. In a case-control study in Southeast China, a consumption of 3 cups of green tea daily lowered the risk of PC [12]. In a large cohort study in Japan, only 5 or more cups per day reduced the risk of advanced PC [48]. Agreeing with some studies [49, 50], our results did not confirm the protective effect of green tea consumption. This can be explained by the fact that green tea is not a popular beverage in Algeria. The studied population reflected the fact that most of the studied individuals rarely or never drink green tea.

The family history of PC was another factor risk in the Algerian population studied. 22% of PC cases had a family history of PC, against only 4% of controls. Family history of PC has been examined in many studies and in different populations, with most studies finding a positive association [51–53]. Steinberg et al. observed increased PC risk among men whose first or second degree relatives also had PC [52]. In a multicenter case-control study performed in the US and Canada in Afro-American, Whites, and Asian-Americans, 13% of cases and 5% of controls reported a father, brother, or son with PC and family history association with a statistically significant 2- to 3-fold increase in risk in each of the three ethnic groups [53]. Also, in a recent study in Brazil, family history was also a PC risk factor [51]. In addition to the positive association found between family history and PC risk, the latter increased with the number of relatives affected [52].

Our results suggest that alcohol intake is associated with increased PC risk. No current users were found in the population studied since cases and control individuals never drink alcohol or just drank a few times during their youth, with the rest (29% cases and 7% controls) being former users. Most studies showed no association between alcohol intake and PC [54–56] with a few studies reporting an inverse association [57, 58]. In a follow-up study of alcohol and PC, a significant inverse association was shown between distant past heavy drinking (25 drinks/week) and PC [58]. Similarly, an inverse association was observed in another study with the total number of drinking years but no association was found with lifetime intake of total and beverage specific ethanol [57]. In these studies, the protective role of alcohol observed has been interpreted by the finding that alcohol may increase metabolic clearance of testosterone [59]. Nevertheless, our study is comparable with many others supporting the hypothesis that alcohol increases PC risk. In a recent case-control study, current alcohol intake was not associated with PC but lifetime intake increased significantly the risk of both aggressive and nonaggressive PC [60] while, in the cohort of US health professionals, men who drank large amounts of alcohol in a short time were also found to be at higher risk of PC [61]. Other studies suggested that heavy alcohol consumers or alcohol abusers have higher PC incidence, with 4 alcoholic drinks per day being associated with about a 21% increased risk of PC [62]. In our study, we did not have enough information from all cases and controls to examine the association between the amount and the period of alcohol intake and PC risk.

In contrast to several studies which did not support that smoking is a factor risk of PC [17, 24, 55], we found out that smoking was associated with increased risk. Our results support the finding of other studies which found a positive association between smoking and PC risk. In the study of Plaskon et al. current smokers were associated with an increased risk in contrast to former smokers, with a dose-response relation noted between number of pack-years smoked and PC risk [63]. In another study, the relative risk for PC was significantly elevated among cigarette smokers but no clear dose-response relation for was observed [64]. Another study found that early (before age 30), late (within recent 10 years), and lifetime cumulative smoking history were unrelated to the risk of total PC. While men who had smoked 15 or more pack-years of cigarettes within the preceding 10 years were at higher risk of distant metastatic and fatal PC, the excess risk among smokers was eliminated after 10 years of quitting [65]. Two pathogenic mechanisms were proposed to link cigarette smoking and increased PC risk. The first one is related to carcinogenic substances found in cigarettes such as cadmium which can indirectly induce PC through the interaction with androgen receptor [66]. A second factor relates to the effect of smoking on sex hormones level. Male smokers are reported to have elevated level of circulating testosterone [67].

5. Conclusion

Our study indicates that dietary habits, family history of PC, alcohol intake, and smoking may influence the development of PC in East Algerian population. Risk factors for PC appeared to be the intake of red meat, high consumption of animal fat and dairy product, family history of PC, alcohol intake, and smoking. A protective effect was found for high intake of fruit and vegetables. Green tea reduced, although

not statistically significant, the risk of PC. However the number of meals intake per day and fish consumption was found to be independent factors for PC.

Competing Interests

The authors declare that they have no conflict of interests.

Authors' Contributions

Somia Lassed performed research study, collected and analyzed the data, and wrote the manuscript. Cláudia M. Deus, Albert A. Rizvanov, Nuno Lourenço, and Paulo J. Oliveira analyzed the data and revised the manuscript. Abderrezak Dahdouh and Djamila Zama designed and supervised the study and revised the manuscript.

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