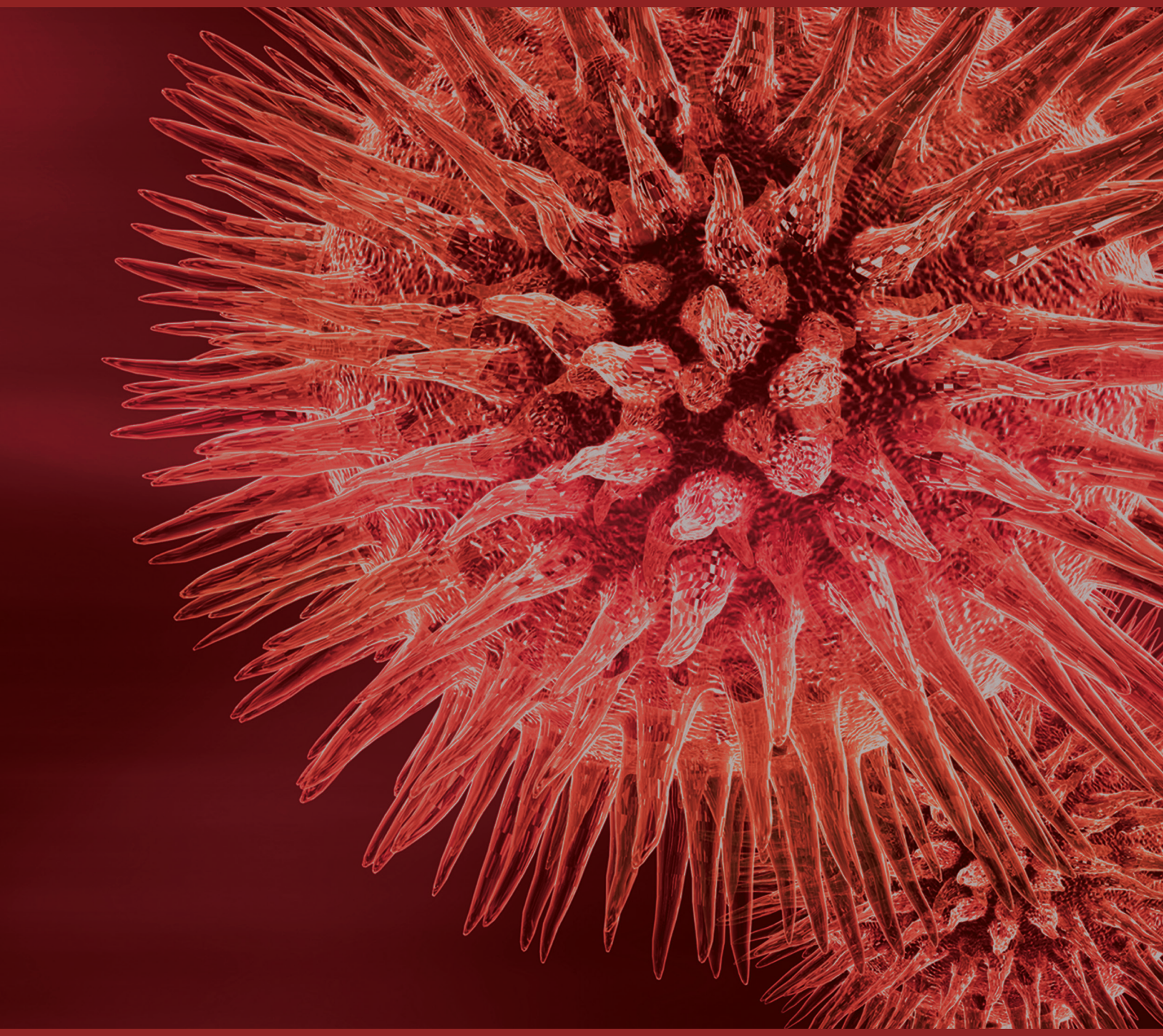
BioMed Research International

Biomarker and Translational Prostate Cancer Research

Guest Editors: Andreas Doll, Jeremy Clark, and Colleen Nelson



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Editorial

Biomarker and Translational Prostate Cancer Research

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The existing clinical biomarkers for prostate cancer (PCa) are not ideal, since they cannot specifically differentiate between those patients who should be treated immediately and those who should avoid overtreatment. Current screening techniques lack specificity, and a decisive diagnosis of PCa is based on prostate biopsy. Although PCa screening is widely utilized nowadays, two-thirds of the biopsies performed are still unnecessary. Thus, the discovery of noninvasive PCa biomarkers remains an urgent unmet medical need. Once metastasized, there is still no curative therapy. A better understanding of sustained androgen receptor signalling in castration resistant prostate cancer (CRPC) has now led to the development of more effective therapies. We need a better understanding of the molecular and cellular aspects of prostate carcinogenesis and progression. Identification of cancer initiating cells and therapies against these populations is a promising way forward to fight this disease.

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The editors thank the authors for their efforts and time spent for each manuscript. The lead editor would like to thank all editors for the time spent in reviewing, assigning reviews, and commenting on submitted manuscripts. The editors hope that this special issue will prove useful to investigators, policy makers, and veterinarians involved in the study of respiratory diseases of small ruminants. At last but not least, the expertise and cooperation of editorial team are highly acknowledged.

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Jeremy Clark
Colleen Nelson

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Research Article

Identification and Validation of Potential New Biomarkers for Prostate Cancer Diagnosis and Prognosis Using 2D-DIGE and MS

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This study was designed to identify and validate potential new biomarkers for prostate cancer and to distinguish patients with and without biochemical relapse. Prostate tissue samples analyzed by 2D-DIGE (two-dimensional difference in gel electrophoresis) and mass spectrometry (MS) revealed downregulation of secernin-1 ($P < 0.044$) in prostate cancer, while vinculin showed significant upregulation ($P < 0.001$). Secernin-1 overexpression in prostate tissue was validated using Western blot and immunohistochemistry while vinculin expression was validated using immunohistochemistry. These findings indicate that secernin-1 and vinculin are potential new tissue biomarkers for prostate cancer diagnosis and prognosis, respectively. For validation, protein levels in urine were also examined by Western blot analysis. Urinary vinculin levels in prostate cancer patients were significantly higher than in urine from nontumor patients ($P = 0.006$). Using multiple reaction monitoring-MS (MRM-MS) analysis, prostatic acid phosphatase (PAP) showed significant higher levels in the urine of prostate cancer patients compared to controls ($P = 0.012$), while galectin-3 showed significant lower levels in the urine of prostate cancer patients with biochemical relapse, compared to those without relapse ($P = 0.017$). Three proteins were successfully differentiated between patients with and without prostate cancer and patients with and without relapse by using MRM. Thus, this technique shows promise for implementation as a noninvasive clinical diagnostic technique.

1. Introduction

Prostate cancer is the most commonly occurring cancer among men in economically developed countries. In 2008, 62 out of 100,000 men were diagnosed with the disease [1]. Worldwide, 248,500 men died of prostate cancer in 2008 [1], although most men diagnosed with prostate cancer die from causes other than prostate cancer [2]. Some prostate cancers are clinically relevant from the start, while others will acquire clinical significance over the years [3, 4]. High-grade prostatic intraepithelial neoplasia often develops into prostate cancer [5–7], although many prostate cancers may remain indolent for 10–15 years or longer [8]. Today, the "gold standard" for the treatment of prostate cancer is prostatectomy, but

approximately one-third of all prostatectomy patients will develop a "biochemical relapse" [9, 10], which is defined as the elevation of prostate specific antigen (PSA). Almost 100% of patients who show a biochemical relapse will later develop a clinical relapse [11], with metastasis ultimately causing death [12, 13].

Today, prostate cancer is most often diagnosed through positive palpatory findings within a digital rectal examination and/or a high PSA value during PSA-screening; although specificity is low [14–16], histopathological findings from punch biopsies are used for verification. These findings together with clinical data are used for prognosis using so called "nomograms" [17], whereas the accuracy is only 70% [18, 19]. Even postoperative nomograms have accuracies of only 75% [18, 19].

PSA is a suitable biomarker to identify recurrent prostate cancer subsequent to treatment. However, PSA remains questionable as a diagnostic and prognostic marker [20–23], because specificity and sensitivity are low for the current diagnostic cutoff levels of 4 ng/mL [24]. Unfortunately, high levels of blood PSA (>4 ng/mL) are not necessarily caused by the presence of prostate cancer [24]. PSA can be elevated due to inflammation, benign prostate hyperplasia (BPH), and/or infections [25–27]. Moreover, 70% of patients with PSA > 4 ng/mL and <10 ng/mL do not actually have prostate cancer, while 5% with PSA < 0.5 ng/mL actually do have prostate cancer [24]. On the other hand, patients who are diagnosed with prostate cancer are often overtreated [28], as many prostate cancers are indolent, and because reliable biomarkers for the aggressive form of the disease are currently not available. Thus, new biomarkers are urgently needed.

Proteomic approaches are very promising for the discovery of new biomarkers (as reviewed in [29]). 2D-DIGE (two-dimensional difference gel electrophoresis) is an accurate method for the relative quantitation of human proteins, as this technique reduces intergel variability and simplifies gel analysis of small sample amounts [30, 31].

Unfortunately, despite intense research, no clinical biomarker panel for recurrent prostate cancer is available yet as most published biomarkers for prostate cancer are limited to the discovery phase, are still waiting for validation, or could not be validated in independent studies [32]. A huge problem is the availability of prostate cancer patients' tissue. Many prostate cancer biomarker studies used suboptimal sample sets where samples in the study groups were not matched to age, stage, or grade, tissues were not dissected into tumor and tumor-free tissue, or there were not enough followup data available. As an example Pang et al. analyzed lymph node metastatic prostate cancer and benign prostate cancer tissue using 2D-DIGE and MALDI-TOF/TOF-MS to identify potential new biomarker candidates for lymph node metastatic prostate cancer [33]. Unfortunately, the sample sets were not matched with regard to patients' age, tumor stage, and tumor grade. Other studies are working with tissue samples of patients, which already have metastasis at the time of biopsy [34, 35]. Unfortunately, comparison of those retrospective samples does not forcibly lead to biomarkers which are useful to stratify patients without recurrence at the time of diagnosis. Further limitations of publicized studies are the use of a 2D-DIGE minimal labeling system (e.g., [33, 36]), which is not suitable for the detection of proteins with low abundance. Therefore, in the present study, a 2D-DIGE saturation labeling system was used, allowing labeling of 1.000–5.000 cells [37, 38] or 0,5 fmol protein [39] whereby this sensitivity could not be reached by other techniques so far [40].

Multiple reaction monitoring (MRM) is a mass spectrometry technique that provides accurate absolute quantitation of selected proteotryptic peptides [41]. For the most accurate quantitation, a synthetic stable isotope-labelled (SIS) peptide at a known concentration is spiked into the sample. Quantitation of the natural peptide takes place through comparison of the peaks from the natural and the chemically identical SIS-peptides. MRM has been shown to fulfill the requirements needed for the verification of biomarker candidates, as it has the capability to quantify proteins consistently, simultaneously, accurately, and reproducibly in complex samples [41]. Compared to ELISA, lead time is shorter and costs are reduced [41]. As an example, Percy et al. and Domanski et al. have developed multiplexed MRM-based assays for the quantitation of cardiovascular disease biomarkers and cancer biomarkers in human plasma [42, 43]. Until now, these assays are developed to fulfill the requirements for preclinical application for evaluating potential useful biomarkers [42]. But hopefully, MRM-based methods for quantitation of cancer-related protein biomarkers will soon be approved by the US FDA [42], moving this technique one step closer to clinical application [44].

In the present study, patients sample sets for both analyzed patient groups (patients with biochemical relapse versus patients without biochemical relapse) were matched with regard to age, tumor stage, and tumor grade as far as possible. Additionally, manual microdissection of the tissue ensures that the percentage of tumor glands in the analyzed tissue were >80%.

Potential new prostate cancer biomarkers were found in a 2D-DIGE study of prostate cancer tissues from patients with and without relapse, with tumor-free tissue samples as controls. The deregulated proteins were identified using mass spectrometry (MS). Ingenuity pathway analyses were accomplished in order to perform functional analysis of the identified proteins. Promising potential biomarker candidates were chosen for further validation with immunohistochemical staining of an independent tissue microarray, Western blots of tissue and urine proteins, and MRM-MS analysis of patients' urine. The detailed study design is shown in Figure 1.

2. Material and Methods

2.1. Analysis of Tissue Samples

2.1.1. Clinical Specimens. Twelve cancer samples from prostatectomy specimens without relapse, 11 cancer samples with relapse, and 14 tumor free prostate samples corresponding to the tumor samples were analyzed with 2D-DIGE. The same samples were used for Western blot analysis. Where possible, matched patient samples with respect to age, tumor

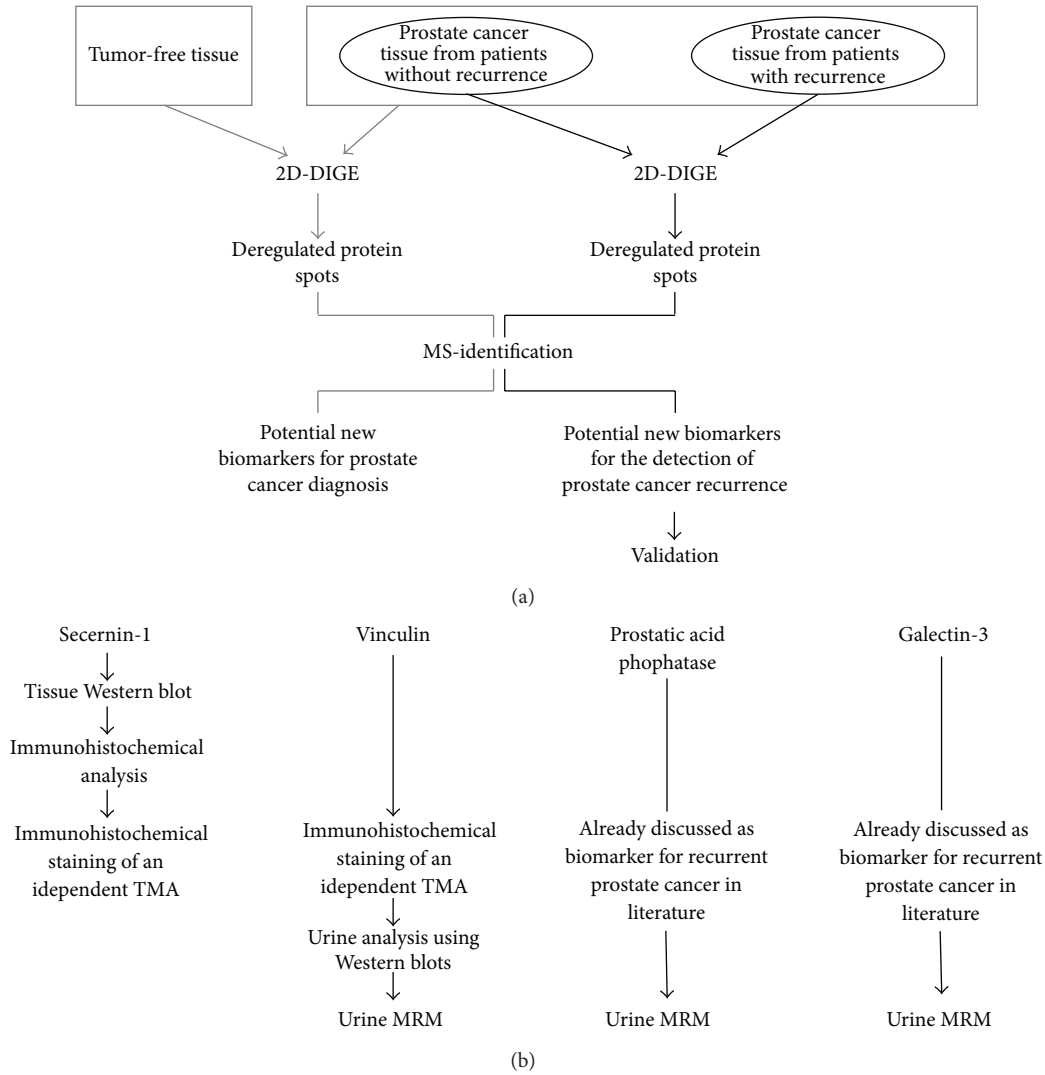


Figure 1: Study design and workflow of prostate cancer biomarker candidate identification (a) and validation (b). (a) Prostate cancer tissue from patients with and without recurrence as well as tumor-free tissue was analyzed using two-dimensional differences in gel electrophoresis (2D-DIGE) and mass spectrometry (MS). (b) Identified potential new biomarker candidates were validated using Western blots, immunohistochemistry, tissue microarrays (TMA), and multiple reaction monitoring (MRM).

grade, and Gleason score were used for both tumor patient groups (with *versus* without relapse). Only patients without hormonal therapy prior to prostatectomy were included in the study. Samples were obtained from patients treated at the Departments of Urology at the University Hospitals Dresden and Aachen between 1998 and 2010. The study was approved by the local ethics committee (ethics approval Aachen: EK 206/09 and ethics approval Dresden: EK194092004 and EK195092004). Written informed consent was obtained for all specimens. Samples were snap-frozen in liquid nitrogen, and the classification of tumors was done by pathologists in accordance with the UICC TNM System [45].

For details see Table 1. Due to sample limitations, Western blot validation could not always be performed with the identical sample set. Details are listed in Supplementary Table 2 available online at http://dx.doi.org/10.1155/2014/454256.

Validation of potential prostate cancer biomarker candidates by immunohistochemical analysis was done with samples obtained from the Department of Urology in Dresden. Samples were formalin-fixed and paraffin-embedded at the Department of Urology in Dresden. Detailed patient information is listed in Supplementary Table 3.

For validation of an independent sample set, tissue microarrays (TMA) were obtained from the Institute of Pathology, University Hospital Bonn. The study was approved by the Institutional Review Board (IRB) at the University Hospital Bonn, and the IRB waived the need for written informed consent of the participants. Patients underwent surgery between 2004 and 2007 at the University Hospital Bonn and TMA preparation was done as previously described [46, 47]. Detailed patient information is listed in Supplementary Table 4 and Supplementary Table 5.

Table 1: Sample sets used in the experiments.

Experiment	Sample set			
	Frozen tissue (obtained from University Hospitals Dresden and Aachen)	FFPE tissue (obtained from University Hospital Dresden)	TMA (obtained from University Hospital Bonn)	Urine samples (obtained from University Hospital Aachen)
IDENTIFICATON				
2D-DIGE and MS-analysis	X			
VALIDATION (TISSUE)				
Western blot secernin-1	X			
IH secernin-1		X		
TMA secernin-1			X	
TMA vinculin			X	
VALIDATION (URINE)				
Western blot vinculin				X
MRM vinculin				X
MRM PAP				X
MRM galectin-3				X

Samples for Western blot analysis of vinculin in urine were obtained from the University Hospital Aachen. Detailed patient information is listed in Supplementary Table 6.

For the MRM-MS analysis, urine samples from the University Hospital Aachen were used (ethics approval Aachen: EK 206/09). Urine samples were obtained between 2005 and 2010 from patients treated at the Department of Urology, University Hospital Aachen. Samples were snap-frozen in liquid nitrogen and stored at −80°C at the Institute of Pathology, University Hospital Aachen, until use. Only samples from patients without neoadjuvant therapy were included in this study. Detailed information is listed in Supplementary Table 7.

Detailed information which sample set was used for which experiment is listed in Table 1.

2.1.2. Manual Microdissection and Tissue Preparation for 2D-DIGE and Western Blot Analysis. All tissue samples were stored at −80°C prior to protein isolation. Proteins for 2D-DIGE analysis were isolated from 4 mm^2 of a 14 μm thin cryoconserved section with a minimum of 80% of prostatic glands. *TissueTec* from the embedding and freezing process was removed using 70% ethanol. The sample sections were stained in a series of ultrapure water, haematoxylin, ultrapure water, and 70% ethanol. All liquids were used with *Complete Protease Inhibitor Cocktail Tablets* (Roche, Mannheim, Germany). The areas of interest were marked using the PALM Axiovert 200M (Carl Zeiss Microscopy, Göttingen, Germany) laser and were manually microdissected. Proteins were dissolved in 10 μL lysis buffer (30 mM Tris-HCl, 2 M thiourea, 7 M urea, 4% (w/v) CHAPS; pH 8.0). The extracts were sonicated on ice and centrifuged at 4°C for 15 min and 16,000 ×g. Supernatants were stored at −80°C.

2.1.3. Protein Labeling and Two-Dimensional Difference in Gel Electrophoresis (2D-DIGE). Protein lysates were labeled with 2 mM Cy5 dye using the GE CyDye DIGE Fluor Labeling Kit (GE Healthcare, UK) according to the manufacturer's instructions. As an internal standard, proteins from all patient samples were pooled and 5 μg were labeled with 2 mM Cy3 dye. Labeled samples were combined. Rehydration buffer (7 mM urea, 2 M thiourea, 2% (w/v) CHAPS, 1% DTT, 1% IPG buffer pH 3-11 NL (GE Healthcare, UK), 0.002% bromphenol blue) was added to give a total volume of 450 μL. Rehydration, isoelectric focusing and gel electrophoresis were performed as described by Labbus et al. [48].

2.1.4. Gel Image Analysis. 2D-DIGE gels were visualized using a Typhoon 9410 fluorescence scanner (GE Healthcare) with excitation/emission at 554/575 nm (Cy3) and 648/663 nm (Cy5). Scanning resolution was 100 microns and the photomultiplier tube was set to 550 V. Gel image and statistical analyses were done using the Delta2D 4.0 Software (Decodon, Greifswald, Germany). The Delta2D data set was first normalized by dividing each spot volume by the sum of all spot volumes on the respective gel image. By opening the analysis tool of Delta2D logarithmic function is performed automatically; furthermore data is standardized (resulting in means of zero and standard deviations of one). The recommended workflow includes fusing all images and detecting the spots on the resulting fused image, which contains all spots of the original images. The spot pattern is then transferred to all original images. Therefore, in this approach, no missing values appear. Additionally detailed Delta2D workflow information is described by Berth et al. [49]. Spots showing a quantitative difference of a ≥1.5-fold change between nontumor and tumor groups and between the two tumor groups (i.e., with or *versus* without a relapse), respectively, were included in further analyses. Additionally, either a Student's t-test (as a parametric test) or a Mann-Whitney U-test (as a nonparametric test) with a P value of <0.05 was accepted as statistical relevant. The U-test and t-test were used because of uncertainty concerning presence of normal distribution (t-test [50]; U-test [51, 52]).

2.1.5. Protein Identification Using MALDI-TOF MS/MS and LC-MS/MS. Trypsin digestion and protein identification using MALDI-TOF MS and MS/MS were done as previously described [48]. All 2D-DIGE protein spots that were not identified using MALDI-TOF MS were further analyzed using LC-MS/MS as follows. Trypsin-digested proteins were extracted from the gel spot using 10–20 μL extraction solution (0.1% TFA/Acetonitrile 1 : 1) and sonicated on ice for 15 min. The supernatant containing the extracted peptides was transferred to a new glass tube. For a second extraction step, the gel spot was once more incubated and sonicated with 10–20 μL extraction solution for 15 min. The supernatants were combined and remaining acetonitrile was removed in a vacuum Speedvac concentrator 5301 (Eppendorf, Germany). Peptides were diluted with 0.1% TFA to a final volume of 17 μL. Peptide concentration was determined by amino acid analysis as described elsewhere [53]. Mass spectrometric analysis was done using a LTQ Orbitrap Velos (Thermo Scientific, San Jose, USA) online, coupled to an Ultimate 3000 RSLCnano system (Dionex, Idstein, Germany). Samples were preconcentrated on a trap column (Acclaim PepMap 100, 300 μm × 5 mm, C18, 5 μm, and 100 Å) and separated on an Acclaim PepMap 100, 75 μm × 25 cm, C18, 3 μm, and 100 Å analytical column. The flow rate was 0.4 μL/min with a linear gradient of 4–35% buffer B (84% acetonitrile and 0.1% formic acid) for 65 min. MS-analyses were done in FT-master scan mode. The collision energy was 35 eV with an activation time of 10 seconds. The intensity counts for MS/MS were set to 500 counts with a dynamic exclusion time of 35 seconds. Five most intense precursor ions were selected for fractionation in a data-dependent acquisition approach (TOP5). Columns were washed after each sample. Protein identification was achieved using Proteome Discoverer 1.3 (Version 1.3.0.399; Thermo Scientific, Bremen) with Mascot database (Version 2.3) as search engine and UniprotKB/Swiss-ProtDatabase (Uniprot/Swissprot-Release 2012_02; 534.695 entries) with the following search criteria: protease trypsin, one missed cleavage, 400–10,000 *m/z*, 1.5 signal-to-noise threshold, mass tolerance of 5 ppm, and a fragment and precursor mass tolerance of 0.4 Da. FDR (false discovery rate) were calculated using the proteome discoverer application's decoy database search feature (Reference: Xcalibur Proteome Discoverer Version 1.1 User Guide XCALI-97276 Revision A October 2009, http://sjsupport.thermofinnigan.com/TechPubs/manuals/Discoverer_UG.pdf), and the FDR was set to a threshold of 0.01. A decoy approach was used for identification. The used protein inference algorithm was used as stated in the Mascot Manual (http://www.matrixscience.com/help/interpretation_help.html#GROUPING): First, Mascot takes the protein with the highest protein score and calls this hit number 1. Then it takes all other proteins that share the same set of peptide matches or a subset and includes these in the same hit. In the report, they are listed as same-set and subset proteins. With these proteins removed from the list, Mascot now takes the remaining protein with the highest score and repeats the process until all the significant peptide matches are accounted for (Mascot Manual, http://www.matrixscience.com/help/interpretation_help.html#GROUPING, paragraph "Protein inference"). Protein identification relied on proteins and unique peptides. For more sensitive analysis, an LTQ Velos Pro (Thermo Scientific, San Jose, USA) was used. The instrument was online coupled to an Ultimate 3000 RSLCnano System (Dionex, Idstein, Germany) equipped with an Acclaim PepMap RSLC, 75 μm × 25 cm, C18, 2 μm, and 100 Å column. All LC and analysis methods remained constant between the two MS platforms.

Ten proteins (Supplementary Table 12 and Supplementary Table 13) were identified using the Maxis 4G (Bruker Daltonik, Bremen, Germany) controlled by Compass 1.3 for micrOTOF-SR1 Software (Bruker Daltonik, Bremen, Germany). The MS instrument was online coupled to an U3000 LC system (Dionex, Idstein, Germany), controlled by *Chromeleon 6.8 SR8*, and equipped with a 25 cm long C18 analytical column (ID 75 μm) heated up to 50°C. Thirty μL of each sample in 0.1% trifluoroacetic acid was injected and analyzed at a flow rate of 350 nL/min with a linear gradient of 5 to 40% acetonitrile achieved through dilution with buffer B (84% acetonitrile and 0.1% formic acid). Capillary voltage was 4800 and flow of dry gas was 4 L/min. Protein identification was performed using ProteinScape 2.0 (Bruker Daltonic, Bremen, Germany) with Mascot (Version 2.3) as search engine and UniprotKB/Swiss-Prot Database (Uniprot/Swissprot-Release 2012_02; 534.695 entries) with the following search criteria: protease trypsin and one missed cleavage, and variable methionine oxidation was allowed. The mass tolerance was 15 ppm for peptides and 0.1 Da for MS/MS identification. FDR and protein inference were calculated as described above.

2.1.6. Ingenuity Pathway Analysis. Ingenuity pathway analysis (IPA, QIAGEN Redwood City, http://www.qiagen.com/ingenuity) was used to determine *Top Diseases*, *Biofunctions*, and *Localization* of the identified proteins. Direct and indirect relationships were included in the analysis. Molecules and relationships were considered as long as the species was human and molecules and the relationships were experimentally observed. The number of molecules for type, localization, molecular, and cellular functions, as well as the role of the identified proteins in development and function of the physiological systems, were counted.

2.1.7. Protein Selection for Further Validation. Based on IPA and—more importantly—on literature review we selected four proteins for further validation using Western blot analysis, immunohistochemical analysis, and/or MRM analysis. For study design, see Figure 1.

2.1.8. Bradford-Assay. Unless otherwise specified, protein concentrations were determined using the Bio-Rad Bradford assay (Bio-Rad Laboratories, Hercules/California, USA). Forty μL of ultrapure water was mixed with 10 μL Bradford reagent and 1 μL protein sample. Forty μL ultrapure water mixed with 10 μL Bradford reagent was used as a blank. Samples were measured with an ELISA Reader Infinite M200 (Tecan, Männedorf, Switzerland) at an extinction of 595 nm. Protein concentrations were determined by comparing the absorption at 595 nm with dilution series consisting of 10 μL Bradford reagent and 1 μg, 2 μg, 3 μg, 4 μg, or 5 μg of bovine

Table 2: Antibodies used for Western blot analysis.

Antibody	Host	Type	Company	Dilution
β-Actin (A5441)	Mouse	Monoclonal	Sigma Aldrich, St. Louis, USA	1 : 500
Secernin-1	Rabbit	Polyclonal	Sigma Aldrich, St. Louis, USA	1 : 500
Vinculin	Mouse	Monoclonal	Fitzgerald, North Acton, USA	1 : 1,000
Anti-mouse + HRP (P0447)	Goat	Polyclonal	DAKO, Hamburg, Germany	1 : 5,000
Anti-rabbit + HRP (P0448)	Goat	Polyclonal	DAKO, Hamburg, Germany	1 : 5,000
Peroxidase anti-Mouse IgG (PI-2000)	Horse	Polyclonal	Vector Laboratories, USA	1 : 10,000
Peroxidase anti-Rabbit IgG (PI-1000)	Goat	Polyclonal	Vector Laboratories, USA	1 : 20,000

Table 3: Antibodies used for immunohistochemistry.

Target protein	Species	Type	Company	Dilution	Incubation time and temperature	Positive control
Secernin-1	Rabbit	Polyclonal	Sigma Aldrich, St. Louis, USA	1 : 1000	1 h, 37°C	Testis
Vinculin	Mouse	Monoclonal	Fitzgerald, North Acton, USA	1 : 1000	Overnight, 4°C	Testis

serum albumin (BSA), respectively, filled up to 40 μL ultrapure water.

2.1.9. Western Blot. Ten μg protein samples were mixed 1 : 4 (v : v) with SDS sample buffer (4% SDS, 0.5 M Tris-HCl pH 6.8, 40% glycerol, 10% β-mercaptoethanol, and 0.002% bromophenol blue). Samples were incubated for 5 min at 95°C and loaded onto a Novex NuPAGE 4–12% Bis-Tris gel (Invitrogen, Carlsbad, CA, USA). After electrophoresis at 130 V until the bromophenol blue front reached the end of the gel, proteins were electrotransferred onto polyvinylidene fluoride membranes (Millipore Corporation, Bedford, MA, USA). Blots were blocked with 10% milk powder in TBS-T (0.5 M NaCl, 1 M tris pH 7.5, 0.5% tween). Antibodies used for immunodetection of desired proteins are listed in Table 2. For visualization, the membrane was incubated with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific, Rockford, IL, USA) and exposed to Amersham Hyperfilm (GE Healthcare). Densitometric analyses of the results were performed using ImageJ 1.45 (Oracle Corporation, National Institute of Health, USA).

2.1.10. Immunohistochemistry. For immunohistochemistry, FFPE tissues were dewaxed 3 times with xylene (15 min each), 2 times with 100% ethanol (10 min each), 2 times with 96% ethanol (5 min each) and 70% ethanol (5 min each), and 3 times with ultrapure water (5 min each). For antigen retrieval, slides were incubated in 20% citrate buffer pH 6.0 for 30 min in a 98°C water bath and then cooled for 30 min and washed 5 times with phosphate buffered saline (PBS). Blocking of endogen peroxidase was done with 3% H_2O_2 for 15 min. Slides were washed 5 times with PBS prior to blocking of unspecific antibody binding with DAKO protein block serum-free (Dako, Hamburg, Germany) for 20 min at 37°C. Primary antibodies were diluted in 1% milk powder in PBS. The incubation conditions are listed in Table 3.

Slides were washed with PBS and incubated at 37°C with DAKO Envision premade biotin-free enhancer solution (for detection of mouse and rabbit primary antibodies) for 3 min and then were washed and incubated in PBS for one hour. A DAKO Liquid DAB Substrate Chromogen System Kit (DAKO) was used for development: 1 mL buffer was mixed with 20 μL DAB. Slides were incubated with this solution for 5 to 10 min and then were washed with ultrapure water and incubated in PBS for 5 min. Incubation in hematoxylin for 10 min was used for counterstaining. Slides were incubated in tap water for 10 min and dehydrated once in 70% ethanol for 1 min, once in 96% ethanol for 1 min, twice in 100% ethanol for 2 min, and twice in xylene for 5 min. The stained tissue samples were mounted with vitroclud (R. Langenbrinck, Germany) and glass cover slides in case the Remmele score were used for scoring of immunohistochemical stainings. Scoring was done as described elsewhere [54] whereby the described scoring of nuclear staining was adapted to the cytoplasmatic staining (adapted Remmele score).

2.2. Analysis of Urine Samples

2.2.1. Preparation of Urine Samples for Vinculin Western Blot Analysis. Urine proteins were precipitated with 10 volumes of ice cold methanol. Samples were incubated for 30 min at −20°C followed by centrifugation at 16,000 ×g at 4°C for 20 min. The supernatant was discarded and the sediment was dried at room temperature. Proteins were suspended in 10 μL lysis buffer (30 mM Tris-HCl, 2 M thiourea, 7 M urea, and 4% (w/v) CHAPS; pH 8.0).

2.2.2. Preparation of Urine Samples for MRM-MS Analysis. A total of 23 urine samples from the University Hospital Aachen were used for MRM analysis. All samples were prepared as follows: 2 mL urine, corresponding to 160 μg of protein, was centrifuged at 3,900 ×g for 30 min at 4°C to remove cells and cell debris. Each supernatant was transferred to a Millipore Amicon Ultra-4 centrifugal filter unit (10,000 MWCO (molecular weight cutoff)) and centrifuged at 3,900 ×g until concentrated 4-fold. The concentrated protein solution was

washed on filter with 4 mL 20% acetonitrile and 25 mM ammonium bicarbonate and centrifuged at 3,900 ×g until the volume was reduced to 500 μL. In a second washing step, the protein solution was diluted with 2 mL of 25 mM ammonium bicarbonate and centrifuged at 3,900 ×g until the volume was reduced to 100 μL. A 18.75 μL sample was removed and denatured with 81.25 μL of 8 M Urea containing 0.1 M ammonium bicarbonate.

Trypsin digestion was based on the protocol by Selevsek et al. [55]. Briefly, 100 μL of the concentrated and denatured protein solution was reduced with 1.2 M DTT (final concentration of 12 mM) for 30 min at 37°C. Proteins were alkylated with 0.5 M iodoacetamide (final concentration of 40 mM) and incubated for 30 min at 37°C. The samples were diluted with 0.1 M ammonium bicarbonate (Sigma-Aldrich, USA) to a final urea-concentration below 2 M. The proteins were then digested with Worthington TPCK trypsin (0.9 mg trypsin in 1 mL 10 mM $CaCl_2$-dihydrate containing 25 mM ammonium bicarbonate) at a 20 : 1 (protein to enzyme) ratio and incubated at 37°C overnight.

2.2.3. Development of the MRM Assay. The development of an MRM assay involves several stages, the first being the selection of the target peptides that will represent each target protein. The selection rules for these peptides has been discussed in several previous papers [42] and will not be repeated here. Briefly, peptide selection involves optimizing the peptide mass spectrometric detectability by taking into account factors such as the peptide length, the absence of oxidizable residues, and other factors such as the avoidance of residue combinations such as RK and KK, which can lead to missed cleavages. These are avoided because they could lead to a reduction in sensitivity by multiple isoforms of the target peptides. The efficiency of tryptic digestion <95% was verified with ExPASy Peptide-Cutter (http://web.expasy.org/peptide_cutter/). If all of the above criteria were met, peptides were ranked based on their previous detection using both The GPM (http://gpmdb.thegpm.org/index.html) and Peptide Atlas (https://db.systemsbiology.net/sbeams/cgi/PeptideAtlas/Search) databases. All of the SIS-peptides used in this study are listed in Supplementary Table 8.

2.2.4. Synthesis and Purification of Isotopically Labeled Standard Peptides. Synthesis and purification of SIS-peptides were done as previously described [43].

2.2.5. MRM Q1/Q3 Ion Pair Selection Using Direct Infusion (Peptide Optimization). Prior to MRM analysis, the ion pairs (called "transitions") for protein quantification had to be selected. This "peptide optimization" was done as previously described [43], with the following changes: the nebulizer gas flow was 60 psi and the scanning time was 500 ms. A list of all possible b- and y-ion series for 2+ and 3+ precursor ion charge spanning a range of m/z from 200 to 1100 was generated using the Agilent MassHunter Optimizer For Peptides Software (Version B.05.00, Agilent). Product ions within 1 Da were excluded to ensure that only a single targeted product-ion was measured. All +2 or greater product ions were eliminated from the method using Mathew Monroe's Molecular Weight Calculator Freeware (http://www.alchemistmatt.com/). For each peptide the top 5 transitions, defined as those transitions with the most abundant signals, were selected for chemical interference screening.

2.2.6. Interference Screening of SIS-Peptides in Urine Samples. Interference testing has been described elsewhere for plasma [43], and a similar process is followed for urine. Basically, interference testing requires examining the ratios of each endogenous and SIS-peptide's transitions in buffer and in urine. If there are no interferences, the ratio in buffer and in urine should be the same.

A pooled urine sample from 5 female and 5 male donors collected from first void, midstream, and with sodium azide added to a produce a final concentration of 0.05% (Bioreclamation LLC, Westbury NY, USA; Lot No BRH683580), was used as the matrix for the interference testing.

Urine samples were prepared for tryptic digestion as described above. 100 fmol/μL of each measured SIS-peptide was added to tryptic-digested urine and the samples were desalted on a Waters Positive Pressure Manifold using Waters Oasis 96-well μElution Plates 30 μg HLBa sorbent (batch number 115B) according to the manufacturer's instructions. Briefly, μElution plates were activated with 200 μL of 100% methanol and equilibrated with 200 μL 0.15% formic acid. Samples were diluted 1 : 1 with formic acid before adding them to sorbent. The sorbent was washed twice with 200 μL 0.1% formic acid prior to elution with 100 μL of 50% acetonitrile/0.1% formic acid. After a short centrifugation step, samples were frozen at −80°C and lyophilized overnight. Before LC/MRM-MS analysis, samples were rehydrated in 0.1% formic acid (mobile phase A). Both urine digest and matrix-free samples were analyzed in triplicate.

All LC/MRM-MS measurements were carried out on an Agilent 1290 infinity UHPLC system coupled to an Agilent 6490 triple quadruple mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) with MassHunter Workstation Software (Agilent, B.04.01). Twenty μL of each sample was injected and separated at a flow rate of 400 μL/min on an Agilent Zorbax RRHD Eclipse Plus C18, 2.1 × 150 mm and 1.8 μm analytical column using a mobile phase gradient from 3 to 90% phase B (90% acetonitrile/0.1% formic acid) in a 43 min analysis. The gradient was as follows: 0 min, 3% B; 1,5 min, 7% B; 16 min 15% B; 18 min, 15,3% B; 33 min, 25% B; 38 min, 45% B; 29 min, 90% B; 43 min, 3% B. All acquisition methods used have previously described acquisition parameters [43] and scheduled retention times with a minimum dwell time of 20 ms to allow for the maximum number of peptides to be analyzed per injection.

The most intense interference-free signal producing transition was later used for peptide quantification (the quantifier) while other two transitions were used for quality control (the qualifiers). All quantifier and qualifier transitions are listed in Supplementary Table 8.

2.2.7. Concentration Balancing of SIS-Peptides in Urine Samples. For the highest quantitation accuracy, the concentration

of each SIS-peptide should match as closely as possible to the concentration in the sample [56]. Abundance of each SIS-peptide should be at least between 1×10^3 and 1×10^4 to ensure optimal peak shape and therefore correct integration. The difference in peak areas between the natural and SIS peaks should be no more than a factor of 10 for optimal quantitation. Supplementary Table 9 shows the final SIS-peptide concentrations.

2.2.8. MRM-Based Quantitation of Vinculin, Galectin-3, and Prostatic Acid Phosphatase in Urine and MRM Data Analysis. Urine samples for scheduled MRM analysis were prepared as described above. Concentration-balanced SIS-peptides were spiked in (Supplementary Table 9) and the samples were desalted and lyophilized and then reconstituted in 20 μL 0.1% formic acid prior to analysis as described in Interference Screening of SIS-Peptides in Urine Samples section.

MRM data was processed and evaluated with Agilent's MassHunter Quantitative Analysis Software (Agilent B.04.00) and Agilent's Integrator Algorithm for Peak Integration. All peaks were verified for correct chromatographic peak selection and integration. The ratio between the natural peptide peak area and SIS-peptide's peak area was calculated as the response ratio (RR). Natural peptide concentrations were calculated by multiplication of the RR by the concentration of the SIS-peptides that had been spiked into the sample. The accuracy of the calculation was further increased by verifying the purity of the SIS-peptides by amino acid analysis (AAA) and capillary zone electrophoresis (CZE); data are listed in Supplementary Table 9.

2.2.9. Statistical Evaluation. Statistical analyses for Western blot analysis, immunohistochemical analysis, and MRM analysis were done using SPSS 15.0 (SPSS, Chicago, IL). P values of <0.05 were defined as statistically significant. Two-sided Mann-Whitney U-test was used to detect differences in abundance levels among the various groups studied, based on the Western blot, immunohistochemical, and MRM-MS results.

3. Results

3.1. Identification of Novel Potential Biomarkers for Prostate Cancer in Tissue Using 2D-DIGE with MS Identification. For the identification of differentially regulated proteins in prostate cancer, 12 prostatectomy samples from prostate cancer patients without biochemical relapse, 11 prostatectomy samples from patients with biochemical relapse, and 14 corresponding tumor-free prostate cancer tissue samples were comparatively analyzed by 2D-DIGE saturation labeling. Comparison of all samples revealed 1000 gel spots common to all gels by using the Delta2D software. Tumor and tumor-free samples as well as tumor samples from patients with and without biochemical relapse could be distinguished from each other using principal component analysis (Figure 2). Comparison of the tumor-free *versus* the tumor samples revealed 37 protein spots with bigger normalized volume and 27 protein spots with smaller normalized volume in the tumor samples compared to the tumor-free samples (Supplementary Table 10). Of these, 14 protein spots were identified using MALDI-MS and LC-MS/MS (Figure 2, Table 4, and Supplementary Table 12).

In addition, 12 prostatectomy samples from patients without biochemical relapse and 11 prostatectomy samples from patients with biochemical relapse were compared to reveal proteins involved in tumor aggressiveness. The analysis resulted in 22 protein spots which showed bigger normalized volumes and 13 protein spots which showed smaller normalized volumes prostatectomy samples of patients with biochemical relapse compared to samples of patients without biochemical relapse (Supplementary Table 11). Of these, 29 deregulated protein spots were identified using MALDI-MS and LC-MS-MS (Supplementary Table 13). Among these, prostatic acid phosphatase (PAP), vinculin, secernin-1 (SCRN1), lamin A/C, and gelsolin were identified. All of the identified proteins are listed in Table 5.

3.2. Ingenuity Pathway Analysis (IPA) of the Identified Proteins. Ingenuity pathway analysis of the potential new biomarkers identified using 2D-DIGE and MS revealed that most of the deregulated proteins are located in the cytoplasm. As shown Figure 3, 60.0% of the differentially-expressed proteins in the tumor *versus* tumor-free sample set, and 53.3% within the aggressive *versus* non aggressive tumor sample set, were located in the cytoplasm. Proteins that are differentially expressed between tumor-free tissue and prostate cancer tissue were mostly associated with cellular assembly (5 proteins), cellular development (4 proteins), cell morphology (3 proteins), cellular compromise (i.e., associated with damage or degeneration of cells; 1 protein), and carbohydrate metabolism (1 protein). The differentially expressed proteins in tumors with or without relapse were mostly associated with cellular growth and proliferation (12 proteins), cellular development (10 proteins), cellular movement (8 proteins), cell morphology (5 proteins), and carbohydrate metabolism (2 proteins). Detailed results, as well as the classification of the identified proteins and their functions in development and in the physiological system, are shown in Table 6 and Figure 3.

3.3. Validation of Potential Tissue Biomarker Candidates Found by DIGE. Some candidates found by 2D-DIGE and MS (secernin-1, vinculin, prostatic acid phosphatase (PAP), and galectin-3) were selected for further validation. Two of those, PAP and galectin-3, have already been suggested as potential biomarkers for prostate cancer. *Secernin-1* 2D-DIGE analysis also revealed that secernin-1 shows significantly lower abundance in recurrent prostate cancer tumors compared to prostate cancer tumors without biochemical relapse. For Western blot validation, eight tumor-free tissue samples and four prostatectomy samples from patients without and six prostatectomy samples from patients with relapse were analyzed (Figure 4). Secernin-1 showed a significant downregulation in tumors ($P = 0.001$) but no deregulation between tumors with and without relapse ($P = 0.762$). Further immunohistochemical analysis of the 13 prostatectomy samples from prostate cancer patients without relapse, the 14 samples from patients with relapse, and the 43 tumor free tissue samples (kindly provided by the University Hospital

Table 4: Deregulated proteins in prostate cancer identified with 2D-DIGE and MS.

Spot		Acc. no.	Protein name	Ratio Tf versus Tu	*P* value (*t*-test) Tf versus Tu	*P* value (*U*-test) Tf versus Tu
Down 01		P22626	Heterogeneous nuclear ribonucleoproteins A2/B1	−21.8	0.043	0.035
Down 05		P17661	Desmin	−15.7	0.156	0.024
Down 06		P17661	Desmin	−9.1	0.085	0.009
Down 04		P17661	Desmin	−3.8	0.037	0.012
Down 09		P09493	Tropomyosin alpha-1 chain	−2.9	0.035	0.042
Down 10		P09493	Tropomyosin alpha-1 chain	−2.9	0.009	0.020
Down 08		P09493	Tropomyosin alpha-1 chain	−2.8	0.010	0.019
Down 07		P12277	Creatine kinase B-type	−2.7	0.045	0.009
Down 11		Q05682	Caldesmon	−2.2	0.115	0.026
Down 02		P08670	Vimentin	−1.7	0.053	0.024
Down 03		P17661	Desmin	−1.6	0.039	0.110
Up 01		COEA1	Collagen alpha-1(XIV) chain	2.4	0.104	0.042
Up 03	Mix	ANXA5	Annexin A5	3.7	0.038	0.033
		A1BG	Alpha-1B-glycoprotein	3.7	0.038	0.033
		P04217	Alpha-1B-glycoprotein	3.7	0.038	0.033
Up 02		TCPA	T-complex protein 1 subunit alpha	46.7	0.405	0.049

2D-DIGE: two-dimensional differences in gel electrophoresis; MS: mass spectrometry; Acc. no.: accession number; Tf: tumor free; Tu: tumor; *U*-test: two-sided Mann-Whitney *U*-test; ratio: division of the mean; mean: normalized spot volume.

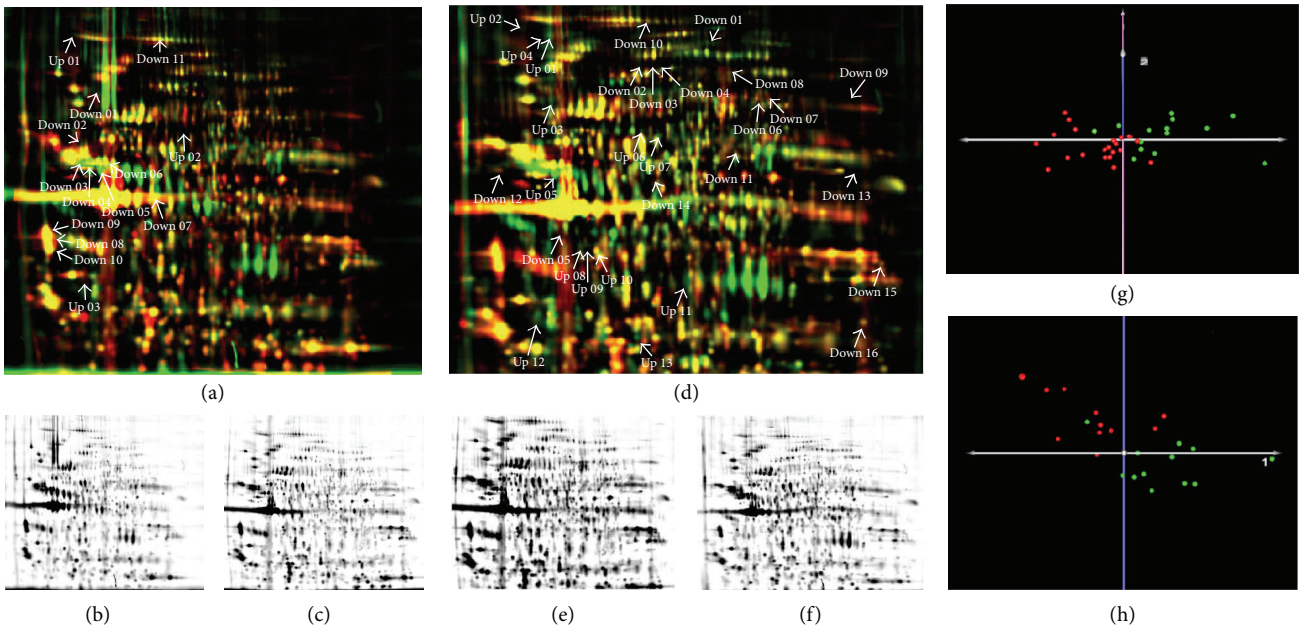


Figure 2: 2D-DIGE analysis of 14 tumor free prostate tissue samples, 12 prostatectomy samples from prostate cancer patients without relapse in a 5-year followup, and 11 prostatectomy samples from patients with relapse. (a) Overlay of 2D-DIGE gels of prostatectomy samples from tumor-free tissue areas ((b), green) and patients with prostate cancer ((c), red). (d) Overlay of 2D-DIGE gels from prostatectomy samples from patients without ((e), red) and with ((f), green) relapse. Downregulated spots in prostate cancer and prostate cancer with relapse, respectively, are annotated with "down." Upregulated spots in these samples are annotated with "up." Principal component analysis (PCA) of prostate cancer (red) and tumor-free tissue (green) (g) and prostate cancer samples without (red) and with relapse (green) (h).

Table 5: Deregulated proteins in recurrent prostate cancer identified with 2D-DIGE and MS.

Spot		Acc. no.	Protein name	Ratio + versus − rec	P value (t-test) + versus − rec	P value (U-test) + versus − rec
Down 13		P01857	Ig gamma-1 chain C region	**−274.3**	0.334	0.041
Down 01		FLNA	Filamin-A	**−12.0**	0.269	0.031
Down 12		SCRN1	Secernin-1	**−8.9**	0.088	0.044
Down 04		O95394	Phosphoacetylglucosamine mutase	**−7.5**	0.228	0.012
Down 08		PYGB	Glycogen phosphorylase, brain form	**−5.6**	0.176	0.031
Down 03		P06396	Gelsolin	**−5.3**	0.092	0.023
Down 02		P06396	Gelsolin	**−3.5**	0.109	0.019
Down 16		LEG3	Galectin-3	**−3.4**	0.272	0.033
Down 05		PTGR2	Prostaglandin reductase 2	**−3.2**	0.047	1.169
Down 06		LMNA	Lamin-A/C	**−2.6**	0.036	0.023
Down 14		TTC38	Tetratricopeptide repeat protein 38	**−2.4**	0.087	0.036
Down 15	Mix	MDHM	Malate dehydrogenase, mitochondrial	**−2.3**	0.233	0.014
		P51911	Calponin-1	**−2.3**	0.233	0.014
Down 09		P16870	Carboxypeptidase E precursor	**−2.2**	0.122	0.049
Down 10		CO6A2	Collagen alpha-2(VI) chain	**−1.9**	0.018	0.031
Down 07		LMNA	Lamin-A/C	**−1.7**	0.133	0.036
Down 11		G6PD	Glucose-6-phosphate 1-dehydrogenase	**−1.7**	0.255	0.036
Up 08	Mix	Q9UBR2	Cathepsin Z precursor	**1.2**	0.529	0.951
		P20774	Mimecan precursor	**1.2**	0.529	0.951
Up 10		CAZA1	F-actin-capping protein subunit alpha-1	**1.5**	0.209	0.042
Up 05		PAPP	Prostatic acid phosphatase	**1.8**	0.048	0.056
Up 01		VINC	Vinculin	**2.2**	0.027	0.031
Up 03		GRP78	78 kDa glucose-regulated protein	**2.5**	0.025	0.036
Up 02		COEA1	Collagen alpha-1(XIV) chain	**2.5**	0.075	0.049
Up 09		KCD12	BTB/POZ domain-containing protein KCTD12	**2.6**	0.158	0.045
Up 11		GLO2	Hydroxyacylglutathione hydrolase, mitochondrial	**3.2**	0.047	0.042
Up 12	Mix	ANXA5	Annexin A5	**3.2**	0.032	0.074
		A1BG	Alpha-1B-glycoprotein	**3.2**	0.032	0.074
Up 04		SYNEM	Synemin	**4.9**	0.055	0.027
Up 13		PRDX4	Peroxiredoxin-4	**4.9**	0.022	0.004
Up 06		ANXA4	Annexin A4	**6.4**	0.288	0.042
Up 07		TCPA	T-complex protein 1 subunit alpha	**51.9**	0.275	0.031

2D-DIGE: two-dimensional differences in gel electrophoresis; MS: mass spectrometry; Acc. no.: accession number; + rec: prostate cancer with recurrence; − rec: prostate cancer without recurrence; U-Test: two-sided Mann-Whitney U-Test; ratio: division of the mean; mean: normalized spot volume.

Table 6: Ingenuity pathway analysis of the deregulated proteins in prostate cancer, identified by 2D-DIGE and MS localization; type and top 5 functions of the identified proteins are listed.

ID	Entrez gene name	Localization	Type	Functions (top 5)
P04217	Alpha-1-B glycoprotein	Extracellular space	Other	
P08758	Annexin A5	Plasma membrane	Other	Cellular assembling and organization, carbohydrate metabolism, and cellular compromise
Q05682	Caldesmon 1	Cytoplasm	Other	Cellular assembling and organization, cell morphology, and development of connective tissue
P12277	Creatine kinase, brain	Cytoplasm	Kinase	
Q05707	Collagen, type XIV, alpha 1	Extracellular space	Other	Cellular development and development of connective tissue
P17661	Desmin	Cytoplasm	Other	Cellular assembling and organization, development and function of the cardiovascular system, and organ morphology
P22626	Heterogeneous nuclear ribonucleoprotein A2/B1	Nucleus	Other	Cellular development
P17987	T-complex 1	Cytoplasm	Other	
P09493	Tropomyosin 1 (alpha)	Cytoplasm	Other	Cellular assembling and organization, cell morphology, cellular development, development of connective tissue, development and function of the cardiovascular system, and organ morphology
P08670	Vimentin	Cytoplasm	Other	Cellular assembling and organization, cell morphology, cellular development, development of connective tissue, development and function of the cardiovascular system, and tumor morphology
P04217	Alpha-1-B glycoprotein	Extracellular space	Other	
P15309	Acid phosphatase, prostate	Extracellular space	Phosphates	Cellular growth and differentiation, cellular development, and development and function of the hematopoietic system
P09525	Annexin A4	Plasma membrane	Other	
P08758	Annexin A5	Plasma membrane	Other	Carbohydrate metabolism, organ development, and development and function of the hematopoietic system
P52907	Capping protein (actin filament) muscle Z-line, alpha 1	Cytoplasm	Other	Cellular growth and differentiation
P51911	Calponin 1, basic, smooth muscle	Cytoplasm	Other	Cellular growth and differentiation and cellular development
Q05707	Collagen, type XIV, alpha 1	Extracellular space	Other	Cellular growth and differentiation, cellular development, and development and function of connective tissues
P12110	Collagen, type VI, alpha 2	Extracellular space	Other	Cellular growth and differentiation
P16870	Carboxypeptidase E	Cytoplasm	Peptidase	
Q9UBR2	Cathepsin Z	Cytoplasm	Peptidase	Cellular growth and differentiation, cellular development, cell morphology, cellular movement (migration), and development and function of the hematopoietic system
P21333	Filamin A, alpha	Cytoplasm	Other	Cell morphology, cellular movement (migration), and development and function of the cardiovascular system
P11413	Glucose-6-phosphate dehydrogenase	Cytoplasm	Enzyme	Cellular development, carbohydrate metabolism, organ development, and development and function of the hematopoietic system
P06396	Gelsolin	Extracellular space	Other	Cellular growth and differentiation, cellular movement (invasion), organ development, and development and function of connective tissue
Q16775	Hydroxyacylglutathione hydrolase	Cytoplasm	Enzyme	Organ development
P11021	Heat shock 70 kDa protein 5 (glucose-regulated protein, 78 kDa)	Cytoplasm	Enzyme	Cellular growth and differentiation, cellular development, cellular movement (migration), development and function of the hematopoietic system, and development and function of the cardiovascular system
P01857	Immunoglobulin heavy constant gamma 1 (G1m marker)	Extracellular space	Other	Cellular growth and differentiation, cellular development, cellular movement (migration, invasion), development and function of the hematopoietic system, and development and function of the cardiovascular system
Q96CX2	Potassium channel tetramerization domain containing 12	Plasma membrane	Ion channel	
P17931	Lectin, galactoside-binding, soluble, 3	Extracellular space	Other	Cellular growth and differentiation, cellular development, cell morphology, cellular movement (migration, invasion, and chemotaxis), organ development, development and function of the hematopoietic system, development and function of connective tissue, and development and function of the cardiovascular system
P02545	Lamin A/C	Nucleus	Other	Cell morphology
P40926	Malate dehydrogenase 2, NAD (mitochondrial)	Cytoplasm	Enzyme	
P20774	Osteoglycin	Extracellular space	Growth factor	

Table 6: Continued.

ID	Entrez gene name	Localization	Type	Functions (top 5)
O95394	Phosphoglucomutase 3	Cytoplasm	Enzyme	Cellular growth and differentiation, cellular development, and development and function of connective tissues
Q13162	Peroxiredoxin 4	Cytoplasm	Enzyme	
Q8N8N7	Prostaglandin reductase 2	Cytoplasm	Enzyme	
P11216	Phosphorylase, glycogen; brain	Cytoplasm	Enzyme	
Q12765	Secernin 1	Cytoplasm	Other	Cellular growth and differentiation, cellular development, cell morphology, and cellular movement (migration)
O15061	Synemin, intermediate filament protein	Cytoplasm	Other	
P17987	T-complex 1	Cytoplasm	Other	
Q5R3I4	Tetratricopeptide repeat domain 38	unknown	Other	
P18206	Vinculin	Plasma membrane	Enzyme	Cellular movement (migration) and development and function of connective tissues

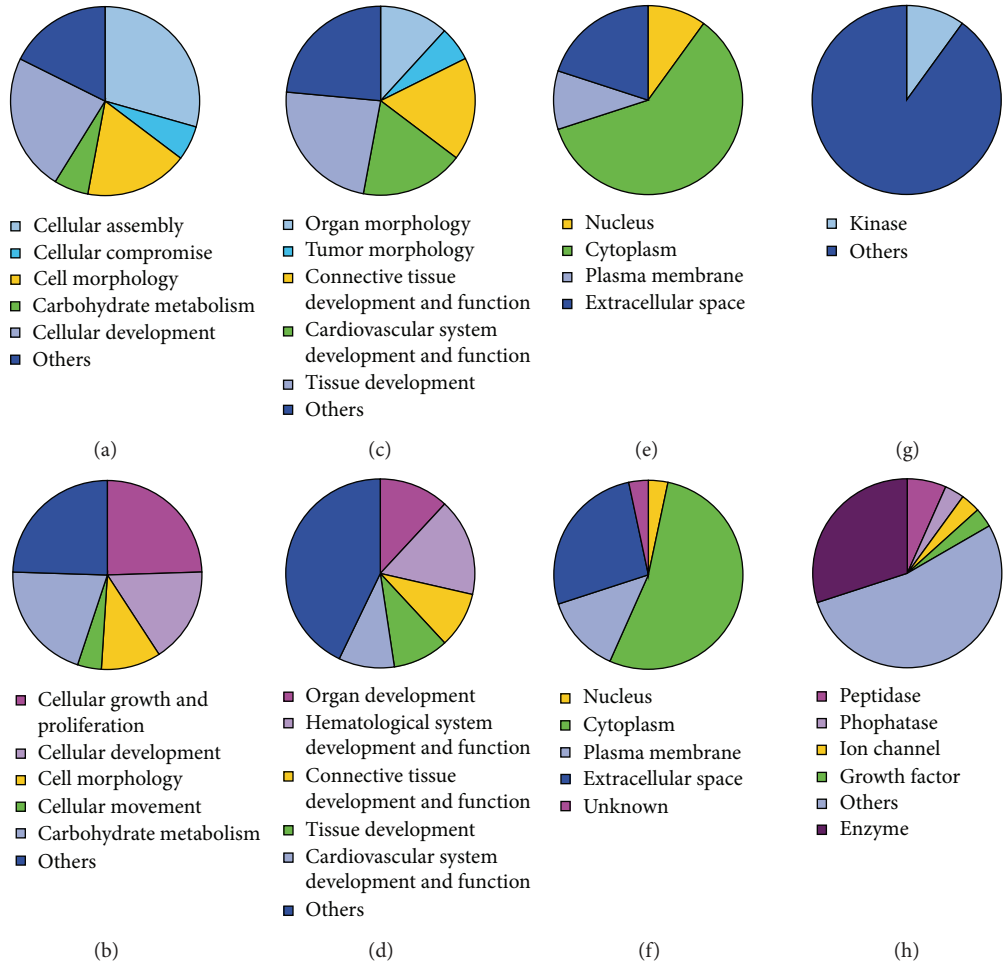


Figure 3: Ingenuity pathway analysis of the proteins that were deregulated between tumor-free samples and prostate cancer samples ((a), (c), (e), and (g)) as well as proteins that were deregulated between prostate cancer samples from patients with and without relapse ((b), (d), (f), and (h)). Distribution of molecular and cellular functions ((a) and (b)), role of the identified proteins in development and function of the physiological systems ((c) and (d)), localisation ((e) and (f)), and type of the identified proteins ((g) and (h)) are shown.

Dresden) validated the results of the Western blot: tumors from patients with and without relapse showed a significantly lower immunohistochemical score (median 1.0 for tumor tissue) than tumor-free tissue (mean 3.00; $P < 0.001$; Supplementary Figure 1). Differences in expression levels between tumors with and without relapse could not be shown. Moreover, immunohistochemical staining showed secernin-1 expression in the basal cell layer but not in the luminal cells itself. To further examine the potential use of secernin-1 as a potential biomarker candidate for prostate cancer, and to discriminate between prostate cancer and prostatitis, five tissues with prostatitis were analyzed. In these experiments, the areas with prostatitis showed no difference in secernin-1 expression levels compared to the noninflamed, tumor-free tissue areas (Figures 5(i)–5(k)). These results were further validated with immunohistochemical staining of an independent tissue microarray (TMA) of prostate cancer patients kindly provided by University Hospital Bonn. To test for secernin-1 expression, 124 tumor free samples, 49 intraepithelial neoplasia lesions (PIN), 52 patients without biochemical relapse, and 16 patients with biochemical relapse were analyzed. The results of this TMA showed the same regulation of secernin-1 as the previous immunohistochemical analysis: tumor-free tissues showed significantly higher secernin-1 expression than tumors ($P < 0.001$), but no difference was found between patients with and without relapse (Figures 5(a)–5(h)). Immunohistochemical secernin-1 staining could detect prostate cancer with a sensitivity of 98.0% and a specificity of 99.2% for a threshold score of ≥1 versus <1. For a more precise scoring, an adapted Remmele score was used to classify the secernin-1 expression, and the results are shown in Figures 5(a)–5(h) and Supplementary Table 14. The PIN showed higher secernin-1 expression levels than the tumors but lower secernin-1 expression than the tumor-free tissues ($P < 0.01$).

Vinculin. Because secernin-1 is downregulated in prostate cancer, it was considered to be a potential biomarker candidate for the diagnosis of prostate cancer but was not suitable for the early detection of a recurrence.

Vinculin was validated as a potential biomarker candidate for recurrent prostate cancer. Immunohistochemical analysis

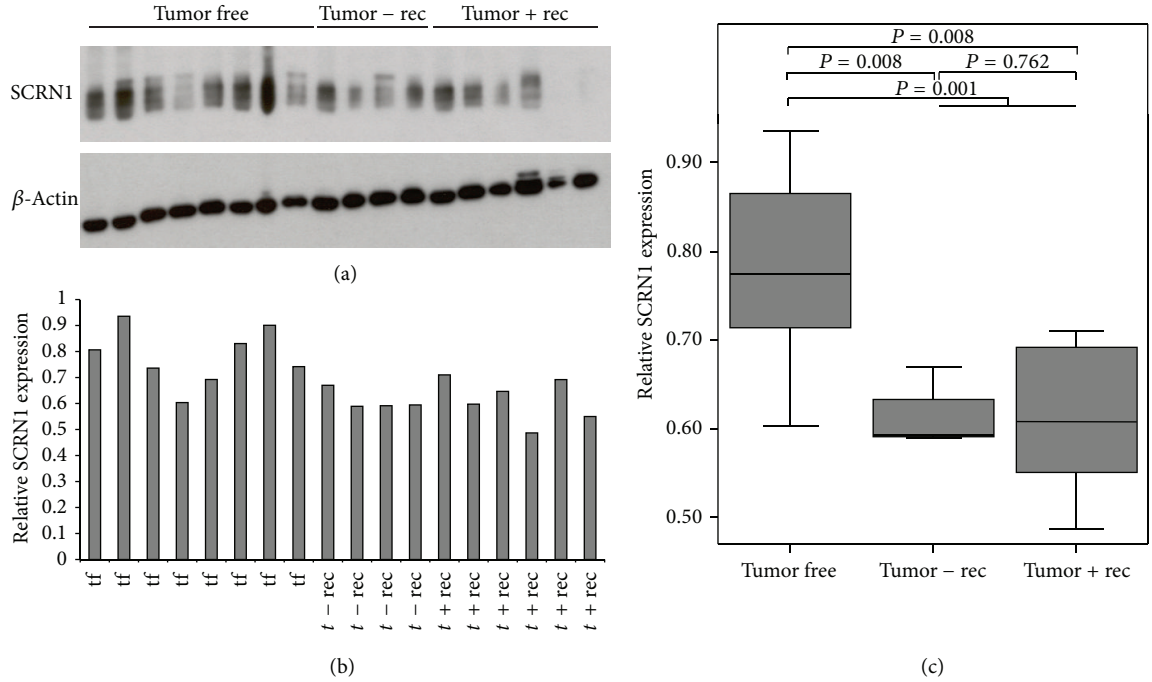


Figure 4: Western blot analysis of secernin-1 (SCRN1) and β-actin as a house keeping protein in prostate cancer tissue and tumor-free tissue samples. (a) Western blot analysis. (b) Relative SCRN1 expression levels were calculated densitometrically in reference to the β-actin expression level. (c) Boxplot of the densitometrically determined SCRN1 expression levels. A significant difference between tumors without ($t-$ rec) and with recurrence (t + Rec) is not detectable ($P = 0,762$) but SCRN1 is significantly downregulated in prostate cancer tissue compared to tumor-free tissue samples (tf) ($P = 0.001$).

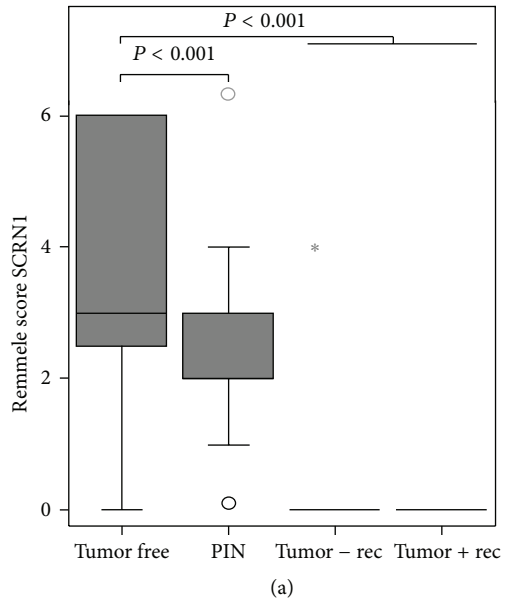
of the previously described TMA set showed a significant upregulation of vinculin in PIN and prostate cancer compared to tumor-free tissue ($P < 0.001$ for tumor-free *versus* PIN and $P < 0.001$ for tumor-free *versus* prostate cancer patients without relapse and $P = 0.013$ for tumor free *versus* prostate cancer patients with relapse). Immunohistochemical vinculin staining could detect prostate cancer with a sensitivity of 38.0% and a specificity of 56.9% for a threshold score ≤1 versus >1. Biochemical prostate cancer recurrence could be detected with specificity of 65.5% and a sensitivity of 50.0%. Detailed scoring information as well as representative examples of immunohistochemical staining are shown in Figure 6 and Supplementary Table 15.

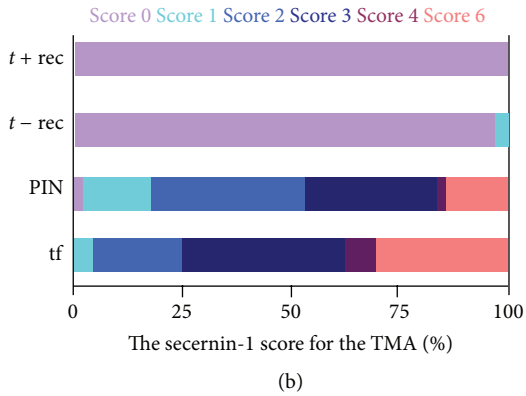
3.4. Validation of Potential Prostate Cancer Biomarker Candidates in Urine

Vinculin. To validate vinculin as a potential noninvasive biomarker candidate for prostate cancer, we determined the abundance of vinculin in the urine of prostate cancer patients. Urine from 14 control patients without prostate cancer, 33 prostate cancer patients without relapse, and 15 patients with relapse were analyzed using Western blot. Vinculin expression was scored from 0 (no vinculin antibody signal detectable) to 4 (strong vinculin antibody signal detectable). Again, vinculin expression was significantly higher in the urine of prostate cancer patients than in controls ($P = 0.006$, Figure 7). Most importantly, vinculin levels in urine from prostate cancer patients with relapse were higher than in urine from patients without relapse (median score without relapse 1.00; median with relapse 1.75; $P = 0.229$; median vinculin score in the control urine 0.250; $P = 0.006$). Moreover, 62.5% of the patients without relapse showed no vinculin or low vinculin levels (score 0-1) while only 37.5% of these patients showed high vinculin levels (score 2–4). In contrast, only 40% of patients with relapse showed low vinculin levels (score 0-1) while 60% of these patients showed high vinculin levels (score 2–4). Vinculin levels in Western blots of urine >1 could detect prostate cancer with a sensitivity of 54.2% and a specificity of 85.7%. Biochemical prostate cancer recurrence could be detected with specificity of 60.6% and a sensitivity of 40.0%.

Because Western blot analysis is not practical for daily routinely use in the clinic, we tested the ability of multiple reaction monitoring (MRM) to detect vinculin abundance in urine. In the MRM-MS analysis of vinculin, 16 prostate cancer patients (nine without relapse and seven with relapse) and seven control urine samples were used. Vinculin could be detected in concentrations up to 0.55 pmol/mg protein in patients' urine. Moreover, vinculin levels were higher in prostate cancer patients' urine (median 0.109 pmol/mg) than in the urine of the control group (median 0.090 pmol/mg). Notably, the vinculin levels in urine from patients with relapse were higher (median 0.120 pmol/mg) than the vinculin levels from patients without relapse (median 0.100 pmol/mg) (Figure 7).

3.5. Validation of Additional Proteins as Potential Biomarker Candidates in Urine Using MRM-MS. PAP, Galectin-3, and Secernin-1. Three proteins in our initial 2D-DIGE and MS





Patient	Secernin-1 score in inflammation	Secernin-1 score in normal glands
1	2	2
2	1	1
3	1	1
4	3	3
5	2	2

(i)

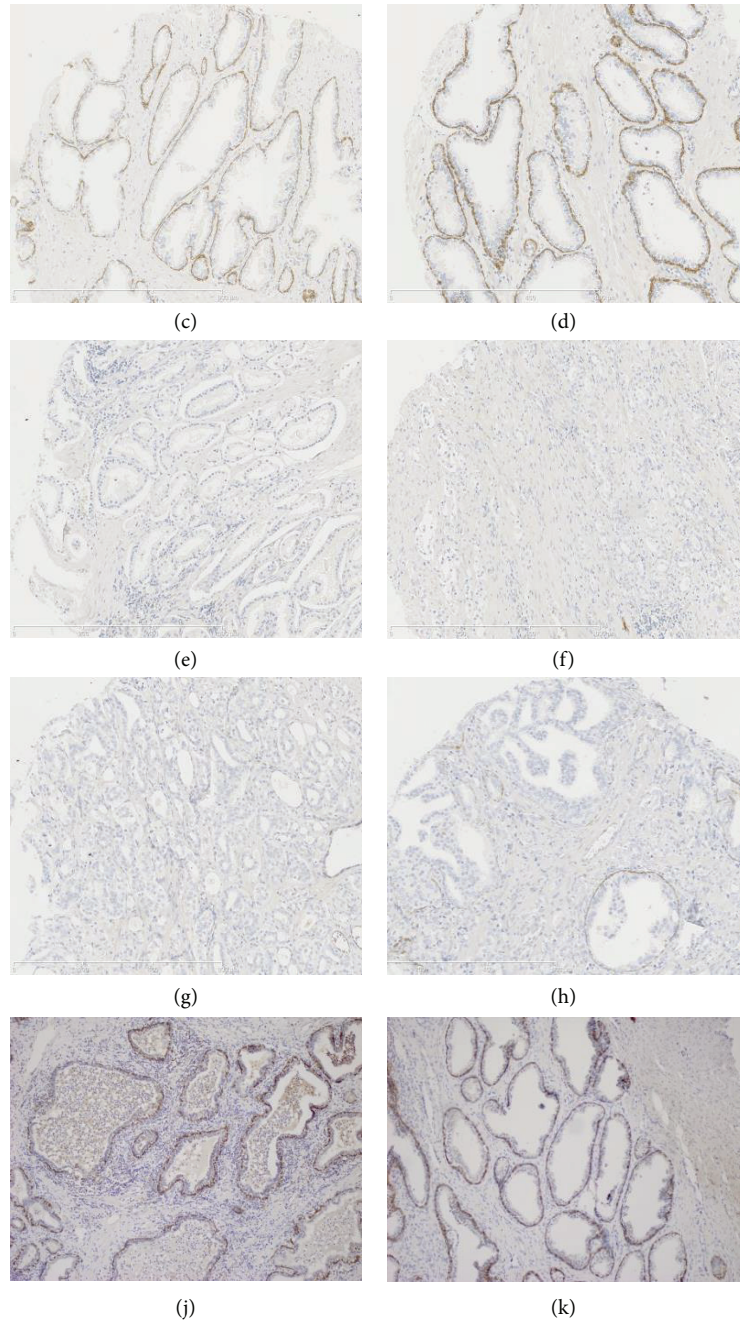


FIGURE 5: (a)–(h) Representative immunohistochemistry of secernin-1 in an independent tissue microarray (TMA) obtained from the University Hospital Bonn. (a) Boxplot of the secernin-1 expression levels in the analyzed patient groups. (b) Percentages of each score in each analyzed patients group. For more detailed information, an adapted Remmele score was used for classification of the secernin-1 expression. (c) and (d) Tumor-free prostatectomy samples. (e) and (f) Prostatectomy samples of prostate cancer patients without relapse. (g) and (h) Prostatectomy samples of prostate cancer patients with relapse. Secernin-1 expression is significantly downregulated in prostate cancer tissue compared to tumor-free tissue samples ($P < 0.001$). Downregulation of secerin-1 starts in the peri-intraepithelial neoplasia (PIN) as PIN lesions showed less secerin-1 expression than tumor-free tissue samples ($P < 0.001$) but stronger secernin-1 expression than prostate cancer tissue ($P < 0.001$). (i)–(k) Representative immunohistochemical staining of 5 prostatectomy samples of patients with prostatitis (j) and corresponding normal prostate tissue (k) as well as a table (i) of the results of all 5 analyzed patients obtained from the University hospital Aachen. Secernin-1 staining intensity is not affected by inflammation: the five analyzed tumor-free tissue samples showed the same staining intensity for secernin-1 as the corresponding inflamed tissue.

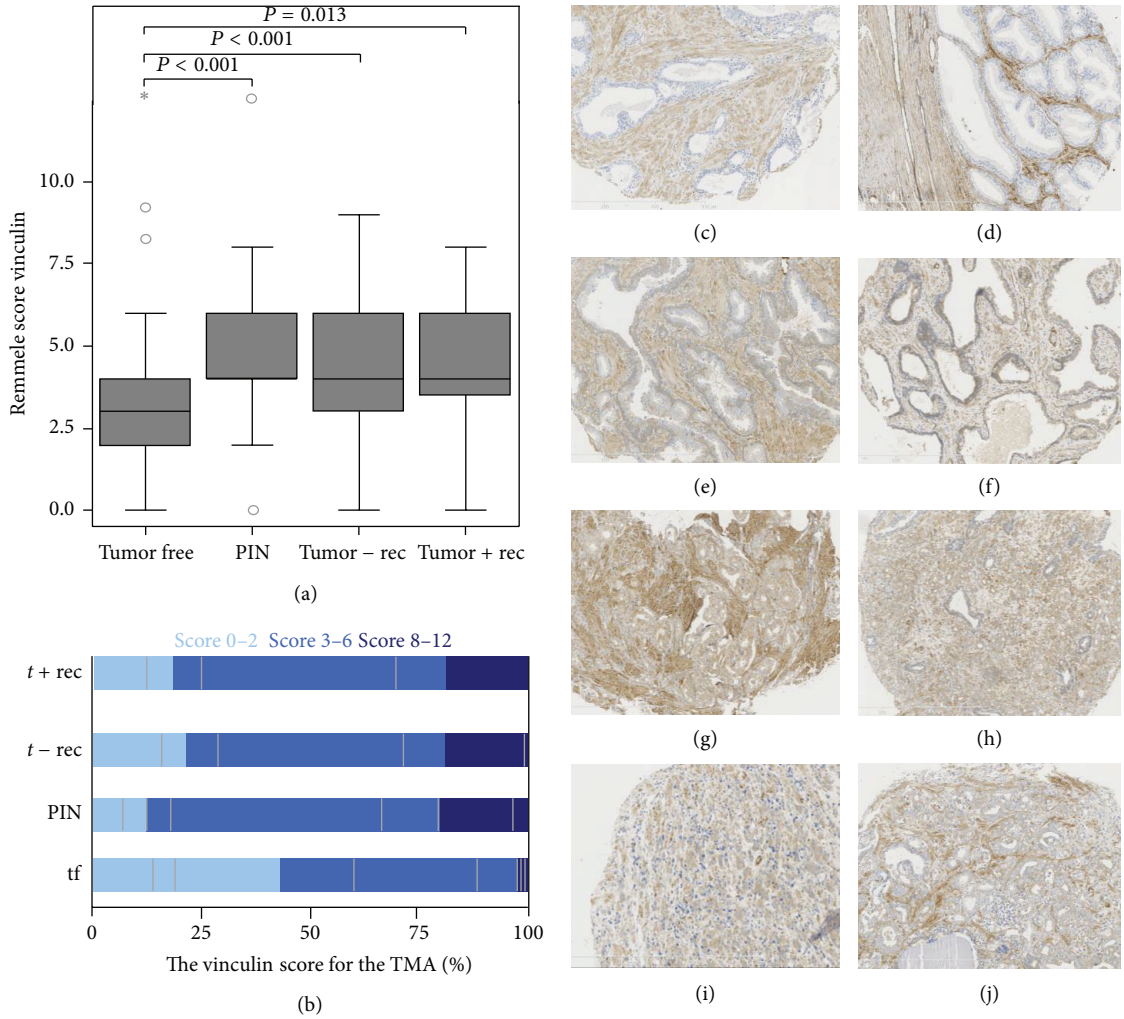


FIGURE 6: Representative immunohistochemistry of vinculin in an independent tissue microarray (TMA) obtained from the University Hospital Bonn. 116 tumor-free tissue samples, 54 prostatic intraepithelial neoplasia (PIN) lesions, 54 prostatic samples from patients without relapse (− rec), and 16 prostatectomy samples from prostate cancer patients with relapse (+ rec) were analyzed. Boxplots of the immunohistological scores of the stained tissue. (b) Percentage of each score in each analyzed patients group. For more detailed information, an adapted Remmele score was used to classify the vinculin expression. (c)–(j) Immunohistochemically stained tissue: (c) and (d) tumor-free prostatectomy samples, (e) and (f) prostatectomy samples of PIN, (g) and (h) prostatectomy samples of prostate cancer patients without relapse, and (i) and (j) prostatectomy samples of prostate cancer patients with relapse. Vinculin is significantly upregulated in peri-intraepithelial neoplasia (PIN) and prostate cancer compared to tumor-free tissue samples ($P < 0.001$).

experiments were found to be associated with prostate cancer (secernin-1, PAP, and galectin-3) were chosen for a proof of principal MRM-MS study, using urine from nine prostate cancer patients without relapse and seven patients with relapse. Urine samples from seven patients without prostate cancer were used as controls. In MRM-MS results, PAP was found to show higher protein levels in the urine of prostate cancer patients compared to the PAP concentrations in the urine of the control group (median control urine = 1.21 pmol/mg median prostate-cancer patient urine = 6.26 pmol/mg; $P = 0.012$, Figure 8). However, no significant difference in PAP concentration in the urine of patients with and without relapse was found. Galectin-3 showed significantly lower protein levels in urine from prostate-cancer patients with relapse compared to urine from patients without relapse (median control urine = 0.27 pmol/mg, median in urine of prostate-cancer patients without relapse = 0.48 pmol/mg; median in urine of prostate-cancer patients with relapse = 0.13 pmol/mg; $P = 0.017$, Figure 8). Secernin-1 was not detected in the patient urine samples.

4. Discussion

4.1. Identifying Potential New Biomarker Candidates for Prostate Cancer. Ten potential biomarker candidates for prostate cancer diagnosis and 32 potential prognostic biomarker candidates to discriminate nonrecurrent from recurrent prostate cancer were identified using 2D-DIGE and MS. Ingenuity pathway analysis (IPA) was performed in order to classify the identified proteins. A comparison of tumor and tumor-free tissue revealed ten general prostate cancer biomarker candidates. These ten proteins were differentially regulated

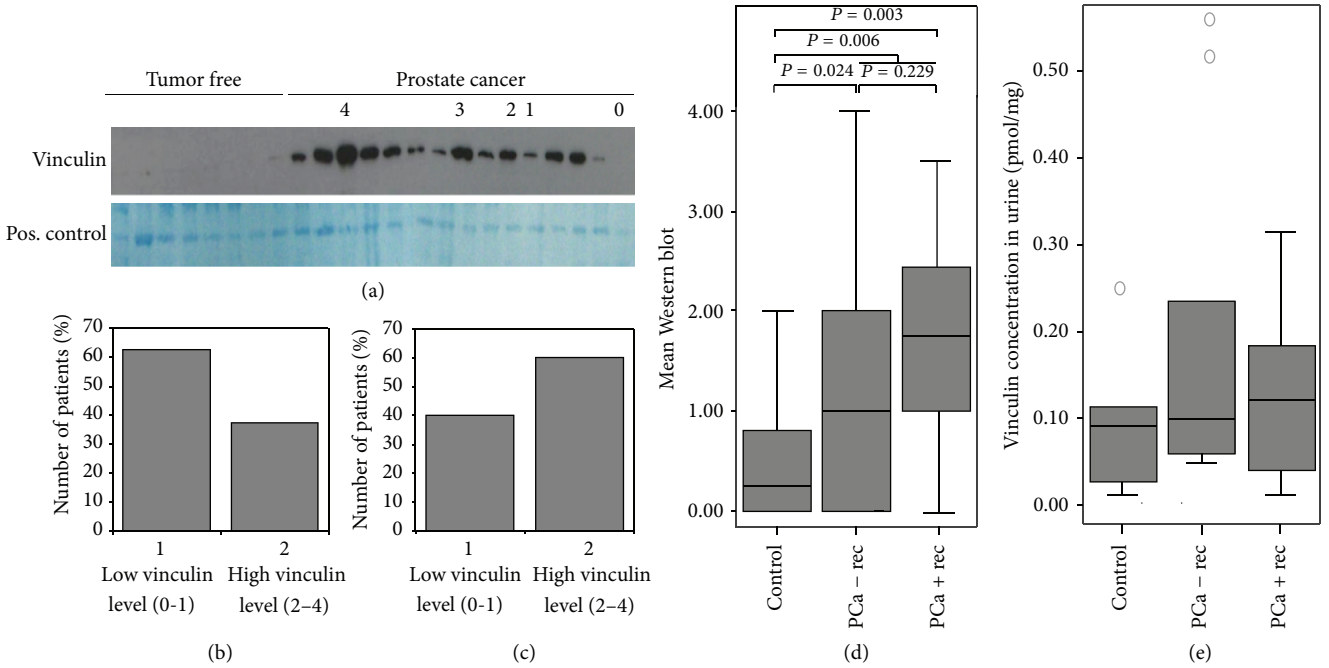


Figure 7: Validation attempts of vinculin levels. (a) Representative Western blot results of the vinculin levels in urine of prostate cancer patients and control patients. Coomassie brilliant blue stained gel as a positive control (pos. control). (b) Percentage of patients without recurrence with high and low vinculin levels in urine. (c) Percentage of patients with recurrence with high and low vinculin levels in urine. (d) Results of all 34 analyzed patients without recurrence, 15 prostate cancer patients with relapse and 12 analyzed control urines: boxplot of the vinculin levels in prostate cancer patients without (− rec) and with recurrence (+ rec) compared to control patients. Vinculin shows a tendency to be upregulated in prostate cancer patients with recurrence compared to patients without recurrence ($P = 0.229$). Moreover, vinculin is significantly upregulated in prostate cancer patients compared to control patients ($P = 0,006$). (e) MRM analysis of seven urine samples from control patients without prostate cancer, nine prostate cancer patients without recurrence and seven prostate cancer patients with recurrence. Vinculin (peptide SLGEISALTSK) is upregulated in prostate cancer patients urine compared to the urine of control patients ($P = 0.438$).

between tumor and tumor-free tissue and are mostly associated with cellular assembly, cellular development, cell morphology, cellular compromise, and carbohydrate metabolism. The 32 identified potential biomarker candidates for recurrence in prostate cancer are associated with cellular growth and proliferation, cellular development, cellular movement, cell morphology, and carbohydrate metabolism.

Potential biomarker candidates from both comparisons are associated with cellular development, cell morphology, and carbohydrate metabolism. Cellular assembly and cellular compromise are more associated with general prostate cancer biomarker candidates than with specific biomarker candidates for recurrence. In recurrent prostate cancer, proteins involved in cellular movement, especially invasion and migration, as well as cellular growth and proliferation, are often deregulated. This is in agreement with well-known features of cancer: activating invasion is crucial for the spread of cancer [57], and cellular growth and proliferation are arguably the most fundamental traits of cancer cells [57]. This study showed that cellular movement, particularly invasion and migration, cell growth, and proliferation play a more important role in recurrent prostate cancer than in prostate cancer in general.

The fact that these functions are less associated with general prostate cancer biomarker candidates than with recurrent prostate cancer might reflect the heterogeneity of the disease: as mentioned previously, many prostate cancers are indolent and are not clinically relevant due to very slow proliferation [8]. Therefore, proteins characteristic for proliferation and cellular movement are more especially suitable biomarker candidates for recurrence.

Principal component analysis (PCA) led to the detection of several potential prostate cancer biomarker candidates that have already been discussed as potential prognostic prostate cancer biomarkers in the literature. This underlines the quality of our study. PCA allowed us to detect a clear separation between benign and malignant prostate tissue as well as tissue from recurrent and nonrecurrent prostate cancer in patients. In addition, we also found differential expression levels of PAP and galectin-3, proteins which have already been discussed in literature as potential biomarker candidates for recurrent prostate cancer [58–63].

A. B. Gutman and E. B. Gutman identified increased PAP levels in patients with prostate cancer [64]. Thus, PAP was the first serum biomarker for prostate cancer to be used in clinical practice, although it lacked sufficient sensitivity to be a reliable biomarker for response to systemic therapy or recurrence [65]. Therefore, PAP was replaced by the more sensitive marker PSA. However, there is currently new interest in serum PAP as a possible prognostic marker, particularly

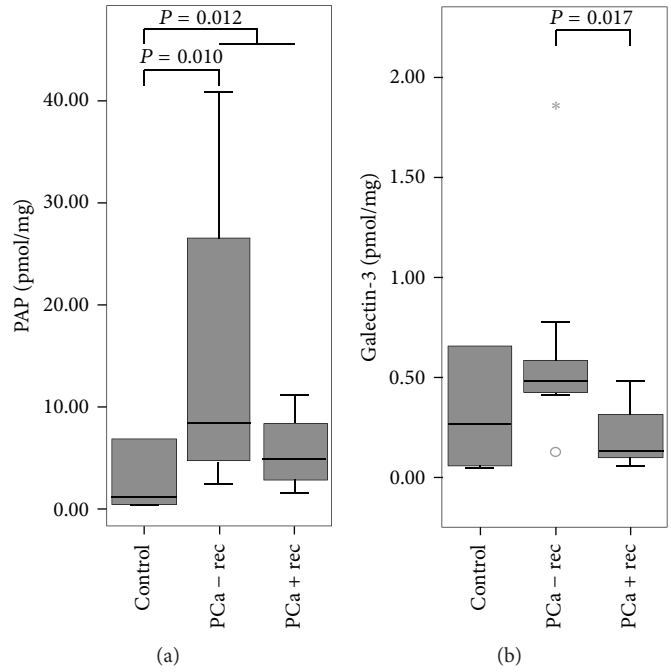


Figure 8: MRM analysis of prostatic acid phosphatase (PAP) peptide FQEELESETLK and galectin-3 peptide IALDFQR in nine urine samples obtained from prostate cancer (PCa) patients without relapse (− rec), seven urine samples from patients with relapse (+ rec), and seven samples from control patients. (a) PAP showed significantly higher protein levels in urine of prostate cancer patients compared to control patients urine ($P = 0.012$), while galectin-3 showed significantly lower protein levels in urine of prostate cancer patients with recurrence compared to urine of prostate cancer patients without recurrence ($P = 0.017$) (b).

in the prognosis of intermediate to high-risk prostate cancer [59, 60]. Fang et al. showed that, in higher-risk prostate cancer, PAP could better predict cancer-specific survival after treatment than PSA concentration and the Gleason score [58]. Surprisingly little is known about PAP as a potential biomarker for prostate cancer in urine. In 1991, Bogdanowicz et al. published a study which compared PAP and PSA levels in urine of prostate cancer patients. They found that PSA and PAP levels increased with each advancing clinical stage. PAP was elevated in 77% of patients with metastatic cancer [66]. Because we found a higher PAP abundance in recurrent prostate cancer compared to nonrecurrent prostate cancer in our initial 2D-DIGE study of prostate cancer tissue, we used MRM-MS to determine the PAP concentrations in the urine of prostate cancer patients with and without relapse. The detected concentration of PAP was significantly higher in urine of patients with recurrence compared to urine of patients without recurrence and in the control urine. Our results therefore support the hypothesis that PAP might be a good noninvasive prognostic biomarker candidate for prostate cancer. Moreover, PAP might be a noninvasive, urinary biomarker candidate for recurrent prostate cancer. As our patients' cohort was small, however, further analysis is needed to validate PAP as a promising noninvasive, urinary biomarker candidate for recurrent prostate cancer.

The 2D-DIGE experiments revealed galectin-3 as another potential new biomarker candidate for recurrent prostate cancer. Galectin-3 is similar to PAP which is already discussed as a potential biomarker candidate for recurrent prostate cancer in the literature. Galectin-3 levels have been found to be significantly decreased in primary carcinomas and metastatic disease compared to normal and premalignant prostatic tissue [61]. Normal glands show moderate galectin-3 expression in the nucleus and cytoplasm. In contrast, galectin-3 is not expressed or its expression is decreased in prostatic cancer cells and it is only present in the cytoplasm [62, 67]. Moreover, cytoplasmic expression of galectin-3 was associated with PSA relapse in univariate analysis [62]. Multivariate analysis revealed galectin-3 as an independent predictor for PSA relapse after the Gleason score and pathological stage [62]. Thus, galectin-3 promotes tumor progression when expressed in the cytoplasm but shows antitumor activity when expressed in the nucleus [62, 68]. Additionally hormone-refractory tumors showed lower galectin-3 expression than hormone-sensitive tumors [63]. Interestingly, galectin-3 is downregulated in the serum of prostate cancer patients compared to serum of healthy controls [69].

To the best of our knowledge, galectin-3 levels in urine of prostate cancer patients have not yet been studied. The only published data for galectin-3 in urine is a combined assay of galectin-3 methylation specific PCR in combination with GSTP1 (Glutathione S-transferase P1) promoter methylation. In that study, galectin-3 promoter methylation had 100% specificity and could be detected in 22/22 urine samples of patients [70]. In our study, we analyzed galectin-3 levels in urine of prostate cancer patients using MRM. Consistent with our 2D-DIGE results and other published data, we were able

to detect galectin-3 downregulation in the urine of prostate cancer patients with recurrence compared to patients without recurrence. Thus, galectin-3 levels in urine are a promising biomarker candidate for the prediction of prostate cancer progression.

2D-DIGE analysis also revealed lower secernin-1 abundance in recurrent prostate cancer compared to nonrecurrent prostate cancer. Validation of secernin-1 expression using tissue Western blots, immunohistochemical staining, and immunohistochemical staining of an independent TMA set showed a significant downregulation of secernin-1 in prostate cancer tissue compared to tumor-free tissue. The downregulation of secernin-1 already starts at the point of PIN. Secernin-1 is described as being deregulated in different carcinomas: secernin-1 is upregulated in colon cancer [71–73], in gastric cancer [73], and in Barrett's esophagus associated with high-grade dysplasia compared to nondysplastic Barrett's esophagus [74]. Moreover, the upregulation of secernin-1 in colorectal cancer compared to normal colorectal tissue correlates with clinicopathological data and is associated with poor prognosis [72]. In contrast to these results, Suehara et al. described secernin-1 as being upregulated in synovial sarcoma with good prognosis compared to synovial sarcoma with poor prognosis [75]. In prostate cancer, Ashida et al. found KIAA0193 (secernin-1) to be downregulated during the transition from PIN to prostate cancer using genome-wide gene expression profiling [6]. In summary, secernin-1 is up regulated in many tumors, but we and others have demonstrated that secernin-1 is downregulated in prostate cancer tissue compared to tumor-free prostatic tissue. Thus, secernin-1 is a novel potential biomarker candidate for recurrent prostate cancer.

Our immunohistochemical analysis revealed secernin-1 expression in the basal layer, while prostatic luminal cells showed no secernin-1 expression. Notably, some of the tumor glands of the immunohistochemically analyzed tumor sample set in the explorative phase showed a weak secernin-1 expression (data not shown). This secernin-1 expression could not be confirmed in the independent TMA obtained from the University Hospital Bonn. Thus, it appears that secernin-1 is mostly expressed in the basal layer and could possibly be lost during the transition from benign prostate tissue to PIN and prostate cancer. As secernin-1 expression is identical in inflamed and normal prostate tissue, secernin-1 might be a good biomarker candidate for prostate cancer. Secernin-1 level in urine has not been detected by MRM-MS thus far, so the potential of secernin-1 as a noninvasively obtained biomarker candidate in urine from prostate cancer patients is still part of our ongoing work.

Our 2D-DIGE analysis showed that vinculin abundance is higher in recurrent prostate cancer compared to nonrecurrent prostate cancer tissue. Ruiz et al. showed that vinculin is not expressed or only expressed at a very low level in benign prostate hyperplasia. In 92% of the localized prostate cancer samples, vinculin showed a low expression level. Vinculin showed high expression levels in 16% of castration-resistant prostate cancer samples and a moderate expression level in 34% of castration resistant prostate cancer samples [76]. Our TMA analysis also showed that vinculin is significantly upregulated in prostate cancer tissue compared to tumor-free prostatic tissue. This upregulation can already be found in PIN lesions. The Western blot analysis of urine confirmed the significant upregulation of vinculin in prostate cancer patients. Moreover, vinculin showed higher protein levels in the urine of prostate cancer patients with recurrence compared to the urine from patients without recurrence. Thus, future analysis of a larger number of patient samples may validate the potential of vinculin as a biomarker candidate for recurrent prostate cancer. Pang et al. found that vinculin isoform 2 was downregulated in the lymph nodes of metastatic cancer patients compared to lymph node-negative cancer tissues [33]. Thorsen et al. described an alternative splicing variant of vinculin in colon, bladder, and prostate cancer where exon 19 of the vinculin gene is skipped in the cancer tissue, and the long isoform was downregulated in metastatic prostate cancer compared to localized cancer [77]. These two studies showed that different vinculin isoforms may be differentially expressed in prostate cancer. Thus vinculin expression and vinculin isoforms in prostate cancer should be analyzed carefully in the future.

5. Conclusion

2D-DIGE combined with MS is a labor-intensive and expensive method with a low throughput of the 2D-DIGE. But the advantages of the proteomic methods compared to DNA methods are magnificent: most functional information about cancer-related genes are placed in the proteome [78] and the discordance of mRNA and protein level elucidates the advantage of proteomic methods [79, 80]. Moreover, most of FDA-approved diagnostic and prognostic tests for cancer (therapies) are protein-based, which makes it easier to transfer results of protein studies to clinical useful tools than results of genome-based studies [81]. 2D-DIGE enables the separation of thousands of proteins [82], including protein isoforms [32] and posttranslational modifications [83]. Moreover, it has a linear detection rate of >10.000 of protein abundances [39, 82]. Unfortunately, very big or very small proteins, plasma membrane-associated proteins, hydrophobic, very alkaline, or very acidic protein scan not be detected using 2D-DIGE [84]. Moreover, gel-to-gel variability is around 20%. Therefore, 3–5 biological replicates per analyzed group are needed to detect expression differences of a protein from 1.5 to 2 [85]. These disadvantages can be minimized by separating one sample and a standard in each gel [86]. Label-free proteomic approaches are an alternative to 2D-DIGE for analyzing the proteome. But Megger et al. could show that different proteins can be detected by 2D-DIGE and label-free methods. Therefore, both methods complement to each other [53].

In conclusion, we have successfully demonstrated that 2D-DIGE combined with MS is a powerful tool to identify differentially expressed proteins in prostate cancer tissue. Galectin-3 was verified as a potential prognostic biomarker candidate by analyzing prostate cancer patient urine samples with MRM-MS. MRM-MS was also found to be suitable for quantifying PAP levels in urine. Secernin-1 has been successfully validated as a potential diagnostic biomarker candidate

for prostate cancer in tissue using Western blot analysis. Vinculin expression has been shown to be upregulated in prostate cancer patient tissue samples, while the urine of recurrent prostate cancer patients showed higher vinculin levels than nonrecurrent prostate cancer patients. Although larger-scale validation studies involving more patients are still needed, vinculin and galectin-3 seem to be promising urinary biomarker candidates for recurrent prostate cancer, while secernin-1 seems to be a useful tissue diagnostic biomarker candidate for prostate cancer.

Conflict of Interests

The authors declare that they have no conflict of interests regarding the publication of this paper.

Acknowledgments

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Clinical Study

Simultaneous Treatment with Statins and Aspirin Reduces the Risk of Prostate Cancer Detection and Tumorigenic Properties in Prostate Cancer Cell Lines

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Nowadays prostate cancer is the most common solid tumor in men from industrialized countries and the second leading cause of death. At the ages when PCa is usually diagnosed, mortality related to cardiovascular morbidity is high; therefore, men at risk for PCa frequently receive chronic lipid-lowering and antiplatelet treatment. The aim of this study was to analyze how chronic treatment with statins, aspirin, and their combination influenced the risk of PCa detection. The tumorigenic properties of these treatments were evaluated by proliferation, colony formation, invasion, and migration assays using different PCa cell lines, in order to assess how these treatments act at molecular level. The results showed that a combination of statins and aspirin enhances the effect of individual treatments and seems to reduce the risk of PCa detection (OR: 0.616 (95% CI: 0.467–0.812), $P < 0.001$). However, if treatments are maintained, aspirin (OR: 1.835 (95% CI: 1.068–3.155), $P = 0.028$) or the combination of both drugs (OR: 3.059 (95% CI: 1.894–4.939), $P < 0.001$) represents an increased risk of HGPCa. As observed at clinical level, these beneficial effects *in vitro* are enhanced when both treatments are administered simultaneously, suggesting that chronic, concomitant treatment with statins and aspirin has a protective effect on PCa incidence.

1. Introduction

Nowadays prostate cancer (PCa) is the most common solid tumor in men from industrialized countries and the second leading cause of death [1, 2]. For this reason, the discovery of modifiable risk determinants would provide an opportunity to prevent or delay the onset of this common disease. Established risk factors, such as ancestry or family history, are not modifiable, and the evidence for dietary and lifestyle risk factors is inconclusive [3].

At the ages when PCa is usually diagnosed, mortality related to cardiovascular morbidity is high [4]. Associations between coronary heart disease and PCa risk have even been suggested [5]; men at risk of PCa frequently receive chronic lipid-lowering and antiplatelet treatment [6, 7]. Statins and aspirin are two of the most frequently used drugs, and they both have been implicated in prostate carcinogenesis, albeit not without some controversy [8, 9].

Statins (3-hydroxy-3-methyl-glutaryl-coenzyme A reductase inhibitors) are the most commonly used drugs for

Table 1: Characteristics of study groups.

	Nontreated cells	Statins	Aspirin	Statin + aspirin
Men (*n*)	1504	440	160	304
Age (years)	67	68	68	67
BMI (Kg/m^2)	26.9	27.6	27.2	27.8
Serum PSA (ng/mL)	6.9	6.7	6.9	6.8
Positive DRE (%)	540 (36)	160 (36)	50 (31)	106 (35)
Gleason score < 7 (%)	112 (7.4)	32 (7.3)	14 (8.8)	18 (6)
Gleason score = 7 (%)	273 (18.2)	76 (17.3)	38 (23.8)	45 (14.8)
Gleason score > 7 (%)	155 (10.3)	43 (9.8)	18 (11.2)	24 (7.9)

BMI: body mass index, PSA: prostate specific antigen, and DRE: digital rectal examination.

lowering cholesterol [10]. In recent years, a growing amount of evidence on the anticancer effects of statins has become available from laboratory and epidemiological studies. *In vitro* and *in vivo* studies [11] suggest that statins are able to arrest cell cycle progression [12], induce apoptosis [13], reduce inflammation, and impede angiogenesis [14].

Currently, observational studies support that statin users have an unchanged overall risk of PCa; however, some well-designed epidemiological studies have reported a reduced incidence of advanced PCa in this population. Moreover, statins showed an inhibitory effect on the growth of PCa in both *in vitro* and *in vivo* studies [15]. On the other hand, in recent years the role of inflammation in cancer etiology has gained attention, and several studies have suggested that nonsteroidal anti-inflammatory drugs (NSAIDs) may have chemopreventive activity. NSAIDs have been associated with a reduced risk of detection for different malignancies: a reduction of the relative risk of 63% for colorectal cancer, 39% for breast cancer, 36% for lung cancer, and up to 39% for PCa [16]. In particular, the use of acetyl salicylic acid (ASA), the active metabolite of aspirin and a well-known NSAID, is associated with a 15%–55% risk reduction for PCa [17–20]; however, two other studies associated aspirin with increased risk of PCa [21, 22], while others reported no association [23, 24]. Although some controversy exists, the protective effect of aspirin treatment is reinforced by a recent meta-analyses study that showed a decreased overall mortality from cancer in a population using a continuous low dose of aspirin daily. This effect increased according to the treatment time [25]. This effect could be related to the potential role of the inflammation pathway in the development of PCa.

There are many studies based on the influence of statins and aspirin on PCa risk. However, even though it is common clinical practice [26], there is no report describing how a chronic treatment combining both treatments influences the risk of PCa detection. Therefore, the aim of our study was to analyze how the chronic treatment with statins, aspirin, and their combination influenced the risk of PCa detection in a cohort of patients undergoing prostate biopsy (PB) due to elevated prostate specific antigen (PSA) or abnormal digital rectal examination (DRE). We also evaluated the effect of these treatments on tumorigenic properties using different PCa cell lines, in order to corroborate clinical observations at *in vitro* level.

2. Materials and Methods

2.1. Design. We conducted a retrospective case-control study at a single academic center. All participants received detailed information about the study procedure and provided written informed consent before study entry. All procedures were in accordance with the ethical standards established in our country. Internal review board approval was not required for such a nonexperimental study.

2.2. Patients. Between January 2006 and December 2011, 2,408 consecutive Mediterranean men, who had been referred for prostate biopsy due to abnormal DRE and/or serum PSA levels higher than 4 ng/mL, were included in this study.

A total of 1,830 men (76%) were undergoing their first PB, while, for 578 of them, it was a repeat PB procedure. All patients were assessed by anamnesis about cardiovascular comorbidities and risk factors, life style (smoking habit and physical activity), and pharmacological treatments. A total of 1,504 men (62.5%) had not been treated with statins or aspirin, while 440 of them (18.3%) had been treated with statins, 160 (6.6%) with aspirin, and 304 (12.6%) with a combination of statins and aspirin. All of the men who received prior treatment had received it chronically for more than 1 year when this study began, and no significant differences existed among the groups, either in the initial PSA levels, in DRE results, or in the Gleason scores (see Table 1).

2.3. Prostatic Biopsy Technique. The PB was done as an ambulatory procedure with local anesthesia. An end-fire ultrasound transducer (Falcon 2101, B-K Medical, Inc.) and a 16-gauge automatic biopsy needle (Bard, Inc.) were used to perform the biopsy. At least 10 cores, plus 2 to 8 additional ones, according to age and prostate volume, were obtained, according to a modified Vienna nomogram [27].

2.4. Cell Culture. All cell lines were obtained from the American Type Culture Collection (Rockville, MD). PCa cell lines PC3 (isolated from bone metastasis of human prostate carcinoma) and LNCaP (established from a biopsy of a lymph node metastasis of hormone refractory PCa) were maintained in RPMI 1640 medium (Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal bovine

serum (FBS), 2 mM l-glutamine, 100 U of penicillin/mL, 100 μg of streptomycin/mL, and 0.1 mM nonessential amino acids. Cells were grown at 37°C in an atmosphere of 5% CO_2-95% air and 99% humidity.

2.5. Drug Concentrations. Doses of the drugs used in this study were chosen based on previous studies by Murtola et al. [28], where, moreover, the same prostate cancer cell lines were used. Standard doses of simvastatin (STA) have been reported to obtain a peak serum value in the range of 10–100 nM; a concentration slightly above the therapeutic range had been chosen for this study (1 μM) (Sigma-Aldrich). Acetylsalicylic acid (ASA) (Sigma-Aldrich) was used at a concentration of 1 mM based on common plasma salicylate levels with therapeutic doses of aspirin [29].

2.6. Proliferation Analysis. A number of 5×10^4 cells per well were seeded on six-well plates with the appropriate medium. The cells were allowed to attach and were subsequently treated with the indicated drugs (STA, ASA, or STA + ASA) for 7 days, renewing growth medium and treatments daily. Cells were counted by using Neubauer chamber.

2.7. Colony Formation Assay. For the colony formation assay, cells were seeded at low density (250 cells/well in a 12-well plate) and were allowed to grow for 10 days, renewing growth medium and treatments every 2 days. Then, the cells were fixed in paraformaldehyde 4% and stained with crystal violet. Colony formation was quantified by OD reading at 590 nm after 2-hour extraction of the crystal violet stain.

2.8. Migration Assays

Wound Healing. The PC3 cells were allowed to reach confluent monolayer in 24-well plates and were incubated overnight. A straight line (wound) was then gently performed at the bottom of the dish with a 0.5 mm plastic pipette tip. Afterwards, cells were washed, incubated in medium with 2% FBS, with or without treatments, and kept in a computer controlled mini-incubator, which provided a stabilized temperature of 37°C with 95% humidity, 5% CO_2, and optical transparency for microscopic observations. The incubator was fastened to an inverted microscope (Live Cell Imaging CellR, Olympus, Japan) to monitor cell migration. Images were taken with the 4x objective every 30 minutes at predetermined wound sites (3 sites per condition, performed in triplicate) and were analyzed using the Image J software (Wright Cell Imaging Facility, USA). Initial wound area (μm^2) and time needed to close the wound (hr) were the variables used to calculate the migration speed of the cells.

Transwell Migration Assay. LNCaP cells were seeded (10^5 cells/insert) onto transwell inserts (BD Bioscience, USA) with 8 μm diameter pore membranes in medium supplemented with 2% FBS in the presence or absence of treatments. After 48 hours, cells from inserts were fixed with 4% paraformaldehyde (PFA) and stained with 0.1% crystal violet. Migration was quantified by OD reading at 590 nm after 2-hour extraction of the crystal violet stain.

2.9. Transwell Matrigel Invasion Assay. PCa cells were seeded (10^4/insert) onto Matrigel Invasion Chamber inserts (BD Bioscience, USA) with 8 μm diameter pore membranes in triplicate in medium supplemented with 2% FBS in the presence or absence of treatments and with the lower chamber containing medium supplemented with 10% FBS. After 48 hours, the number of invading cells was determined as described above (*transwell migration assay*).

2.10. Western Immunoblotting. Cultured cells, washed with cold PBS, were scratched with RIPA buffer (Tris 20 mM pH 8.8, NaCl 150 mM, EDTA 5 mM, Triton X-100 1%, and protease inhibitors) and supernatant boiled at 100°C for 5 min with Laemmli buffer. The same amount of protein was resolved by 10% SDS-PAGE and transferred onto a PVDF membrane (Immobilon P^{SQ}, Millipore, Massachusetts, USA). Membranes were blocked in TBS with 5% nonfat dried milk and 0.1% Tween 20 for 1 h prior to incubation at 4°C overnight with the appropriate primary antibody (1 : 200 α2-integrin, Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1 : 400 cyclin D1, Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1 : 50 vimentin, Sigma-Aldrich, Saint Louis, Missouri, USA; 1 : 2000 E-cadherin, BD Transduction Laboratories, Palo Alto, CA, USA; 1 : 1000 α-tubulin, Sigma-Aldrich, Saint Louis, Missouri, USA). Detection was done using horseradish-peroxidase-conjugated secondary antibodies and enhanced chemiluminescence reagents (Amersham ECL, GE Healthcare, Buckinghamshire, UK).

2.11. Statistical Analysis. Quantitative variables were expressed as medians, interquartile range, and range. Qualitative variables were expressed as percentages. Mann-Whitney *U* test, Wilcoxon test, and Kruskal-Wallis test were used to evaluate the significance of differences, comparing quantitative variables in two or more groups. Chi-square and Cochran tests were used to assess the normal distribution of patients, according to categorical variables and their correlation. A multivariate analysis through a binary logistic regression was performed to detect independent PC or HGPCa predictors. Odds ratios and 95% confidence interval were estimated. All analyses were calculated using SPSS v.20 (IBM, Inc.) statistical software. Statistical analysis of the cell culture data was performed by means of one-way analysis of variance (ANOVA) (Tukey's posttest).

3. Results

3.1. Observational Clinical Study. Once the final diagnosis was obtained, PCa was detected in 848 of the 2,408 patients included in this study (35.2%), 240 of whom presented with high grade PCa (HGPCa) (28.3%).

The distribution of PCa and, specifically, the HGPCa cases in relation to aspirin and statins treatments is shown in Table 2. Among the 1,504 nontreated men, PCa was detected in 552 (36.7%) without statistical differences when compared

Table 2: Distribution of patients according to the diagnosis of PCa and HGPCa and the treatment with statins, aspirin, and the combination of both treatments.

	PCa detection (%)	*P* value	HGPCa detection (%)	*P* value
Nontreated cells	552/1504 (37)	—	136/552 (24.6)	—
Statins	152/440 (34.5)	0.879	40/152 (26.3)	0.673
Aspirin	64/160 (40)	0.536	24/64 (37.5)	0.034
Statins + aspirin	80/304 (26.3)	0.003	40/80 (50)	<0.001

Table 3: Multivariate analysis of PC risk and HGPCa risk according to the treatment with statins and aspirin respect no treatment.

Treatment	PCa detection*	*P* value	HGPCa detection*	*P* value
Age (years)	1.066 (1.049–1.084)	0.001	1.100 (1.071–1.130)	0.001
BMI (Kg/m^2)	1.018 (0.001–1.047)	0.189	0.989 (0.948–1.032)	0.616
PSA (ng/mL)	1.024 (1.008–1.041)	0.004	1.038 (1.027–1.049)	0.001
DRE (positive versus negative)	1.219 (1.028–1.428)	0.001	1.266 (0.879–1.822)	0.205
Statin (yes versus no)	0.910 (0.728–11.137)	0.430	1.092 (0.725–1.646)	0.637
Aspirin (yes versus no)	1.150 (0.824–1.604)	0.439	1.835 (1.068–3.155)	0.028
Statins + aspirin (yes versus no)	0.616 (0.467–0.812)	<0.001	3.059 (1.894–4.939)	<0.001
Time on statins (months)	0.998 (0.955–1.000)	0.063	1.005 (1.000–1.010)	0.034
Time on aspirins (months)	0.984 (0.979–0.990)	<0.001	1.033 (1.020–1.047)	<0.001

BMI: body mass index, PSA: prostate specific antigen, and DRE: digital rectal examination. *Values expressed as odds ratio (95% confidence interval).

to men who were only treated with statins or those who were only treated with aspirin. In the statin-only subset, PCa was detected in 152 of 440 patients (34.5%) (*P* value = 0.879), and in the aspirin-only subset 64 PCa cases were detected (40%) (*P* value = 0.536). Interestingly, in the group of 304 patients that received concomitant treatment with statins and aspirin, PCa was detected in significantly fewer cases (26.3%) when compared to the nontreated group (*P* value = 0.003).

Regarding the rate of PCa patients who presented with HGPCa, we observed that, in the nontreated group, 24.6% of the PCa patients presented with HGPCa (136/552), whereas the aspirin group presented a significantly higher incidence rate of HGPCa, 37.5% (24/64) (*P* value = 0.034). This increase was especially noted in men who had been administrated the concomitant treatment with statins and aspirin, achieving a rate of 50% (40/80) (*P* value < 0.001). On the other hand, no statistical difference was observed in PCa patients treated with statins with HGPCa cases having a rate of 26.3% (*P* value = 0.673).

After the analysis of the above results, a binary logistic regression was conducted to evaluate if the treatment with statins and aspirin could be an independent predictor of the PCa risk and HGPCa incidence. Age, serum PSA, DRE and body mass index (BMI), statins, aspirin, and the combination between statins and aspirin treatments as well as the duration of those were included as predictive variables in this analysis (Table 3). As expected, age and serum PSA were PCa predictors. The reduction in overall PCa incidence was statistically significant for current users of both drugs. Statins and aspirin used as chronic concomitant treatment were an independent predictor factor of reduction of PCa detection risk, OR: 0.616 (95% CI: 0.467–0.812), $P < 0.001$.

Regarding the risk of high grade tumors detection, treatment with aspirin, OR: 1.835 (95% CI: 1.068–3.155), $P = 0.028$, and combined therapy, OR: 3.059 (95% CI: 1.894–4.939), $P < 0.001$, were independent factors of HGPCa. Furthermore, the length of aspirin treatment was a significant predictor of PCa, OR: 0.998 (95% CI 0.979–0.990), $P < 0.001$, while the length of statins treatment, OR: 1.005 (1.000–1.010), $P = 0.034$, and aspirin, OR: 1.033 (1.020–1.047), $P < 0.001$, was a predictor of HGPCa.

3.2. In Vitro Effects of Simvastatin, Acetylsalicylic Acid, and Their Combination. Considering the clinical observations that have been mentioned above, STA and ASA were used to treat different PCa cell lines, in order to unveil by which mechanisms the chronic treatment with these drugs significantly decreased the PCa incidence.

3.2.1. Effect on the Proliferation of Prostatic Cancer Cells. Firstly, after 7 days of treatment the effects of STA, ASA, and their combination on proliferation were studied in PC3 and LNCaP PCa cell lines. The results showed a significant decrease in the proliferation rate of PC3 treated with STA (62%; *P* value = 0.001) and with the combination of both (68%; *P* value < 0.001); however, no effects were observed due to the presence of ASA in the culture media compared to control cells (cells treated only with DMSO). On the other hand, only the combination of STA and ASA was able to markedly reduce the proliferation in LNCaP cells (90%; *P* value = 0.002) (Figures 1(a)-1(b)).

To further study the effect of these treatments on the cell cycle, we analyzed the levels of cyclin D1 protein, a key protein in regulation of cell cycle. As expected, cyclin D1 showed a significant decrease in protein levels in those groups in which we also obtained a most marked decrease in cell proliferation (Figures 1(c)-1(d)).

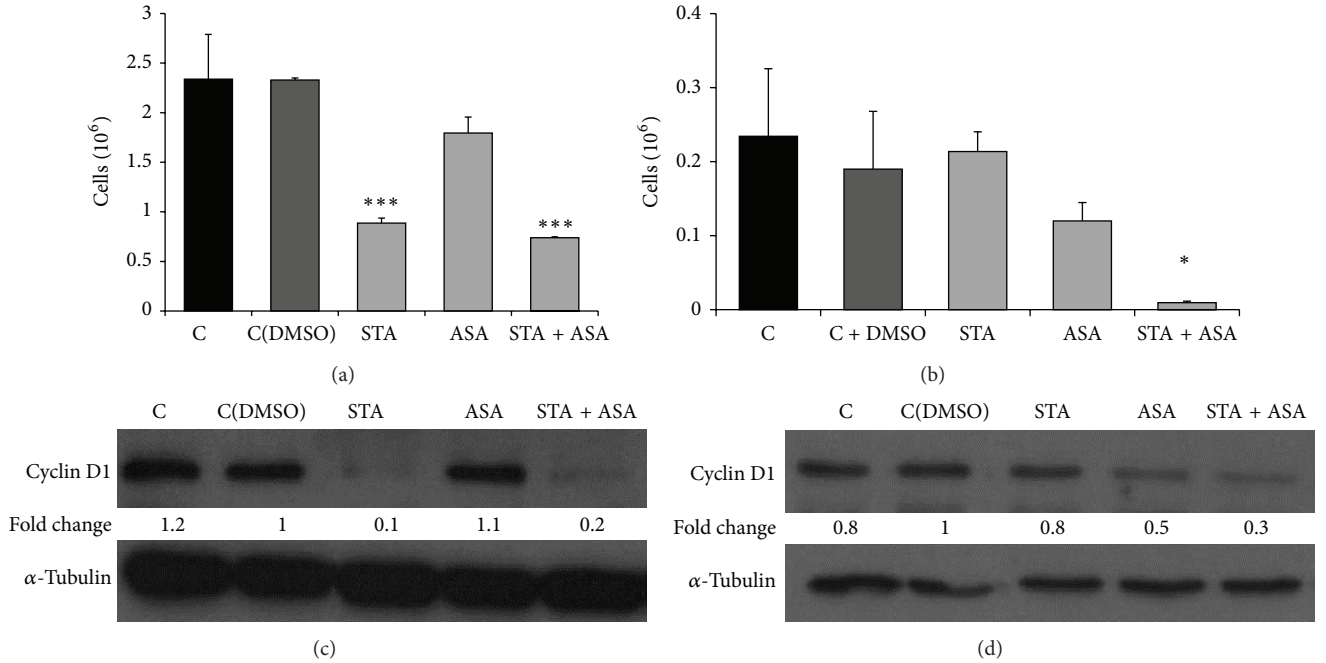

Figure 1: Effect of STA, ASA, and the combination of both treatments on prostate cancer cell lines proliferation. Values for proliferation are presented as number of cells ($*10^6$) after 7 days of treatment for (a) PC3 and (b) LNCaP cell lines with different conditions: nontreated cells, control (C) and control with DMSO (C(DMSO)), and cells treated with statin (STA), aspirin (ASA), and a combination of both statin and aspirin (STA + ASA) (for further details, see Section 2). The bars represent the mean ± SEM. Values that are significantly different by one-way analysis of variance (ANOVA) from the C(DMSO) group are indicated by $^{*}P < 0.05$, $^{***}P < 0.001$. Western blots of cyclin D1 (37 KDa) and the control α-tubulin (52 KDa) performed on total cell extracts after 7 days of treatment are presented for PC3 (c) and LNCaP (d) cell lines. Densitometry analysis using Image J software is represented below bands as fold change values (versus C(DMSO)).

3.2.2. Effect on Colony Formation Capacity. PC3 cells were grown at low cell density (250 cells/well) in the absence or presence of the indicated concentration of STA, ASA, and the combination of both STA and ASA for 10 days. PC3 cells formed colonies, as described by others [30]; however LNCaP cells did not show the same ability to grow from so low cell density. Regarding treatment effects, long-term ASA treatment inhibited cell colony formation ability compared to control cells which had been receiving DMSO in 36% (P value < 0.001); furthermore, this effect was even higher in those cells treated with STA (91%; P value < 0.001) and the combination of both drugs (98%; P value < 0.001) (Figure 2).

3.2.3. Effect on Migration and Invasion Capacity. In addition to cell growth and proliferation, cell mobility was studied as an important step in tumor progression; therefore, we analyzed the invasion and migration abilities of PC3 and LNCaP cells.

First, using transwell assay the invasion capacity was assessed on PC3 and LNCaP cell lines. The number of PC3 that migrated to the bottom of the transwell chambers significantly decreased compared to the nontreated group (cells with DMSO) after being treated for 48 h with ASA (48%) and STA + ASA (48%) (Figures 3(a) and 3(c)). Unexpectedly, no evident changes were found for LNCaP invasiveness (Figures 3(b) and 3(d)).

Second, the migration ability of both tumoral cell lines was analyzed by using wound-healing assays in PC3 line because of their ability to form a monolayer and transwell assay in LNCaP. STA and the combination of STA and ASA caused significant inhibition of PC3 migration after 48 h (61% and 57%, resp.) (Figure 4(a)) as these treatments do not allow the cells to close the wound (Figure 4(c)). On the contrary, no effect for the different treatments was observed in LNCaP cell lines (Figure 4(b)).

The lethal consequences of HGPCa are related to its metastasis to other organ sites, especially bones. Epithelial-to-mesenchymal transition (EMT) has received considerable attention as a paradigm to explain invasive and metastatic behavior during PCa progression as well as other tumors. Cells undergoing EMT lose their epithelial morphology, reorganize their cytoskeleton, and acquire a motile phenotype through the up- and downregulation of several molecules including tight and adherent junctions proteins and mesenchymal markers [31]. Bearing this in mind, the final aim of the present investigation was to see which proteins could be related to these effects on cell invasion and migration. The levels of some of those proteins, which play an important role during EMT, were analyzed (α2-integrin, vimentin, and E-cadherin for PC3 and α2-integrin for LNCaP). The results showed that, after 7 days of treatment with ASA and the combination of STA and ASA, the levels of α2-integrin increased slightly in PC3 cells. In the case of LNCaP,

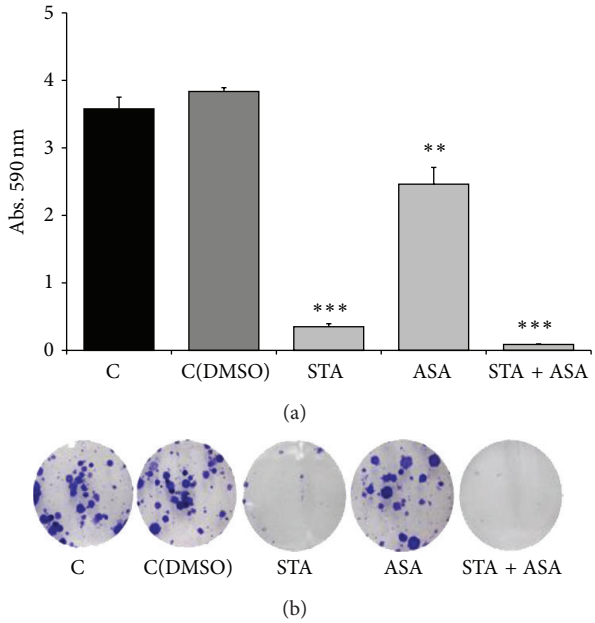

Figure 2: Effect of STA, ASA, and the combination of both treatments on PC3 cell line colony formation capacity. Values for colony formation capacity are presented as crystal violet absorbance/well after 10 days of treatment for PC3 cell line with different conditions: nontreated cells, control (C) and control with DMSO (C(DMSO)), and cells treated with statin (STA), aspirin (ASA), and a combination of both statin and aspirin (STA + ASA) (for further details, see Section 2). (a) The bars represent the mean ± SEM. Values that are significantly different by one-way analysis of variance (ANOVA) from the C(DMSO) group are indicated by $^{**}P < 0.01$, $^{***}P < 0.001$. (b) An inverted microscope with phase contrast at 10x magnification was used to take images of stained colonies.

α2-integrin slightly increased in cells treated with ASA and increased more markedly with the combination of both treatments compared to the control cells (Figures 3(c) and 3(d)). On the other hand, no change was observed in the other proteins analyzed.

4. Discussion

PCa predominantly affects older men, who are likely to have comorbidities, such as cardiovascular conditions, that often indicate treatment with aspirin and statins. This find is in keeping with some authors, who have reported a significant correlation between PCa and coronary disease prevalence [5] as well as the fact that cardiovascular events are usually the first cause of death in patients with PCa [4].

In the last years some publications on this field investigated the relation between the uptake of statins and aspirin and PCa incidence; however, the impact of chronic treatment with statins and aspirin in prostate carcinogenesis is controversial. In 2008, a meta-analysis that included 6 randomized trials, 6 cohort studies, and 6 case-control studies concluded that there was neither association between statin therapy and PCa risk nor the time of treatment administration [9]. However, other studies observed a protective effect of statins on diagnosing PCa in advanced stages [9]. A similar study, where 4,202 men who had undergone PB were analyzed [32], showed that 24% of the patients that had been treated with statins at the time of PB presented a PCa detection rate of 45% compared to 42% in those patients who had not received statins. Furthermore, the Gleason score was 7 or higher in 72% of the tumors detected in chronic statins users compared to 62% in those patients not using this drug. However, the rate of patients with a Gleason score 8 or higher in radical prostatectomy specimens was 14% in both groups. This study did not take into account treatment with aspirin. With the same objective, Murtola et al. [33] analyzed the impact of statins in a series of 23,320 men (28% of them received statins) who underwent PSA screening between 1996 and 2004. The authors concluded that this treatment reduced the overall incidence of PCa, even after removing the bias produced by its effect on serum PSA. Moreover, they observed an association between time of statins therapy and the risk of PCa detection, without any relation to tumor aggressiveness. This study also monitored the use of other drugs, such as aspirin (17% of patients) and other potential confounders of PCa risk, which did not change the benefit of statins. However, the impact of combination therapy with statins and aspirin was not analyzed in this study. Aspirin has been associated with a reduced risk of PCa by some authors, while others have questioned this correlation. The chemopreventive effect of aspirin and other nonsteroidal anti-inflammatory drugs has been reported in recent data. This effect ranges from 10 to 55% [17, 18, 20, 34–37]. A recent meta-analysis that included 24 studies, 9 case controls with 5,795 men, and 15 cohorts with 31,657 patients has suggested a chemopreventive effect of aspirin on PCa [38].

With the aim of clarifying these controversial studies, we conducted a clinical observational study on 1,504 men undergoing PCa screening. Our study shows that more than a third of the population at screening of PCa was receiving some treatment to prevent cardiovascular disease. Specifically in this cohort, around 38% were chronic patients with 48% receiving statins, 18% receiving aspirin, and 34% receiving both drugs.

We observed that the detection rates of PCa in patients treated with statins or aspirin were statistically similar to the rate observed in patients who were not receiving any of these drugs. However, the detection rate of PCa observed in patients that were treated simultaneously with statins and aspirin was significantly lower (P value = 0.003). Furthermore, with relation to histological grade, our study suggests that chronic treatment with aspirin could be an independent predictor of HGPCa detection as well as the combination therapy with statins and aspirin. This is because, according to our analysis, there was a higher incidence of HGPCa in chronic aspirin (P value = 0.034) users or men receiving concomitant treatment (P value < 0.001).

This is not the first time that an epidemiological study has related the increase of HGPCa tumors in chronic users of aspirin [38]. However, other authors have found an inverse correlation between aspirin and HGPCa rates [39, 40]. Besides that, some studies have shown that statins are able to decrease serum PSA levels [41]. This could be considered

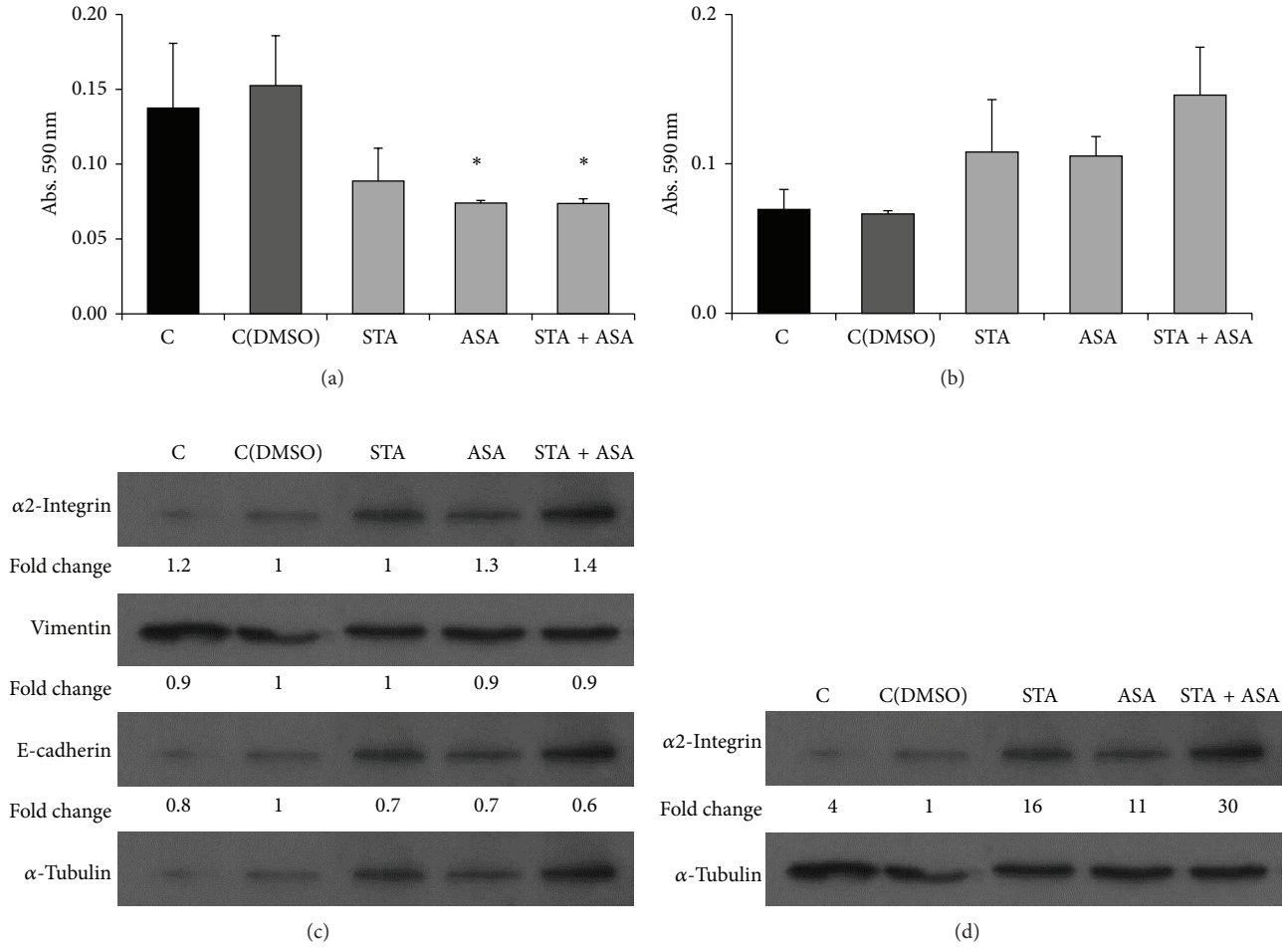

Figure 3: Effect of STA, ASA, and the combination of both treatments on prostate cancer cell lines invasion capacity. Values for invasion are presented as crystal violet absorbance/well for PC3 (a) and LNCaP (b) cell lines after 48 h of treatment with different conditions: nontreated cells, control (C) and control with DMSO (C(DMSO)), and cells treated with statin (STA), aspirin (ASA), and a combination of both statin and aspirin (STA + ASA) (for further details, see Section 2). The bars represent the means ± SEM. Values that are significantly different by one-way analysis of variance (ANOVA) from the control (C = nontreated) group are indicated by $^{*}P < 0.05$ (versus C(DMSO)). Western blot of α2-integrin (150 KDa), vimentin (58 KDa), E-cadherin (120 KDa), and the control α-tubulin (52 KDa) was performed on total cell extracts after 7 days of treatment for PC3 cell line (c) and α2-integrin (150 KDa) and the control α-tubulin (52 KDa) for LNCaP (d). Densitometry analysis using Image J software is represented below bands as fold change values (versus C(DMSO)).

as one possible explanation that could clarify the controversy on the effect of statins in PCa detection. The serum PSA level directly impacts the indication for PB [42, 43], and it may cause a masking bias on the possible protective effect of statins, since a population subgroup would not have biopsy criterion. In this case, the treatments may decrease the risk of PCa but also delay the diagnosis, which could explain the increased detection of HGPCa.

The present study had some limitations, which were also common in several other studies, where the effects of statins and aspirin were evaluated, for example, not being a prospective study or the effect of statins in the serum PSA hindering the evaluation of the influence of this drug in PCa. Then, there is the idea of the chronic treatment concept or not analyzing each drug dose separately, which would not allow us to draw distinct conclusions.

As a secondary objective, the tumorigenic properties of statins and aspirin on different PCa cell lines were studied, as a way to better understand our clinical observations. Thus, taking into account the results obtained *in vitro* in which the proliferation decreased dramatically as well as colony formation capacity after treating the cells with STA and ASA, we were able to hypothesize that statins and aspirin do have antitumoral effects in PCa, as other authors have observed previously [44–46]. Specifically, it could be that either aspirin treatment alone and/or simultaneous treatment may enhance the preventive effect of statins on prostate carcinogenesis at the expense of an increase in the detection of HGPCa. As we mentioned before, we have seen a direct effect of statins on proliferation, which can probably be explained by a marked decrease of cyclin D1 levels. The combination of both treatments could enhance this effect. These results,

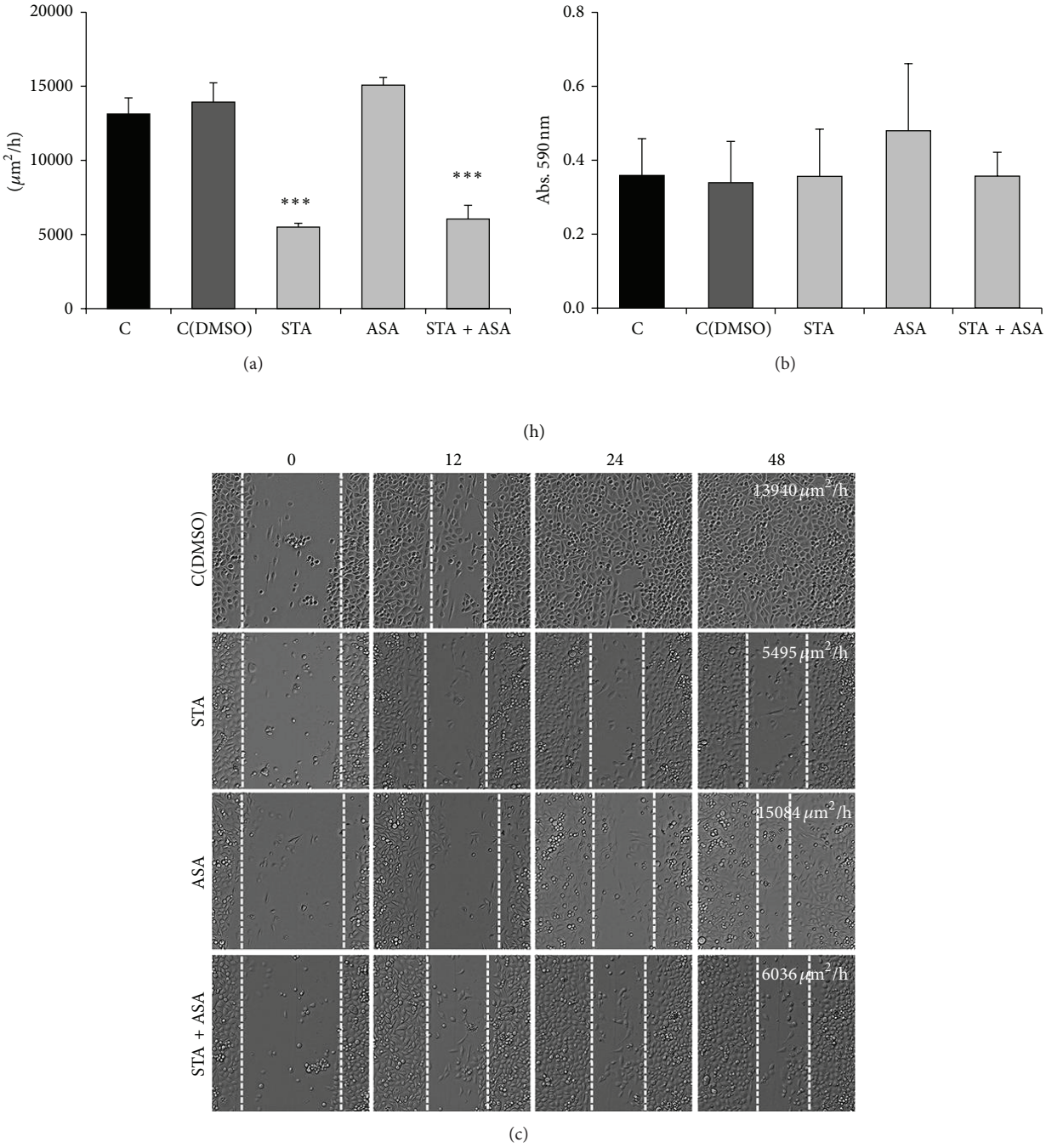

Figure 4: Effect of STA, ASA, and the combination of both treatments on prostate cancer cell lines invasion capacity. Values for PC3 cell line migration capacity (a) are presented as the difference between the wound-healing speed (μm^2/h) before and after 48 h of treatment calculated using Image J software and values for LNCaP migration (b) are presented as crystal violet absorbance/well after 48 h of treatment. Both cell lines were treated with different conditions: nontreated cells, control (C) and control with DMSO (C(DMSO)), and cells treated with statin (STA), aspirin (ASA), and a combination of both statin and aspirin (STA + ASA) (for further details, see Section 2). The bars represent the mean ± SEM. Values that are significantly different by one-way analysis of variance (ANOVA) from the C(DMSO) group are indicated by $^{***}P < 0.001$. (c) Phase contrast 10x images from migration mode during wound-healing assay at 48 h after treatment for PC3 cell line.

in addition to those based on the significant effect of the three treatments being higher when the cells received a concomitant treatment and on colony formation capacity, could be related to clinical observations of patients referred for PB and the incidence of PCa.

The mechanism by which statins influence prostate carcinogenesis could be mediated by the reduction of serum cholesterol and, consequently, by a decrease of intraprostatic dihydrotestosterone levels. Prostate tissues lose homeostatic control over cholesterol levels with age [47]. This effect

could explain the observed differences in the results for each cell line used in our study because, as it is well known, PC3 prostate cancer cells are hormone independent and LNCaP prostate cancer cells are hormone dependent and these properties may influence the cell line response although several studies would be needed to clarify this question. The overall consequence of this cholesterol accumulation on prostate physiology is unknown, but a role for high levels of serum cholesterol in PCa incidence and progression has been suggested by a number of epidemiological and preclinical studies [10–14, 48].

In order to find an explanation for the increased incidence of HGPCa in patients treated chronically with aspirin and simultaneously with statins and aspirin, we should perform more extensive studies. However, in cancer, it is known that the expression of integrins, which are involved in cell adhesion, is frequently altered, leading to cell proliferation, migration, and metastasis. Previous studies have shown that integrin expression levels were correlated to the different stages of human cancer progression [49–51], and it has also been suggested that $\alpha 2$-integrin may be a metastasis suppressor [50]. In contrast, in PCa, $\alpha 2$-integrin was found to induce PCa cell metastasis to the bone [51, 52]. Therefore, integrin function is cell type and context dependent. Keeping in mind what we obtained, a moderate increase of these protein levels *in vitro* after ASA and concomitant treatment of STA and ASA in PC3 cell line, it could be related to those patients with a higher incidence of HGPCa. On the other hand, after performing invasion and migration assays on both PCa cell lines, the concomitant treatment seemed to present antitumorigenic effects. No changes in vimentin and E-cadherin were observed *in vitro,* which makes us think that the decrease in migration might not be related to changes in phenotype EMT. Therefore, a different approach is necessary to clarify the role of integrin and how other proteins could be implicated in the positive properties of statins and aspirin in PCa and HGPCa.

5. Conclusions

Although only a randomized, prospective trial that controls for patient age, comorbidities, adjuvant treatments, and dose stratification will prove the benefits of statins and aspirin treatment, the collection of all of the results presented in this study suggests that chronic concomitant treatment with statins and aspirin does have a protective effect for PCa incidence. Statins and aspirin have antitumorigenic properties, and these beneficial effects are enhanced when both treatments are administered simultaneously.

Conflict of Interests

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

Authors' Contribution

A. Doll and J. Morote contributed equally to this work.

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Review Article

From Bench to Bedside: Immunotherapy for Prostate Cancer

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The mainstay therapeutic strategy for metastatic castrate-resistant prostate cancer (CRPC) continues to be androgen deprivation therapy usually in combination with chemotherapy or androgen receptor targeting therapy in either sequence, or recently approved novel agents such as Radium 223. However, immunotherapy has also emerged as an option for the treatment of this disease following the approval of sipuleucel-T by the FDA in 2010. Immunotherapy is a rational approach for prostate cancer based on a body of evidence suggesting these cancers are inherently immunogenic and, most importantly, that immunological interventions can induce protective antitumour responses. Various forms of immunotherapy are currently being explored clinically, with the most common being cancer vaccines (dendritic-cell, viral, and whole tumour cell-based) and immune checkpoint inhibition. This review will discuss recent clinical developments of immune-based therapies for prostate cancer that have reached the phase III clinical trial stage. A perspective of how immunotherapy could be best employed within current treatment regimes to achieve most clinical benefits is also provided.

1. Introduction

Prostate cancer (PCa) is the most frequently diagnosed cancer in men and the sixth leading cause of death from cancer in men worldwide [1]. An estimated 903,000 men were newly diagnosed, and 258,000 died from PCa in 2008 [1]. When diagnosed at its early stages, PCa can be effectively treated by surgery or radiation. However, up to one-third of patients with organ-confined PCa eventually fail local therapy and ultimately progress to advanced-staged or metastatic disease within 10 years [2]. Approximately 4% of all newly diagnosed patients present with metastatic disease, and up to 85% of patients diagnosed with CRPC have metastases [3].

2. Current Therapeutic Options for PCa

The normal prostate and PCa require androgens for growth and optimal function of cell survival pathways [4]. After local surgery or radiation, cancer control is monitored by Prostate Specific Antigen (PSA), a prostate epithelial-specific protein detected from secretions into the blood stream. The suppression of androgens by androgen deprivation therapy (ADT) alone or together with androgen receptor (AR) antagonists initially induces tumour regression and a period of cancer control, accompanied by nondetectable or exceedingly low PSA levels [5]. Ultimately, patients relapse, signalled by a rise in PSA, and develop CRPC. Docetaxel (Taxotere) was the first chemotherapy drug to show improved survival for patients with CRPC, compared to the then standard of care, mitoxantrone [6, 7]. More recently, cabazitaxel was FDA-approved for patients who fail docetaxel therapy on the basis of prolonged survival [8]. The seminal finding that PCas can undertake *de novo* steroidogenesis and synthesis of androgens and other steroids that reactivate the AR [9] has underpinned the rationale for developing the steroid

synthesis CYP17A1 inhibitor, abiraterone; this was approved for clinical use after landmark trials showing extended survival after docetaxel failure [10, 11]. The new AR antagonist, enzalutamide, is the latest drug to be FDA-approved in CRPC, again on the basis of prolongation of survival compared to placebo [12]. Cancer immunotherapy has recently been introduced into the therapeutic landscape for CPRC following the approval of the dendritic-based cancer vaccine sipuleucel-T (Provenge) by the FDA in 2010 [13]. The goal of immunotherapy is to harness the powerful capabilities of the immune system, comprising both the adaptive and innate arms, to effectively recognise and kill transformed cells whilst sparing healthy tissues. For excellent reviews on the mechanisms of antitumour immunity induction, see [14–16]. Currently, various forms of immunotherapy are being investigated in clinical trials for PCa including dendritic cell-based vaccines, immune checkpoint inhibition, viral-based vector, and whole cell-based vaccines.

3. Immunotherapy Is Rational for PCa Treatment

3.1. Androgen Deprivation and Immune Changes. Over the past decade, strong evidence that PCa is inherently immunogenic has emerged, which underpins the rationale for using immune-based therapies for this disease. PCa can stimulate immune responses, as evidenced by induction of T cell responses to cancers by various immunotherapies (see later sections), and by histological data revealing the presence of CD4+ T cells, CD8+ T cells, natural killer (NK) cells, dendritic cells, and macrophages within tumours. Early studies reported that prostate tumours with a dense infiltration of lymphocytes correlated with longer patient survival and that high grade prostatic adenocarcinomas have significantly less infiltration of T cells and macrophages as compared to benign nodular prostatic hyperplasia [25, 26], suggesting that tumour progression could be associated with defects in cell-mediated immune responses. Although subsequent studies found that greater tumour infiltration of CD4+ T cells can predict poorer prognosis [27], we now appreciate that a proportion of these were likely regulatory T cells (Tregs), which suppress immune responses through their inhibitory actions at both the induction and effector phases. High tumour infiltration of forkhead box P3- (foxp3-) expressing cells (Tregs) was also found to correlate with higher baseline PSA levels [28]. A high prevalence of regulatory T cells within tumours is associated with more lethal PCa [29], suggesting that therapeutic blockade of these cells may induce beneficial clinical responses, by facilitating the generation of effective cytotoxic immune responses. Increased NK cell infiltrate within tumours was also found to be associated with a lower risk of progression, providing evidence that these innate immune cells may have a protective role against PCa in humans [28]. A recent study reported that in organ-confined PCa, the prevalent macrophage phenotype was M1 (tumour-inhibitory, promotes Th1 responses, proinflammatory; defined as CD68+ by those authors) whereas in PCas with extracapsular extension, M2 macrophages (tumour-promoting, proangiogenic, promotes Th2 responses; defined as CD163+) were more prevalent [30]. These findings, together with another observation of reduced infiltration of CD68+ macrophages associated with lymph node positivity and higher clinical stage [31], indicate that reduced numbers of macrophages with cytotoxic capabilities parallel more aggressive disease; hence immune interventions that shift the balance of macrophages to the M1 phenotype may have antitumour effects. Despite this, it is important to note that other studies have reported infiltration of CD68+ macrophages to correlate with poorer patient outcomes [28, 32]. While there is strong evidence that PCa is immunogenic, it is also known that these cancers employ numerous immune escape strategies that contribute to their growth and progression. Such escape mechanisms include defects in antigen presentation (e.g., downregulation of HLA class I antigens and beta-2 microglobulin to escape killing by cytotoxic T cells), induction of T cell death (e.g., expression of Fas ligand to trigger apoptosis of Fas-expressing immune cells), production of immunosuppressive cytokines (e.g., transforming growth factor-β), and recruitment of regulatory T cells, which limit immune responses [33]. These are challenges that immunotherapies need to overcome, in order to deliver clinical benefits to PCa patients.

Realistically, if immunotherapies are to be incorporated into treatment regimens for PCa, they would most likely be employed as adjunct therapies to ADT, the mainstay approach for both high-risk early PCa and recurrent or metastatic disease. Further evidence in support of the rational use of immune-based therapies for PCa is that ADT seems to enhance the influx of immune cells into tumours. Androgen-depleted patients were reported to have a 5-fold increase in CD4+ T cell infiltration, 2-fold increase in CD8+ T cell infiltration, and 3-fold increase in macrophages, as compared to untreated tumours [34]. Moreover, hormone ablation resulted in an increase in the number of tumour-resident cells expressing the costimulatory molecules B7.1 and B7.2, which are necessary for effective T cell activation [34]. A significant proportion of T cells from patient tumours expressed IFNγ, the proliferation marker Ki67, and exhibited a restricted pattern of T cell receptor (TCR) beta-chain variable region (Vβ) gene usage, an oligoclonal response consistent with an antigen-specific response [34]. Similar findings were demonstrated in another report whereby androgen-depleted tumours had a greater density of total T cells, as well as the CD8+ T cell subset [28]. These results suggest that ADT may augment antitumour immunity by enhancing T cell activation as well as their trafficking to tumours. The relative timing of androgen ablation with immunotherapy would be crucial and warrants investigation.

3.2. Tumour Associated Antigens and Their Role in the Immune Response. The existence of several tumour-associated antigens (TAAs) provides further evidence in support of the view that PCa would be amenable to immunotherapy; these include the serine protease prostate specific antigen (PSA; kallikrein 3), prostatic acid phosphatase (PAP), prostate specific membrane antigen (PSMA), prostate stem cell antigen (PSCA), mucin-1 (MUC-1), and NY-ESO-1 [2]. Although in a steady state these self antigens do not provoke strong

immune responses, due to multiple layers of tolerance mechanisms, nevertheless, several immunotherapeutic strategies have been shown to induce or enhance responses to these antigens [2]. PSA, in addition to being selectively expressed by prostate epithelial cells, is also a well-characterised serum biomarker for PCa progression; therefore it also has utility in the assessment of patient treatment response. Another characteristic of PCa which renders it attractive for immunotherapy is that it is a relatively slowly progressing disease, allowing sufficient time for the immunologic intervention to take effect. In addition, the prostate gland is nonessential for life; therefore even if the immune response destroys normal prostatic tissue, it would not be life-threatening or likely to cause significant morbidity. Thus, immunotherapy is a rational approach for PCa treatment. This review will discuss recent clinical development of immune-based therapies for PCa that have reached the phase III clinical trial stage, to highlight the range of strategies currently being explored and that show promise. These are sipuleucel-T (dendritic cell-based vaccine), ipilimumab (immune checkpoint inhibitor), Prostvac-VF (viral-based vaccine), and GVAX (whole cell-based vaccine) (Table 1).

4. Sipuleucel-T (Dendritic Cell-Based Vaccine)

The rationale for using autologous dendritic cells in cancer immunotherapies is based on their efficient activation of antigen-specific cytotoxic T cells to kill cancer cells. Sipuleucel-T is a dendritic-based immunotherapy in which autologous peripheral blood mononuclear cells (PBMCs) are incubated *ex vivo* for 36–48 hours with a fusion protein (PA2024) of PAP and granulocyte-macrophage colony-stimulating factor (GM-CSF) [35]. An analysis of culture supernatant during the manufacture process showed an increase in antigen presenting cell (APC) activation cytokines (interleukin (IL)-1a, IL-23, macrophage inflammatory protein (MIP)-1a and -1b), T cell activation markers (IL-2, -3, -4, -5, -10, and -17) as well as APC/T cell activation-associated cytokines (IL-12, IFNγ, tumour necrosis factor (TNF)) [36]. After sipuleucel-T treatment of autologous cells, the product containing increased activated APCs and T cells is reinfused into the patient and contains at least 50 million autologous activated CD54+ dendritic cells, and a variable number of T cells, B cells, natural killer cells, and others [37]. Sipuleucel-T immunotherapy targets cells which express PAP, a secreted glycoprotein enzyme that is expressed in 95% of prostate tissue and PCa; expression levels correlate with tumour staging [38, 39]. Moreover, high serum PAP levels are associated with significantly shorter survival and lower responsiveness to radiation therapy [40]. The GM-CSF component of the fusion protein is an immune modulatory cytokine that stimulates the development and maturation of APCs, including type 1 dendritic cells (DC1), the subset responsible for initiation of cytotoxic immune responses [41]. A key preclinical study which laid the groundwork for the clinical development of sipuleucel-T demonstrated that mice immunised with APCs incubated with a fusion protein of rat PAP and rat GM-CSF developed profound prostatitis (marked lymphocyte infiltrate), indicating that this approach could break tolerance to the self-antigen [42, 43]. In a follow-up experiment, coculture of PAP-expressing tumour cells with splenocytes from animals immunised with APCs pulsed with PAP and GM-CSF inhibited tumour cell proliferation [42], showing the potential antitumour effect of the cancer vaccine. These preclinical studies formed the basis for clinical trials of sipuleucel-T.

In a sequential phase I/II clinical trial involving 31 men with progressive disease despite ADT (12 with metastatic disease in the phase I cohort and 19 with nonmetastatic disease in phase II), sipuleucel-T was found to be well-tolerated, with preliminary evidence of clinical efficacy [44]. 100% patients developed T cell proliferative responses to the fusion protein, PA2024, and 38% against native PAP, following sipuleucel-T administration (none had preexisting responses to either antigen), suggesting that immunological tolerance against PA2024 and PAP could be broken by this therapy [44]. Importantly, patients that developed an immune response against PAP, whether in T cell proliferation or antibody development, had significantly longer median time to disease progression as compared to those who did not (34 versus 13 weeks, resp.; $P < 0.027$) [44]. In total, 6 patients had PSA value declines of greater than 25% from baseline. In another phase I clinical trial of 13 patients with similar clinicopathological characteristics, sipuleucel-T was again well-tolerated, and all patients developed measurable antigen-specific T cell responses and antibodies against PA2024 after immunisation [45].

These promising findings led to the commencement of three phase III clinical trials, all of which have now been completed. In the first study (D9901), 127 patients with asymptomatic metastatic CRPC were assigned in a 2 : 1 ratio of sipuleucel-T ($n = 82$) or placebo (infusion of APCs not pulsed with PA2024; $n = 45$) [17]. While there was no difference in the primary endpoint of time to progression (TTP) between the 2 treatment groups (11.7 weeks for sipuleucel-T and 10.0 weeks for placebo), there was a significant increase in the secondary endpoint of overall median survival for those given sipuleucel-T (25.9 months versus 21.4 months) [17]. Moreover, the survival rate at 36 months was significantly higher ($P = 0.005$) for sipuleucel-T treated patients (34%) when compared to placebo (11%). The clinical efficacy of this dendritic-based immunotherapy was confirmed in another similarly designed clinical trial of 98 patients (65 for sipuleucel-T and 33 for placebo) (D9902A) [18]. No difference in TTP was observed between the two groups, although the median survival time was greater for sipuleucel-T-treated patients over placebo (19.0 months versus 15.7 months, resp.), consistent with the prior clinical trial [18]. The 36-month survival rates were 33% (sipuleucel-T) and 15%, (controls). A third phase III clinical trial known as the Immunotherapy for Prostate Adenocarcinoma Treatment (IMPACT) trial was undertaken; 512 patients were involved (341 on sipuleucel-T and 171 on placebo) [19]. Consistent with previous trials, sipuleucel-T treatment provided a 4.1 month improvement in median survival (25.8 months versus 21.7 months), and the 36-month survival rate was 31.7% versus 23.0% [19]. The time to disease progression was similar between the treatment

Table 1: Key clinical trials based on the 4 selected immunotherapies for PCa.

Drug	Trial design	Number of patients	Phase	Key finding	Reference
Sipuleucel-T	Randomized, double blind, placebo controlled trial for asymptomatic metastatic CRPC	127	III	Improved OS by sipuleucel-T compared to placebo (25.9 versus 21.4 months)	[17]
	Randomized, double blind, placebo controlled trial for asymptomatic metastatic CRPC	98	III	Improved OS by sipuleucel-T compared to placebo (19 versus 15.7 months)	[18]
	Randomized, double blind, placebo controlled trial for asymptomatic metastatic CRPC	512	III	Improved OS by sipuleucel-T compared to placebo (25.8 versus 21.7 months)	[19]
Ipilimumab	Randomized, double blind, placebo-controlled trial for metastatic CRPC after docetaxel	799	III	No difference in OS between the 2 groups, but trend of improved PFS rate by ipilimumab at 6 months (30.7% versus 18.1%)	[20]
Prostvac-VF	Randomized placebo-controlled trial of Prostvac-VF for metastatic CRPC	125	II	Improved OS by Prostvac-VF compared to control vector placebo (25.1 versus 16.6 months)	[21]
	Nonrandomized trial for chemotherapy-naive CRPC	32	II	Improved OS by Prostvac-VF compared to historical controls (Halabi nomogram): (26.6 versus 17.4 months)	[22]
GVAX	Randomized trial of GVAX with docetaxel versus docetaxel with prednisone in taxane-naïve patients with symptomatic CRPC	408	III	Trial terminated early due to excess deaths in GVAX plus docetaxel group compared to control (docetaxel plus prednisone) (67 versus 47), and shorter median OS (12.2 versus 14.1 months).	[23]
	Randomized trial of GVAX with docetaxel versus docetaxel with prednisone in taxane-naïve patients with asymptomatic CRPC	626	III	Trial terminated early based on futility analysis showing <30% chance of meeting primary endpoint (improved OS)	[24]

groups. Interestingly, multivariate analysis showed that baseline PSA levels provided a strong prognostic factor, whereby the overall survival hazard ratio in the lowest baseline PSA quartile (<22.1 ng/mL) was 0.51 (0.31–0.85) compared with the highest PSA quartile (<134 ng/mL) of 0.84 (0.55–1.29) [46]. This finding suggests that patients with lower tumour burden are more likely to benefit most from sipuleucel-T and provides a rationale to employ this immunotherapy as an early treatment strategy. Since the 3 aforementioned phase III clinical trials were similarly designed, and recruited patients with similar characteristics, an analysis for immune parameters with overall survival was performed on pooled data ($n = 737$) [36]. Overall survival significantly correlated with development of at least one postbaseline peripheral immune response (T cell proliferation, IFNγ ELISPOT, or antibody production) to PA2024 or PAP (HR = 0.47) (95% CI: 0.29–0.78) [36]. In sipuleucel-T treated patients, a positive correlation between overall survival and cumulative APC activation (increase in CD54 expression on APCs; from pre- to postculture with the fusion protein), APC count and total nuclear cell (TNC) count were observed [36]. Whilst these studies have collectively confirmed the clinical utility of sipuleucel-T for the treatment of advanced PCa, some questions remain as to why progression-free survival is unaffected. One possible explanation relates to how progression is defined, which in these studies is an increase in measurable disease based on radiographic imaging, new cancer-related pain associated with radiographic correlation, or other clinical events consistent with progression such as spinal cord compression or pathological fracture. The development of new lesions could have been interpreted as progression yet the disease was responding to treatment. Another speculation is that the treatment gradually slows down progression, which is reflected in prolongation of overall survival, but short-term improvements are not apparent. In addition, since the mode of action of Sipuleucel-T is to activate the immune system, a delay in treatment effect is expected. It is also important to note that PSA levels generally do not alter in response to Sipuleucel-T, further highlighting the difficulty in assessing the therapeutic response.

5. Ipilimumab (Immune Checkpoint Inhibitor)

T cell responses are initiated through the interaction of the T cell receptor (TCR) with antigen presented in major histocompatibility complex (MHC) molecules on antigen presenting cells (APCs). However, the quality and magnitude of the response are dependent on a fine balance of costimulatory and coinhibitory signals delivered to the T cell; for an excellent review on T cell costimulation see [47]. CD28 (a costimulatory receptor) and cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) (a coinhibitory receptor) are homologues that compete for their ligands, CD80 (B7-1) and CD86 (B7-2) expressed on APCs [47]. CTLA-4 is a key negative regulator of T cell responses and is upregulated following T cell stimulation to attenuate the response. It therefore functions as an immune checkpoint molecule. CTLA-4 is also constitutively expressed on regulatory T cells and mediates their immune suppressive effects [48]. From a therapeutic point of view, CTLA-4 blockade could prevent immune inactivation, leading to robust antitumour immune responses. In a preclinical model of PCa, *in vivo* blockade of murine CTLA-4 with an antibody significantly delayed the growth of subcutaneous TRAMP-C1 tumours [49], derived from Transgenic Adenocarcinoma of the Mouse Prostate (TRAMP) mice [50, 51] which spontaneously develop PCas due to prostate-specific expression of Simian virus 40 (SV40) large T antigen (Tag). In a follow-up study, CTLA-4 blockade prevented and/or slowed the outgrowth of subcutaneous TRAMP-C2 tumours, which spontaneously metastasise to the regional lymph nodes [52]. Moreover, anti-CTLA-4 antibody treatment after surgical resection of the primary tumour facilitated the elimination of lymph node metastases, suggesting that this immunotherapy has potential to be used as an adjunct therapy to eliminate residual metastatic PCa after surgery [52]. Based on these preclinical studies, clinical trials of CTLA-4 blockade for PCa treatment have been initiated.

Ipilimumab is a fully human IgG1 monoclonal antibody that binds to and blocks the activity of CTLA-4. It was approved by the FDA in 2011 for the treatment of advanced melanoma on the basis of prolonged survival [53, 54]. Ipilimumab is currently being trialled for the treatment of non-small cell lung cancer (NSCLC) [55], metastatic renal cell cancer [56], ovarian cancer [57, 58], and PCa. In a phase I clinical trial comprising 14 patients with progressive metastatic CRPC (some of whom previously also received chemotherapy or other investigational drugs), ipilimumab treatment at 3 mg/kg was well tolerated without the development of significant pathological autoimmunity [59]. A trend observed across the cohort was a gradual increase in the percentage of CD4+ HLA DR+ T cells in peripheral blood following ipilimumab treatment [59], consistent with its mode of action in modulating immune activation. Two of 14 patients also had a >50% decline in PSA, which lasted for 135 and 60 days, respectively. In another phase I/II clinical trial involving a similar cohort of PCa patients, ipilimumab was administered alone or in combination with radiotherapy (8 Gy per bone target, up to 3 lesions per patient) [60]. The maximal tolerable dose was determined as 10 mg/kg, with common immune-related adverse events such as diarrhoea, colitis, and rash usually of grade 1/2 [60]. For patients that received this dosage (with or without radiotherapy) ($n = 50$), eight had PSA declines of >50%, one had complete response, and six had stable disease. These observations indicated potential clinical antitumour activity of ipilimumab and led to a phase III clinical trial that compared ipilimumab against placebo after radiotherapy of mCRPC patients who have failed docetaxel ($n = 799$) [20]. While there was no significant difference in terms of overall survival (primary endpoint) between the ipilimumab (11.2 months; 95% CI 5–12.7) and placebo (10 months; CI 8.3–11) groups, there were signs of activity with the drug [20]. Progression-free survival at 6 months was 30.7% (95% CI 26–35.3) for ipilimumab, and 18.1% (14.3–22) for placebo. A decline in PSA levels (>50% at any time) was also more frequent with the ipilimumab

group (13.1%; 9.5–17.5) than placebo group (5.2%; 3–8.4). However, an exploratory and post-hoc subgroup analysis showed overall survival benefit for ipilimumab after radiotherapy in a subset of patients with favourable prognostic features (no visceral metastases, nonraised or mildly raised alkaline phosphatase, and without anaemia). The median overall survival was 22.7 months for ipilimumab ($n = 146$), as compared to 15.8 months for placebo ($n = 142$). This apparent benefit from ipilimumab in patients with lower tumour burden warrants further investigations, as it has significant implications for further treatment studies using ipilimumab for this disease.

6. Prostvac-VF (Viral-Based Vaccine)

Viral vectors are attractive for use in cancer immunotherapies as they can mimic natural infection and lead to the induction of robust immune responses against the tumour antigen(s) that they encode [61]. Prostvac-VF (or PSA-TRICOM) is a recombinant viral vaccine currently being trialled as an immunotherapy for PCa that involves a prime and multiple booster injections with attenuated strains of *Vaccinia* and *Fowlpox* viruses, respectively [62]. Both recombinant viruses are engineered to encode the entire PSA gene with a modified agonist epitope (increases immunogenicity) and 3 costimulatory proteins: B7-1 (facilitates T cell activation), lymphocyte function-associated antigen 3 (LFA-3; CD58), and intercellular adhesion molecule-1 (ICAM-1; CD54) (both are cell adhesion molecules that strengthen interactions between APCs and T cells). The rationale behind this approach is that the virus will directly infect the APCs (resulting in expression of the costimulatory molecules), or somatic cells (epithelial and/or fibroblasts) at the site of injection, leading to cell death and subsequent uptake of cellular debris containing PSA by the APCs [63]. The transduced APCs, or antigen-loaded APCs, upon interaction with CD4+ and CD8+ T cells, will effectively promote the development of T cell-mediated immune responses that destroy PSA-expressing cancer cells. The viral vectors are nonreplicative and do not integrate into the genome of host cells, thus allowing for controlled administration of the transgenes [62]. Sequential injection of the *Fowlpox*-based booster vaccine is designed to overcome the reduced immunisation response following the development of neutralising antibodies against the *Vaccinia*-based priming vaccine, so that a level of immunity is maintained. Several preclinical studies have shown that the 3 costimulatory molecules, commonly referred to as TRI-COM, act synergistically to enhance antitumour immune responses to tumour self-antigens [64]. For example, vaccination of transgenic mice expressing human carcinoembryonic antigen (CEA-Tg) with recombinant *Vaccinia* and *Fowlpox* vectors encoding for CEA and TRI-COM can facilitate rejection of CEA-expressing murine colon carcinomas, an effect dependent on CD4+ and CD8+ T cells but not NK cells [64]. These mice had a longer survival time and did not develop pathologic autoimmunity based on biochemical, immunological, and histological criteria, demonstrating the therapeutic potential of this vaccine approach with minimal toxicity. Such studies have formed the basis for the development of Prostvac-VF.

A number of phase I clinical trials have established the safety and tolerability of Prostvac-VF [65, 66], and a phase II study was used to optimise the sequence of vector administration (one *Vaccinia* prime followed by three *Fowlpox* boosts) [67]. These findings led to the commencement of a phase II randomised controlled trial of 125 patients with minimally symptomatic CRPC (82 given Prostvac-VF and 40 control empty viral vectors) [21]. Prostvac-VF treatment was associated with longer overall survival time (25.1 months) as compared with control (16.6 months)—an improvement in median survival of 8.5 months [21]. The 3-year survival rate was almost double (30.5% versus 17.5%). However, no difference in progression-free survival was observed between the 2 arms (primary endpoint). Consistent with these findings, another phase II trial (nonrandomised) reported that Prostvac-VF treatment correlated with improved overall survival as compared with historical controls (the Halabi nomogram) (26.6 versus 17.4 months); the Halabi nomogram is a prognostic model developed on historical data that is designed to predict survival of mCRPC patients given docetaxel [22]. Importantly, patients with a Halabi who predicted survival of <18 months had an actual overall survival median of 14.6 months following Prostvac-VF treatment, whereas for those with a Halabi prediction of ≥18 months, it was ≥37.3 months (not yet reached) [22]. This suggests that patients with less aggressive or earlier-stage disease are more likely to benefit most from this vaccine; this could influence how Prostvac-VF may be employed clinically. Moreover, this study showed that 12 of 32 evaluable patients (37.5%) showed a decline in serum PSA after vaccination, and 2 of 12 patients with soft tissue metastases exhibited a measurable decrease in index lesions by CT [22]. Recently, an analysis of immunological impact by Prostvac-VF on pooled data from several clinical trials conducted similarly was published [68]. Collectively, 59 of 104 patients (57%) had an increase in the number of PSA-specific T cells following vaccination (median of 5-fold), and interestingly, 19 of 28 patients (68%) exhibited evidence of immune responses mounted against other tumour-associated antigens not found in the vaccine, for example, MUC1; a phenomenon known as antigen spreading [68]. This could be another mechanism that mediates the antitumour effect of Prostvac-VF. Moreover, the number and function of regulatory T cells were deceased following vaccination, although no effect on NK cell numbers was seen [68]. Due to these promising results, Prostvac-VF is currently being investigated in phase III clinical trials.

7. GVAX (Whole-Cell Based Vaccine)

GVAX is a whole-cell vaccine comprised of a mixture of 2 human PCa cell lines, LNCaP (androgen-sensitive; derived from a lymph node metastasis) and PC3 (androgen-insensitive; derived from a bone metastasis), which are modified to constitutively express GM-CSF and irradiated to prevent cell replication [69]. The rationale behind this vaccine approach is to mount immune responses against shared tumour antigens between the two allogeneic cell lines and the host cancer, with GM-CSF enhancing this process by functioning as a chemoattractant for dendritic cells as

well as facilitating their maturation and antigen-presentation to T cells to elicit robust antitumour effects [69]. The use of allogeneic tumour cells as the core component also has advantages in being faster and less expensive to manufacture as compared to autologous tumour cells, which are technically more difficult to harvest. Several preclinical studies have demonstrated that this whole-cell vaccine approach can induce potent antitumour effects in a number of cancer types. For example, subcutaneous injection of irradiated murine B16 melanoma cells retrovirally modified to express GM-CSF was associated with lower tumour take rate and longer survival time as compared to nontransduced cells, an antitumour effect mediated by both CD4+ and CD8+ T cells [70]. In addition, mice injected with irradiated GM-CSF-expressing tumour cells rejected a subsequent challenge with nontransduced B16 cells, but not Lewis lung carcinoma cells, indicating the vaccination induced strong protective tumour-specific immune responses [70]. Similar results were found in a preclinical model of PCa, whereby rats implanted with irradiated MatLyLu prostate carcinoma cells engineered to express GM-CSF exhibited longer disease-free survival compared to those challenged with nontransduced cells but also received soluble GM-CSF [71], indicating that the continual tumour secretion of this cytokine facilitates the development of antitumour immunity. These early studies collectively formed the foundation for clinical trials of GVAX for PCa.

A number of phase I/II clinical trials have demonstrated the safety and tolerability of GVAX [72]. Early evidence for clinical responses was observed in a phase II trial involving 55 men with chemotherapy-naive metastatic CRPC; 34 with radiologic metastases (24 with high dose GVAX and 10 with low dose); and 21 with rising PSA (all low dose) [73]. In the radiologic group, the median time to PSA progression was 2.3 months for those given low dose, 3.7 months for high dose, and for the PSA-rising group, it was 3.9 months [73]. The overall median survival time after treatment initiation was 24 months (low dose) and 34.9 months (high dose) in the radiologic group, whereas the estimated media survival of these patients based on the Halabi nomogram was 19.5 months, showing a trend of increased survival time by GVAX in a dose-dependent fashion. Moreover, there was evidence for induction of antibodies to PC3 cell lysate (18/28; 64.3%) and LNCaP (12/28; 64.3%) by patients in the radiologic group, as shown by immunoblots with patient sera. In another phase II clinical trial comprised of 80 men with the same clinicopathological characteristics, treatment with high dose was associated with longer median survival time (35 months) as compared with those given medium dose (20 months) and low dose therapy (23.1 months) [74]. The proportion of patients that generated an antibody response to either cell line was 89%, 72%, and 43%, respectively [74]. Collectively, those who exhibited antibody induction had a median survival of 34 months ($n = 30$), compared to 16 months for those who did not ($n = 6$), suggesting that immune reaction is associated with better clinical outcomes. These promising results led to the two phase III clinical trials which recruited men with metastatic CRPC comparing GVAX with docetaxel-prednisone with (trial named VITAL-1) or without (VITAL-2) cancer-related pain [75]. Unfortunately, VITAL-2 ($n = 408$) was terminated early as preliminary analysis demonstrated an excessive number of deaths (67 versus 47) and shorter median survival (12.2 months versus 14.1 months) for GVAX and docetaxel patients, respectively [23]. Similarly, VITAL-1 ($n = 626$) was also terminated early following a futility analysis showing that GVAX was unlikely to meet its primary endpoint of improved overall survival [24]. Despite these disappointing phase III results of GVAX, this immune-based agent is currently being trialled in combination with other immunotherapies, for example, Ipilimumab for PCa [76].

8. A Perspective on the Future of Immunotherapy for PCa

Since ADT plays a central role in the treatment of high-risk early PCa and recurrent or metastatic disease, it is crucial that immune-based therapies are compatible and, preferably, show synergistic activity. There is already preclinical and clinical evidence suggesting that ADT augments antitumour immunity through enhancement of T cell activation and trafficking to tumours [28, 34, 77, 78]. In addition, a reduction in tumour burden achieved through ADT (or surgery, chemotherapy, or other agents) may increase tumour sensitivity to immune-mediated destruction, as tumours can be highly immunosuppressive [79]. Moreover, the immune system is more likely to eliminate residual disease/low tumour volume than bulky lesions, a concept supported by the trend of longer survival time by sipuleucel-T or Prostvac-VF in patients with lower tumour burden [22, 46]. Recently, a phase II trial comprising nonmetastatic CRPC patients reported that Prostvac-VF, used in combination with the AR antagonist flutamide, was associated with longer median time to progression as compared to flutamide alone (233 days versus 85 days, resp.) [80], highlighting the potential of this combined approach. In terms of treatment sequencing, two phase II clinical trials provided early evidence that administration of ADT (nilutamide) after the immunological agent is associated with longer median survival [81] and median time to treatment failure [82], as compared to the reverse sequence. A possible explanation is that the immune modulatory effects of ADT augmented the antitumour immune responses initiated by Prostvac-VF. The optimal timing and sequence of Sipuleucel-T with novel hormone therapies such as abiraterone and enzalutamide are currently being investigated in phase II and III clinical trials, with the results being much anticipated [37, 83]. Similarly, the development of PD1 and PDL1 immune checkpoint inhibitors has attracted much interest [63]. Whilst occasional PCa patients have been enrolled in the phase I trials of these compounds, there has been no particular signal of activity in CRPC to date. Nevertheless, learning to harness PD1 and PDL1 inhibitors into the therapeutic armamentarium for CRPC would seem to be a priority.

Combination immunotherapy is another rational strategy to substantially enhance clinical outcomes by employing different agents that work via varied mechanisms of action and are potentially synergistic. A strategy that is receiving

increasing attention is the combination of ipilimumab with Prostvac-VF. The goal is to reduce or eliminate regulatory elements during the induction and/or effector phases of antitumour immunity (ipilimumab), to further boost responses to the target antigen PSA (Prostvac-VF). A recent phase I trial of ipilimumab/Prostvac-VF combination reported encouraging results, with men treated with both agents having an overall survival of approximately 34 months. This compares favourably with historical controls of previous trials which used vaccine alone (26 months) [62, 84]. Moreover, there was evidence for antigen cascade induction, that is, immune responses developed against antigens not encoded in the vaccine, and that the combination did not seem to exacerbate the immune-related adverse events associated with ipilimumab. There are also trials now investigating the combination of GVAX with ipilimumab [76, 85]. While GVAX as a monotherapy had limited efficacy in phase III trials, it is an attractive candidate for combined usage with ipilimumab as the latter may augment immune responses to multiple tumour antigens. A recent phase I trial of ipilimumab and GVAX was well tolerated, and 25% of patients exhibited decline in PSA levels [85].

Chemoimmunotherapy is also a potentially useful approach for CRPC. In the past, chemotherapy was thought to compromise immune responses by way of lymphocyte lysis as a bystander effect of its mode of mechanism on actively dividing cancer cells. However, there is growing evidence that chemotherapy can induce immune modulatory effects that could facilitate the induction of antitumour immunity. For example, docetaxel has been reported to increase the production of proinflammatory cytokines [86], cyclophosphamide to decrease Tregs [87], cisplatin to upregulate the apoptosis ligand Fas [88], and doxorubicin to enhance cytotoxic T cell antitumour immunity [89]. However, the feasibility of chemoimmunotherapy for PCa remains to be determined, as early phase clinical trial results have so far been inconclusive. A phase II clinical trial involving men with metastatic CRPC reported that those given Prostvac-VF concurrently with docetaxel had longer progression-free survival than patients given the vaccine alone (3.2 months versus 1.8 months) [90], but this clearly needs to be validated in large prospective trials. On the other hand, a phase III trial that investigated the effects of GVAX plus docetaxel was terminated early following preliminary findings that the combination treatment was associated with shorter survival and had a higher death rate compared to the docetaxel plus prednisone control group [91]. To properly assess the clinical utility of chemoimmunotherapy, it is necessary to consider the optimal timing and scheduling of treatments.

9. Summary

While enzalutamide [12], abiratirone [10], and cabazitazel [8] have been shown to be efficacious in the postdocetaxel setting by prolonging survival, they are not curative; hence, novel therapies need to be integrated into treatment regimes to further improve patient outcome. Immunotherapy has the potential to meet this need, following the demonstration that some approaches can improve patient survival [19] and that other immune therapies may synergise with ADT [80] or with each other in their anticancer activity. The realisation that PCa is inherently immunogenic and, more importantly, can be targeted by the immune system through immune modulatory agents is driving intense research in this field. Various approaches are being explored, although only dendritic cell-based vaccines, immune checkpoint inhibition, viral vector, and tumour-based vaccines have reached phase III clinical trial endpoints to date. Key questions remain, including the mode of achieving immune modulation (vaccine? immune suppression modulation? TAAs?), timing of immune therapy according to stage of disease, sequence of treatments, and which candidate treatments might best be paired with immune therapy.

Conflict of Interests

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

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Review Article

Role of miRNA *Let-7* and Its Major Targets in Prostate Cancer

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Prostate cancer is worldwide the sixth leading cause of cancer related death in men thus early detection and successful treatment are still of major interest. The commonly performed screening of the prostate-specific antigen (PSA) is controversially discussed, as in many patients the prostate-specific antigen levels are chronically elevated in the absence of cancer. Due to the unsatisfying efficiency of available prostate cancer screening markers and the current treatment outcome of the aggressive hormone refractory prostate cancer, the evaluation of novel molecular markers and targets is considered an issue of high importance. MicroRNAs are relatively stable in body fluids orchestrating simultaneously the expression of many genes. These molecules are currently discussed to bear a greater diagnostic potential than protein-coding genes, being additionally promising therapeutic drugs and/or targets. Herein we review the potential impact of the microRNA *let-7* family on prostate cancer and show how deregulation of several of its target genes could influence the cellular equilibrium in the prostate gland, promoting cancer development as they do in a variety of other human malignant neoplasias.

1. Introduction

Prostate cancer (PC) is a heterogeneous disease ranging from an asymptomatic to a fatal systemic malignancy [1]. According to the World Health Organization (WHO) 1,111,689 men were estimated to be diagnosed with PC in the year 2012 (http://globocan.iarc.fr/). Accounting worldwide for 6.6% (307,471) of all cancer death in men in 2012, PC is one of the most common malignant neoplasias and the sixth leading cause of cancer related death in men (http://globocan.iarc.fr/).

The development of PC is considered to be a multistep process initiated by genetic and epigenetic changes [1]. Human PC is commonly accepted to be an androgen dependent malignancy.

An analysis of PC related metastatic pattern in 1,589 patients by Bubendorf et al. revealed that 35% of the analyzed tumors spread to other organs with preference to the bones (90%), lungs (46%), liver (25%), pleura (21%), and adrenals (13%) [2].

The androgen deprivation therapy is actually the most effective palliative standard treatment for primary advanced PCs with bone metastasis (effective in up to ~90% of patients). However, the great majority of patients relapse subsequently due to the development of castration resistance [3].

Since the introduction of the prostate-specific antigen (PSA) test in the 1990s, the number of diagnosed cases has been rapidly rising being initially associated with a reduced mortality. However, the recent decline in PC related mortality rates is now being discussed to be partially explained by the improved treatment and earlier diagnosis due to a broad standard PSA screening in economically developed countries [4, 5]. As the standard PSA screening in the early diagnosis of human PC remains a very controversial issue, novel, reliable molecular PC markers are needed [6–8].

A promising marker candidate gene is the miRNA *let-7*, which was reported to be down regulated among others in human PC [9–11]. Further, the reconstitution of the *let-7* expression resulted in suppression of PC cell proliferation [10, 12]. In general a single miRNA is able to regulate a huge number of genes. Concerning *let-7* the respective acting ways are actually not entirely deciphered.

Nevertheless, it is to be expected that a deeper understanding of the molecular interactions of *let-7* and associated genes will significantly contribute to the development of novel diagnostic and therapeutic treatment modalities for PC.

Due to the complex regulation mechanisms of *let-7* and its potential role in PC development and relapse the present review highlights *let-7* and its direct and downstream targets in the context of PC.

2. Micro RNA *Let-7* Family

MicroRNAs (miRNAs) are small, non-protein-coding RNAs derived from long, endogenously expressed primary RNA (pri-miRNA) molecules. These pri-miRNAs are processed by the nuclear enzyme Drosha to precursor RNAs (pre-miRNAs), exported by Exprotin-5 [13] and maturated by the cytoplasmic enzyme Dicer [14]. Finally the guide strand of the mature miRNA is incorporated into the RNA-induced silencing complex (RISC), which blocks the translation of the target mRNA by binding to its $5'$-, $3'$-prime, or exon regions [15, 16]. The passenger strand is usually degraded [17] (Figure 1).

Mature miRNAs are known to be part of the gene expression regulating machinery at transcriptional [18, 19] and as well posttranscriptional level [13]. It was reported that a single miRNA can orchestrate the expression of several genes and a single gene can be regulated by a set of different miRNAs [20–22]. Several observations suggest that more than 60% of all protein coding genes are regulated by miRNAs [23].

One of the first described members of the large class of non-protein-coding RNAs is *let-7* which was the second miRNA discovered and designated as *lethal-7* (*let-7*) according to the phenotype of a *let-7* deficient *C. elegans* mutant [20]. Soon thereafter, further *let-7* homologs were identified in a variety of species ranging from vertebrates to mollusks [24].

In contrast to "less complex" organisms such as worms, vertebrates show a higher number of *let-7* isoforms coded by different genes [16]. In humans, 13 *let-7* family precursor miRNAs were described (*let-7a-1*, *let-7a-2*, *let-7a-3*, *let-7b*, *let-7c*, *let-7d*, *let-7e*, *let-7e*, *let-7f*, *let-7g*, *let-7i*, *miR-98,* and *mir-202*) which code for 10 different mature *let-7* miRNA isoforms [25].

Although the role of *let-7* is still not fully understood, it is evident that the *let-7* family members have a distinct expression pattern in animal development [26]. In the embryonic stage the *let-7* miRNAs were found to be barely detectable, but having an increased expression in differentiated cells [20, 27]. Furthermore, aberrant *let-7* expression was associated with a variety of human diseases as, for example, cardiovascular diseases [28], liver fibrosis [29], lung diseases [30], and cancer [9–12, 26, 31–34]. Interestingly several *let-7* family members were found to be located at fragile sites of human chromosomes potentially contributing to aberrant *let-7* transcript levels [35].

Cancer initiation, progression, and aggressiveness are hypothesized to be driven by cancer stem cells (CSCs) [36, 37]. Inflammatory microenvironment [38] as well as epithelial-to-mesenchymal transition (EMT), which is tightly linked with CSC biology [39], seems to play a substantial role in cancer etiology as well. Remarkably, a linkage between these factors is the *let-7* miRNA family. As described above *let-7* was shown to be downregulated in prostatic CSCs [36] whereas reconstitution of the *let-7* suppressed the growth of PC cells [10, 12]. Additionally, a direct causal link between cancer and inflammation is given by the association of *let-7*, IL6, and NFκB, which are major players involved in the epigenetic switch from inflammation to cell transformation [31]. The connection between EMT and *let-7* is represented by the *HMGA1* and *HMGA2* genes, which are directly regulated by *let-7* and were found to be implicated in EMT [40, 41].

Further, miRNAs of the *let-7* family were reported to directly, negatively regulate *IL6* [24], *NRAS* [42], *c-Myc, HMGA1* [43, 44], *HMGA2* [45], and *CCND2* [11]. Notably, these *let-7* targets are involved in a wide range of diverse cellular processes interwoven with *let-7* and each other in a fine balanced way (Figure 10).

The c-Myc protein regulates the biogenesis of *let-7* by stimulating *Lin28* [46], Lin28 in turn blocks the maturation of *let-7* [47]. Additionally, c-Myc stimulates the expression of *HMGA1* [48], *AR* [12], and *IL6* [49]. NRAS is suggested to have an impact on *HMGA2* biogenesis [45]. *HMGA2* on the other hand influences *HMGA1,* its gene product in turn regulates the expression of *c-Myc* [50] and *HMGB1* [51]. HMGB1 was found to bind the *AR* promoter [52], AR protein was described itself to stimulate *let-7* expression [53] (Figure 10).

Interestingly, the *let-7* family [10, 11] and some of its above mentioned targets were already found to be implicated in PC. As *let-7* is linked with all these protein-coding genes a deeper insight into these connections is of great interest. Thus, these interactions are reviewed more detailed in the following parts.

3. *HMGA1*

The high mobility group proteins (HMG) are chromatin associated nonhistone proteins constituting three superfamilies (HMGA, HMGB, and HMGN) which are classified by their characteristic functional DNA-binding motifs [54]. Expression of these proteins was described to be involved in a variety of biological processes as, for example, transcription, embryogenesis, differentiation, neoplastic transformation, apoptosis, and inflammation [52, 55, 56].

In human neoplasias the HMGA genes are among the most commonly rearranged genes [57]. Deregulation of the *HMGA1* expression was described in human PC [58, 59], lung cancer [60], and breast cancer cells [61]. Its oncogenic property is speculated to be partly mediated through the cytoplasmic relocalization of the HIPK2 which is a proapoptotic activator of the tumor suppressor p53 [51] (Figures 2 and 10). HMGA1 was reported to enhance the proliferation rate and invasion of PC cells [62, 63] potentially through the implication in epithelial-to-mesenchymal transition (EMT) [61]. In line with this, *HMGA1* knock down in the human triple-negative breast cancer cell lines MDA-MB-231 and Hs578T

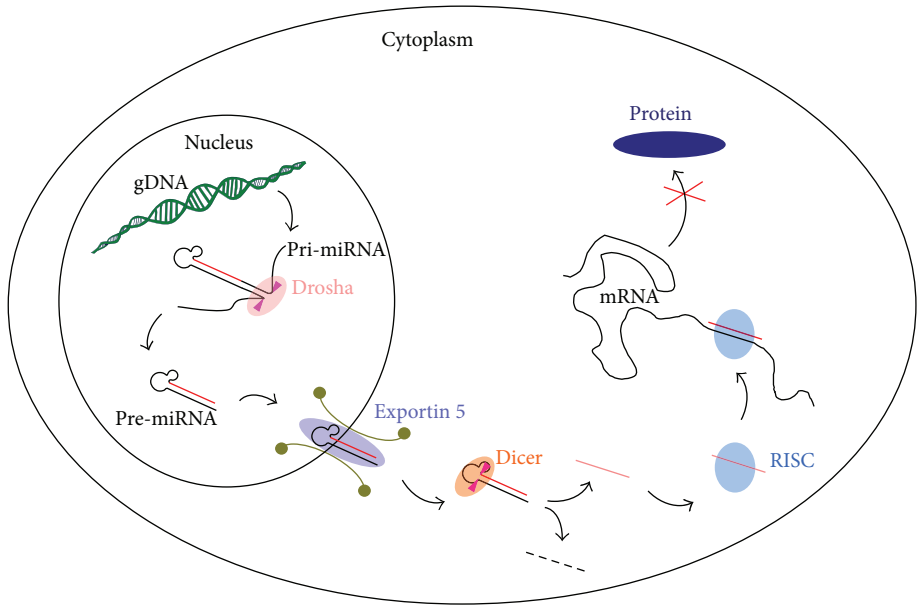

Figure 1: Schematic overview of the miRNA maturation and the way of function in eukaryotic cells. The endogenously expressed primary miRNA (pri-miRNA) is processed in the nucleus of a cell by the enzyme Drosha. The emerging precursor miRNA (pre-miRNA) product is exported into the cytoplasm by Exportin-5 and maturated by Dicer. Finally the guide strand is incorporated into the RNA-induced silencing complex (RISC), which blocks the translation of the target mRNA.

repressed the mesenchymal gene *SNAI1* and stimulated *CDH1* expression [40] (Figures 2 and 10), both of which are involved in EMT [64, 65]. Furthermore, HMGA1 was reported to drive tumor progression by reprogramming cells to a stem-cell-like state [40]. In accordance, Shah et al. reported in human embryonic stem cells (hESCs) a significant downregulation of the stemness-associated genes *OCT4*, *Sox2*, *Lin28*, and *c-Myc* 96 h after *HMGA1* repression [50]. Interestingly HMGA1 is not only stimulating *c-Myc* expression [50] it was also reported to be itself induced by c-Myc [48] (Figures 2 and 10). It is remarkable that HMGA1 is implicated in the upregulation of several miRNAs in murine embryonic fibroblasts. Among these miRNAs is the *miR-196a-2*, which in turn is predicted to target its sister gene *HMGA2* [66] (Figures 2 and 10). Furthermore, Hillion and colleagues reported a positive correlation between HMGA1 and STAT3 in a subset of primary human acute lymphoblastic leukemia samples [67]. In line with this, HMGA1 was described to bind the STAT3 promoter and to upregulate its expression in malignant human hematopoietic cells [67] (Figures 2 and 10). The transcription factor STAT3 mediates uncontrolled growth, angiogenesis, and survival of cells and has a great potential as target in cancer therapies [68]. Remarkably, Iliopoulos et al. identified STAT3 binding sites in the promoters of the miRNAs *miR-181b* and *miR-21* [69] (Figures 2 and 10). These tiny regulators in turn where found to block *PTEN* (Figures 2 and 10), stimulating the activity of NFκB [69]. The tumor suppressor PTEN functions as an antagonist of PI3K by dephosphorylating its product PIP3 [70] (Figure 10).

The *HMGA1* and *HMGA2* genes were reported to be highly expressed during embryogenesis, reexpressed in several cancer types but to be absent or not detectible in most of the adult healthy tissues [57, 71]. The expression of both

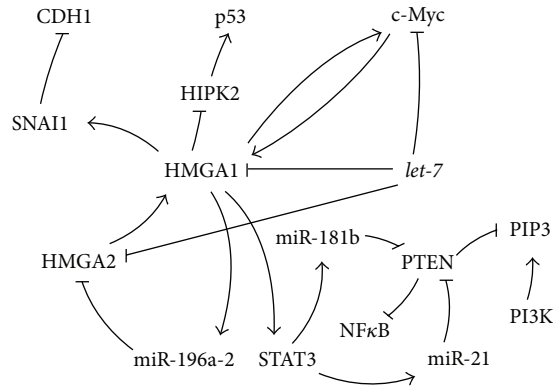

Figure 2: Overview of the described interactions between *let-7* and its direct target *HMGA1* with other genes.

HMGA1, *HMGA2*, and of its regulator *let-7* was shown to be negatively correlating in gastroenteropancreatic neuroendocrine tumors [44] and retinoblastomas [72]. In accordance they were found to be directly, negatively regulated by *let-7* [45, 73, 74] (Figures 2 and 10).

4. *HMGA2*

Comparable to the described *HMGA1* knock down, the repression of *HMGA2* in the human PC cell line PC-3 induced an upregulation of *CDH1* indicating an important role in EMT [41]. *SNAI1* and *SNAI2* are repressors of *CDH1* and were shown to be directly activated by HMGA2 [45] (Figures 3 and 10).

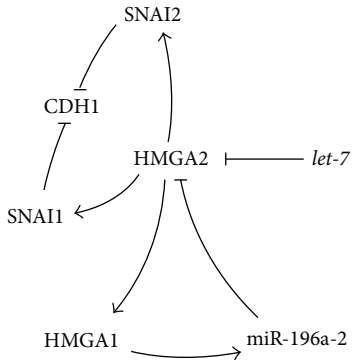

Figure 3: Association of the HMGA2/*let-7* axis with the regulation of genes involved in EMT and miRNAs.

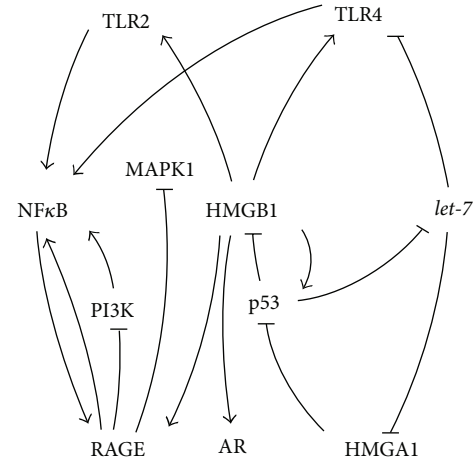

Figure 4: Schematic overview over the HMGB1 and *let-7* regulatory pathways affecting each other's activity.

Similar to *HMGA1*, an upregulation of *HMGA2* was reported in human lung and breast cancers [75, 76] as well as in a subset of canine PCs [77]. Furthermore, *HMGA2* was recently described to modify gene expression not only as protein but as well as a competing endogenous RNA (ceRNA) by acting as a decoy for mature *let-7* miRNAs [78]. Interestingly, a stimulating HMGA2 influence on the expression of its sister protein HMGA1 was found in rat epithelial thyroid cells [79], thus constituting a feedback loop by the stimulation of its suppressor, the *miRNA-196a-2* [66] (Figures 3 and 10). Remarkably *HMGA2* was described to bear seven *let-7*-binding sites in its 3′-untranslated region (3′-UTR) [33]. Aberrations of the chromosomal region 12q14-15 that affect *HMGA2* were frequently found in human cancers [80–82]. Moreover, the disrupted pairing between *let-7* and *HMGA2* by mRNA truncations of the 3′UTR was reported to induce HMGA2 overexpression leading to tumor formation [33].

5. *HMGB1*

The high mobility group box 1 (HMGB1) is one of the HMGB superfamily members which was also shown to be implicated in inflammation exercising cytokine like functions [83]. In line with its multiple roles it can be located in the nucleus as well as in the cytoplasm and can even be released passively by necrotic cells or actively secreted in response to inflammatory signals by certain cell types [83, 84].

This proinflammatory cytokine exerts its function by interacting with the toll-like receptors (TLR) 2, and TLR4 and RAGE [85–87] (Figures 4 and 10). Interestingly, the receptor coding gene *TLR4* was found to be a direct *let-7i* target (Figure 4), presenting a mechanism to modify the HMGB1 signaling [88]. The activation of the HMGB1 receptor RAGE results among others in deactivation of MAPK1 and PI3K [89]. PI3K in turn was shown to stimulate NFκB [90]. Furthermore, the TLRs and RAGE were demonstrated to activate NFκB thus, inducing the secretion of angiogenic factors, growth factors, and cytokines [85, 91].

Remarkably, NFκB is able to stimulate RAGE expression by binding to its promoter constituting a positive feedback loop [92] (Figures 4 and 10). Blockade of the RAGE/HMGB1 signaling decreased growth and metastasis of implanted and as well of spontaneously developing tumors in susceptible mice [93].

HMGB1 was described to be involved in all proposed hallmarks of cancer and is thus a potential target for therapeutic and diagnostic approaches [94]. Kuniyasu et al. observed the secretion of HMGB1 in primary cultured human prostatic stromal cells after androgen deprivation [95]. *In vitro* suppression of HMGB1 was demonstrated to block the invasion of PC-3 cells which was reversed by culturing the cells in conditioned medium of the above-mentioned stromal cells deprived of androgen [95, 96]. Additionally, HMGB1 was found to stimulate DNA binding of several steroid receptors including the *let-7* downstream target *AR* (Figure 10) [97]. These facts indicate that *HMGB1* may be a molecular marker for advanced prostate cancer [95, 96].

Although *HMGB1* was not shown to be a direct *let-7* target, its expression is modulated by the direct *let-7* target HMGA1 [51]. Interestingly HMGB1 was also shown to be involved in the p53 network by facilitating the binding of the tumor suppressor p53 to its cognate DNA [98]. As mentioned before p53 can be inactivated by the HMGB1 sister protein HMGA1 by translocation of the p53 activator HIPK2 [51] (Figures 4 and 10). The tumor suppressor p53 in turn was found to downregulate the activity of the HMGB1 promoter [99] and to trigger the radiation induced decrease of *let-7a* and *let-7b* expression (Figures 4 and 10) in the human colon cancer cell line HCT116 [100].

6. *CCND2*

Many tumor cells accumulate mutations resulting in uncontrolled proliferation due to direct or indirect deregulations of the cyclin-dependent kinases (CDKs). Cyclins are known regulating subunits of CDKs being expressed at specific time points during the cell cycle. Consequently cyclin deregulations induce uncontrolled cell proliferation [101].

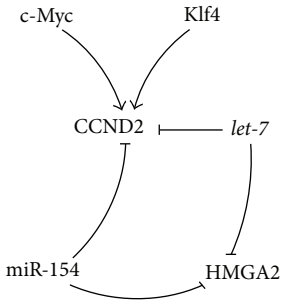

Figure 5: *Let-7* and CCND2 mediated gene regulation.

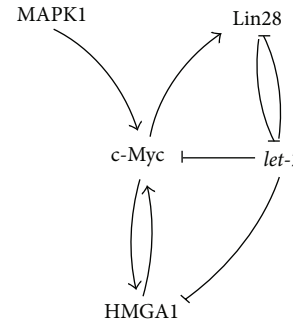

Figure 6: Interactions of the oncogene c-Myc with *let-7* and MAPK1, Lin28, and HMGA1.

The cyclin D2 (CCND2) is one of the cell cycle regulating factors. This gene, which is highly conserved among mammals, has been associated with human prostate cancer [11], gastric cancer [102], colon cancer [103], and leukemia [104].

Interestingly, *CCND2* was shown to be a direct *let-7* and *miR-154* target like *HMGA2* [11, 41, 45, 105] (Figures 5 and 10). Additionally the *let-7* regulated oncogene *c-Myc* and the stem cell marker *Klf4* were reported to stimulate the *CCND2* transcription [106, 107] (Figures 5 and 10).

Dong et al. described that ectopically overexpressed *let-7a* induced cell cycle arrest at the G1/S phase by suppressing among others the cyclin CCND2 and additionally inhibited the proliferation of the human prostatic cell lines PC-3 and LnCap [11]. The same group reported that in nude xenograft mice, inoculated with *let-7a* transfected PC-3 cells, the tumor was 80% lighter after 4 weeks of growth compared to controls [11].

7. *c-Myc*

c-Myc is an oncogene frequently activated in human cancers, but is low expressed or absent in quiescent cells [108–110]. In contrast, its overexpression has been connected with PC formation and progression [111, 112]. This gene encodes a transcription factor that has a great impact on the global gene expression pattern and, thus, influences cell-cycle progression, glucose and glutamine metabolism, lipid synthesis, and many other processes, which contribute to tumor progression [109].

Mitogen activated protein kinases (MAPK), glycogen synthase kinase 3 (GSK3), and other CDKs play a key role in the biological function and half-life of c-Myc proteins by posttranslational phosphorylation of the Thr58 end Ser62 sites [113] (Figures 6 and 10). Apart from various posttranslational protein modifications and transcriptional regulations of the *c-Myc* gene products, this gene was reported to be directly negatively regulated by members of the *let-7* family [114, 115] (Figures 6 and 10). Additionally, elevated MAPK1 activity, which was associated with advanced, androgen independent human PCs, [116] was demonstrated to influence the c-Myc protein, resulting in prolonged function in a human muscle-derived rhabdomyosarcoma cell line [117]. In line with the functions of c-Myc, MAPK1 controls diverse cellular processes as growth, differentiation, migration, and apoptosis, its deregulation has often been described to be associated with cancer [118]. Furthermore, c-Myc was shown to transcriptionally activate *Lin28* [119], which in turn inhibits the biogenesis of its regulator *let-7* constituting a double negative feedback loop [47] (Figures 6 and 10). Interestingly the expression of the direct *let-7* target *HMGA1* is as well induced by c-Myc [48], which constitutes a positive feedback loop, stimulating *c-Myc* expression [50] (Figures 6 and 10).

8. *IL6*

Chronic inflammation of the prostate gained major attention as it is considered to account to the factors contributing to PC [120]. In previous reports a direct causal link between cancer and inflammation has been described with IL6, *let-7*, Lin28, and NFκB being the major players involved in the epigenetic switch from inflammation to cell transformation [31].

Originally identified as an inducer of the terminal differentiation of B-cells into antibody-producing cells [121] interleukin-6 (IL6) appears to be a major regulator of prostate cancer progression [122]. Notably, IL6 is not only released by inflammatory cells but also found to be released by hormone insensitive cell lines DU145 and PC-3 but not by the hormone sensitive LNCaP cells [123]. Furthermore, this pleiotropic cytokine stimulates growth and survival of human PC and promotes its progression [123, 124]. In accordance, increased IL6 levels were found in epithelial cells of PC compared to benign tissues [125]. Moreover, Giri et al. reported a ~18 times higher IL6 expression in malignant prostate tissues compared to "normal" prostate specimens [126]. Michalaki and colleagues described significantly higher IL6 serum levels in patients with metastatic prostatic disease [127].

The biological activities of IL6 are mediated by binding to the α-subunit receptor IL6R and the following association with the ubiquitously expressed signal-transducing β-subunit gp130 [128]. Upon engagement of gp130 various Janus tyrosine kinase (JAK) family members (JAK1, JAK2, JAK3, and Tyk2) [129] are activated by ligand induced receptor oligomerization phosphorylating themselves and the intracellular domains of the receptors [130]. Once gp130 is phosphorylated the second protein family, the signal transducer and activator of transcription (STAT), binds to the intracellular domain of the receptor. This leads to the activation of STATs and the subsequent dissociation, allowing

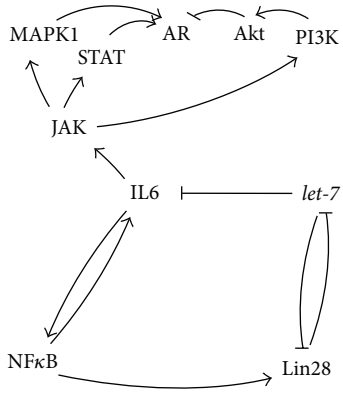

Figure 7: IL6 regulated genes.

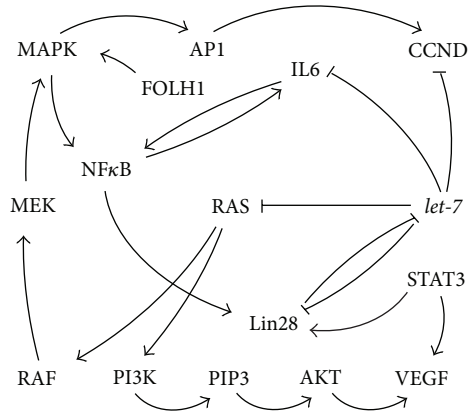

Figure 8: Schematic overview over some of the numerous pathways modified by RAS and *let-7*.

STAT dimerization and translocation into the nucleus where they act as transcription factors [131]. Additionally IL6 was shown to stimulate the PI3K and MAPK pathways by signaling trough activated gp130 [132, 133].

Interestingly, LnCaP cells stimulated with IL6 presented an enhanced AR activity in the absence of a ligand [134, 135]. The IL6 mediated activation of the human AR was indicated to be mediated by STAT3 and MAPK signaling [134, 136], which potentially contribute to recurrence of hormone refractory PCs. Whereas the AR transactivation can be suppressed by the PI3K/AKT pathway. Thus, these three pathways are suggested to coordinately regulate AR activation [136].

Acquiring resistance to apoptosis appears an important feature for the development of hormone resistant and aggressive human prostate cancer. Furthermore, IL6 was shown to act as a survival factor, blocking apoptosis induced by Bcl-xl, p53, TGF1β [137], and cytotoxic agents such as doxorubicin [138] and enzalutamide [139]. Whereas siRNA or STAT3-inhibitor-AG490 mediated suppression of the downstream acting STAT diminished the IL6 induced antiapoptotic function [138, 139].

NFκB is a regulator of the transcription of *IL6* [140] and *Lin28B* [31, 141] (Figures 7 and 10). Lin28B was demonstrated to block the maturation of *let-7* [46]. Additionally, members of the miRNA *let-7* family directly target *IL6*, which in turn constitutes a positive feedback loop on NFκB [31, 49] (Figures 7 and 10).

Remarkably, while only a few cells express membrane bound IL6R all cells display gp130 on their surface [132]. This is an interesting feature as IL6 can also bind to a soluble IL6R (sIL6R) variant, which interacts in an IL6R agonistic manner with gp130, thus, enabling the stimulation of cells lacking endogenous IL6R [142].

9. *RAS*

The founding members of the *RAS* gene superfamily *N-RAS*, *H-RAS,* and *K-RAS* are coding for small GTP-binding proteins [143]. Originally identified as retroviral oncogenes in rat sarcomas, *RAS* were the first human oncogenes discovered, shown to be mutated in around 30% of all human tumors [144, 145]. The very common mutations in the residues G12, G13, and Q61 lock RAS in a constitutively activated state by impairing the intrinsic GTP hydrolysis [145, 146]. RAS proteins are active when they have bound GTP. By hydrolyzing GTP to GDP they become inactive. The intrinsic GTPase activity of the RAS proteins is very low relying on the help of specialized GTP hydrolysis accelerating factors called GTPase activating proteins (GAP) which increase the hydrolysis by more than 100,000 fold [145].

RAS-GTPs are acting as signal transducers across membranes by binding various effector proteins to stimulate signaling pathways [143, 147]. Among these factors are the Raf serine/threonine MAPKK kinases (ARAF, BRAF, and RAF1) which in turn activate MEK-MAPK cascades [148] (Figures 8 and 10). Accordingly, the mammalian MAPK pathways are estimated to be deregulated in one-third of all human cancers [149]. MAPKs activate cytosolic and nuclear factors like JUN and ELK1, which are regulating FOS expression. JUN and FOS are forming the activator protein 1 (AP1) and, thus, influencing the expression of proteins such as CCNDs which are involved in cell-cycle progression [150] (Figures 8 and 10).

Furthermore, RAS-GTPs induce the translocation and subsequent activation of phosphatidylinositol 3-kinase (PI3Ks) by binding to its catalytic subunit [151] (Figures 7 and 10). PI3K signaling is one of the most often deregulated systems in human cancer [152]. Taylor et al. described that the PI3K expression is altered in 42% of the primary and in 100% of the metastatic cases in the analyzed set of human prostatic cancers [153]. PI3Ks belong to one of the main effector molecules of RAS [151]. This enzyme type phosphorylates primarily to the 3′-OH group of the membrane bound phosphatidylinositol-4,5-biphosphate (PIP) to generate the messenger phosphatidylinositol-3,4,5-biphosphate (PIP3) [154]. PIP3 activates itself several pleckstrin homology domain-containing proteins as Akt by directly binding and recruiting it to the plasma membrane [154] (Figures 8 and 10). The activated Akt promotes many processes contributing to a malignant tumor phenotype [155]. Ectopic expression of a constitutively active Akt in the thyroid cell line SW579 was reported to significantly increase VEGF levels [156] (Figures 8 and 10). Neovascularization and angiogenesis are essential

features for the progression of a growing tumor VEGF is one of the most important inducers of angiogenesis [157]. Niu et al. demonstrated a positive correlation between VEGF and a constitutively active STAT3 [157]. In accordance, it was found that STAT3 binds to the *VEGF* promoter [157] (Figures 8 and 10). Additionally STAT3 was reported to bind the promoter of the *let-7* biogenesis regulating gene *Lin28*, resulting in the concomitant upregulation of the *let-7* targets *RAS*, *c-Myc,* and *HMGA2* [158].

In human tissues the activation of RAS and Rac-MAPK pathways was described to be induced by the extracellular signal transducer FolH1 [159] (Figures 8 and 10). FolH1 is expressed in most of the human prostate cancers and is thus a potential target for diagnostic and therapeutic strategies [160]. The elicited phosphorylation of MAPK1 and MAPK14 induces in turn the activation of the transcription factor NFκB (Figures 8 and 10) which controls the expression of various genes including the *let-7* biogenesis-controlling Lin28 [47] and the cytokine IL6 [31, 161] (Figures 8 and 10). Additionally, NFκB was also shown to enhance the endogenous transcription of the primary miRNAs *let-7a-3* and *let-7b* through NFκB responsive binding sites in the promoter regions [141] (Figures 8 and 10).

Remarkably, Johnson et al. reported numerous *let-7* binding sites in the 3′-UTR of the RAS genes [42]. In conclusion the expression of the oncogenes *NRAS*, *KRAS,* and *HRAS* was described to be negatively regulated by several members of the *let-7* family [42, 162] (Figures 8 and 10).

10. *Androgen Receptor* (*AR*)

The gene of the steroid receptor family member *AR* [163] is located on the human chromosome X and codes for a ligand-dependent transcription factor [164, 165]. Upon ligand binding it translocates into the nucleus and regulates its target genes by binding to the androgen response elements (AREs) [166, 167]. Expressed in nearly all primary human PCs, AR plays a pivotal role in carcinogenesis of the prostate. At the initial diagnosis the majority of PCs depends on androgens and progress after hormone therapy to an androgen-independent disease [3, 168].

Continuous androgen expression is required to drive prostate gland formation during embryogenesis and later to maintain the normal function and glandular anatomy in adults [169]. In general the androgen mediated effects in prostate gland development are driven by the interaction with ARs [169]. The bypass mechanisms of *AR* upregulation include among others the HMGB1 enrichment on the *AR* promoter, which enhances the transcription [52] (Figures 9 and 10), an intracrine androgen production [170, 171] additionally ligand independent AR activation by cytokines or growth factors were reported as well [172]. Furthermore, altered specificity or sensitivity as for example by alternative splicing is discussed [173].

However, the activated AR stimulates the expression of its targets as, for example, the above mentioned VEGF [174] and PSA [175]. PSA is a pivotal downstream target of AR, which is used as biomarker for human PC progression [175]. Interestingly, the frequently observed rising of serum PSA in

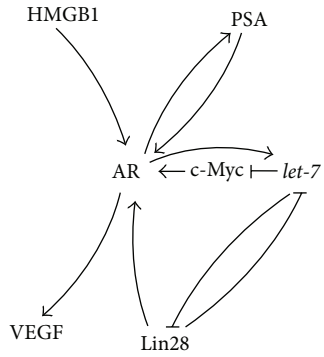

Figure 9: Potential AR interaction in prostate cancer development.

castrate-resistant PC patients could in parts be explained by AR activity, which is reexpressed/reactivated in advanced PCs [176]. Remarkably, PSA constitutes a positive feedback loop stimulating *AR* expression as was demonstrated *in vitro* [175] (Figures 9 and 10).

Furthermore, Tummala et al. highlighted the impact of the Lin28/*let-7*/Myc axis on PC and demonstrated that Lin28 activates the AR (Figures 9 and 10) and promotes growth of PC [177].

Remarkably *AR* was reported to be regulated in a negative way by the miRNA *let-7c* which suppresses its transcriptional activator c-Myc [12] (Figures 9 and 10). Additionally Lyu et al. described an AR induced upregulation of *let-7a, let-7b*, *let-7c*, and *let-7d* (Figures 9 and 10) in the breast cancer cell lines MDA-MB-231 and MDA-MB-453. At least in the case of *let-7a* this upregulation is indicated to be triggered by AR binding to AREs located at the *let-7a* promoter [53] (Figures 9 and 10). Furthermore, it was shown that in these cell lines the expression of the direct *let-7a* targets c-Myc and KRAS was decreased upon treatment with 5α-dihydrotestosterone and increased after an additional suppression of the miRNA *let-7a* [53].

The spatiotemporal expression of genes and functions depend highly on the cellular and developmental context. Thus, the impact of a single gene can be completely different between diverse tissues and at different time points in development. Nevertheless elucidation of the above described interactions in PC bears great potential due to the ubiquitous existence of the cellular regulatory elements and the potential interactions in each somatic cell of an organism. This idea is supported by the already found implication of each of the described genes in various human cancers. Furthermore several of the reviewed genes are already used as targets for diagnostic, prognostic, and therapeutic approaches. Thus, the master regulator family *let-7* is as well a promising target in cancer of the prostate gland.

For a better overview all described interactions between the master regulator family *let-7* and its major targets are summarized in Figure 10.

11. Conclusion

Although the knowledge of the genetic and epigenetic alterations in prostate cancer has significantly increased in the last

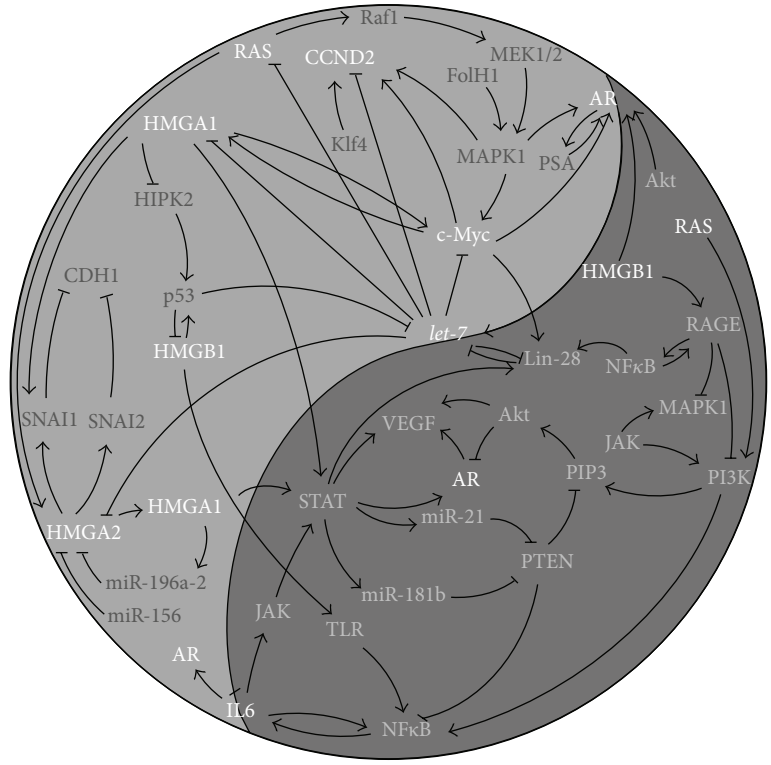

Figure 10: This figure represents the described interactions between *let-7* and the reviewed *let-7* associated targets (in white letters) with other genes which are as well commonly deregulated in human cancers (in gray letters). The indicated interactions are on transcriptional, posttranscriptional or posttranslational level.

decades, its diagnosis and therapy still remains a major challenge. The actually described genetic alterations in prostate cancer give more questions than answers. As we could highlight, the genes reviewed in the present paper are not acting in solitude but are closely interwoven with each other (Figure 10). Remarkably, the miRNA *let-7* family members are major players in the regulation of gene expression and appear to contribute greatly to the maintenance of the Ying and Yang in "normal" prostatic cells. However, their impact can be modified greatly by other factors. For that reason the complex intra- and intercellular genetic interactions of *let-7* family members and associated genes must be further investigated and will likely have an impact on diagnostic, prognostic, and treatment modalities in future.

Conflict of Interests

The authors have no conflict of interests.

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Review Article

Noncoding RNAs as Novel Biomarkers in Prostate Cancer

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Prostate cancer (PCa) is the second most common diagnosed malignant disease in men worldwide. Although serum PSA test dramatically improved the early diagnosis of PCa, it also led to an overdiagnosis and as a consequence to an overtreatment of patients with an indolent disease. New biomarkers for diagnosis, prediction, and monitoring of the disease are needed. These biomarkers would enable the selection of patients with aggressive or progressive disease and, hence, would contribute to the implementation of individualized therapy of the cancer patient. Since the FDA approval of the long noncoding *PCA3* RNA-based urine test for the diagnosis of PCa patients, many new noncoding RNAs (ncRNAs) associated with PCa have been discovered. According to their size and function, ncRNAs can be divided into small and long ncRNAs. NcRNAs are expressed in (tumor) tissue, but many are also found in circulating tumor cells and in all body fluids as protein-bound or incorporated in extracellular vesicles. In these protected forms they are stable and so they can be easily analyzed, even in archival specimens. In this review, the authors will focus on ncRNAs as novel biomarker candidates for PCa diagnosis, prediction, prognosis, and monitoring of therapeutic response and discuss their potential for an implementation into clinical practice.

1. Introduction

1.1. Prostate Cancer Diagnosis. The prostate is an exocrine gland in the male reproductive system that is responsible for the production of 50–70% of the seminal fluids. In men, prostate cancer (PCa) is the second most common diagnosed malignant disease and the sixth leading cause for cancer related death among men worldwide, with an estimation of 899.000 new cases and 258.000 deaths in 2008 [1, 2]. The rate of PCa diagnosis increased over the past decades due to an aging population, increased awareness, and the use of prostate-specific antigen (PSA) in serum for screening and diagnosis [3]. Upon abnormal digital rectal examination (DRE) and/or elevated serum PSA values, the diagnosis of PCa is usually obtained by pathological evaluation of transrectal ultrasound-guided prostate needle biopsies. However, this procedure is limited by false-negative biopsies and overdiagnosis of clinically insignificant malignancies [4, 5]. PCa is a heterogeneous disease and the clinical behavior ranges from slow-growing tumors with no or little clinical significance to aggressive metastatic and lethal diseases. By definition, clinically insignificant PCa does not contribute to PCa mortality [6] and the treatment of indolent PCa can result in side effects that reduce quality of life of the patient for no or little benefit.

The use of biomarkers has the potential to improve the diagnosis of cancer, especially to identify cancer at an early stage of disease with potentially curative therapy options. The currently used PSA test has some well-known limitations [7]. Although PSA is prostate-specific, it is not cancer-specific due to elevated levels of serum PSA under benign conditions, like benign prostate hyperplasia, urinary retention, prostatitis, trauma, or physical manipulation [7]. Approximately, 30% of men with a serum PSA of 5–10 ng/mL and >50% of men with a PSA >10 ng/mL will have prostate cancer. More importantly, clinical trials have shown that the PSA testing and screening is associated with an overdiagnosis and as a consequence an overtreatment of patients with indolent disease [4, 5, 8].

One of the current clinical priorities includes the identification of biomarkers that discriminate between indolent and aggressive PCa so that patients with an indolent disease with low risk of progression may better benefit from avoiding unnecessary treatments. The aim should as well be to identify patients with an aggressive, rapidly lethal PCa at an early stage, for which potential curative therapy options are available. Without any doubt, there is a need for new diagnostic and predictive biomarkers, and these markers would enable individualized therapeutic management for the cancer patient.

1.2. Prostate Cancer Monitoring. Beside screening and diagnosis, serum PSA is currently also used for monitoring disease progression. PCa depends on androgen receptor activity at all stages. Standard therapy of disseminated prostate cancer in hormone-naive patients is based on androgen-deprivation therapy or androgen receptor antagonists. Unfortunately, successful treatment effects are often followed by recurrence of PCa, resulting in the so-called castration-resistant prostate cancer (CRPC). Currently, there are no curative treatments for CRPC available. Docetaxel is the first-line chemotherapy for CRPC, providing modest survival benefits [9–11]. Response to treatment is usually determined by changes in serum PSA levels and reduction of tumor burden on radiological scans. The response rate is ~50%, and many patients suffer from significant toxicity [9, 11]. Metastasis and chemoresistance are reasons for the mortality of PCa patients. There are advances in the development of alternative effective therapies (e.g., abiraterone acetate, enzalutamide, cabazitaxel, and radium-223), but there is still a lack of useful biomarkers for the management or monitoring of patients with CRPC. Absolute serum PSA values are not suitable for and predictive of CRPC patients. Predictive biomarkers would be used to identify patients who most likely benefit from a particular treatment, enabling personalized medicine and hence treatment failure including side effects could be avoided.

1.3. Biomarkers. The heterogeneous nature of PCa and CRPC is coupled with genetic and epigenetic alterations that occur during disease progression and response to therapy. These changes can lead to the expression or production of novel disease-specific macromolecules, which could serve as novel biomarkers. Biomarkers are molecules that can provide information about the disease. In general, beside their role in diagnosis, they could be useful for the evaluation of the disease predisposition, screening, prognosis, prediction of drug response, monitoring, and pharmacodynamic properties (e.g., for a determination of the most effective dose) in combination with clinical history and parameters [12]. Biomarkers should be detectable in tissue, obtained by biopsy or surgical resection, or in bodily fluids like blood, urine, and semen. Potential biomarkers can be specific cells, proteins (enzymes, hormones, etc.), metabolites, DNA or an epigenetic modification of DNA, and expression levels of (novel) RNA transcripts, including noncoding RNAs (ncRNAs) [13].

Human prostatic acid phosphatase (PAP) was the first reported biomarker for PCa [14]. Proteomics and genomic technologies have enhanced the discovery of potential novel candidates. Many excellent reviews have summarized novel candidate biomarkers including proteins (e.g., α-methylacyl-CoA racemase, endoglin, prostate-specific membrane antigen [PSMA], caveolin-1, interleukin-6, CD147, TGF-β 1, and human kallikrein-2), genetic biomarkers (e.g., *TMPRSS2:ETS* gene fusions, *BRCA1/2* mutations), epigenetic modification (e.g., methylation of the glutathione S-transferase [*GSTP1*] gene and histone modifications), and expression of (novel) mRNA transcripts in PCa [6, 15–19]. Examples are shown in Table 1. Despite technological advances that contributed to the identification of novel biomarker candidates in serum, the use of proteins as biomarkers is still limited by the wide range of protein concentrations. In addition, it is often difficult to detect low-abundant proteins, due to interfering compounds, the masking effects of high-abundance proteins, high levels of salts, and big variations between individuals [6]. In contrast, for example, RNA-transcripts can be reproducibly detected and quantified in all kinds of specimens, even when present at very low levels due to possibility to include amplification steps.

Common genetic alterations in tumors also have an impact on specific noncoding RNAs (ncRNAs). NcRNAs (e.g., microRNAs) have received increasing attention of investigators as they can target multiple signaling pathways related to tumor progression, invasion, metastasis, and chemoresistance. Recent evidence suggests that ncRNAs represent useful and promising markers for diagnostic and prognostic purposes alone or in addition to other candidate markers. NcRNAs have the potential to improve the current tests or even to be superior to established protein-based biomarkers. Therefore, the authors of this review focus on ncRNAs and discuss their potential as new biomarkers for PCa diagnosis, prognosis, prediction, and monitoring of PCa patients.

1.4. Noncoding RNAs. Only about 25,000 protein-coding genes (covering approximately 2% of the human genome) have been recognized by the International Human Genome Sequencing Consortium [20]. Most of the human genome does not code for any protein and therefore is called noncoding DNA (ncDNA). Most of the ncDNA, estimations ranging from 60 to 90%, is transcribed into functional ncRNAs. These ncRNAs are not translated into proteins [21, 22] but still act as important mediators of gene regulation in physiological as well as pathological processes. Cumulative evidence points towards an important role of ncRNAs in cancer initiation, development, and progression [22]. NcRNAs are classified in small and long ncRNAs (lncRNA) based on their size and function (Figure 1).

1.4.1. Long Noncoding RNAs. Long noncoding RNAs (lncRNAs) have a length greater than 200 nucleotides (nt) and are located in the nucleus or in the cytoplasm. The number of lncRNAs is not clear. It has been estimated that approximately 15,000 lncRNAs are present in the human genome, but the GENCODE v19 catalog of human lncRNAs contains

Table 1: Overview of prostate cancer biomarkers.

Specimens	Category	Examples	Up/down	Summary/description	Reference
Tissue	**Noncoding RNAs:**				
	lncRNAs	*PCA3*	↑	PCa-specific, 66-fold upregulated in PCa-tissue compared to nonmalignant prostate tissue (in >95% of PCa patients)	[6, 18, 40–43]
		CBR3-AS1, MALAT1, PCAT1, 6, 7, 18, PCGEM1, PRNCR1, and *SCHLAP1*	↑	Identified overexpressed lncRNAs in PCa tissue	[23, 44–51]
	microRNAs	miR-21, miR-183/96/182	↑	Well-known oncomirs, upregulated miRNAs in several cancer tissues	[52–71, 108]
		miR-221/222	↑	Upregulated in different cancer tissue, highly expressed in CRPC tissue	[66, 72, 73]
		miR-375	↑	Upregulated in PCa tissue compared to nonmalignant tissue	[66, 109]
		miR-143/145	↓	Downregulation is associated with progression of cancers	[91–93]
		miR-205, miR-200-family	↓	Well-known tumor suppressor, downregulated in many cancer tissues, involved in EMT	[66, 74–90]
	Protein-coding genes:				
	mRNAs/proteins	AMACR, caveolin-1, CD147, endoglin (CD105), human kallikrein-2, interleukin-6, PSMA, and TGF-β1		Upregulated in PCa tissue compared to nonmalignant tissue; AMACR: upregulated in 88% of PCa, CRPC and metastasis strongly positive; CD147: overexpressed in many solid tumors; increased expression is associated with PCa progression and poor prognosis; PSMA: transmembrane glycoprotein, upregulated in PCa tissue compared to benign tissue; TGF-β1: growth factor; increased expression correlates with tumor invasion, metastasis and biochemical recurrence; human kallikrein-2: serine protease activates pro-KLK3 to its active form (PSA)	[6, 18]
	DNA modifications:				
	Genetic modification	*TMPRSS2-ERG* fusion, BRCA1/2 mutation		*TMPRSS2-ERG* gene fusion: expressed in malignant prostate tissue, independent marker of disease progression and poor prognosis; BRCA1/2 are tumor suppressors; BRCA2 mutation is associated with aggressive PCa and poor survival outcome	[6, 18]
	Epigenetic modification	*GSTP1, RASSF1A*		*GSTP1* hypermethylation was detected in 90% of prostate cancer; *RASSF1A* is a tumor suppressor gene; *RASSF1A* hypermethylation has been observed in 60–74% of PCa and in 18.5% of BPH	[18, 19]

Table 1: Continued.

Specimens	Category	Examples	Up/down	Summary/description	Reference
Body fluids (blood and/or urine)	**Noncoding RNAs:**				
	lncRNAs	*PCA3*	↑	PCA3 score (*PCA3/KLK3* ratio) FDA approved as diagnostic biomarker for PCa (sensitivity 66%, specificity 76%)	[6, 18, 41–43]
	microRNAs (circulating)	miR-141, miR-375, miR-21, miR-221/222	↑	upregulated in plasma/serum of PCa patients with advanced disease (metastasis and/or CRPC)	[108–130]
		miR-200-family	↑	Upregulated in serum of CRPC patients; high levels were found in serum of nonresponders to docetaxel prior treatment, associated with shorter survival	[9, 111]
		miR-107, miR-574-3p	↑	Upregulated in urine of PCa patients compared to healthy controls	[112]
		miR-205, miR-214	↓	Downregulated in urine of PCa patients compared to healthy controls	[134]
	DNA modifications:				
	Genetic modification	*TMPRSS2:ERG* fusion		Detection of TMPRSS2:ERG fusion transcript in urinary sediments, obtained after DRE (specificity 93%), combined test: PCA3 score + *TMPRESS2:ERG* fusion in urine after DRE—improved sensitivity from 66% (PCA3 alone) to 73% (combined)	[6, 18]
	Epigenetic modification	*GSTP1*		*GSTP1* hypermethylation was found in postprostate massage urine sediments of 68% of PCa patients with early confined disease, 78% of patients with locally advanced PCa, 29% of patients with PIN, and 2% of patients with BPH.	[18, 19]
	Cells/vesicles:				
	CTCs			Detection of CTCs in blood has the potential to evaluate disease progression and for monitoring of therapy response. The Veridex CellSearch Assay has received FDA approval for the enumeration of CTCs in prostate cancer.	[16, 165–170]
	Extracellular vesicles	Exosomes, apoptotic bodies, microvesicles, and prostasomes		Extracellular vesicles are cell-derived vesicles that can be isolated from urine and blood and have the potential as biomarker for PCa. They also contain specific DNA, RNA, and protein molecules that are unique to the cells they originate of, and these could also serve as biomarker(s).	[125, 142–150, 157]

AMACR: α-methylacyl-CoA racemase; CTCs: circulating tumor cells; *GSTP1*: glutathione S-transferase pi 1; KLK3: kallikrein-3; lncRNAs: long noncoding RNAs; PCa: prostate cancer; PSA: prostate-specific antigen; PSMA: prostate-specific membrane antigen.

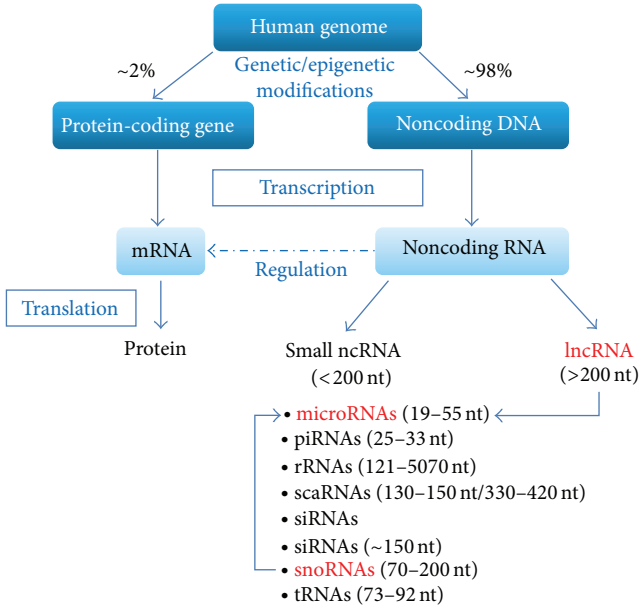

Figure 1: The human genome consists of approximately 2% protein-coding sequences, which can be transcribed into messenger RNAs (mRNAs) and then translated into proteins. The majority of the human genome exists in nonprotein-coding DNA, which can be transcribed in (functional) noncoding RNAs (ncRNAs). According to their size and function, ncRNAs can be grouped into long noncoding RNAs (lncRNAs) and small ncRNAs. The group of small ncRNAs, which are less than 200 nucleotides (nt) in length, consists of microRNAs (miRNAs), piwi-interacting RNAs (piRNAs), ribosomal RNAs (rRNAs), small Cajal body-specific RNAs (scaRNAs), small-interfering RNAs (siRNAs), small nuclear RNAs (snRNAs), small nucleolar RNAs (snoRNAs), and transfer RNAs (tRNAs). Beside their biogenesis from hairpin precursor molecules, miRNAs can also be derived from lncRNAs and snoRNAs (highlighted in red).

13,870 lncRNA genes that produce 23,898 lncRNAs [23]. However, only a few lncRNAs are expressed in a cell type-specific manner [24, 25]. Recent studies have demonstrated that lncRNAs regulate many processes such as transcription, translation, cellular differentiation, gene expression regulation, cell cycle regulation, chromatin modification, and nuclear-cytoplasmic trafficking [20, 26–28]. LncRNAs can function as oncogenes or as tumor suppressors. Examples for oncogenic lncRNAs are *CDKN2B-AS1* (ANRIL), which is described in tissue of melanoma and PCa, HOX antisense intergenic RNA (*HOTAIR*), which was found in breast and colon cancer, and *CCAT1*, which is increased in gastric carcinoma tissue. LncRNAs that function as tumor suppressors are, for example, growth arrest-specific 5 (*GAS5*), which is aberrantly expressed in several cancers including PCa and the pseudogene *PTENP1* [20]. Several PCa-specific lncRNAs have been identified, such as *PCA3*, *PCAT1*, and *PCGEM1*. The identification and function of these PCa-specific lncRNAs are excellently reviewed by Walsh et al. [23]. The most interesting lncRNA candidates as PCa biomarkers are discussed in the chapter "lncRNAs and prostate carcinogenesis" (see Section 2.1).

1.4.2. Small Noncoding RNAs. Small ncRNAs, in general, have a size of less than 200 nucleotides. Based on their size and function the small ncRNAs can be subdivided into microRNAs (miR, miRNAs), piwi-interacting RNAs (piRNAs), ribosomal RNAs (rRNAs), short interfering RNAs (siRNAs), small Cajal body-specific RNAs (scaRNAs), small nuclear RNAs (snRNAs), small nucleolar RNAs (snoRNAs), and transfer RNAs (tRNAs, Figure 1).

MiRNAs are the best characterized class of small ncRNA transcripts. Currently, there are 2,578 mature human miRNAs listed in the miRBase catalog of human miRNAs (v20, June 2013) [22, 29], and the number of identified miRNAs is still rising. Many excellent articles have described the biogenesis and function of miRNAs and their role in human diseases such as cancer. MiRNAs are fragments of single-stranded ncRNAs of 19–25 nt, derived from hairpin precursor molecules [30, 31]. They are reported to regulate more than 50% of all protein-coding genes in mammalian cells [31, 32]. Predominantly, they repress protein expression by inhibiting translation or by degradation of the target mRNA [33]. Genes can be targeted by multiple miRNAs and each miRNA is able to target hundreds of mRNAs directly or indirectly. According to their important role in the regulation of genes that are involved in many physiological processes, it is not of a surprise that they are also involved in the initiation and progression of cancer [34]. The dysregulation of miRNAs has been demonstrated in all types of human malignancies. Like, for proteins, both miRNAs with oncogenic functions, also called oncomirs, and tumor suppressive miRNAs have been found [34, 35]. The expression of miRNAs can be influenced by chromosomal rearrangements (deletions, amplifications, and mutations), by DNA methylation, or by other types of transcriptional control.

It is becoming evident that also another class of small ncRNAs, the small nucleolar RNAs or snoRNAs, may be dysregulated in cancer. Most snoRNAs are ubiquitously expressed and function in the maturation and modification of other ncRNAs such as rRNAs [36]. SnoRNAs can be divided into two structural classes, C/D-box (SNORD) and H/ACA-box (SNORA) RNAs [37]. Until now, approximately 400 snoRNA species have been identified in the human genome [37]. It is also known that some miRNAs originate from snoRNAs and these snoRNA-derived miRNAs are termed sno-miRs, sno-miRNAs, or sdRNAs. Very recently it was reported that snoRNAs and fibrillarin, which is an enzymatic small nucleolar ribonucleoprotein (snoRNP), are frequently overexpressed in human breast and prostate cancer tissues [38]. Specific cancer-related snoRNA signatures in blood were described for nonsmall-cell-lung cancer [37, 39]. Further investigations are necessary to confirm these findings, to describe the role of snoRNAs and sdRNAs in (prostate) cancer, and to evaluate their potential as biomarker.

2. What Is Known about Noncoding RNAs in Prostate Cancer?

2.1. Long Noncoding RNAs and Prostate Carcinogenesis. A well investigated lncRNA in PCa is *PCA3*. *PCA3* was found

to be strongly overexpressed (66-fold in PCa tissue compared to normal prostate tissue) in more than 95% of primary PCa specimens and metastasis [40, 41]. It is not expressed in other normal human tissues and, therefore, *PCA3* is so far the most PCa-specific gene known. *PCA3* can be identified in post-DRE voided urine, and based on this it was claimed to be a novel PCa biomarker. The PROGENSA PCA3 test is the first FDA-approved urine-based molecular diagnostic test for men with elevated serum PSA and a previous negative biopsy. A urine PCA3 score (*PCA3*-to-*PSA* ratio) with a cut-off of ≥35 had an average sensitivity of 66% and a specificity of 76% for the prediction of PCa at biopsy (area under the curve (AUC) 0.75), while serum PSA had only a specificity of 47% [18, 42]. The PCA3 score is not influenced by age, inflammation, prostate volume, or 5α-reductase inhibitors [18]. The use of the *PCA3* ncRNA as biomarker in clinical practice has been extensively reviewed [18, 43]. However, in independent studies, no significant association of PCA3 score in urine with prognostic or predictive parameters was found.

Additional PCa-specific lncRNAs have been described (Table 1). Overexpression of oncogenic lncRNAs may promote tumor-cell proliferation and metastasis, and aberrant expression of lncRNAs in PCa is associated with disease progression. The lncRNA metastasis-associated lung adenocarcinoma transcript 1 (*MALAT1*) is overexpressed in several human cancers, including PCa. In primary PCa, *MALAT1* overexpression is correlated with markers of poor prognosis (high Gleason-score, higher tumor-node-metastasis (TNM) stage, and serum PSA >20 ng/mL) and its expression significantly increases from hormone sensitive PCa to CRPC [44]. Small interfering RNA- (siRNA-) mediated knockdown of *MALAT1* in PCa cell lines 22Rv1 and LNCaP inhibits cell growth, invasion, and migration and results in cell cycle arrest in the G0/G1 phase, demonstrating its functional role in PCa [44].

The lncRNA prostate cancer associated ncRNA transcript- (*PCAT*-) 1 is highly prostate-specific and is upregulated in a subset of high-grade localized (Gleason-score ≥7) and metastatic PCa [23]. *PCAT1* induces cell proliferation and has a repressive effect on gene expression, for example, on the tumor suppressor *BRCA2* [45]. By Affymetrix array-based expression profile analysis 213 lncRNAs were found to be significantly differentially expressed in PCa tissue compared to benign prostate tissue. Based on these results, two novel clinically relevant lncRNAs have been identified in PCa, *PCAT6*, and *PCAT7.* The expression of both lncRNAs increased from normal prostate tissue to primary to metastatic PCa [23]. Knockdown of *PCAT6* and *PCAT7* reduces cell growth and soft agar colony formation in LNCaP cells.

Second chromosome locus associated with prostate-1 (*SCHLAP1*) is a lncRNA that is highly expressed in 25% of prostate tumors. *SCHLAP1* expression is revealed as a predictor for PCa aggressiveness, with highly significant hazard ratios for predicting biochemical recurrence, clinical progression, and PCa-specific mortality in a large cohort of localized PCa ($n = 235$, median follow-up 8.1 years). *SCHLAP1* has the potential as prognostic marker due to a significant association between biochemical recurrence and overall survival and high expression levels of *SCHLAP1* [23, 46].

The lncRNA prostate cancer gene expression marker 1 (*PCGEM1*) was first described by Srikantan et al. in 2000 [47]. Recently, Yang et al. reported about two lncRNAs, prostate cancer noncoding RNA1 (*PRNCR1*) and *PCGEM1*. *PCGEM1* is overexpressed in more than half of PCa tissues [48]. The authors reported that these lncRNAs regulate AR-mediated gene transcription in PCa. In CRPC, both lncRNAs *PCGEM1* and *PRNCR1* activate the transcription of AR splicing variants, even in the absence of ligand binding [23, 48]. However, Prensner et al. confirmed the association of *PCGEM1* and *PRNCR1* with PCa, but the authors found no interaction with AR or components of AR signaling. Furthermore, they demonstrated that *PCGEM1* and *PRNCR1* are not useful as prognostic marker after analysis of 230 high-grade PCa patients and their clinical outcome [49]. It is of note that it is also recently reported that *PCAT18* is a highly prostate-specific transcript, upregulated in prostate cancer, and that the expression of *PCAT18* is induced by AR signaling [50]. Furthermore the lncRNA CBR3 antisense RNA 1 (*CBR3-AS1*) has been reported to be associated with changes in AR activity [51].

So, in addition to *PCA3*, several novel PCa-specific or PCa-associated lncRNAs are on the horizon, but none of these, so far, made it to a clinical test for PCa. In initial studies, the presence of lncRNA fragments in blood specimens has been analyzed. For example, *MALAT1* levels in plasma were determined and these were found to be able to distinguish biopsy-positive from biopsy-negative PCa patients (AUC 0.767; $P < 0.001$). However, the sensitivityof the plasma *MALAT1* test was 58.6% and therefore lower than that for serum PSA [44]. Research concerning lncRNAs as biomarkers for PCa is still in its infancy, and further investigations as well as large validation studies are necessary before a translation into the clinical setting will be possible.

2.2. Dysregulated microRNAs in Cancer Tissue. Many miRNA profiling studies in PCa have been performed, using technologies such as microarray analysis and next-generation RNA sequencing (NGS) [52]. These methods allow the analysis of many miRNAs simultaneously, but because of their low sensitivity and their high throughput-screening nature a validation in independent samples using quantitative technologies such as qRT-PCR is needed.

Some miRNAs (Table 1) have been shown to be dysregulated and functionally relevant in certain cancer types [53, 54]. Volinia et al. reported about 21 miRNAs that were upregulated in six solid cancer tissues [55]. MiR-21 is well known to play an important role in normal and pathological processes including development, inflammation, cardiovascular function, and cancer. Many researchers found high expression of miR-21 in almost all types of solid cancer tissues including PCa and, therefore, it was classified as an oncomir [56]. MiR-21 targets tumor suppressor genes, such as phosphatase and tensin homolog (*PTEN),* tumor suppressor gene tropomyosin 1 (*TPM1*), programmed cell death 4 (*PDCD4*), maspin, and reversion-inducing cysteine-rich protein with

Kazal motifs (*RECK*) [57–60]. The activation of miR-21 might enhance general processes of tumor progression, invasion, and metastasis [17]. Furthermore, Wang and Zhang reported that miR-21 was elevated in serum of patients with breast cancer, colorectal cancer, lung cancer, esophageal cancer, and gastric cancer compared to healthy controls and concluded that miR-21 has the potential as a broad-spectrum serum-based biomarker for the detection of some solid cancers. However, the authors found no correlation with gender, clinical stage, and lymph node metastasis [61].

Zhang et al. [54] systematically reviewed 49 studies that investigated the expression of miR-183 family members, consisting of miR-96, miR-182, and miR-183 in different tumors (HCC, gastric, pancreatic, colon, rectal, breast, gynecologic, lung, bladder, and prostate cancer) compared to noncancerous tissues. Several studies reported that these miRNAs are directly involved in human cancer processes, such as cellular differentiation, tumorigenesis, proliferation, apoptosis, and metabolism [54, 62–64]. All miRNAs from the miR-182-96-183 cluster, located on chromosome 7q32.2, were upregulated in 14 human cancers. The miR-183 family members were most upregulated in colorectal and prostate cancer tissue [65–68], followed by bladder, lung cancer, and HCC tissue [54]. The results for breast and gastric cancer were inconsistent and miR-183 was downregulated in osteosarcoma [69]. These miRNAs were so far not described in the circulation of cancer patients, but higher levels of miR-96, miR-182, and miR-183 were found in urine specimens of bladder cancer patients compared to healthy controls [70, 71].

Upregulation of miR-221 and miR-222 has been observed in several cancer cells [72] and it is also reported that the miR-221/miR-222 cluster is highly expressed in metastatic CRPC tissue. A transient overexpression of miR-221/miR-222 in LNCaP cells promoted the development of a CRPC-like phenotype. As a result of elevated expression of miR-221, expression of many cell cycle genes was altered and pathways promoting epithelial-to-mesenchymal transition (EMT) and tumor metastasis were activated [72]. Upregulated levels of circulating miR-221 were identified in serum of PCa patients and also in serum or plasma of patients, for example, with lung cancer, ovarian cancer, melanoma, and lymphoma, compared to their healthy controls [31, 73].

MiR-205 is frequently downregulated in different cancers, including glioblastoma [74, 75], melanoma [76], breast cancer [77, 78], renal cell carcinoma [79], and prostate cancer [80–83]. The presence of miR-205 suppresses cell proliferation and metastasis. In prostate cancer, miR-205 functions as a tumor suppressor through downregulation of multiple targets like BCL2 [81], protein kinase C epsilon [84], and androgen receptor [85]. Genes regulated by miR-205 are enriched in, for example, the MAPK/ERK, toll-like receptor, and IL-6 signaling pathways [85]. Furthermore, miR-205 is observed to be downregulated in cells that have undergone EMT, a process that is accompanied by a decrease in E-cadherin and fibronectin expression [86]. Inversely, expression of miR-205 is upregulated in mesenchymal cells that initiated mesenchymal-to-epithelial transition (MET) associated with an upregulation of E-cadherin and a reduction of tumor cell migration and cell invasion [82, 85]. MiR-205 inhibits tumor invasion through several pathways; for example, low-density lipoprotein receptor-related protein 1 (LRP-1) promotes cancer cell migration and invasion by inducing the expression of matrix metalloproteinases (MMP) 2 and 9 [87, 88]. Schaefer et al. reported about an inverse correlation between prostate tissue miR-205 levels and the occurrence of metastases and shortened overall survival of PCa patients [66, 85]. Wang et al. confirmed these results and they reported a stronger downregulation of miR-205 in advanced and/or metastatic PCa [89]. MiR-205 was significantly downregulated in serum of breast cancer patients compared to healthy controls [90], but studies on serum of PCa patients are lacking. Nevertheless, circulating miR-205 may be a promising biomarker for PCa prognosis.

In addition to miR-205, miRNAs from the miR-143/miR-145 cluster are also downregulated in (prostate) cancer [91]. MiR-143 and miR-145 are transcribed after TGFβ1 pathway activation and inhibition of this pathway will lead to a decrease of these miRNAs [92, 93]. MiR-143 targets Kirsten rat sarcoma viral oncogene homolog (KRAS), ELK1, myosin 6, B cell lymphoma 2 (BCL-2), and extracellular signal-regulated kinase 5 (ERK). A loss of miR-143 expression causes an upregulation of ERK5, which induces cell proliferation, survival, and invasion and as a consequence it leads to the development of more aggressive forms of PCa [92]. The downregulation of miR-145 also leads to enhanced cell proliferation. MiR-145 targets MYO6 and fascin homolog 1 (FSCN1), proteins also associated with PCa progression [92]. Whether miR-143 and miR-145 can serve as biomarker for PCa still has to be investigated.

While the above described miRNAs are dysregulated in many types of cancer, some miRNAs seem to be tissue-specific. MiRNA expression profiles specific for PCa have been discovered [17, 55, 66, 91, 94–98]. These profiling studies showed differences in the expression of miRNAs in localized or metastatic prostate cancer compared to benign prostate epithelium or BPH, but in these studies also a large number of non-tissue-specific miRNAs were found. The first profiling of miRNAs in PCa was published in 2007. Porkka et al. [94] observed a downregulation of 37 miRNAs and an upregulation for 14 miRNAs in PCa. Schaefer et al. identified 10 miRNAs (miR-16, miR-31, miR-125b, miR-145, miR-149, miR-181b, miR-184, miR-205, miR-221, and miR-222) to be downregulated and 5 miRNAs (miR-96, miR-182*, miR-183, and miR-375) that were upregulated in PCa tissues compared to the matched normal tissues [66]. Ozen et al. found a widespread downregulation of miRNAs in prostate cancer tissue [96] and Ambs et al. reported about miR-32 to be upregulated in PCa tissue [95]. Many of these differentially expressed miRNAs are described only in one study and, thus, have to be validated in independent investigations.

2.3. Circulating microRNAs as Diagnostic and Prognostic Biomarkers in Prostate Cancer. For most of the deregulated miRNAs in tissue, it is still unclear whether they can serve as novel diagnostic and/or prognostic biomarkers. However, some of the dysregulated ncRNAs in cancer tissue can be

analyzed in body fluids and are therefore promising as putative biomarkers for PCa (Table 1).

MiRNAs can be released into the blood circulation (Figure 2) as a result of apoptotic and necrotic cell death as well as by active secretion [99]. Extracellular miRNAs may be involved in cell-cell communication and immune regulation [100]. Cell-free miRNAs are detectable in all body fluids. This accessibility makes them attractive as promising biomarker candidates for PCa, as well as for other malignancies. Mitchell et al. showed that cell-free circulating miRNAs are highly stable and reproducibly detectable in serum and plasma of humans [101, 102]. The stability of miRNAs in noncell environments (i.e., protection from RNase activity) may be because they form complexes with RNA-binding proteins such as AGO1, AGO2 or high-density lipoprotein (HDL) for the transport of miRNAs to recipient cells via the bloodstream (Figure 2). Beside other protective mechanism, they are also incorporated in microvesicles, like exosomes and apoptotic bodies [103–105]. Circulating miRNAs also may originate from the tumor surrounding tissue, from other tissues in the human body that are not involved in cancer or from blood cells [106, 107]. Studies are ongoing to characterize potential cancer-specific circulating miRNAs.

Some studies described an upregulation of miR-21, miR-200c, and miR-375 in prostate cancer tissue compared to nonmalignant prostate tissue [66, 95, 108, 109]. Higher levels of miR-21, miR-141, miR-200a, miR-200b, miR-200c, and/or miR-375 were also observed in serum, plasma, or circulating microvesicles in patients with metastatic disease compared to localized PCa or healthy controls [109–113]. The latter findings suggest that these miRNAs are derived from tumor tissue and may act as circulating miRNA biomarkers for the detection of metastatic disease. Circulating miRNAs can originate from tumor cells, from other affected organs involved in tumor invasion and metastasis, or from inflammatory responses [114]. MiRNAs have been associated with prostate cancer; for example, miR-21 and miR-221 and their deregulation were described in tumor tissue [55, 66, 94, 95, 98] and serum of PCa patients [110, 115, 116]. The differences in serum miRNA levels between PCa patients ($n = 51$) and healthy controls ($n = 20$) were highly significant for miR-21 ($P < 0.001$, AUC 88%) and for miR-221 ($P < 0.001$, AUC 83%). In patients with metastatic disease the serum levels of miR-21, miR-221, and miR-141 ($P < 0.001$, AUC 75.5%) were significantly higher than in patients with a localized PCa [115]. Furthermore, circulating miR-21 and miR-221 were reported to discriminate PCa patients with intermediate risk from those with low risk CAPRA scores with a sensitivity of 38.1% and a specificity of 94.2% (AUC 0.801) [116].

Across independent studies, circulating miR-141 and miR-375 were the most promising miRNAs, suggested as diagnostic and prognostic markers for high risk PCa, and two miRNAs that were also decribed to be higher expressed in prostate cancer tissue compared to nonmalignant prostate tissue [66, 91, 95, 113]. Four independent studies analyzed circulating miRNA levels in about 240 PCa patients and 70 healthy controls in total and found either miR-141 or miR-375 or both as a diagnostic and prognostic marker(s) [17, 101, 113, 115, 117]. Mitchell et al. [101] reported, as the first, that tumor-derived miRNAs can enter into the circulation and can be measured in serum and plasma as a blood-based biomarker for human cancer. They observed that circulating miR-141 was significantly elevated in sera of prostate cancer patients (sensitivity 60%, specificity 100%) [101]. Brase et al. confirmed that circulating miR-141 and miR-375 were highly detectable in serum from patients with an advanced disease, and their levels correlated with high-risk tumors (Gleason-score ≥8 or metastasis) [113].

Independent studies evaluated the increased serum levels of miR-141 in patients with aggressive PCa [113, 115, 118, 119], indicating its diagnostic potential. However, Westermann et al. recently analyzed serum miR-141 in a multicenter study and reported that miR-141 did not qualify as an early diagnostic marker for PCa (AUC 0.49). In this study, serum samples were collected from 170 patients who underwent a prostate biopsy, of which 54 patients were diagnosed with PCa. MiR-141 levels were not increased in serum from patients with evident PCa compared to patients with a PCa-negative biopsy, but the authors reported that miR-141 levels were significantly increased in patients with a higher Gleason-score ($P = 0.049$). However, there was no association with clinical tumor stage or PSA [120]. The function of miR-141 and miR-375 in prostate cancer is still unclear. Waltering et al. found that miR-141 was upregulated by androgens and that an overexpression of miR-141 in LNCaP cells increased cell proliferation, suggesting that miR-141 could be involved in PCa progression [121]. Recently, it was reported that upon androgen treatment increased levels of miR-141 and miR-375 were released in the cell culture medium of LNCaP cells [122]. The authors concluded that an increased release of miR-141 and miR-375 from androgen-stimulated cells may explain their higher levels in the blood of patients with advanced PCa or CRPC [122, 123].

Beside single miRNAs, Chen et al. reported about a panel of five circulating miRNAs (miR-30c, miR-622, miR-1285, miR-let7c, and miR-let-7e) to discriminate PCa from healthy individuals and BPH patients with a sensitivity of 61% to 90% and a specificity of 57% to 75%, respectively (PCa versus healthy control AUC 0.86 and PCa versus BPH AUC 0.924) [124].

Larne et al. [68] identified four miRNAs that discriminate PCa from nonmalignant tissue. They combined these four miRNA levels into a miRNA index quote (miQ): (miR-96-5p × miR-183-5p)/(miR-145-5p × miR-221-5p). The advantages of such a miQ are the increased discriminatory power of the test and omitting the need for housekeeping genes. The described miQ test predicts the presence of PCa ($P < 0.0001$) with high accuracy (AUC 0.931), which was verified in four independent cohorts. miQ has also a prognostic power to predict aggressiveness of tumors (AUC 0.895), metastatic status (AUC 0.827), and overall survival ($P = 0.0013$, hazard ratio 6.5) [68]. In this pilot project, miQ was used with miRNAs identified in tissue specimens, but it has also the potential for analysis of a panel of miRNAs in serum/plasma and urine. The value of miQ as a PCa biomarker has to be validated in independent multicenter validation studies.

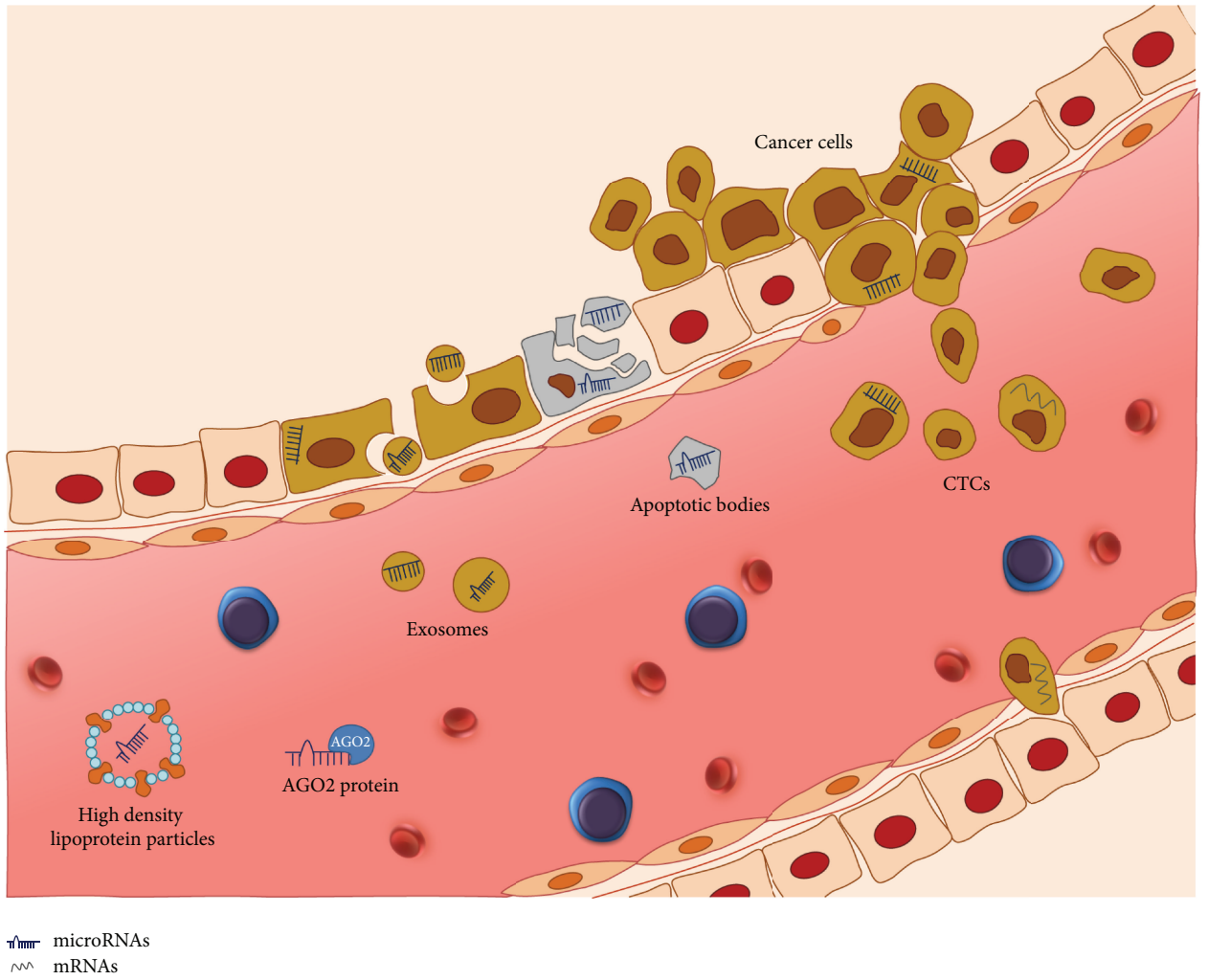

Figure 2: microRNAs can be released from normal and malignant cells in the blood circulation. This process can be passive, for example, after apoptosis, or as active secretion. In plasma or serum, cell-free microRNAs are protected against RNase activity, for example, through their binding to AGO1, AGO2 proteins or to high density lipoproteins. Furthermore, microRNAs can also be incorporated into microvesicles, such as exosomes, apoptotic bodies, or circulating tumor cells (CTCs). Specific microRNAs released from prostate cancer cells in body fluids, such as blood or urine, can serve as novel biomarkers for diagnosis, prognosis, prediction, or monitoring of cancer patients.

In recent investigations, additional up- or downregulated miRNAs in serum or plasma were described. Most of these miRNAs were only reported in one study, and therefore large validation studies are needed to verify these putative candidates [112, 116, 124–130].

In summary, miR-141 and miR-375 are the most consistently reported circulating miRNA candidates to be associated with high risk PCa. However, inconsistent findings based on miRNA levels are also notable. Major reasons for the variations and the lack of consistency in the data of circulating miRNAs are potentially variability in methodology of extraction and quantification of (micro) RNAs and data analysis [131]. There is no consensus on suitable reference RNAs that could be used as internal controls. Current protocols, using spiked-in synthetic nonhuman (e.g., *Caenorhabditis elegans*) miRNAs only correct for technical variability [101, 132]. Furthermore, contamination of miRNAs due to haemolysis or during sample processing is possible. The studies, published until now, are a solid basis for discovery and establishing new biomarkers in PCa, but for further studies better, widely accepted, standardization, and normalization protocols are needed.

2.4. Urinary microRNAs as Diagnostic and Prognostic Biomarkers. Due to its noninvasive and easily attainable nature, urine is a promising substrate for biomarker testing. It is known that prostate cells and ncRNAs can be directly released into the urethra through the prostatic ducts after DRE. Cellular and extracellular miRNAs in urine and urinary microvesicles may be derived from urological cancers (e.g., prostate, bladder, or renal cancer), but they can originate from normal epithelium, the glomerulus, or renal tubules [133]. Therefore, specific markers and good normalization

procedures are needed. The PROGENSA urine test for PCa makes use of such a PCa-specific biomarker, the *PCA3* lncRNA, and a normalization procedure using the prostate epithelium-specific marker *KLK3* (see Section 2.1).

Until now, only 4 studies reported miRNA levels in urine of PCa patients [112, 134–136]. Bryant et al. found that miR-107 and miR-574-3p were present at significantly higher levels in urine of men with PCa compared to controls [112]. Srivastava et al. analyzed miR-205 and miR-214 in tissue and urine of PCa patients and reported that these two candidates were present in detectable levels in urine samples but significantly lower in the cancer group compared to healthy controls. In this study, urinary miR-205 and miR-214 were able to discriminate PCa patients and healthy controls with 89% sensitivity and 80% specificity [134]. In a profiling study using microarray analysis and validation by qRT-PCR, miRNA levels in whole urine of PCa patients were compared to patients with BPH and healthy controls. The investigators identified miR-1825 and miR-484 as potential urinary biomarkers for PCa diagnosis [135]. Unfortunately, this study was limited by a very small number of samples (8 PCa patients, 12 BPH patients, and 10 healthy men) [135]. In a profiling study, Sapre et al. identified miR-16, miR-21, and miR-222 as predictors of high risk PCa, but the authors were not able to validate the results in an independent cohort [136].

Due to their high stability and easy detection, urinary miRNAs have a high potential to become noninvasive biomarkers. Further investigations, using standard procedures for preanalytical processing and data normalization, followed by validation of candidate miRNAs in large clinical trials are required to translate incidental finding(s) into a clinically applicable test.

2.5. microRNAs as Predictive Biomarkers in Prostate Cancer. MiRNAs have also the potential to serve as predictive markers. In a profiling study, Selth et al. detected increasing levels of three circulating miRNAs (miR-141, miR-146b-3p, and miR-194) in serum of patients who experienced a rapid biochemical recurrence after radical prostatectomy, but only miR-146b-3p and miR-194 were also associated with disease progression in a validation cohort [119]. Santos et al. found higher levels of miR-221 and miR-7 in blood of patients diagnosed with high Gleason-score PCa and those patients with high miR-221 levels developed CRPC much earlier (10 versus 46 months) [137]. Furthermore, the authors observed a significantly lower overall survival in patients with high levels of miR-7 and, therefore, it is suggested that miR-221 and miR-7 can serve as potential predictive biomarkers in advanced prostate cancer [137]. Independently, Kneitz et al. reported that miR-221 in prostate cancer tissue is an independent predictor for cancer-related death and suggested that miR-221 offers a novel tissue-based predictive biomarker and possibly therapeutic target in high-risk PCa [138].

Recently, Huang et al. demonstrated that single-nucleotide polymorphisms (SNPs) in microRNAs or miRNA target sites can act as a useful predictive biomarker. The authors found two SNPs (rs2043556 in miR-605 and rs3737336 in the 3′UTR of *CDON*) to be associated with biochemical recurrence after radical prostatectomy ($P < 0.05$) [139]. SNP rs3737336 lays in *in silico* analysis within putative target sites of *CDON* for miR-181a, miR-181b, miR-181c, miR-181d, miR-4262, and miR-5007 [139]. Furthermore, the authors observed an increased risk for biochemical recurrence with cumulative number of risk alleles; two risk alleles and 3 or 4 risk alleles had 1.55-fold ($P = 0.009$) and 2.53-fold ($P < 0.001$) increased risk of biochemical recurrence, respectively [139]. Hulf et al. found that miR-205 DNA methylation is significantly associated with biochemical recurrence and the authors suggest that miR-205 is an epigenetically regulated tumor suppressor [83].

2.6. microRNAs as Therapeutic Response. The improvement of therapy modalities leads to a need for markers that can help decide the best therapy for the individual patient. In addition, markers are needed for monitoring, that is, to evaluate early therapeutic response, to monitor effectiveness of a treatment, and to predict chemoresistance [140, 141]. Only a few studies have investigated the levels of (circulating) miRNAs as therapeutic markers. The investigations that evaluated potential biomarkers in metastatic PCa and/or CRPC are limited by the number of patients and tested miRNAs [9, 109–111, 118]. In these studies, the researcher investigated upregulated circulating miRNAs in serum of CRPC patients that are applicable candidates as markers for therapeutic response. Nguyen et al. [109] demonstrated that miR-375, miR-378*, and miR-141 were significantly higher in serum of CRPC patients compared to serum of low risk patients with a localized PCa. Zhang et al. [110] reported that serum levels of miR-21 are higher in patients with CRPC compared to patients with an androgen-dependent PCa and these patients with low levels of serum PSA had also levels of miR-21 similar to patients with a localized PCa or BPH [110]. Furthermore, Cheng et al. [111] confirmed the previous results and found that miR-141 ($P < 0.0001$), miR-200a ($P = 0.007$), miR-200c ($P = 0.017$), miR-375 ($P = 0.009$), and miR-210 ($P = 0.022$) were significantly elevated in serum of CRPC patients compared to age-matched controls [111].

Until now, only Lin et al. determined an association of circulating microRNAs and docetaxel chemotherapy outcome of CRPC patients ($n = 97$) [9]. The authors identified 14 miRNAs out of 46 to be associated with overall survival or PSA response to chemotherapy. Nonresponders to docetaxel and patients with a shorter survival had high levels of miR-200 family members prior to chemotherapy or decreased/unchanged levels of miR-17 family members after docetaxel treatment and therefore the authors associated high levels of miR-200 family members in serum with poor chemotherapy outcome of CRPC patients [9]. Furthermore, Gonzales et al. [118] analyzed miR-141, which is a member of the miR-200 family, together with circulating tumor cells (CTCs), lactate dehydrogenase (LDH), and PSA in serum of 21 PCa patients and examined the utility of miR-141 alone or in combination with CTCs, LDH, and PSA as a predictive marker and response to therapy. The authors found a strong correlation between clinical course according to progression

and nonprogression of PCa and miR-141 levels. MiR-141 levels predicted a clinical progression with an odds ratio of 8.3 and had a high correlation with changes of PSA ($R = 0.77$; $P < 0.001$) and CTCs ($R = 0.76$; $P < 0.001$) [118]. However, independent studies are necessary to confirm these findings and to validate treatment response.

2.7. Extracellular Vesicles as Biomarkers in Prostate Cancer. It is known that cells are able to release several types of extracellular vesicles, which are involved in many physiological and pathological processes, such as immune response and cellular differentiation [142]. Extracellular vesicles differ mainly in their cellular origins and sizes. The most important extracellular vesicles released from cells are apoptotic bodies, exosomes, and shed microvesicles (MVs). Apoptotic bodies are released from the cell membrane as a final consequence of cell fragmentation during apoptosis [142]. They have an irregular shape and a size of 1–5 μm [143, 144]. Exosomes have a size of 30–100 nm and are released by the fusion of multivesicular bodies (MVB) with the plasma membrane [143, 144]. Shed MVs, which are 100–1000 nm in size, are released by outward budding or blebbing of the plasma membrane [142]. Extracellular vesicles can be isolated from all body fluids, for example, blood, urine, semen, ascites, and malignant pleural effusion [125, 142] and they contain specific nucleic acids (e.g., miRNAs, mRNAs) and proteins, including enzymes, which represent their tissue origin.

Recent findings indicate that extracellular vesicles are not only waste products from cells. Exosomes and MVs that are released from viable cells are involved in intercellular communication in physiological as well as in pathological processes (e.g., cancer) [143]. Exosomes, actively secreted *in vitro* and *in vivo,* are involved in immune system modulation, regulation of neuronal cell functions, and cancer progression. Exosomes released from tumor cells can contribute to metastasis, stimulate angiogenesis, and can deliver prooncogenic miRNAs to target cells [142, 145–147]. All exosomes contain characteristic surface protein markers which enable their identification [6], such as CD9 [148], CD81 [149], and PDCD6IP [150].

Also MVs, which are characterized with high levels of phosphatidylserine in their membranes, contribute to cancer progression. MVs are, for example, able to modify the extracellular matrix through the involvement of lytic enzymes that are present in MVs [142, 151]. Through the transfer of enzymes MVs are also able to transform fibroblasts and epithelial cells to adopt typical cancer characteristics [152] and MVs can be involved in drug resistance through accumulation of antitumoral drugs [153].

In the literature another subpopulation of microvesicles, called prostasomes, has been described. Prostasomes are vesicles, derived from the prostate gland, that have a size of 50–500 nm (mean diameter 150 nm) and are present in high concentrations in seminal and prostatic fluids [154]. Some authors have hypothesized that prostasomes are just prostate-derived exosomes. Prostasomes are secreted by normal and malignant prostate acinar cells [155] after MVB fusion with membranes like exosomes, but they differ from exosomes according to their size and composition (e.g., membrane lipids). Like all extracellular vesicles, prostasomes are also involved in exchange of information. In physiological conditions, the recipient cells are mainly spermatozoa [156]. In addition, prostasomes are also involved in PCa progression [156] through an inhibition of the immune system, inhibition of the complement system, induction of migration by fibrinogen phosphorylation (protein kinases A and C), induction of invasion, and induction of angiogenesis [142]. Tavoosidana et al. evaluated the use of prostasomes as biomarkers and found elevated levels of these vesicles in semen of PCa patients, which were correlated with a higher Gleason-score of the tumor [157]. Furthermore, prostasomes were also found in plasma from PCa patients [142] suggesting their potential as biomarker.

Despite a clear difference in cell origin and size, extracellular vesicles are often overlapping according to their function and features. The currently available isolation techniques make it difficult to separate the subpopulations [142]. It was recently reported that tumor-derived circulating exosomes also contain miRNAs, which can downregulate their target genes in recipient cells [142]. Circulating exosomal miRNAs were correlated with miRNAs identified in tissue of ovarian cancer [158, 159] and lung cancer [160]. These results suggest that circulating exosomal miRNAs could potentially be used as diagnostic markers also in PCa [161]. The PCa specific gene fusion *TMPRSS2-ERG,* which is found in 50% of clinically localized PCa and which is associated with lethal PCa, is also present in exosomes isolated from VCaP cells (an androgen responsive PCa cell line) [162, 163]. Beside blood also urine, which contains exfoliated PCa cells and PCa-secreted products, is a promising resource for diagnostic markers. It is reported that urine from PCa patients contains more exosomes compared to patients with an indolent PCa or nonmalignant disease. In PCa patients, both *PCA3* and *TMPRSS2-ERG* were reported in urinary exosomes, showing that microvesicles may contain disease-specific molecules which then can serve as promising novel biomarkers [164]. However, *PCA3* and *TMPRSS2-ERG* were not detectable in exosomes from patients with ADT or in patients with bone metastasis after radical prostatectomy [164]. The currently used protocols for exosome isolation are time-consuming, including a series of (ultra) centrifugation steps and nanomembrane ultrafiltration. Further studies are necessary to establish new and faster methods for exosome isolation to enable fast and simple detection in routine clinical practice. The clinical relevance of circulating and urinary exosomes and prostasomes in semen as biomarkers for PCa needs to be evaluated.

2.8. Circulating Tumor Cells in Prostate Cancer. The importance of circulating tumor cells (CTCs) detection and molecular characterization is becoming evident. These cells can provide significant information for a better understanding of tumor biology and tumor cell dissemination [165]. CTC detection may be a useful approach for monitoring disease progression. CTCs have been shown to be the strongest independent predictor of overall survival in comparison to

posttherapy PSA. CRPC patients with more than 5 CTCs in 7.5 mL blood have a significantly reduced overall survival compared to patients with less than 5 CTCs in 7.5 mL blood [166]. These results were confirmed in other studies [167, 168]. CTC detection remains a major technical challenge. Currently, the use for diagnosis and monitoring is limited by costly CTC isolation procedures and the low number of CTCs in blood. However, technical advances could lead to novel possibilities.

The clinical utility of monitoring CTCs is currently being tested in large phase III trials with PCa patients being treated with the novel antiandrogen abiraterone acetate or the novel androgen receptor antagonist enzalutamide (a.k.a. MDV3100). Molecular determinants can be identified and characterized in CTCs as potential predictive biomarkers of tumor sensitivity to a therapeutic modality [165, 169]. Potential miRNA biomarkers were also identified in CTCs. Sieuwerts et al. found that, for example, miR-183 is expressed in CTCs of metastatic breast cancer patients [170]. Whether CTCs of PCa patients express specific ncRNAs remains to be investigated.

3. Conclusion

To improve the management of PCa patients, novel diagnostic, predictive, and prognostic biomarkers are needed. The ideal biomarker should be able to detect the presence of a disease and predict its progression (i.e., identify high risk individuals), predict recurrence, and monitor therapy response. PCa and especially CRPC are heterogeneous diseases and, therefore, it is not expected that a single biomarker for all stages of the disease will be identified. Multiple biomarkers, or panels of markers, will be needed to address each particular clinical question.

The lncRNA *PCA3* has already been successfully translated into clinical setting, especially to predict (repeat) biopsy outcome of patients with elevated serum PSA. Recent studies showed the potential of other ncRNAs, especially the miRNAs, as novel biomarkers for PCa. Across independent studies, elevated serum levels of miR-141 and miR-375 have been consistently found and confirmed for metastatic PCa and/or CRPC. These miRNAs are associated with further prognostic parameters like higher Gleason-score and positive lymph node status. There are a number of additional promising miRNAs and other biomarkers, such as exosomes and CTCs, for PCa on the horizon, but these await further validation in independent studies. Only then, these biomarkers can be translated into clinical practice.

Disclosure

Jack A. Schalken and Gerald W. Verhaegh are inventors on PCA3 related IP. The IP is owned by their employer, Radboudumc, who has licensed the technology and receives royalty payments.

Conflict of Interests

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

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Review Article

Novel RNA Markers in Prostate Cancer: Functional Considerations and Clinical Translation

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The availability of ultra-high throughput DNA and RNA sequencing technologies in recent years has led to the identification of numerous novel transcripts, whose functions are unknown as yet. Evidence is accumulating that many of these molecules are deregulated in diseases, including prostate cancer, and potentially represent novel targets for diagnosis and therapy. In particular, functional genomic analysis of microRNA (miRNA) and long noncoding RNA (lncRNA) in cancer is likely to contribute insights into tumor development. Here, we compile recent efforts to catalog differential expression of miRNA and lncRNA in prostate cancer and to understand RNA function in tumor progression. We further highlight technologies for molecular characterization of noncoding RNAs and provide an overview of current activities to exploit them for the diagnosis and therapy of this complex tumor.

1. Introduction

Prostate cancer (PCa) is the most frequent tumor in men and a major cause of cancer-related morbidity and mortality. The diagnostic application of the prostate specific antigen (PSA) has led to widespread overdiagnosis and subsequent overtreatment of clinically insignificant tumors. This problem will extensively aggravate in the future: with rising life time expectancy, the number of men diagnosed with PCa will increase since the frequency of PCa is steeply rising in elderly men. Thus, there is a huge demand for novel markers for improved PCa diagnosis, prognosis, and therapy decision.

Facilitated by the implementation of ultra-high throughput sequencing technologies, the recent discoveries of the ENCODE consortium (http://encodeproject.org/ENCODE/) confirmed that 80% of the human genome is transcribed [1–4]. The GENCODE effort has provided the annotation of more than 9000 lncRNAs [5], as well as micro- (mi-) RNA, small nucleolar (sno) RNA, piwi-interacting (pi) RNA, and others. In parallel, the number of investigations covering the functional analysis of these molecules has increased substantially. Based on these data, it is becoming obvious that many noncoding RNAs are involved in the regulation of gene expression in health and disease, including cancer (Figure 1). For example, global analyses of miRNA expression in cancers revealed robust signatures differentiating between malignant and benign tissues [6], between tumors of several origins, and between primary tumors and metastases [7]. The characterization of miRNA and lncRNA involvement in PCa is likely to result in a better understanding of tumor progression and the identification of novel therapy targets. Here, we provide a summary of recent progress in analyzing noncoding RNA in PCa and in attempting to advance miRNAs and lncRNAs into diagnostic and therapeutic markers.

2. miRNA in Prostate Cancer

MiRNAs are ~20–22 nt short noncoding RNAs, which regulate gene expression at the posttranscriptional level by mRNA repression and/or degradation. MiRNAs are transcribed as pri-miRNAs by RNA polymerase II (RNA Pol II) and processed by the RNase III enzyme Drosha to generate precursor miRNAs (pre-miRNAs; Figure 1) [8]. Dicer, another RNase III enzyme, then cleaves the pre-miRNA into ~20–22 long RNA duplexes. Subsequently, the strand with the stable

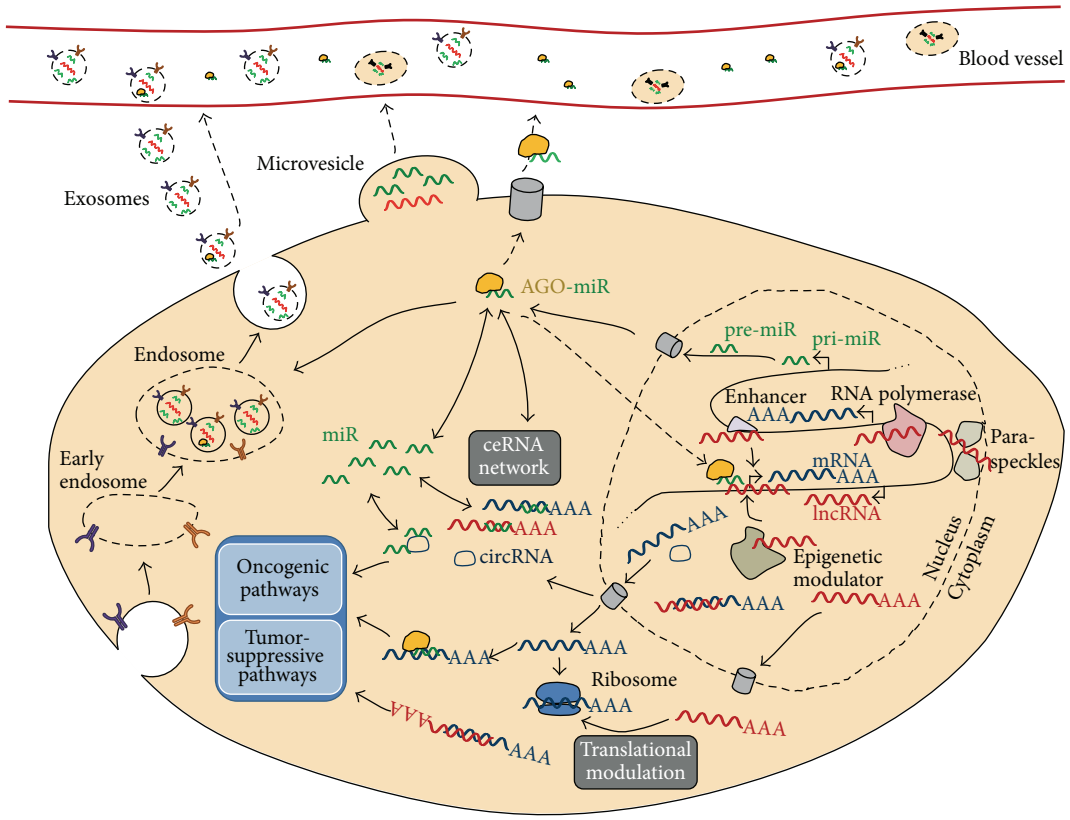

Figure 1: Schematic view of functional networks of different RNA species in cancer. Hypothetical interactions are symbolized with dashed arrows. AGO: Argonaute protein (yellow); miR: miRNA (green); lncRNA: long noncoding RNA (red); mRNA: messenger RNA (blue); circRNA: circular RNA (blue).

5′ end is removed, whereas the complementary strand with the less stable 5′ end is incorporated into Argonaute (AGO) proteins to form the RNA induced silencing complex (RISC). MiRNAs guide the RISC to target mRNAs via sequence complementarity [8–11].

Many miRNAs are known to be deregulated in cancer [6]. For instance, miRNAs are differentially expressed between benign prostate and PCa tissues: in a genomewide microarray-based microRNA expression study, 25 miRNAs were found to be deregulated [12]. Here, *miR-375* and *miR-200c* showed highest upregulation in PCa tumor tissue, whereas *miR-221* and *miR-222* were the most downregulated miRNAs [12]. Using a sequencing-based approach, the same group identified 33 miRNAs being up- or downregulated >1.5-fold in PCa tissue [13]. Here, *miR-143* and *miR-145* were the highest downregulated ones in PCa [13]. Consistent with these data, an independent study found *miR-145*, *miR-221*, and *miR-222* among the five most significantly downregulated miRNAs [14], and we identified *miR-375* and *miR-141* as significantly upregulated in PCa tissue [15].

Other analyses revealed the association of miRNA expression with distinct PCa: for instance, *miR-375* expression was reported to be dependent on pT and pN status (TNM staging system) of PCa patients, with the highest enrichment in pN1 tumors [16]. Moreover, ten miRNAs (including *miR-145*, *miR-221*, and *miR-222*) were downregulated and five (including *miR-375*and *miR-96*) upregulated in advanced PCa, whereas *miR-96* was associated with tumor recurrence after radical prostatectomy [17]. MiRNAs are also known to correlate with Gleason score: in high-grade PCa, increased expression of *miR-31*, *miR-182*, and *miR-205* was identified when compared to intermediate-grade PCa [18].

The functional consequences of differential expression of various miRNAs have been studied *in vitro* by many groups. Here, we can address only the most prominent examples: *miR-375* was found upregulated in several PCa studies and was known to target Sec23 homolog A (*SEC23A*) [19], which was involved in trafficking from ER to Golgi [20]. The authors suggested that *miR-375*-mediated *SEC23A* downregulation impairs cellular immunogenicity of PCa by reducing *HLA* class I cell surface receptors [19]. *MiR-375* was also shown to downregulate the phosphatase *PHLPP2* and to strongly increase prostate carcinoma cell growth [16]. *MiR-141,* which is upregulated in PCa tissue, was found to downregulate the gene coding for the nuclear receptor subfamily 0, group B, member 2 (*NR0B2*) in prostate epithelial cells. In this way, *miR-141* prevented the inhibitory effects of NR0B2 on androgen receptor- (*AR*-) regulated genes [21]. In contrast, *miR-143* and *miR-145* were found to be downregulated in PCa tissue and to be involved in PCa development via regulation of myosin VI (*MYO6*) [13]. MYO6 was involved in cancer cell dissemination and described as a potential PCa marker [22, 23].

Interestingly, miRNAs, which have not been described as highly deregulated in PCa tissues, were also found to play crucial roles in prostate carcinogenesis. For instance, *miR-200b* attenuated PCa cell growth and invasiveness [24, 25]. Studies in the PCa cell line PC-3 suggested that *miR-200b* mediates the reversal of the epithelial-to-mesenchymal transition (EMT) by targeting the prometastatic transcription factors Zinc finger E-box binding homeobox 1 and 2 (*ZEB1* and *ZEB2*) [25]. Overexpression of *miR-200b* led to increased levels of the epithelial markers E-cadherin, cytokeratins 8 and 18, as well as downregulation of the mesenchymal markers fibronectin and vimentin [25, 26]. Another study found *miR-1* to be reduced with PCa progression. SLUG is a regulator of mesenchymal differentiation and was assumed to repress *miR-1* and *miR-200* transcription whereas *miR-1* and *miR-200* are also able to regulate *SLUG* by a mutually inhibitory feedforward loop [27]. Moreover, *miR-1* was found to act as a mediator of oncogenic pathways in the PCa cell lines PC-3 and LNCaP: the heat-shock protein HSPB1 reduced *miR-1* levels leading to an activation of AR and TGFB1 signaling cascades [28].

MiRNAs are also able to regulate the expression of important *epigenetic marker genes.* For instance, *miR-34b* targeted methyltransferases and deacetylases in PCa [29]. *MiR-34b* is also an example for epigenetic miRNA regulation via a positive feedback loop. *MiR-34b* was found to directly target the AKT pathway like *AKT* itself and its downstream genes *GSK3B*, *CTNNB1*, *MYC*, and *CCND1*, which were involved in cell proliferation and survival [29]. In a PCa xenograft model, *miR-34b* exhibited antitumor properties by decreasing prostate tumor growth [29]. This was probably due to affecting EMT since *miR-34b* expression decreased the mesenchymal markers vimentin, ZO1, N-cadherin, and SNAIL, and increased E-cadherin [29]. Furthermore, *miR-152* was reported to downregulate DNA (cytosine-5)-methyltransferase 1 (*DNMT1*) in LNCaP, PC-3, and MDA-PCa-2b cells. *DNMT1* siRNA caused an increase of *miR-152* expression, suggesting a reciprocal relationship between *DNMT1* and *miR-152*. Increased *DNMT1* and depletion of *miR-152* were assumed to arise during the progression to advanced PCa [30]. The *miR-200* family was downregulated by the histone methyltransferase enhancer of zeste homolog 2 (EZH2) [24]. EZH2 is a component of the polycomb repressive complex 2 (PRC2), which is involved in epigenetic regulation of cell homeostasis. *EZH2* expression was elevated in aggressive forms of PCa [31]. *MiR-200* family on the other hand represses PRC1 proteins BMI1 and RING2. Therefore, increased PRC2 levels lead to an increase of PRC1 expression due to miRNA linkage, suggesting crucial roles for miRNA in regulating the epigenetic silencing machinery [24].

MiRNAs are primarily known as key regulators of active genes. However, it has recently been shown that the expression of the *PTEN* pseudogene (*PTENP1*) is able to rescue the expression levels of its ancestral gene *PTEN* by competing for the same set of miRNAs (*miR-20a*, *miR-19b*, *miR-21*, *miR-26a*, and *miR-214*) [32]. Similarly, the pseudogene *KRASP1* derepressed *KRAS* in DU145 cells, probably by sharing common sites for *miR-143* and *let-7* [32]. Thus, expressed pseudogenes can display tumor suppressive or oncogenic properties by acting as *competitive endogenous RNAs (ceRNAs)* for their corresponding active genes: the increased number of miRNA binding sites upon pseudogene expression was able to titrate the pool of available miRNAs in PCa cells, and miRNA concentrations are regulated by the abundances of their target genes. In this concept, target RNAs are not only passive substrates of miRNAs but also active players in the regulatory networks leading to PCa progression.

The identification of their target genes is essential to estimate the potential of miRNAs as biomarkers. Many *bioinformatic tools* are able to predict miRNA target genes (Table 1). However, since these tools are based on certain assumptions (e.g., base complementarity in the 3′UTR, thermodynamic stability, target-site accessibility, and evolutionary conservation of miRNA binding sites), their predictions are accompanied by large numbers of false positives. Thus, experimental approaches for targetome identification *in vitro* are required. For example, using AGO-RNA immunoprecipitation, followed by sequencing *(AGO-RIP-Seq)* AGO2/miRNA/RNA target complexes are captured from cells stably expressing a miRNA of interest. MiRNA-associated targets are then identified and quantified by deep transcriptome sequencing. AGO-RIP-Seq also enables the identification of novel nonannotated RNA targets like lncRNAs or pseudogenes. Other methods (*AGO-HITS-CLIP* [33, 34], *AGO-PAR-CLIP* [35], and *CLASH* [36]) use crosslinking of the AGO complex to obtain more information about miRNA binding to target genes. These techniques have revealed that some miRNAs can also bind their targets in the coding regions of genes and that other miRNAs do not require base complementarity at all in order to regulate target gene expression [33–36]. These methods are rather novel, and systematic studies reporting the experimental analysis of genomewide miRNA targetomes in PCa have not been described to the best of our knowledge.

MiRNAs are also known to be present in the nucleus [37–40]. Nuclear miRNAs are considered as regulators of gene expression at the transcriptional level (reviewed in [41]). For instance, miRNAs are supposed to bind to promoter sequences in an AGO-dependent manner to regulate gene transcription in the PCa cell line PC-3. This hypothesis was supported by the finding that AGO1-bound promoter sequences contain putative miRNA binding sites [39] and suggest a role for miRNAs in PCa not only in the cytoplasm, but also in the nucleus.

3. Long Noncoding RNA in Prostate Cancer

Long noncoding RNAs (lncRNAs) are defined by their length, which ranges from 200 bases up to 100 kb. LncRNAs have adopted the transcriptional machinery from protein-coding genes (Figure 1): they are primarily transcribed by RNA Pol II, frequently contain a 5′ terminal methylguanosine cap, and are often spliced and polyadenylated [42]. However, a set of nonpolyadenylated lncRNAs has also been identified to be transcribed from RNA polymerase III promoters [43, 44]. The fact that lncRNAs have been found to bind to ribosomes raises the possibility that they are potentially translatable into proteins. Indeed, ribosome profiling [45]

Table 1: Databases and tools for functional annotation of miRNAs and lncRNAs.

Database name	Description	URL
miRNA		
TargetScan	miRNA target prediction based on sequence complementarity	http://www.targetscan.org/
PITA	miRNA target prediction based on target site accessibility	http://genie.weizmann.ac.il/pubs/mir07/mir07_data.html
MiRecords	Collection of miRNA target prediction programs	http://mirecords.biolead.org/
MicroPIR	Prediction of miRNA-promoter interactions	http://www4a.biotec.or.th/micropir/
miRvestigator	Identification of miRNAs responsible for coregulated gene expression patterns discovered through transcriptome profiling	http://baliga.systemsbiology.net/drupal/content/mirvestigator-web-application-identify-mirnas-responsible-co-regulated-gene-expression
DIANA miRPath v.2.0	Combinatorial effect of microRNAs in pathways	http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=mirpath/index
miRGator v2.0	Integrated system for functional investigation of microRNAs	http://mirgator.kobic.re.kr/
mirConnX	Condition-specific mRNA-microRNA network integrator	http://mirconnx.csb.pitt.edu/job_config
MAGIA	Tool for miRNA and genes integrated analysis	http://gencomp.bio.unipd.it/magia/start/
lncRNA		
ChIPBase	Platform decoding transcription factor binding maps, expression profiles, and transcriptional regulation of ncRNAs from ChIP-Seq data	http://deepbase.sysu.edu.cn/chipbase/
DIANA-LncBase	Database for experimentally verified and computationally predicted miRNA targets on lncRNAs	http://www.microrna.gr/LncBase
lncRNome	Biologically oriented knowledge base for lncRNAs in humans	http://genome.igib.res.in/lncRNome
starBase v2.0	RNA interaction networks based on CLIP-Seq data	http://starbase.sysu.edu.cn/
NONCODE v3.0	Expression and functional lncRNA data obtained from reannotated microarray studies	http://www.noncode.org
lncRNAdb	Database of annotated eukaryotic lncRNAs	http://www.lncrnadb.org
LncRNADisease database	lncRNAs associated with a number of diseases	http://www.cuilab.cn/lncrnadisease
Functional lncRNA Database	Mammalian long non-protein-coding transcripts that have been experimentally shown to be both noncoding and functional	http://www.valadkhanlab.org/database
LNCipedia 2.0	Database for annotated human lncRNA transcript sequences and structures	http://www.lncipedia.org
ncRNA Expression Database (NRED)	Integration tool for annotated expression data of ncRNAs from mouse and human	http://jsm-research.imb.uq.edu.au/nred/cgi-bin/ncrnadb.pl
circBase	Database of circular RNAs	http://www.circbase.org
Linc2GO	Functional annotation of human lincRNA based on the ceRNA hypothesis	http://www.bioinfo.tsinghua.edu.cn/~liuke/Linc2GO/index.html
ceRDB-Competing Endogenous mRNA DataBase/CEFINDER	Web-server for identification of ceRNA for a given mRNA target based on the homology of miRNA binding sites present in the 3′UTR sequence	http://www.oncomir.umn.edu/cefinder/

and mass spectrometry approaches [46] detected small peptides potentially derived from lncRNAs. However, the lack of conserved long open reading frames supports the idea that lncRNAs do not encode proteins but rather exert regulatory functions. LncRNAs are cataloged according to their genomic localization: long *intergenic noncoding RNAs (lincRNAs)* are derived from regions that are devoid of other genes, whereas *intronic lncRNAs* are transcribed from introns of protein coding genes. LncRNAs originating from opposite strands of the same genomic locus are called *sense/antisense pairs* and are divided into convergent (head-to-head), divergent (tail-to-tail), and fully overlapping transcripts [47]. LncRNAs are expressed at much lower levels and with much higher cell-type specificity than mRNAs [5]. Although lncRNAs are observed in many species, their degrees of evolutionary conservation are low, and some of the human lncRNAs are thought to have arisen solely in primates [5].

In PCa, the most prominent and clinically relevant RNA biomarker is the antisense lncRNA *PCA3*, which is overexpressed in >95% of primary tumors [48]. *PCA3* is a diagnostic biomarker detectable in urine [49], with higher specificity compared to PSA [50]. One of the first lncRNAs suggested as a marker for PCa incidence, androgen-dependent proliferation, and antiapoptotic signaling in cell lines was the PCa gene expression marker 1 (*PCGEM1*) [51–54]. Overexpression of *PCGEM1* in tumor cells inhibited PARP (poly-ADP-ribose-polymerase) cleavage and delayed the induction of p53 and p21 [52], resulting in increased chemoresistance. *PCGEM1* was found to bind to AR at methylated K349 and to regulate the expression of AR target genes, which stimulated tumor growth in a PCa xenograft mouse model [54]. Additionally, two single-nucleotide polymorphisms (SNP) contributing to cancer risk were identified in *PCGEM1* [55].

In a more recent and comprehensive study, Prensner and colleagues, by analyzing the transcriptomes of 102 prostate tumors and cell lines using next generation sequencing [56], identified 121 so far nonannotated PCa-associated noncoding RNA transcripts (PCATs). *PCAT-1* was upregulated in a subset of metastatic and high-grade localized prostate tumors. *PCAT-1*, a transcriptional target of *EZH2*, stimulated cell proliferation [56] and impaired homologous recombination by repressing of the *BRCA2* gene [57]. PCa noncoding RNA 1 (*PRNCR1*; *PCAT-8*), which is located immediately adjacent to *PCAT-1* and the *c-MYC* oncogene at the well-known PCa risk locus on chromosome 8q24 [58, 59], was identified to be upregulated in aggressive prostate tumors [54]. *PRNCR1*-bound DOT1-like histone H3K79 methyltransferase (DOT1L) methylated the AR at position K349, which mediated the interaction of AR with *PCGEM1*, thereby resulting in ligand-independent activation of AR signaling and cell proliferation [54]. *PCAT-18* was recently suggested as a biomarker for metastatic PCa [60]. Knockdown of *PCAT-18* decreased cell proliferation, migration, and invasion but also triggered apoptosis by promoting caspase activity [60]. In another study, *PCAT-114* (*SCHLAP1*) expression correlated with high-grade, metastatic PCa (see below). By analyzing the transcriptomes of 14 prostate tumors and adjacent benign tissues in another genomewide approach, 137 new lncRNAs were identified [61]. One of these, the metastasis associated in lung adenocarcinoma transcript 1 (*MALAT-1*), was suggested to be a novel marker for PCa diagnosis [62]: high expression of *MALAT-1* correlated with high Gleason score, PSA, tumor stage, and castration-resistant PCa (CRPC).

The majority of PCa are driven by androgen signaling. Various studies have highlighted the functions of androgen-responsive or AR-interacting lncRNAs: Takayama and colleagues identified the antisense lncRNA C-terminal binding protein (*CTBP1-AS*) as highly upregulated in PCa, by repressing its sense gene *CTBP1*, an AR corepressor. This is achieved by recruitment of the transcriptional repressor SFPQ (splicing factor proline/glutamine-rich) and histone deacetylases to the promoter of *CTBP1* [63], thereby promoting androgen-dependent and castration-resistant tumor growth [63]. The PCa-upregulated lncRNA *PlncRNA-1* modulates apoptosis and proliferation through regulation of the AR [64]. Recently, *PCAT-29* was identified as an androgen-repressed suppressor of growth and metastases of prostate tumors [65]. The same group contradicted the prognostic value of *PCGEM1* and *PRNCR1* in aggressive PCa [54]: in a large high-risk PCa patient cohort, Prensner and colleagues could not find any evidence for the binding of these lncRNAs to the AR [66].

As lncRNAs do not encode proteins, their functions are closely associated with their abundance. Bioinformatic tools such as the *"guilt by association analysis"* [67] are capable of predicting the function of an lncRNA. This method uses coexpression analysis of lncRNAs with protein coding genes in signaling pathways to predict the functions of the lncRNAs. This approach has led to the prediction of various roles for lncRNAs, ranging from stem cell pluripotency to cancer [42]. Besides such bioinformatic approaches and databases listing annotated lncRNAs and functional assignment (Table 1), several crosslinking methodologies have been developed to experimentally elucidate the interactomes of lncRNAs *in vitro*. Chromatin isolation with RNA purification *(ChIRP)* [68] and capture analysis of RNA targets *(CHART)* [69] are able to copurify lncRNA-bound proteins and DNA in juxtaposition. In DHT- (dihydrotestosterone-) stimulated LNCaP PCa cells, ChIRP uncovered thousands of PCGEM1 occupancy sites, colocalizing with ARE and H3K4me1-marked enhancers [54].

The regulatory potential of lncRNAs, in particular lincRNAs, has been best characterized in the context of their roles as *epigenetic modulators*. It was observed that 20% of cellular lncRNAs are associated with specific chromatin modification complexes, such as PRC2, which mediates histone H3 lysine 27 trimethylation (H3K27me3) [70, 71]. *ANRIL*, an lncRNA overexpressed in PCa, mediated epigenetic silencing of the adjacent tumor suppressor *p15* via H3K27 trimethylation of the *CDKN2A* (*INK4A/ARF*) locus by directly interacting with SUZ12, a component of PRC2, and CBX7, a PRC1-associated chromodomain-containing protein [72, 73]. In aggressive PCa, aberrantly high expression of *PCAT-114* inhibited SWI/SNF-mediated epigenetic regulation, due to direct disruption of SNF5 activity, a core unit of the SWI/SNF complex [74]. As it has been shown that the expression of PCa-associated lncRNAs can be regulated by long terminal repeats (LTRs) [75], *PCAT-114* might be regulated through an LTR in its 5′UTR (own unpublished observations).

Currently, there is little evidence for a direct interaction between lncRNAs and DNA [68]. In contrast, the crosstalk of lncRNA with other RNA species has been studied in detail [35, 76]. Antisense lncRNAs can regulate the stability and translation of complementary mRNAs. For example, translation of the *UCHL1* mRNA was found to be regulated by its antisense lncRNA through complementary 5′ overlap and an embedded inverted *SINEB2* element [77]. In another example, imperfect base pairing between *Alu* elements in lncRNAs and the 3′UTR of translationally active mRNAs resulted in a double-stranded RNA, which is bound by staufen double-stranded RNA binding protein 1 (STAU1) and subsequently targeted for degradation [78].

The ceRNA concept [32] also suggests that lncRNAs—like mRNAs—act as natural sponges (Figure 1). In this context, a long noncoding *circular RNA (circRNA)* was identified to harbor more than 70 target sites for *miR-7*. Binding of this miRNA to the sponge circRNA suppressed miRNA activity, resulting in miRNA target derepression [79, 80]. CircRNA datasets are publically available via http://www.circbase.org/ (Table 1).

The noncoding part of the human genome offers comprehensive prospects to identify the genetic factors and mechanisms, which are critical for PCa initiation, development, and progression. However, a recent study suggests that a particular lncRNA may exert both oncogenic and tumor-suppressive functions in PCa [81]. This indicates that lncRNA action is highly context-dependent, and further efforts are required to understand the increasing complexity of lncRNA function in health and disease.

4. PCa Diagnosis and Treatment Monitoring Using Circulating RNA Markers

The remarkable stability of noncoding RNAs prompted efforts to evaluate their potential as diagnostic or prognostic molecular markers. In particular, genetic material released from tumor cells into the blood or other body fluids ("liquid biopsy") could provide a rich source of such markers that can be exploited to gain insights into the molecular alterations associated with disease progression [82]. In PCa, liquid biopsies could be highly valuable since the heterogeneity of the tumors often precludes precise staging and grading. In addition, a faithful representation of tumor aggressiveness in easily accessible liquids could lead to an improved therapy decision.

MiRNAs circulating freely in blood have been studied as potential biomarkers for PCa [83, 84]. Several miRNAs were demonstrated to discriminate between PCa and healthy controls or patients with benign prostate hyperplasia (BPH) [83, 85], as well as between localized and metastatic PCa [86]. Brase et al. observed an overall higher level of circulating miRNAs in serum samples of patients with advanced PCa compared to those with primary PCa [15]. Of 667 miRNAs screened, 69 miRNAs were found to be more abundant in sera of lymph-node positive versus negative PCa patients. *MiR-375* and *miR-141* showed statistically significant correlation with clinical patient parameters and were identified as the best serum markers for aggressive PCa [15]. Significantly higher abundance of *miR-141* in localized PCa compared to metastatic disease in plasma samples was also seen by other groups [87, 88], while *miR-21* distinguished best between early-stage disease and healthy controls and was correlated with resistance to docetaxel [89]. Serum *miR-194* correlated with biochemical recurrence and poor prognosis [88]. The first clinical studies are ongoing which examine the benefit of circulating miRNAs for PCa diagnosis and disease monitoring (Table 2). These include investigations to determine whether specific miRNA profiles are related to PCa outcome in high-risk PCa patients as well as screening studies for novel markers (including miRNA and lncRNA) associated with tumor progression in general (e.g., http://www.mdanderson.org/) or with locally recurrent or metastatic PCa. Furthermore, the potential of circulating miRNA markers as tools for companion diagnostics in clinical trials (prediction of therapy response against abiraterone or combination therapies; Table 2) is currently being explored.

Two recent studies suggested the detection of transcript levels of selected mRNAs in whole-blood samples of patients with CRPC as useful prognostic markers [90, 91]: a panel consisting of six *circulating mRNA* markers (*ABL2*, *C1QA*, *SEMA4D*, *TIMP1*, *ITGAL*, and *CDKN1A*) predicted survival when compared to clinical and histopathological parameters of prognostic significance in CRPC [91]. Poor prognosis and poor overall survival of patients with CRPC were correlated with a nine-gene model (*RHAG*, *CA1*, *HEPACAM2*, *SNCA*, *HEMGN*, *SOX6*, *TMEM56*, *OSBP2*, and *RHD*) [90]. The determination of *PCA3* in peripheral blood in combination with increased PSA level (>10 ng/mL) showed high specificity and improved diagnostic accuracy to distinguish BPH from PCa [92]. Since PCa often carries genomic rearrangements involving androgen-regulated genes, the role of rearrangement-associated biomarkers could also be exploited for diagnostic purposes. Urine-based combined detection of *PCA3* and *TMPRSS2:ERG* DNA was used for improved risk stratification of men with elevated serum PSA levels to guide further disease management [93], and changes in *PCA3* and *TMPRSS2:ERG* levels are currently used as marker to evaluate the effect of treatment with the gonadotropin-releasing hormone agonist Triptorelin (Decapeptyl) in a Phase III multicenter study in patients with advanced PCa (NCT01020448; https://clinicaltrials.gov/). A multiplex marker panel consisting of *PCA3*, *TMPRSS2:ERG*, annexin A3, sarcosine, and serum PSA showed improved diagnostic accuracy for PCa [94].

Circulating miRNAs have been found to be present in *microvesicles* such as *exosomes* (Figure 1). Exosomes are nano-sized (40–100 nm) extracellular vesicles secreted from cells via the endosomal pathway. Tumor cells release exosomes at increased levels, which are then detectable in different body fluids. Exosomal miRNAs reflect the miRNA signature of the tumor, and differences in miRNA content and expression were found in exosomes derived from cancerous versus noncancerous cell lines and in tumor-derived exosomes compared to those from healthy individuals [95–97]: in PC-3 cell lines, miRNA profiling identified 31 miRNAs that were

Table 2: MiRNA-based biomarker studies in prostate cancer.

Trial title	Objectives	Study type	Source	Trial number
Micro-RNA Expression Profiles in High Risk Prostate Cancer	Correlation of miRNA expression with prostate cancer outcome	Observational retrospective	Radical prostatectomy	NCT01220427[a]
Blood and Tissue Samples From Patients with Locally Recurrent or Metastatic Prostate Cancer	Sequencing of genomic tumor DNA, genotyping, gene expression, and miRNA/noncoding RNA	Observational prospective	Blood, soft tissue or bone metastases	NCT01050504[a]
MicroRNA in Prostate Cancer Progression: Genetic Variation, Genotype-Phenotype Correlation, and Circulating Biomarker	MiRNAs as predictors of progression	Biomarker screening	Tumor versus normal tissue, plasma	MD Anderson Cancer Center[b]
Abiraterone Acetate in Treating Patients with Metastatic Hormone-Resistant Prostate Cancer	MiRNA expression in tumor metastases as biomarker for sensitivity and resistance; correlation of miRNA with response and progression	Interventional, Phase II clinical trial	Blood (biopsy)	NCT01503229[a]
Bicalutamide and Goserelin or Leuprolide Acetate w/wo Cixutumumab in Patients with Newly Diagnosed Metastatic Prostate Cancer	Correlation of miRNA expression and CTC count	Interventional, Phase II clinical trial	Serum	NCT01120236[a]

[a]http://clinicaltrials.gov.
[b]http://www.mdanderson.org.

exclusively present in exosomes [96]. In larger extracellular vesicles (>200 nm), *miR-141*, *miR-375*, and *miR-205*, known to be associated with PCa progression, and *miR-31* (which targets AR) were differentially expressed [97]. So far, there are only few reports analyzing miRNAs in microvesicles derived from PCa patients. However, a recent study found differential expression of eleven miRNAs, of which *miR-107* exhibited the largest differential expression in circulating microvesicles from PCa patients compared to controls [86]. Additionally, 16 miRNAs were found to be enriched in microvesicles of patients with metastatic PCa compared to patients with organ-confined disease [86]. In this study, *miR-375* and *miR-141* were significantly enriched in plasma-derived microvesicles from patients with metastatic disease. Exosomal *TMPRSS2:ERG* and *PCA3* mRNA from urine samples of untreated PCa patients have also been proposed as a potential diagnostic test [98].

All exosomal content is derived from their parental cells. However, the precise mechanism of miRNA/lncRNA/protein delivery into exosomes is not fully understood. Notably, the RNA and protein cargo within exosomes was shown to be functionally active in recipient cells, which suggests that tumor cells can exploit circulating exosomes as a means to "communicate" with their regional or distal environment [95, 99, 100].

5. Preclinical Studies for ncRNA-Based PCa Therapy

With the identification of PCa-specific miRNA expression patterns and the understanding of their role in PCa biology, novel opportunities for miRNA-based therapies arise. These include targeting the 3′UTRs of oncogenes using specific miRNAs, restoring normal intracellular miRNA pools, or enhancing therapeutic response by combining miRNA with existing anticancer drugs, attempting to exploit the concept of synthetic lethality. In this section, we highlight *in vivo* studies that support the implementation of miRNA-based therapeutics (Table 3).

The ability of *let-7* to inhibit tumor initiation was first demonstrated in a murine CD44-positive xenograft model, where overexpression and therapeutic injection of *let-7* resulted in delayed tumor formation [101–103]. Intratumoral administration of *miR-199a-3p* mimics into a murine PCa xenograft model led to decreased expression of the oncogene Aurora kinase A (*AurkA*), which is associated with a malignant PCa phenotype [104]. In another murine xenograft model, using transfected PCa cell lines [103], *miR-128* showed antiproliferative as well as proapoptotic effects and negatively regulated PCa stem cells by directly targeting the stem cell related genes *Bmi-1* and *Nanog* (Nanog homeobox). Additionally, clonogenic and tumorigenic properties of PCa cells were inhibited in a dose-dependent manner by different levels of endogenous *miR-128*. *MiR-16* replacement therapy in a luciferase PCa xenograft substantially reduced bone metastatic tumor growth compared to untreated mice [105]. *MiR-26a*, which is downregulated in PCa, was shown to inhibit growth and metastatic progression of human-tumor xenografts, probably by targeting lin-28 homolog B (*Lin28B*) and zinc finger CCHC domain containing 11 *(ZCCHC11)* [106]. Finally, downregulation of the lncRNA *MALAT-1*

Table 3: MiRNA-based therapeutics in prostate cancer preclinical trials.

miRNA	Target	Model used	Delivery method	Phenotypes	Reference
Let-7	Myc, RAS	AR-negative mouse xenograft	Let-7c-lentivirus intratumoral injection	Repressed tumor growth	Nadiminty et al. [102]
miR-199a-3p	Aurora kinase A	DU145 mouse xenograft	Agomir intratumoral injection	Reduced tumor growth	Qu et al. [104]
miR-16	CDK1, CDK2	Murine bone metastatic prostate cancer model	Intravenous injections	Reduced tumor development in bone tissue	Takeshita et al. [105]
miR-34a	CD44, SIRT1	$CD44^+$ mouse xenograft	Intratumoral and systemic injection of miR-34a oligo	Reduced tumor volume and lung metastasis, prolonged survival	Liu et al. [107]
miR-221	p27	PC-3 mouse xenograft	Intratumoral injections of miR-221/miR-222 antagomirs	Reduced tumor growth	Mercatelli et al. [110]

delayed tumor growth *in vivo* and reduced metastasis of PCa xenografts in castrated male nude mice [62].

Intratumoral and systemic injection of *miR-34a* into mice grafted with human PCa tumors resulted in reduced tumor volumes and lung metastasis and was associated with prolonged survival by suppressing the adhesion molecule CD44 [107]. Deregulated *miR-34a* expression was also linked to drug resistance, showing a sevenfold decrease in paclitaxel-resistant PC-3 cells compared to wild-type PC-3 cells [108]. Overexpression of *miR-34a* in paclitaxel-resistant PC-3 cells recovered paclitaxel sensitivity, while *miR-34a* antagomir transfection attenuated it [108].

Liu et al. [107] and Kojima et al. [108] presented a rationale for *miR-34a*-based therapy to target PCa stem cells and drug-resistant PCa. *MiR-34a* has emerged as therapeutical agent under the name MRX34, a liposome-encapsulated *miR-34* mimic (http://www.mirnatherapeutics.com) and is currently being studied in a multicenter Phase I clinical trial in patients with unresectable primary liver cancer and liver metastases derived from tumors of other primary origin (NCT01829971; https://clinicaltrials.gov/).

Restoration of *miR-30b* expression by src tyrosine kinase (Src) inhibitors was able to prolong survival and reduce metastatic disease of VCaP-derived xenograft mice by negatively regulating *TMPRSS2:ERG* and downregulating EMT-associated as well as ERG target genes [109]. Consequently, inhibitors of the AR activator Src are currently being tested in Phase I and II clinical trials for the treatment of PCa patients. Similar to the reconstitution of silenced miRNAs, suppression of overexpressed miRNAs was also shown to be a potential therapeutic approach: Mercatelli et al. established an antagomir targeting *miR-221* [110]. Inverse correlation of *miR-221* and p27 protein level was validated in primary cells from 18 patients with stage II-III PCa. In mouse xenograft models, intratumoral injection of a *miR-221* antagomir led to significantly reduced tumor growth, which was correlated with p27 upregulation [110].

There are currently no clinical trials studying miRNAs as therapeutics in human PCa. However, the data from these and other preclinical *in vivo* models using miRNA-based therapeutic approaches provide promising results for potential future applications in human PCa.

6. Summary

The advent of ultra-high throughput sequencing technologies has caused a vast expansion of the catalog of noncoding RNA molecules. Thus, on the one hand, we are closer to an overview of the transcriptional landscapes in health and disease. On the other hand, the complexity of regulatory factors has grown substantially, and we have just started to uncover some underlying principles: although miRNAs are probably the best investigated class of noncoding RNAs, the recent discoveries of complex miRNA and ceRNA networks and nuclear miRNAs indicate that our understanding of miRNAs in PCa progression is far from complete. Similarly, the analysis of lncRNAs has just commenced, and the roles of the majority of *PCATs* and other lncRNAs in PCa remain to be investigated. The reports on novel diagnostic PCa biomarkers from liquid biopsies are encouraging. However, the data are mostly based on discovery-driven approaches that frequently have limited validity due to small sample sizes, nonstandardized settings, and different experimental designs. Thus, the diagnostic and therapeutic benefits of many potential RNA markers in PCa still need to be proven in well-controlled and standardized large-scale prospective clinical studies.

Abbreviations

BPH: Benign prostate hyperplasia
ceRNA: Competitive endogenous RNA
CHART: Capture analysis of RNA targets
ChIP: Chromatin immunoprecipitation
ChIRP: Chromatin isolation with RNA purification
circRNA: Circular RNA
CLASH: Crosslinking, ligation, and sequencing of hybrids
CRPC: Castration-resistant prostate cancer
DHT: Dihydrotestosterone
EMT: Epithelial-to-mesenchymal transition
HITS-CLIP: High throughput sequencing of RNA isolated by UV crosslinking immunoprecipitation

lincRNAs: Long intergenic noncoding RNAs
lncRNA: Long noncoding RNA
miRNA: microRNAs
PAR-CLIP: Photoactivatable-ribonucleoside-enhanced crosslinking and immunoprecipitation
PCa: Prostate cancer
PCAT: Prostate cancer associated transcript (nonprotein coding)
piRNA: piwi-interacting RNA
PRC2: Polycomb repressive complex 2
PSA: Prostate-specific antigen
RIP-Seq: RNA immunoprecipitation sequencing
RISC: RNA-induced silencing complex.

Conflict of Interests

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

Authors' Contribution

Julia M. A. Pickl and Doreen Heckmann contributed equally to this work.

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Review Article

Prognostic Histopathological and Molecular Markers on Prostate Cancer Needle-Biopsies: A Review

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Prostate cancer is diverse in clinical presentation, histopathological tumor growth patterns, and survival. Therefore, individual assessment of a tumor's aggressive potential is crucial for clinical decision-making in men with prostate cancer. To date a large number of prognostic markers for prostate cancer have been described, most of them based on radical prostatectomy specimens. However, in order to affect clinical decision-making, validation of respective markers in pretreatment diagnostic needle-biopsies is essential. Here, we discuss established and promising histopathological and molecular parameters in diagnostic needle-biopsies.

1. Introduction

Prostate cancer is the most common cancer in Western men. Although prostate cancer follows an aggressive course in a significant number of men, most tumors do not cause significant clinical symptoms. Therefore individual assessment of a tumor's aggressive potential is crucial for clinical decision-making in men with prostate cancer.

In general, prostate cancer is diagnosed on needle-biopsies prompted by elevated serum prostate specific antigen (PSA) levels, suspicious digital rectal examination or transrectal ultrasonography findings, and/or clinical symptoms of urinary tract obstruction. In addition to elevated PSA levels and clinicoradiological signs of either local extension or metastasis, detailed histopathological characterization of prostate cancer at needle-biopsies predicts clinical tumor behavior and sustains therapeutic decision-making. In daily practice, the pathology report of prostate cancer includes the grade of differentiation according to the modified Gleason grading system, the number of biopsies infiltrated by prostate cancer, and a quantitative assessment of the tumor volume per biopsy in either length in mm or percentage of tumor [1, 2]. Implementation of novel histopathological and molecular markers is required for several reasons. While only half of the patients has a potentially life-threatening prostate cancer (Gleason score ≥ 7), 55–90% of patients with low-risk disease still undergo radical prostatectomy [3]. Active surveillance has become a widely used alternative for treatment after prostate cancer diagnosis. However, up to 33% of patients on active surveillance need therapeutic intervention after a median follow-up of 1.2–3.5 years [4–8]. Therefore, better stratification of prostate cancer patients with respect to clinical decision-making is necessary, especially in the predominant group of low- to intermediate-risk prostate cancers.

Another reason to implement novel markers in prostate cancer diagnosis and clinical decision-making is the considerable interobserver variability in Gleason grading among pathologists. This interobserver variability is particularly of significance in the large group of low- to intermediate-risk prostate cancer, as it can influence therapeutic approaches [8–10]. Contemporary modified Gleason grading in needle-biopsies demands adding the most common and highest Gleason grade to the final Gleason score, regardless of the amount of "highest" Gleason grade. In practice, when only a few atypical glands considered Gleason grade 4 are present together with a large volume of Gleason grade 3, the Gleason score is 7 and excludes patients from active surveillance in our institute. As considerable interobserver variability exists between the distinction of Gleason grades 3 and 4, important

treatment decisions depend too much on individual pathologist's opinions. Thus, it is important to improve the reproducibility of Gleason grading by more objective parameters. In particular, molecular markers reflecting tumor biology could act as novel threshold in active surveillance or watchful waiting.

Finally, spatial heterogeneity of prostate cancer might lead to under- or, rarely, overestimation of prostate cancer aggressive potential on diagnostic needle-biopsies. In general, prostate biopsies only sample 0.05% to 0.5% of the total prostate volume, which might result in undersampling of the most significant area of prostate cancer tissue. In addition to improved image-guided needle-biopsy procedures, implementation of novel molecular markers might predict the presence of unsampled significant areas in case molecular aberrations precede pathologically discernible patterns.

Last decade, much effort has been put in the identification of novel histopathological and molecular markers to further improve prediction of tumor behavior in prostate cancer patients. The vast majority of research has focused on correlation of novel markers with static clinicopathologic parameters at radical prostatectomy such as Gleason score, pT-stage and surgical margin status, or biochemical recurrence after operation. However, in order to affect clinical decision-making, validation of respective markers in pretreatment diagnostic needle-biopsies is essential. Here, we discuss established and promising histopathological and molecular tissue markers in diagnostic needle-biopsies.

2. Pathologic Markers

2.1. Gleason Grading. The contemporary system for grading prostate cancer was developed by Gleason in the 1960s [11]. The Gleason grading system is solely based on the tumor's architecture. The Gleason score equals the sum of the two most common Gleason grades in radical prostatectomy, and the sum of the most common and highest Gleason grades in needle-biopsies. Up to date, the Gleason score is a strong predictor for disease progression, and one of the most important parameters in therapeutic decision-making.

In 2005, the Gleason grading system was modified at the International Society of Urological Pathology (ISUP) conference [1]. As a result, several tumor growth patterns classically considered as Gleason grade 3 were redefined as Gleason grade 4 [1]. Shortly after, small cribriform and glomeruloid glands have been reconsidered Gleason grade 4 as well [12–14]. As a result of this stage migration, Gleason score 7 has become the most common assigned score on prostatic needle-biopsies [15–17]. For instance, Helpap and Egevad showed in 368 needle-biopsy cases a significant change in distribution of modified Gleason score: Gleason score 2–4 decreased from 2.7 to 0%, Gleason score 5 decreased from 2.7 to 0%, Gleason score 6 decreased from 48 to 22%, but Gleason score 7 increased from 26 to 68% [16]. Generally, modified Gleason grading has improved the predictive value of grading prostate cancer.

2.1.1. Modified Gleason Grading: Up- and Downgrading. The overall concordance of Gleason score between prostate needle-biopsies and radical prostatectomies has improved from 58% in classic Gleason score to 72% in modified Gleason score [16]. Uemura et al. found a decrease in downgrading Gleason score in needle-biopsies in comparison to radical prostatectomy (15% versus 20%), although overall Gleason score concordance rates did not change significantly [18]. To date, the overall upgrading rate at radical prostatectomy using the modified Gleason score ranges between 26 and 50% [19–22]. For instance, a large study containing 7643 radical prostatectomies with corresponding needle-biopsies demonstrated that 36% of cases (1841/5071) were upgraded from a needle-biopsy Gleason scores 5-6 to a higher grade at radical prostatectomy [19]. In the same study 72% (1143/1577) had matching Gleason score 7 on biopsy and radical prostatectomy, and Gleason score 8 on biopsy showed a similar distribution for radical prostatectomy Gleason score 4 + 3 = 7, 8, and 9-10 [19]. In men with Gleason score 9-10 on needle-biopsy, 58% (69/119) had a similar Gleason score on radical prostatectomy [19]. Proposed predictors for upgrading are age, high preoperative PSA, larger tumor percentage per core, number of positive cores, presence of perineural invasion, absence of inflammation, and high prostate volume [19, 21]. On the other hand, predictors for downgrading from any biopsy Gleason score to a lower Gleason score on radical prostatectomy were low preoperative PSA, lower tumor percentage per core, and larger prostate volume on radical prostatectomy [19, 21]. Although these predictors for up- and downgrading all showed strong statistical significance, the effect on survival was still small. If in one biopsy session, multiple biopsies show differing Gleason scores, for instance, Gleason scores 4 + 3 in one biopsy and 3 + 3 in another biopsy, also referred to as presence of ComboGS, patients have lower odds of upgrading at time of radical prostatectomy and decreased risk of prostate cancer specific mortality [23, 24].

2.1.2. Modified Gleason Grading: Correlation with Pathologic Features at Radical Prostatectomy. The relationship between Gleason score on needle-biopsy and pathological stage on radical prostatectomy has improved since the implementation of the modified Gleason score [15, 25]. For instance, 4315/5205 men (83%) with Gleason score 6 on biopsy had organ-confined disease (pT2) at radical prostatectomy, while increasing Gleason score on biopsy was strongly associated with extraprostatic extension (pT3a) and seminal vesicle invasion (pT3b) [25]. Although high pathologic stage is related to positive surgical margins at radical prostatectomy and biochemical recurrence, it should be mentioned that it is not associated with a uniformly poor prognosis [26–28].

2.1.3. Modified Gleason Grading: Predicting Patient Outcome. Billis et al. studied the impact of needle-biopsy Gleason score modification on biochemical recurrence free survival. Here, the modified Gleason score was a better predictor for biochemical recurrence than classic Gleason score [15]. Subsequently, the predictive value of the modified Gleason score

has been validated in other large cohorts [18, 25]. Uemura et al. showed that the Gleason score was strongly associated with biochemical recurrence, only when the modified Gleason score was applied [18]. Furthermore, in a large study (n = 7850) from the Johns Hopkins Hospital, Pierorazio et al. correlated biopsy Gleason score with biochemical recurrence. Here, 95% of the patients with needle-biopsy Gleason score 6 had no biochemical recurrence after 5 years of follow-up [25]. In men with Gleason score 3 + 4 = 7 and Gleason score 4 + 3 = 7 on needle-biopsy the 5 year biochemical recurrence free survival rates were 83% and 65%, respectively. Men with Gleason score 4 + 4 = 8 or 9-10 had the lowest 5 year biochemical recurrence free survival rates, 63% and 35% respectively. Tollefson et al. indicated that distant metastasis and disease-specific death are best estimated by a combination of Gleason score, perineural invasion, and Ki-67 expression [29].

Altogether, modified Gleason grading has generally improved the concordance between biopsy and radical prostatectomy Gleason score, associates better with pathologic parameters at radical prostatectomy, and is more predictive for biochemical recurrence as well as metastasis and disease-specific death.

2.2. Tumor Quantification. Currently, the number of positive core biopsies should routinely be mentioned in pathology reports. Additional measures of prostate cancer volume in needle-biopsies better predict disease outcome. Various parameters have been proposed as measure of tumor extent, for example, tumor percentage in single biopsies, tumor length in single biopsies (mm), and number of negative biopsies. These quantitative assignments are required for most clinical nomograms. For instance, the Steyerberg nomogram incorporates number of positive biopsies, total cancer length (mm), and total "normal" tissue length (mm) to predict indolent disease on radical prostatectomy [30]. In addition, most active surveillance protocols are delimitated by the number of positive biopsies and/or a measure of tumor extent per biopsy [31, 32].

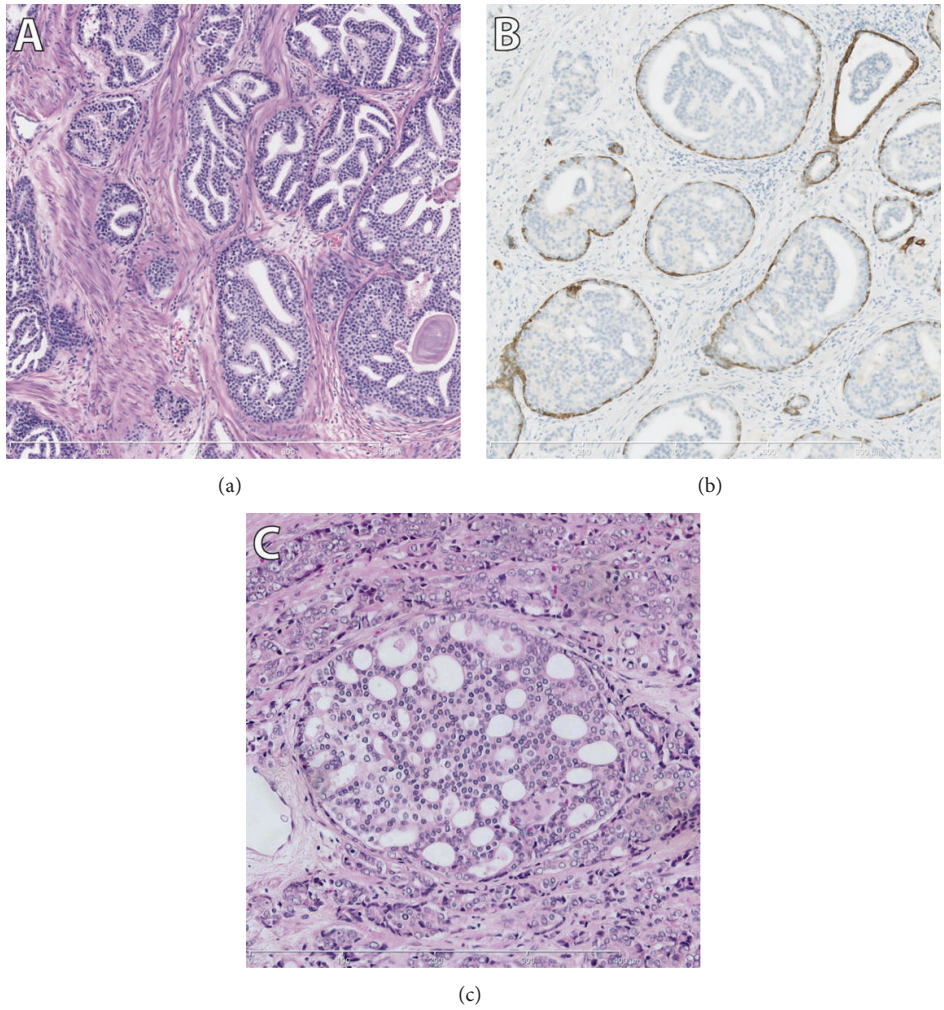
One well-studied example of tumor extent is the percentage of cancer in single biopsies, and many studies have confirmed its prognostic value in biopsies followed by radical prostatectomy [19, 25, 33–37], by dose-escalated external beam radiotherapy [38], or by a combination with hormonal treatment [39]. The location of positive biopsies can additionally be of therapeutic value, for instance for consideration of nerve-sparing surgery. The majority of these studies agree on the predictive value of tumor extent for endpoints such as biochemical recurrence [19, 25, 35–38, 40], metastasis [36–38], and disease-specific death [36, 38]. However, despite its statistical significance, the effect of tumor percentage in biopsies on survival is mostly small. Furthermore, all of the above mentioned publications used different methods to determine the percentage of cancer and different cutoff values. For instance, Vance et al. divided the percentage of cancer in single biopsies into four quartiles (<2.5%, <10%, <25%, and ≥25%) [38], while Nelson et al. categorized it in to 0–10%, 11–59%, and 60–100% [36].

Another frequently assessed measure on prostate biopsies is the percentage of positive biopsy cores, defined as the total number of positive cores divided by the total number of biopsy cores obtained. Studies evaluating its prognostic value have validated the independent predictive value for biochemical recurrence free survival [35, 39, 41, 42]. In 2011 Huang et al. analyzed needle-biopsies of 1056 patients treated with external beam radiotherapy and/or hormonal therapy [43]. Using a cutoff value of >50% positive biopsy cores they found that percentage of positive biopsy cores is a powerful and independent predictor for distant metastasis free and overall survival [43]. However, when the percentage of positive biopsy cores was adjusted for percentage of cancer in needle-biopsies in another study, it did not provide any additional superior risk stratification for biochemical recurrence, distant metastasis, or disease-specific death [38].

While measuring tumor extent is generally straightforward, minor controversies exist for instance for quantifying discontinuous prostate cancer foci in single biopsies. One could regard separate foci as being part of the same tumor and measure the distance between the outermost foci including intervening normal prostate tissue, or only measure malignant areas without intervening stroma. In this case, recent studies show that discontinuous foci of prostate cancer in needle-biopsies should be regarded and measured as one continuous lesion [44, 45].

In short, tumor volume is an important parameter for disease extent, but there is no consensus yet on the best methodology for its assessment. It is clear that assignment of the number of positive biopsies requires identification of separate biopsy cores, even when they are fragmented due to technical procedures. This can be performed by including only one needle-biopsy per cassette, or marking multiple individual cores in one cassette for instance by inking. It is advised that no more than 3 biopsies should be included in one cassette, provided that measures are taken to prevent their curling and floating [46]. The extent of cancer in individual cores is performed by actual measuring of the tumor's length with a ruler or by estimation of tumor percentage by eye-balling. Measuring tumor length is objective and exact, although more time-consuming. If estimation of percentage is applied in daily practice, one should take into account that detection of prostate cancer in short needle-biopsies due to suboptimal technical procedures can result in overestimation of tumor percentage; for instance presence of 2 mm prostate cancer in a 5 mm biopsy results in a tumor percentage of 40%, which might erroneously exclude patients from active surveillance.

2.3. Perineural Invasion. The significance of perineural invasion in prostate cancer biopsies remains questionable. In a systematic review Harnden et al. addressed important limitations of 21 studies on perineural invasion in biopsies followed by radical prostatectomy or radiation therapy [47]. First, the presence of nerves in biopsies was not mandatory for patient inclusion. Second, the number of biopsy cores taken and the number of nerves present ranged widely. Third,



(a) (b)

(c)

Figure 1: (a) intraductal carcinoma of the prostate (100x magnification). (b) 34BE12 immunohistochemistry, demonstrating the presence of basal cells supportive for intraductal carcinoma (100x magnification). (c) Cribriform growth pattern of Gleason grade 4 adenocarcinoma (200x magnification).

pathologists were not obligated to routinely report on presence of perineural invasion; there were striking differences in the frequency of perineural invasion when biopsies were reviewed for study purpose. Interestingly, only 43% (18/42) of surveyed urologists think that presence of perineural invasion on prostate biopsy should influence treatment [48]. Ten out of 18 surveyed urologists (56%) indicated that it helps planning nerve-sparing surgery. In contrast, nerve-sparing surgery was considered as a confounding factor in the studies mentioned by Harnden et al. [47]. However, despite limitations Harden et al. conclude that the weight of evidence in studies ascribing prognostic significance to perineural invasion appears to suggest that perineural invasion should influence clinical decision-making [47]. For instance, Quinn et al. demonstrated in a large cohort ($n = 696$) that perineural invasion was a significant predictor for outcome in a multivariable analysis [49]. Delancey et al. demonstrate that perineural invasion on prostatic needle-biopsy is an independent predictor for biochemical recurrence, disease-specific survival, and overall survival after radical prostatectomy [50].

2.4. Intraductal Carcinoma: A High-Risk Lesion. Intraductal carcinoma of the prostate is defined as a well-circumscribed lesion surrounded by an intact basal cell layer distended by overtly malignant-appearing epithelial populations [51] (Figures 1(a) and 1(b)). Intraductal carcinoma forms a morphologic continuum with high-grade prostate intraepithelial neoplasia (PIN), which is the generally accepted precursor of prostate cancer. While PIN is recognized by the presence of cytologically malignant cells in preexistent prostate glands of normal architecture, these glands should be enlarged in intraductal carcinoma. In contrast to PIN, which can be an isolated finding on prostate biopsy, intraductal carcinoma is associated in 90% of cases with invasive carcinoma, mostly of intermediate or high risk [52]. Up to date, three studies on intraductal carcinoma all demonstrated its independent predictive value for biochemical recurrence after radical prostatectomy, treatment failure, and distant metastasis in biopsies [53–55]. van der Kwast et al. showed that intraductal carcinoma on needle-biopsies prior to external beam radiotherapy with or without androgen deprivation therapy

strongly predicted biochemical recurrence and early distant metastasis in a cohort of 118 intermediate and 132 high risk prostate cancer patients [55].

2.5. Percentage Gleason Grades 4 and 5. A proposed adaption to the Gleason grading system by Vis et al. is the reporting of percentage of Gleason grades 4/5 in prostatic needle-biopsies [37]. In this study, containing 281 patients, amount of high-grade cancer (length in mm, or percentage) in the core biopsy was an independent prognostic factor for biochemical recurrence and clinical relapse of prostate cancer [37]. In addition, when biopsy Gleason score 7 cancers were subcategorized into 3 + 4 and 4 + 3 cancers, the amount of high-grade cancer was the strongest predictor of biochemical recurrence free survival, whereas the Gleason grading system was rejected in the presence of high-grade components [37]. So far, no other studies have yet validated the predictive value of this parameter on prostatic needle-biopsies.

2.6. Gleason Grade 4 Patterns: Individual Prognostic Value. Gleason grade 4 tumors represent a diverse group, containing at least 4 distinctive growth patterns: fused, cribriform, ill-defined, and glomeruloid [1, 14, 56]. Recently, Dong et al. studied the prognostic value of these distinct Gleason grade 4 patterns and showed that cribriform growth, in particular, was strongly associated with biochemical recurrence and distant metastasis after radical prostatectomy [57] (Figure 1(c)). Only one publication has yet demonstrated the adverse prognostic value of cribriform growth pattern in a biopsy series, with radiotherapy as treatment, and biochemical recurrence as clinical endpoint [54].

2.7. Extraprostatic Extension. Rarely, prostate cancer is infiltrating extraprostatic fat tissue, seminal vesicle, or ejaculatory ducts on needle-biopsies. Fat invasion by tumor on prostatic needle-biopsy is considered as extraprostatic extension by 81% of surveyed pathologists from the European Network of Uropathology [58]. If present, these features should be mentioned in diagnostic needle-biopsies. Since signs of extraprostatic extension are mostly seen in voluminous prostate cancer with high Gleason score, these patients could be less eligible for radical treatment.

2.8. Tumor-Associated Macrophages. Solid tumors grow in a complex and dynamic stroma involving various cell types, for example, leukocytes, fibroblasts, and endothelial cells. Tumor-associated macrophages are part of the tumor microenvironment and seem to influence solid tumor progression, for example, in colon, breast, and ovarian cancer [59]. Nonomura et al. were the first to study its prognostic relevance in prostate biopsies [60]. The presence of tumor-associated macrophages, as immunohistochemically identified by the CD68 antibody in a cohort of 71 prostate cancer patients treated with hormones, was associated with disease recurrence after hormonal therapy. Furthermore, tumor-associated macrophages count (>22 at 400x magnification), PSA level, Gleason score ≥7, and extracapsular extension were independent predictors for biochemical recurrence free survival in multivariate analysis [60]. Studies on tumor-infiltrating lymphocytes in prostatic needle-biopsies have not yet been performed.

2.9. Summary

(i) To date, Gleason grading on prostatic biopsy is the most important predictor for biochemical recurrence, distant metastasis, and cancer-specific mortality in prostate cancer.

(ii) Despite the modification in 2005 by the ISUP, Gleason score upgrading at radical prostatectomy still ranges between 26 and 50%.

(iii) Proposed predictors of upgrading are high preoperative PSA, larger tumor percentage per biopsy core, number of positive cores, and presence of perineural invasion.

(iv) Proposed predictors of downgrading are smaller tumor percentage per core and large prostate volume.

(v) Differing Gleason scores on prostate needle biopsies could be a useful tool in decreasing the odds of upgrading.

(vi) Tumor quantification is an important prognostic factor and implemented in clinical nomograms of prostate cancer; there is, however, no consensus on the best methodology for its assessment. Furthermore, despite its fundamental role in clinical nomograms, the effect on survival is relatively small.

(vii) Presence of perineural invasion on needle-biopsies is an independent predictor for survival, and therefore a recommended parameter to add in standardized pathology reporting.

(viii) The amount of Gleason grade 4/5 in needle-biopsies seems to have prognostic value; however, it needs to be validated in large cohorts with strong endpoints.

(ix) Cribriform growth and/or intraductal spread are promising histopathological markers in needle-biopsies.

(x) The prognostic value of tumor-associated inflammation has recently been acknowledged in multiple solid tumors and needs to be further investigated in prostate cancer.

3. Molecular Markers

3.1. Ki-67. The Ki-67 protein is a cell proliferation marker, which is expressed in G_1, S, G_2, and M phases of the cell cycle being absent in resting (G_0) cells. The Ki-67 labeling index as determined by the MIB-1 antibody is the best studied prostate cancer marker in needle-biopsies up to date [61–66]. Ki-67 labeling index shows a strong correlation with Gleason score on diagnostic biopsies [61, 63], on subsequent radical prostatectomy [64–66], or both [62]. In a cohort of 91 patients, Rubio et al. found Ki-67 (cut-off value of 5% positive nuclei) on needle-biopsies to be a marker for disease-free survival after radical prostatectomy in univariate analysis

[62]. Zellweger and colleagues found that Ki-67 labeling index of ≥10% at biopsy cores in 279 patients independently predicted seminal vesicle invasion and Gleason score on subsequent radical prostatectomy [66]. In addition, they found that Ki-67 was the only independent marker for postoperative biochemical recurrence in a subgroup of low-volume (<7%) or low-grade (Gleason score < 7) prostate cancer at needle-biopsies. Tolonen et al. demonstrated that Ki-67 in 247 primarily endocrine-treated prostate cancer patients was associated with progression-free survival [63]. Ki-67 labeling ≥ 10% on 293 microarrayed needle-biopsies had independent predictive value for disease-specific death together with Gleason score and PSA [61]. Recently, Tollefson et al. calculated in a cohort of 451 prostate cancer needle-biopsies that every 1% increase in Ki-67 expression resulted in a 12% increased risk of cancer-specific death after radical prostatectomy [29]. Whereas these studies all show additional value of Ki-67 expression in needle-biopsies with aggressive disease features, two studies focused on the relation of Ki-67 labeling in needle-biopsies and presence of indolent disease on radical prostatectomy. In a well-defined screening cohort Vis et al. were not able to find a significant association of Ki-67 ≥ 10% with significant prostate cancer at radical prostatectomy in 81 patients [64]. Wolters et al. were also not able to find a significant association of high Ki-67 expression (>3%) with significant prostate cancer at radical prostatectomy in 86 patients [65]. Taken together, enhanced Ki-67 labeling at needle-biopsy is associated with adverse clinicopathologic features and disease-specific death in general prostate cancer populations.

3.2. p27. $p27^{kip1}$ is a cyclin-dependent kinase (cdk) inhibitor. It inhibits cell cycle progression in G_1 phase by preventing activation of cyclin E-cdk2 and cyclin D/-cdk4 complexes [67]. Loss of p27 has been widely associated with progression of different tumor types, including breast, colorectal, and lung cancer [67]. Generally, p27 expression in prostate cancer needle-biopsies correlates well with the p27 labeling in radical prostatectomy samples [64, 67]. In prostate cancer, various groups have shown that loss of p27 expression was associated with more aggressive disease parameters. Thomas et al. demonstrated that low expression (<30%) of p27 in needle-biopsies correlated with higher Gleason score and pT-stage at radical prostatectomy [67]. In this relatively small cohort of 44 patients, tumors with low p27 expression showed a trend towards shortened biochemical recurrence free survival after operation. Vis et al. showed that p27 expression in <50% together with Gleason score were the only significant parameters to predict clinically significant disease at radical prostatectomy in a screen-detected cohort of 81 prostate cancer patients [64]. In addition, we found that p27 in <90% in a low-risk prostate cancer cohort was an independent parameter of clinically significant prostate cancer in 86 radical prostatectomy samples [65]. Therefore, loss of p27 is a marker of more aggressive prostate cancer, although the number of patients is limited and different standard cutoff levels have been used by various groups.

3.3. EZH2. Enhancer of zeste homologue 2 (EZH2) belongs to the Polycomb-group proteins and is important in maintaining cell identity and regulation of the cell cycle [68, 69]. EZH2 has been reported to be of both prognostic and therapeutic value in different tumors, such as small cell lung carcinoma [70], breast cancer [71, 72], cervical carcinomas [73], urinary tract carcinoma [74], and lymphoma [75]. Through gene expression profiling, EZH2 was found to be overexpressed in hormone-refractory metastatic prostate cancer [69]. Overexpression of EZH2 in radical prostatectomy samples was associated with poor prognosis [69, 76–78]. In a set of 86 needle-biopsies of screen-detected low-risk prostate cancer, EZH2 expression >1% was associated with clinically significant tumors on radical prostatectomy, defined as presence of extraprostatic extension, Gleason grade 4/5 or tumor volume ≥0.5 mL [65]. In the same study, no prognostic value was found for Polycomb-group protein BMI1. Tolonen et al. found independent predictive value for EZH2 (expression level of >15%) for progression-free survival in 247 hormone-treated biopsies [63]. While enhanced EZH2 expression in prostate cancer biopsies has independent prognostic value, there is no consensus yet on cutoff points in clinical practice.

3.4. TMPRSS2:ERG. Fusion of the androgen-dependent *TMPRSS2* gene to ETS-transcription factor *ERG* (*TMPRSS2 :ERG*) is one of the most common genetic alterations in prostate cancer occurring in 50%–70% of tumors [79]. Many groups have analyzed the presence of *TMPRSS2:ERG* fusion or ERG protein expression in prostate cancer cohorts with variable outcome [80–92]. Barros-Silva et al. used fluorescent in situ hybridization (FISH) to detect *TMPRSS2-ERG* rearrangement in a cohort of 200 biopsies and found an association with low PSA levels at diagnosis and low Gleason score [93]. In needle-biopsies immunohistochemical ERG detection can be used to discriminate prostate cancer from its mimickers, although the additional value to other markers such as p63, basal cell keratin 5, and AMACR is limited [92, 94–99]. In an active surveillance cohort of 265 men, Berg et al. found a strong correlation between ERG protein expression and disease-progression [100]. Likewise, Hagglof et al. showed a shorter survival of prostate cancer patients on watchful waiting when ERG was expressed [101]. They found a cumulative 2-year progression rate of 59% in the ERG-positive group versus 22% in the ERG-negative group. Finally, expression of ERG in high-grade PIN was associated with a higher chance of developing prostate cancer at subsequent biopsies [102]. ERG immunohistochemistry is an easy to perform methodology for detecting TMPRSS2:ERG fusion in prostate cancer. While the clinical relevance of TMPRSS2:ERG fusion on radical prostatectomy specimens is unresolved yet, most reports indicate that ERG expression on biopsy, in surveillance cohorts can select a subgroup with higher chance to progression.

3.5. Neuroendocrine Differentiation. In many prostate cancers, scattered tumor cells show neuroendocrine differentiation as demonstrated by antibodies to Chromogranine,

Synaptophysine, or Serotonine. After hormonal therapy, the relative number of neuroendocrine cells is increased, putatively due to their androgen-independent nature [103–105]. Despite extensive studies on neuroendocrine differentiation in relation to castration-resistance, this feature is rarely studied in pretreatment biopsies. Krauss and colleagues have shown that Chromogranine An expression of >1% in prostate cancer biopsies is an independent predictor for distant metastasis and cause-specific survival after primary radiation therapy [106, 107].

3.6. c-MYC. The oncogene *c-MYC* located at 8q24 encodes a transcription factor involved in cell cycle progression, cell growth, proliferation, protein synthesis, mitochondrial function, stem cell renewal, and DNA replication [108, 109]. *c-MYC* is amplified in approximately 70% of clinical prostate cancer [93, 110, 111]. Ribeiro et al. found that patients with gain of *MYC* gene copy numbers in a group of 60 prostate cancer needle-biopsies using FISH were significantly at risk for disease-specific death [110]. Bastacky et al. showed that amplification of *c-MYC* in needle-biopsies with high-grade PIN was predictive of finding prostate cancer in subsequent biopsies [112]. The potential predictive value of *c-MYC* was confirmed by Zafarana et al. in a cohort of 126 needle biopsies, where they found *c-MYC* gain alone to be prognostic for tumor recurrence after radiotherapy [111]. *c-MYC* gain combined with loss of *PTEN* further increased the predictive value for recurrence after radiotherapy.

3.7. PTEN. Phosphatase and tensin homologue (*PTEN*) is a tumor suppressor gene which is inactivated in many different tumors, including prostate cancer [113]. On large cohorts of radical prostatectomy samples and transurethral resection (TUR) samples, *PTEN* loss has been associated with bone metastases, resistance to radiotherapy and chemotherapy, progression to androgen-independent disease, and disease recurrence after surgery [113, 114]. Zafarana et al. found that *PTEN* loss alone and in combination with *c-MYC* gain were independently associated with biochemical recurrence after radiation therapy in a group of 126 intermediate-risk prostate cancer biopsies [111].

3.8. APC. Using quantitative methylation-specific PCR (QMSP) Henrique and colleagues showed that hypermethylation of *APC*, *GSTP1*, and *RASSF1A* in 83 prostate cancers at sextant needle-biopsies was associated with poor disease-specific survival [115]. Besides clinical stage, hypermethylation of *APC* was independently predictive for decreased disease-free and disease-specific survival. Methylation of *CCND2* and *RARB2* in the same study did not have predictive value for disease outcome.

3.9. Molecular Signatures. Subgroups with unique molecular, pathologic, clinical, and therapy-sensitivity, as have been defined in breast cancer, have not been delineated in prostate cancer yet. Nevertheless, recently gene signatures have been put forth to predict prostate cancer behavior. Klein et al. demonstrated that a 17-gene assay was able to identify patients with high-grade and high-stage disease at radical prostatectomy in a cohort of 395 men with low- to intermediate-risk prostate cancer at biopsies [116]. In addition, Irshad et al. identified a 3-gene signature of *FGFR1*, *PMP22*, and *CDKN1A*, which could accurately predict the outcome of low Gleason score prostate cancer in different cohorts [117]. For clinical implementation and validation this group applied immunohistochemistry for the respective proteins. In a limited matched cohort of 43 low-risk prostate cancer patients on active surveillance they were perfectly able to identify patients with failure upon active surveillance by reduced expression of these 3 proteins. Gene-based signatures therefore are a promising tool for risk stratification and might gain wider application if translation to easy-to-use procedures such as immunohistochemistry is available.

3.10. Biopsy Markers without Prognostic Value. While numerous markers have been shown to correlate with adverse clinicopathologic parameters on radical prostatectomy, just a limited number of these has been investigated in pretreatment needle-biopsies. Briefly, we also want to mention markers that have been investigated on biopsies but did not show additional value. In a group of 91 prostate cancer needle-biopsies, Bax, Bcl-2, and Cox-2 did not show independent predictive value for disease-free survival, although Cox-2 was predictive in univariate analysis [62]. On a large cohort of 247 patients with primary endocrine treatment, Tolonen et al. demonstrated that minichromosome maintenance protein 7 (MCM7) was a significant albeit not independent marker for disease-progression [63]. We validated the prognostic value of Cystein-rich secretory protein 3 (CRISP-3) and β-Microseminoprotein (β-MSP) in a screening cohort of 174 men. We found that expression of these markers was correlated with Gleason score, tumor volume, and pT-stage and significant disease on subsequent radical prostatectomy samples but were not able to predict recurrence [118].

3.11. Summary

(i) The cell proliferation marker Ki-67 (MIB-1) is the best studied immunohistochemical marker in prostate with independent prognostic value in multiple studies.

(ii) Cyclin-dependent kinase inhibitor p27 and Polycomb-group protein EZH2 are both promising immunohistochemical markers for predicting disease outcome.

(iii) The clinical significance of *TMPRSS:ERG* fusion or ERG protein overexpression is still controversial, although some studies demonstrate adverse prognostic value in active surveillance/watchful waiting cohorts.

(iv) Amplification of *c-MYC*, *PTEN* loss, and *APC* hypermethylation are promising markers for predicting disease-specific death, albeit only demonstrated in a small number of biopsy cohorts.

(v) Identification of complex gene signatures offers novel promising platforms for predicting disease-outcome. Routine implementation in local pathology laboratories is currently not applicable.

4. Conclusions

Investigation of potential novel predictive markers in prostate cancer needle-biopsies is of importance to affect clinical decision-making and to be implemented in daily practice. A prerequisite in analyzing novel markers on needle-biopsies is the presence of well-characterized patient cohorts with clinical follow-up and availability of prostate cancer tissue for actual testing. Secondary and tertiary cancer centers often do not have original tissue blocks for further research on site. In addition, small foci of prostate cancer are often not present anymore in the remaining paraffin block. Incorporation of both detailed histopathological prostate cancer features and molecular markers can support optimal therapeutic decision-making in individual patients.

Comprehensive reporting of novel histopathological parameters such as percentage Gleason grade 4/5, intraductal carcinoma and potentially Gleason grade 4 growth pattern is a fast and cheap way to better estimate a prostate cancer's future clinical behavior in daily practice. Various molecular markers such as Ki-67, p27, EZH2, and ERG immunohistochemistry, as well as *c-MYC* and *PTEN* in situ hybridization, can putatively sustain and improve pathologic diagnosis. The variability in patient cohorts, clinical endpoints, technical methodology, and quantification, however, require prospective studies in well-characterized patient groups before implementation in daily practice is feasible. Identification of complex gene-signatures is a recent and promising tool in stratification of prostate cancer patients, though still costly and not easily applicable in daily practice.

Last decade, the diagnosis of prostate cancer has changed significantly. Pathologic Gleason scoring has been modified; sextant biopsies have widely been replaced by 10 to 12 or more biopsy sampling protocols; image-guided biopsies supplement established biopsy schemes and facilitate sampling of biologically relevant tumor areas. It is difficult to interpret the additional value of established prognostic factors of earlier studies in contemporary prostate cancer health care. We advocate that incorporation of novel histopathological parameters such as percentage Gleason grade 4/5, presence of intraductal carcinoma, and Gleason grade 4 growth patterns in daily pathology practice as well as prostate cancer studies offer an inexpensive and short-term opportunity to improve prostate cancer health care and interpret the additional value of promising molecular markers.

Disclosure

A. Marije Hoogland and Charlotte F. Kweldam shared first author.

Conflict of Interests

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

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Review Article

Stem Cell Based Gene Therapy in Prostate Cancer

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Current prostate cancer treatment, especially hormone refractory cancer, may create profound iatrogenic outcomes because of the adverse effects of cytotoxic agents. Suicide gene therapy has been investigated for the substitute modality for current chemotherapy because it enables the treatment targeting the cancer cells. However the classic suicide gene therapy has several profound side effects, including immune-compromised due to viral vector. Recently, stem cells have been regarded as a new upgraded cellular vehicle or vector because of its homing effects. Suicide gene therapy using genetically engineered mesenchymal stem cells or neural stem cells has the advantage of being safe, because prodrug administration not only eliminates tumor cells but consequently kills the more resistant therapeutic stem cells as well. The attractiveness of prodrug cancer gene therapy by stem cells targeted to tumors lies in activating the prodrug directly within the tumor mass, thus avoiding systemic toxicity. Therapeutic achievements using stem cells in prostate cancer include the cytosine deaminase/5-fluorocytosine prodrug system, herpes simplex virus thymidine kinase/ganciclovir, carboxyl esterase/CPT11, and interferon-beta. The aim of this study is to review the stem cell therapy in prostate cancer including its proven mechanisms and also limitations.

1. Introduction

The introduction of stem cells (SCs) in cancer gene therapy is attributed mainly to the powerful advantage of it being a vector or cellular vehicle. Stem cell based cancer gene therapy is based on its tumortropic property. The tumor homing ability of SCs holds therapeutic advantages compared to other vehicles such as proteins, antibodies, nanoparticles, and viruses. Viruses or nonmigratory vector-producing cells have been utilized but demonstrated many shortcomings in effective delivery of the therapeutic agents. Virus-mediated gene therapies have limitation because of the difficulty in tracking cancer cells [1].

Another uprising evidence for using stem cells in cancer therapy is that it is nowadays broadly accepted that cancer is a stem cell disease [2, 3].

Compared with conventional chemotherapy, suicide gene therapy using SCs had no significant adverse effect on the weight gain of the animals. Such safety and efficacy imply a great potential of SC cell line expressing transgenes of interest for biologic study or clinical application [2, 3].

Although there have been large studies, including lung cancer, colon cancer, melanoma, glioma and brain tumors, stem cell based gene therapy in prostate cancer has been less focused on.

In this inspection, we provide an overview of the use of SC based gene therapy for prostate cancer. We identify SC sources and review several possible mechanisms of activity. We offer a summary of the current status of SC based gene therapy of prostate cancer with its limitation and also identify opportunities for further probe. This review contains only preclinical studies because SC based gene therapy for translational clinical therapies is not used until now.

2. Stem Cell Sources

To date, in prostate cancer gene therapy, only mesenchymal stem cell (MSC) and neural stem cell (NSC) were used in preclinical studies.

2.1. Mesenchymal Stem Cells (MSCs). MSCs are an attractive vector for activating the prodrug because they could transport the therapeutic drug directly within the tumor mass; moreover, systemic toxicity could be avoided.

One of the most important advantages of MSCs compared to other types of SCs is its feasibility of acquisition. MSCs can be isolated as a fraction of bone marrow cells or other adult tissues. They possess an extensive proliferative potential and the capacity to differentiate into various cell types [4].

MSC has advantages in its feasibility of isolation and expansions in culture. Isolation of MSCs could be achieved from almost every type of tissue, including bone marrow, adipose tissue, muscles, liver, dental pulp, placenta, amniotic fluid, and menstrual blood, or umbilical cord blood [5, 6].

MSCs from bone marrow (BM-MSCs) reside in small numbers (approximately 10 cells per million of mononuclear cells) but could be well expanded in culture due to their plastic ability.

Adipose tissue for MSCs is about 10 times more abundant compared to bone marrow. Adipose tissue-derived mesenchymal stem cells (AT-MSCs) possess similar properties with BM-MSCs; therefore, BM-MSCs and AT-MSCs are used most frequently in stem cell studies or therapies. Griffin et al. have demonstrated the feasibility of utilizing human fetal bone marrow derived-MSCs for gene therapy [7].

The primary advantage of fetal BM-MSCs over adult BM-MSCs is the prolonged life span *in vitro*, which helps time-consuming enrichment of the MSCs transduction with constitutive expression of the desired transgene by single colony selection [7].

Another merit of MSCs is their immunosuppressive properties, which could be explained by the lacking of major histocompatibility complex (MHC-II) and showing minimal MHC-I expression [7–9]. Through this immunosuppressive property, allogeneic MScs may substitute autologous MSCs in delivering the therapeutic agent in targeted tumor therapy.

Clinical studies using MSCs revealed the prevention of graft-versus-host disease with its safety regardless of the variation of human leukocyte antigen [10].

These findings indicate the possibility of clinical use of premanufactured MSCs, which is conceived to reduce time constraints and increase the accessibility of MSCs in instances of emergency or inherited genetic disorders [2, 11].

2.2. Neural Stem Cells (NSCs). Neural stem cells (NSCs) are self-renewing, multipotent cells that generate the main phenotypes of the nervous system and have tumor-tropic abilities. They have been exploited in preclinical as well as in clinical studies, especially in enzyme prodrug gene therapies. NSCs can be harvested from fetal, neonatal, or postnatal issues [12]. Since it is not feasible to obtain and isolate NSCs in sufficient numbers, immortalized neural progenitor cell lines instead of NSCs were prepared and used in several preclinical studies of prodrug cancer gene therapy [11, 13–15]. The well-characterized NSC line is HB1.F3, which was derived from fetal brain at 15 weeks of gestation and is known to be multipotent, migratory, and nontumorigenic.

Recently, FDA approved the second human NSC clinical trial in recurrent high-grade glioma [16]. Established on the evidence of intrinsic tumor's tropic capacity of NSCs, a genetically modified immortalized human NSC line is expected in its possibility of prostate cancer treatment.

2.3. Other Stem Cells. Endometrial regenerative cells (ERCs) are other optional SCs for utilization in cancer gene therapy, which are isolated from menstrual blood. They showed inhibition of intracranial glioma growth, but no preclinical trials have been reported for prostate cancer treatment. ERCs are a population of mesenchymal-like cells and are characterized by pluripotent differentiation capacity and production of unique growth factors [17].

3. Stem Cell Delivery as a Vector

Gene-directed enzyme prodrug therapy known as well as virus-directed enzyme prodrug therapy or suicide gene therapy did not demonstrate a satisfactory final result in clinical trials in the past. The primary cause for this failure was the missing tumor specificity of this approach. However, SC based prodrug cancer therapy is quite different from the classical prodrug gene cancer therapies. The main differences from earlier versions of cancer gene therapies are tumor homing able of MSCs, *in vitro* preparation of therapeutic SCs by plastic ability, and having prodrug converting genes integrated as DNA provirus in therapeutic SCs. Stable and effective production of prodrug converting enzymes under strong retroviral promoter is a great advantage of stem cell delivery as a vector.

4. Stem Cell Tracking

For CD system, in which the conversion of the 5-FC to 5-FU can be quantified with the nuclear magnetic resonance spectroscopy (NMRS) [18, 19]. The radiolabelled fluoronucleoside analogues of thymidine such as 1-(2-deoxy-2-fluoro-D-arabinofuranosyl)-5-iodouracil (FIAU) and radiolabelled 9-[4-fluoro-3-(hydroxymethyl)butyl]guanine (FHBG) can be used for HSV-tk substrates [20, 21].

It has been shown that MSCs can be easily labeled with super paramagnetic iron oxide (SPIO) nanoparticles [22]. MSCs carrying two kinds of nanoparticles [23] (polylactic acid NPs and LNCs) were shown to retain their viability, differentiation, and tumor homing capacities [22, 24]. Recently, Lee et al. demonstrated the usability of magnetic nanoparticles (MNPs) in the tracking of NSCs (HB1.F3 CD cells).

5. Mechanisms of Stem Cell Based Gene Therapy

Considering the issue that MSCs play dual roles in tumor genesis through potentiating tumor growth, enhancement of neovascularization, or differentiating into tumor stromal fibroblasts, suicide genes are of particular interest because

they facilitate the development of MSCs in antitumor therapy [25–28].

The novel strategy of stem cell based gene therapy in prostate cancer includes silencing gene expression, expression of intracellular antibodies blocking cells' vital pathways, and transgenic expression of caspases and DNases. SC based gene therapy consists of two phases of treatment. In the first step, the gene for a foreign enzyme (bacterial, yeast or viral) is delivered and targeted to the tumor by transduction of SCs. In the second step, the enzymatic activity of the gene product converts less toxic prodrug to cytotoxic substance at the tumor lesion. Therefore, the active cytotoxic substances produced by enzymatic process within transduced MSCs effectively demolish the neighboring or surrounding tumor cells, which is called bystander effect.

In addition, the process of killing tumor and therapeutic stem cells can induce host immune responses mediated by natural killer (NK) cells and T-cells, which is called distant bystander effect [29–31].

The success of SC based the gene therapy depends on several factors, which are the catalytic activity of the enzyme encoded by suicide gene, a suitable combination of prodrug enzyme, the migration ability of the SC vector to target tumor cells, sufficient transgene expression, and the extent of bystander effect [2].

Recently, it was reported indeed that expression of the bifunctional suicide gene *CD::UPRT* increases radio sensitization and bystander effect of 5-FC in PC cells [32].

Using bifunctional suicide gene, researchers have previously shown, in *in vitro* and *in vivo* studies, that the bystander effect mediated by *CD::UPRT* gene directed enzyme prodrug therapy without a direct cell to cell contactor functional gap junctions [32–34].

5.1. Migration and Homing. Tumor sites are a microenvironment with hypoxia and inflammation, which release many cytokines that attract SCs. This tumor-tropic homing capability, together with inhibitory effect on tumor genesis, provides a strong foundation for using the systemic injected SCs for the treatment of distant tumors not easily accessible [35, 36]. With this strong tumor-tropic capability, SCs could be ideal cellular vehicles of antitumor agents.

Although it has been suggested that MSCs could be entrapped passively in highly vascularized tissues, most likely in the lungs, after systemically injected [37], tissue damage in lungs or other organs was not observed after systemic SCs treatment [37]. The migration/homing ability of MSCs has been known to be affected by passage number and high confluence [37–39].

5.2. Bystander Effect. To date, bystander effect is considered to be the potential treatment mechanism in SC therapy. Bystander effect is achieved by the therapeutic consequences of the transfected tumor cells. Although distant tumor cells are not directly transduced, those distant tumor cells could regress by distant paracrine effect. There are currently five mechanisms suggested for bystander effects: (a) release of soluble formulations, (b) passage through gap junctions, (c) passive transportation, (d) stimulation of local microenvironment, and (e) endocytosis of apoptotic vesicles [3]. Among the SC-based prodrug therapy, the CD/5-FU system showed stronger local bystander effect than the herpes simplex virus-(HSV-) thymidine kinase (tk)/ganciclovir (GCV). The 5-FU diffuses efficiently within the tumor cells and could overcome gap junctions that it passages without direct cell-to-cell contact. The GCV triphosphate showed different mechanisms for the bystander effect that the HSV-tk/GCV induces the local inflammation and devascularization, which probably enhance the vascular permeability and the formulation [3].

6. Stem Cell Therapy of Prodrug-Converting Enzymes

Prodrugs are nontoxic, inactive compounds delivered systemically and converted into biologically active cytotoxic agents only at the targeted tumor region. SC based gene therapy using prodrug-converting enzymes enables tracking and infiltrating into the tumor, which represents one of the expected gene therapy approaches [33, 40].

To date, three types of prodrug-converting enzymes have been introduced in prostate cancer (Table 1). First, cytosine deaminase (CD)/5-fluorocytosine (FC) enzyme prodrug therapy has a long history and also has been investigated largely in prostate cancer. The nontoxic 5-FC is converted to the toxic 5-FU in tumor region. The cellular enzymes process the formulation to three cytotoxic antimetabolites: (a) 5-FdUTP, (b) 5-FUTP, and (c) 5-FdUMP. The SC based gene therapy using CD suicide therapy showed cytotoxic effects which are based on three properties: (a) formation of (5-FU) RNA, (b) 5-FU DNA complexes, and (c) thymidylate synthase [3]. Bcl-2 charges for the downregulation of mitochondrial pathways [41].

The CD/5-FU system has been improved by including the gene uracil phosphoribosyltransferase (UPRT) [32, 42]. Finally, the combination of both systems (CD/5-FU and UPRT) delivered simultaneously has also been achieved. The effect of 5-FU on cell growth arrest and apoptosis has been attributed to the ability of its metabolites to induce the level and activity of the tumor suppressor p53 [43].

The basis for the CD/5-FC system is the ability of bacterial or yeast enzyme CD to convert nontoxic prodrug 5-FC into cytotoxic 5-fluorouracil (5-FU). Although bacterial or yeast enzyme could play the role of converting, yeast CD shows much higher efficacy in converting the 5-FC than the bacterial CD. It has been shown that yeast CD produces a 15-fold higher amount of 5-FU than bacterial CD [44].

The ability of yeast CD-expressing AT-MSCs (CDy-AT-MSC) to target tumor sites and micrometastases and to have a low immunogenic potential was reported [32, 33]. Increased efficiency of 5-FC to 5-FU conversion is achieved through bifunctional yeast fusion gene *CD::UPRT* [32]. The gene product of a bifunctional chimeric protein shows at least 100-fold higher activity than native yeast CD [45]. For NSCs, there are two preclinical studies dealing with this system [23, 46]. Lee et al. reported that human NSCs encoding CD (HB1.F3.CD) labeled with MNP showed the

Table 1: Studies with stem cell-based gene therapy in prostate cancer.

Study	Stem cell	Animal	Cancer cell	Transplantation	Prodrug injection	*in vivo* tracking	Prodrug system	Injected dose of therapeutic stem cells	Transduction	Therapeutic stem cell
Lee et al., [23]	Human NSC	C57BL/6 mice	TRAMPC2	Left ventricle injection, 5 weeks after injection of cancer cells	Intraperitoneally, 500 μg/kg/day in three rounds of 5 consecutive days with a break of 2 days	MNP	Bacterial CD/5-FC	1×10^6 cells/100 μL saline	Retrovirus	HB1.F3.CD
Song et al., [51]	Rat BM-MSC	Nude mice	PC3	Intravenous, 10 and 20 days after injection of cancer cells	Intramuscularly, 30 mg/kg each time for 5 consecutive days for 2 cycles,	GFP	HSV-tk/GCV	1×10^6 cells/kg	Lentivirus	TK-BMSC
Cavarretta et al., [32]	Human AT-MSC	Nude mice	Du145, PC3 LNCaP PC3 (only for *in vivo*)	Intravenous, same time with cancer cell injection	Intraperitoneally, 500 μg/kg/day daily, starting on second day after tumor appearance up to 27 or 32 days	None	yeast CD: UPRT/5-FC	2×10^6 cells/200 μL PBS	Retrovirus	CDy-AT-MSC
Ren et al., [52]	Mice BM-MSC	C57BL/6 mice	TRAMPC2	Intravenous, twice, 10 days after injection of cancer cells	None	GFP	None	5×10^5 cells/200 μL saline	Adenoassociated virus	MSC-AAV-IFN-β
Yi et al., [46]	Human NSC	None	LNCaP	None	None	None	Bacterial CD/5-FC, Rabbit CE/CPT-11, Bacterial CD/Human IFN-β	None	Leukemia virus	HB1.F3.CE, HB1.F3.CD, HB1.F3.CD.IFN-β
Lee et al., Immortalized [39]	Human BMSC	Nude mice	Du145, PC3	Intravenous, 10 days after injection of cancer cells	Intraperitoneally, 30 mg/kg in every round for 5 consecutive days	GFP	HSV-tk/GCV	1×10^6 cells/kg	Lentivirus	SV40-TK-hfBMSC

NSC: neural stem cell; AT-MSC: adipose tissue derived mesenchymal stem cell; MNP: magnetic nanoparticles; BM-MSC: bone marrow derived mesenchymal stem cell; BMSC: bone marrow stromal cell; GFP: green fluorescent protein; UPRT: uracil phosphoribosyltransferase; CD: cytosine; CD/5-FC: cytosine deaminase/5-fluorocytosine; HSV-tk/GCV: herpes simplex virus-thymidine kinase/ganciclovir.

favorable treatment outcome. In their report, systemically transplanted HB1.F3.CD SCs migrated toward the tumor and, in combination with prodrug 5-FC, the volume of tumor was significantly reduced. These findings may contribute to the development of a new strategy of target chemotherapy against prostate cancer, including distant metastatic cancer [23].

Another reported prodrug system is HSVtk in combination with GCV. GCV is a nontoxic purine analogue and is phosphorylated by the enzyme HSVtk and further by endogenous kinases. Final phosphorylated substance, GCV-triphosphate, inhibits DNA synthesis and leads to cell death via apoptosis [47].

The HSV-tk/GCV system, induced a delay in the S phase and G2-phase. The observed apoptosis is not a direct result of the system activation, but a result of the delayed proliferation process. In addition, the induction of mitochondrial damage was observed [48], and caspase-8, Chk1 activation was associated with extensive cell death [49, 50].

Song et al. reported that the transduction of BM-MSCs expresses HSVtk using lent virus to inhibit the growth of subcutaneous PC3 prostate cancer xenografts as well as metastastic RIF-1 fibrosarcoma tumor in nude mice in the presence of GCV [51].

Lee et al. found out that the immortalization of human fetal bone marrow-derived mesenchymal stromal cells by simian virus 40 (SV40-hfBMSCs) could be a stable source of MSCs for clinical application of suicide gene therapy [39].

The CD/5-FC system is regarded more efficient than the HSVtk/GCV system for its stronger bystander effects [22]. The main reason is that 5-FU is a small molecule; therefore, it is able to enter neighboring cells through simple diffusion, while GCV requires gap junctions to affect surrounding cells.

Lastly, Yi et al. evaluated the tumor suppression effect of human NSCs genetically modified to express rabbit carboxyl esterase (rCE) enzyme (HB1.F3.CE), which can efficiently convert the prodrug CPT-11 into the cytotoxic drug SN-38 [46].

They have demonstrated that HB1.F3.CE cells increased the conversion rate of CPT-11 into SN-38, thereby inhibiting cancer cell growth more effectively. Therefore, the application of SC based CE gene therapy has the advantage of reducing the effective prodrug dosage. But this study has limitation because the setting of this study does not include *in vivo* study.

7. Stem Cell Therapy of Other Therapeutic Agents

Besides the prodrug therapy, there are also other options of SC gene therapies including other therapeutic agents such as interferon-β (IFN-β), interleukin (IL)-2, IL-7, and IL-12, and IL-18 [2]. Among these agents, genetic modification to overexpress interferon β was used in two preclinical trials (Table 1) [46, 52]. A significant increase in the natural kill cell activity was observed following IFN-β therapy correlating with the antitumor effect in prostate cancer model. Systemic level of IFN-β was not significantly elevated from this targeted cell therapy [52].

7.1. Limitations of Current Preclinical Studies and Future Perspectives. Although it has been well established that systemically administrated MSCs specifically migrate into tumor lesions, the role of recruited MSCs in the tumor microenvironment is still not fully elucidated yet. Several issues have to be clarified before consideration of clinical trials.

Although several preclinical studies showed favorable treatment outcome, the results were affected by transplanting SC amount. To date, appropriate dosages of SCs are not determined yet and it has to be elucidated before clinical trials. Results from preclinical studies suggested that the concentration of CD enzyme is crucial because it affects the reduction of tumor volume in proportion to the 5-FC dosage. Repeated delivery of CD-MSCs successfully suppressed the tumor growth [53].

The amount of SC vectors affects to the sensitivity of effect during suicide gene therapy [2, 33]. Thus, self-limiting the survival of SC vectors is also an important point, which is ideal for chemotherapy applications because the survival of delivered SCs needs to be no longer than sufficient duration for mediating the efficacy.

There is no guideline at the present time on the dosage of systemic injected MSCs as drugs in pharmaceutical sciences because the cellular kinetics of injected MScs is not well elucidated. The number and duration of MSC expressing suicide genes at the tumor sites would be an important determining factor for the therapeutic outcome because it poses the modulatory effects on tumor cells and neovascularization [26, 38, 54].

The different extent of the response to the cytotoxic effect of 5-FU is another issue to be clarified. Those responses observed between cancerous and noncancerous prostatic cells and between hormones sensitive and hormone-refractory PC cells are diverse due to the different enzyme expression in 5-FU metabolism. Indeed, Tanaka et al. have recently demonstrated that the expression of OPRT mRNA, which represents the capability to activate 5-FU, is significantly higher in PC tissue compared to normal prostate tissue [55].

The enzyme thymidylate synthase is also expressed at higher levels in the PC tissue than in the normal prostatic tissue. The possible reason is that the levels of OPRT activity increase in rapidly growing cells including tumor cells [56].

Transplanted SC effect on tumor genesis is also a crucial issue. The acceleration of tumor formation in preclinical studies was observed when MSCs were connected with cancer cells. MSC cells may support subcutaneous tumor growth when connected with tumor cells [57, 58]. The possible mechanisms for this issue include growth factors production, immunosuppressive character of MSCs, and formation of favorable microenvironment for cancer cells. However, other studies showed that MSCs may inhibit tumor growth in preclinical studies [59, 60], and possess antitumor genic effects on some specific tumor such as Kaposi's sarcoma [35].

This controversial effect of transplanted SC to tumor formation could be solved by SC based gene therapy. SC based gene therapy using the CD/5-FC system possesses the advantage to be safe because of suicide gene presence. Tumor

cells were eliminated not only by the administration of 5-FC but also by therapeutic cells themselves [34].

In vitro experiments, cell viability of the noncancerous epithelial prostate cells, were only slightly affected by the presence of the SC vectors. This higher resistance is promising outcome that noncancerous epithelial prostate cells could be spared from the local bystander effect of 5-FU produced by the SC vectors during targeting primary prostate tumors.

CD/5-FC has an additional limitation that 5-FC has a high clearance rate due to its character of an antimycoticum drug. Hence, additional pharmacokinetics studies will be needed to overcome this point.

In all preclinical studies, a total regression of all tumors was not achieved when SC vectors were administered systemically. This is probably due to less number of therapeutic cells reaching the tumor with this delivery route [25] and to the fact that therapeutic cells are administered at a later timepoint. Authors believe that appropriate multiple injections of SC vectors could bring more striking results in terms of growth arrest as well as an earlier beginning of the therapy.

Despite the feasible availability of human adult MSCs from various tissues, there is limitation to use autologous MSCs from patients with osteogenesis imperfect or tumors because of the genetic defeats. Allogeneic MSCs are an alternative, but immune-compatibility is always of concern. Also, the transduction of MSCs and subsequent sterility and identifying tests for tracking and survival of therapeutic SCs require high techniques and the reproducibility in other researchers could be low.

Another immunogenic issue to be solved is the origin of enzymes. The most frequently used enzymes are of nonmammalian origin and differ from any circulating endogenous enzymes in human. Studies are needed to clarify the requirements of nonmamalian enzymes to be expressed in such concentrations for achievement of sufficient conversion of a prodrug.

To promote SC based gene therapy in the treatment of PCs, several things have to be considered. One measure is the administration of adjuvant SC vectors, which increases the expression of gap junction proteins [61]. However, this approach does not increase the number of MSCs at the targeted lesions, and also the involvement of gap junction in SC based gene therapy is not well elucidated yet. Another measure is to induce further inflammation at the tumor sites to promote MSC migration and homing, which is clinically more relevant because current site-specific irradiation induces death of tumor cells and surrounding cells and inflammation and cytokine release [62]. Increasing the amount or frequency of chemotherapeutic agents is not an appropriate method because of possible systemic inflammation and cytotoxicity to the MSCs as well. However, the diverse classes of therapeutic agents lead to the interaction with SC based gene therapy in a synergistic manner by different underlying mechanisms, and other possible options include chemoembolization and tumor-specific chemotherapy.

MSCs were engineered to express the secreted tumor necrosis factor for apoptosis inducing ligand (TRAIL), which induce caspase-mediated apoptosis and could be applied to prostate cancer treatment. Several studies are now reporting the positive effect of this MSCs-TRAIL system in pancreatic cancer treatment [14, 24].

There are favorable results upcoming with the preclinical use of SCs to treat PC. SC based gene therapy, including enzyme prodrug therapy represents a more specific, less toxic and tailored approach to treating PC. Eradication of microscopic metastases that evade current detection techniques would be translated in a longer survival of PC patients with a better quality of life. Importantly, MSCs could be easily derived from the same patient.

8. Conclusions

The previous gene therapy showed unfavorable outcome due to the inability of vectors carrying the suicide gene to reach distant tumor cells and the inefficient spread of the vectors within the tumor. Therefore the SC based suicide gene therapy holds great potential for the possibility of clinical application due to the inherent and privileged migratory and tumortropic nature of MSCs or NSCs. SC based gene therapy is a promising treatment option in heterogeneous tumors like the PC. By further studies, better characterizing of this therapy is needed. It might offer a hope in the treatment of late-stage PC patients, but it may also be applied to prevent formation of metastases in patients with organ-confined PC.

Conflict of Interests

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

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Review Article

Kallikreins as Biomarkers for Prostate Cancer

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The introduction of testing for prostate-specific antigen (PSA), a member of the fifteen-gene family of kallikrein-related peptidases and also known as kallikrein-related peptidase 3 (*KLK3*), in blood has revolutionized both the detection and management of prostate cancer. Given the similarities between PSA and other *KLK* gene family members along with limitations of PSA as a biomarker for prostate cancer mainly in reference to diagnostic specificity, the potential roles of other members of this gene family as well as PSA derivatives and isoforms in the management of prostate cancer have been studied extensively. Of these, approaches to measure distinct molecular forms of PSA (free, intact, complexed PSA, and pro-PSA) combined with kallikrein-related peptidase 2 (*KLK2*), also known as hK2, have been considered holding particular promise in enhancing the diagnosis of prostate cancer. Recently, an integrated approach of applying a panel of four kallikrein markers has been demonstrated to enhance accuracy in predicting the risk of prostate cancer at biopsy. This review presents an overview of kallikreins, starting with the past and current status of PSA, summarizing published data on the evaluations of various *KLKs* as biomarkers in the diagnosis, prognostication, and monitoring of prostate cancer.

1. Introduction

Tissue kallikrein or kallikrein-related peptidase 1 (*KLK1*) is a member of the chymotrypsin family of serine proteases. Since the first identified member of the *KLK* family was the most common protease in pancreas, the name of "kallikrein" is derived from pancreas (kallikreas) in Greek [1, 2]. Subsequently, the *KLK* family of serine protease was defined with the identifications of novel *KLKs, KLK2* (also known as human kallikrein 2: hK2), and *KLK3* (also known as prostate-specific antigen: PSA) [1–3]. Several investigators contributed to the identification of 12 additional novel serine protease genes localized in close proximity to the previously identified *KLK*-encoding genes that overall encompass ≈280 kb at chromosomal region 19q13.4 of the human genome [2]. *KLK* genes share various features, including exon/intron organization, number and length of exonic regions, intron phase, positioning of the methionine start codon, the catalytic-triad residues, and the terminal codons [4]. As the expression of *KLKs* has been detected in tissues and cell lines from many different human organs, *KLK* gene family members have been implicated in a broad spectrum of physiological processes, including blood pressure regulation, skin desquamation, seminal clot liquefaction, tissue remodeling, peptide hormone, and processing and inflammatory cascades [4].

The interest in *KLKs* as biomarker for prostate cancer dates back more than three decades ago when investigators first reported on the ability to detect PSA in serum from prostate cancer cases [5]. Due to the structural similarities between PSA and other *KLKs*, possible roles for the other members of *KLK* family as a biomarker for prostate cancer have also been explored during the past 25 years. This review strives to provide an overview on the clinical applications of PSA and other *KLKs* as the diagnostic and prognostic markers in prostate cancer.

2. History of PSA

In the 1930s, prostatic acid phosphatase (PAP) was first observed to be elevated in the serum of men with metastatic prostate cancer [6]. In the seminal report by Huggins and Hodges, PAP activity was used to indicate the success or failure of hormonal therapy, and, for close to 50 years, PAP

was used as a blood biomarker of disease progression for men with advanced stages of prostate cancer [7]. However, attempts to use PAP for early detection of prostate cancer were not successful [8]. Accordingly, the diagnosis of prostate cancer remained a purely clinical endeavor for a long time.

Then, initially identified in 1966, PSA (*KLK3*), a 33-kDa glycoprotein secreted by prostatic epithelial cells, was first characterized in 1971 by Hara et al. in forensic studies as a marker for human semen [9]. Synthesized in prostate tissue, the proteolytic activity of the active PSA-enzyme catalyzes the degradation of the gel-forming proteins (*SEMG1* and *SEMG2*) in the ejaculate contributed by the seminal vesicles, which results in liquefaction and release of motile sperm [10–13]. Normally confined within the prostate, only a minute fraction ($\approx 10^{-6}$) of the concentration of PSA being present in semen (which ranges from about 10 to 50 μmol/L) can normally be detected in the blood circulation (i.e., 0.5 to 1 ng/mL, which corresponds to ≈15–30 pmol/L). Prostate cancer is histologically characterized by loss of basal cell layer, derangement of the basal lamina, diminished epithelial polarity, and lack of connection of the glandular acini [14]. Although the increased serum level of PSA in prostate cancer patients is commonly considered to be due to disruption of prostate architecture associated with, for example, cancer, the exact molecular mechanism by which serum level of PSA is increased in prostate cancer patients is still unclear as experimental models and data are currently lacking [15, 16]. The use of PSA as a biomarker for prostate cancer was first postulated in the 1970s, when it was isolated from normal, benign hypertrophic, and cancerous prostatic tissues by Wang et al. [17]. Subsequently, other studies recognized the potential value of PSA as a biomarker of prostate cancer, and PAP was rendered largely obsolete [18]. The detection of PSA in serum was first reported by Papsidero et al. [5]. By the mid-1980s, data were accumulated to show that PSA was superior to PAP in monitoring of prostate cancer after treatment, resulting in the approval of PSA serum concentration assay for the follow-up and monitoring of prostate cancer patients from the US Food and Drug Administration (FDA) in 1986 [19]. Several studies showed that PSA was a sensitive marker for detecting residual disease and recurrence after treatment of prostate cancer during follow-up as we still consider an undetectable level of PSA after radical prostatectomy as indicating the absence of postoperative recurrence [20–22]. Similarly, serial PSA measurements were also applied in the contemporary setting to define recurrence following definitive radiation treatment [18, 23].

Subsequent studies in the early 1990s suggested that serum PSA may be useful in early detection of prostate cancer [18, 24, 25]. In a clinical trial of 6,630 men, Catalona et al. showed that combination of PSA ≥ 4.0 ng/mL with other clinical findings, such as those from digital rectal examination (DRE), improved the detection of prostate cancer detection [26]. In consideration of these findings, FDA also approved the usage of PSA for early detection of prostate cancer. This enabled PSA screening to become widely adopted and also led to a dramatic increase in prostate cancer incidences during mid-1990s in the USA [27]. Furthermore, the percentage of patients with clinically localized, early-stage disease increased substantially as documented in the Surveillance, Epidemiology, and End Results (SEER) database [18, 28]. Such stage migration resulted in the decrease of prostate cancer-specific mortality in the USA as well [29].

As for the appropriate PSA threshold for a prostate biopsy, this issue has been debated intensely since the early introduction of PSA testing in the USA. Data reported from the randomly selected, untreated controls in the Prostate Cancer Prevention Trial (PCPT) who underwent end-of-study prostate biopsy showed that PSA levels in blood were associated with evidence of prostate cancer at biopsy (AUC: 0.68) but that there was no PSA threshold in men aged 62–91 below which the presence of any prostate cancer lesion can be ruled out [30]. PSA's ability to detect the presence of Gleason grade 4 cancers at biopsy was higher (AUC: 0.75), but similarly there was no PSA level below which the presence of high grade cancer at biopsy could be excluded with reliable accuracy. With regard to applying the traditional cutoff of 4.0 ng/mL for triggering a biopsy, it has previously been reported that a significant proportion of tumors detected may be already spread to prostatic capsule at the time of diagnosis [31]. Also, it has been reported that 22% of men with a normal DRE and a serum total PSA level between 2.6 and 4.0 ng/mL have prostate cancer [32]. In addition, findings from PCPT revealed that about 15% of men with normal DRE and a serum total PSA less than 4.0 ng/mL may harbor prostate cancer [30]. As such, some advocated decreasing the threshold for biopsy to 2.5 ng/mL [18, 32, 33]. Meanwhile, aforementioned report from PCPT actually showed that 17% of men with low PSA (1.1–2.0 ng/mL) and a normal DRE had prostate cancer, indicating that even the most stringent biopsy criteria would miss a significant proportion of cancers [30]. Another problem with lowering the PSA threshold for biopsy would be that PSA may be elevated as a result of various noncancerous conditions. About 75% of men undergoing biopsy due to PSA in the range of 4.0 to 10.0 ng/mL do not have evidence of prostate cancer at systemic sextant prostate biopsy [34]. Thus, a significant number of patients are rendered to unnecessary cost as well as stress and morbidity. Although lowering PSA threshold and/or decreasing the age for PSA screening may well be beneficial for men who are at increased risk for prostate cancer, such as those with family history of prostate cancer, the determination of the optimal management-guiding threshold for general population should involve not only the clinical and epidemiologic features but also the social and psychological implications [33].

3. Controversy in PSA Screening

Despite the view that PSA screening contributed to significant decrease in prostate cancer-specific mortality rate in 1990s, recent studies have shown that PSA screening may lead to the unnecessary detection and treatment of indolent cancers, which may be associated with significant morbidity for the patients [35, 36]. Hence, the question on the usefulness of PSA screening remains. PSA screening

came under increased scrutiny when two largest prospective screening trials to date demonstrated contradictory findings. Preliminary results of the Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial (PLCO) in the United States comparing annual screening to usual care found no significant difference in cause-specific mortality, although there were numerous methodological limitations to this study [37]. Some of the problems mentioned regarding the PLCO trial include relatively smaller sample size, the higher rate of PSA testing in the control arm (52%), the higher rate of prior history of PSA testing, and lower compliance with prostate biopsy among men with an elevated PSA level [14, 37–39]. In the European Randomized Study of Screening for Prostate Cancer (ERSPC) trial, 182,000 men aged 50–74 years were enrolled from 7 European countries [39]. For this trial, measurement of PSA level, with a threshold for prostate biopsy of 3 ng/mL, was the principal screening method. The interim data from 162,000 men during a median follow-up period of 9 years demonstrated that PSA screening reduced the risk of death due to prostate cancer by 20%. However, ERSPC trial also revealed that screening was associated with substantial overdiagnosis as it was observed from the trial that 1,410 men would need to be screened and 48 additional cases of prostate cancer would need to be treated to prevent one death from the disease. When the noncompliance among the men who were actually screened was adjusted for, screening was assessed to have reduced mortality from prostate cancer by 27% [39]. Overall the performance of PSA testing as a screening tool for prostate cancer is known to be variable. Depending on the PSA cutoff values applied, the specificity and sensitivity of PSA range from 20 to 40% and 70 to 90%, respectively [40]. The area under the curve (AUC) of the receiver operating characteristic (ROC) curve is between 0.55 and 0.70 for the ability of PSA to identify prostate cancer [40, 41]. As aforementioned, one of the explanations for such poor specificity is the fact that several noncancerous causes may increase PSA level. Due to high false-positive rate, PSA screening for prostate cancer demonstrates a positive predictive value of only 25 to 40% [42]. In 2002, the U.S. Preventive Services Task Force (USPSTF) issued a statement mentioning that the evidences are insufficient to recommend routine use of PSA as a screening tool among men younger than age 75 [40]. In 2011, the USPSTF reanalyzed the available evidence and concluded that the population benefiting from PSA screening was inconclusive, recommending against PSA screening at any age [43]. Thus the controversy continues on whether the benefits of PSA screening outweigh its risks.

4. Risk Stratification with PSA

Interestingly, several studies have demonstrated that PSA levels can be used to predict the future risk of prostate cancer, even decades before actual diagnosis [44–47]. It was reported from a large, prospective study that the leadtime between total PSA levels $\geq$ 4 ng/mL and the subsequent clinical diagnosis of prostate cancer was approximately 5.5 years [44]. Extending prediction models to lower PSA ranges and longer follow-up intervals, Lilja et al. reported from examining prostate cancer risk among 21,277 men younger than 50 years that PSA level checked at age 44 to 50 was very strongly associated with the likelihood of developing prostate cancer up to 25 years later [45]. Odds ratio for a prostate cancer diagnosis at a PSA level in the range of 0.51–1.0 ng/mL was 2.51 compared to PSA $\leq$ 0.50 ng/mL. Also odds ratio increased to 7.02 for a PSA of 1.0–1.5 ng/mL and to 19.01 for 2.01–3.0 ng/mL compared with PSA $\leq$ 0.50 ng/mL. In a subsequent study, the same group demonstrated that PSA level at age 44–50 predicted the risk of developing advanced prostate cancer [46]. Another study from the same group found that PSA at age of 60 years is an extremely strong predictor of the risk of prostate cancer metastasis (AUC 0.86) and death (AUC 0.90) by age 85 [47]. In this study, 90% of total mortality occurred in men with PSA > 2 ng/mL, whereas men with PSA < 1 ng/mL had 0.5% risk of metastasis and 0.2% risk of death from prostate cancer by the age of 85. Such finding would indicate that at least half of men can be exempted from PSA screening at age 60, which would allow the early detection efforts to focus on men with elevated risk [33]. Overall, these data show that men who will develop prostate cancer in future have elevated PSA levels many years before the diagnosis.

5. Strategies to Enhance the Accuracy of PSA

As aforementioned, PSA is not a perfect biomarker for the diagnosis of prostate cancer. Hence, the efforts have been made to enhance the diagnostic accuracy of PSA through PSA kinetics. Increasing the specificity would reduce the number of prostate biopsies performed and related burden in men without prostate cancer. One of the tools devised for such purpose is PSA velocity (PSAV). PSAV is defined as the serial evaluation of serum PSA levels over time [48, 49]. PSAV can be calculated via different methods, such as using the first and the last measured values only or applying a regression line through all available measurements. Carter et al. reported that PSAV is only useful if a minimum of 3 consecutive PSA measurements were taken over a two-year period [50]. Prospective studies have found that PSAV does not appear to add diagnostic value for prostate cancer detection beyond that of a single PSA measurement. In a landmark trial of PCPT, it was observed that when PSAV was adjusted for the effect of PSA and other standard variables, it lost its independent predictive value as an independent predictor of prostate cancer [51]. Meanwhile, the PLCO trial showed that although PSAV was an independent predictor of high grade prostate cancer, addition of PSAV only slightly enhanced the prediction of high grade tumor [52]. From analyzing a large cohort of men in early middle age who were likely to have a low incidence of BPH, Ulmert et al. calculated the predictive values of PSAV alone and a single PSA alone for diagnosis of prostate cancer to be 71.2% and 77.1%, respectively, demonstrating no benefit for PSAV [53]. Such result also indicates that PSA levels generally do not rise sharply before the detection of prostate cancer.

Some researchers have shown that a high pretreatment PSAV is strongly associated with lethal disease demonstrating

poor survival following diagnosis. In the Baltimore Longitudinal Study of Aging project, a strong association between survival and higher PSAV as early as 10 to 15 years before diagnosis was observed [54]. Based on such findings, a total PSAV threshold of 0.35 ng/mL/year was also proposed to be used in screening men with low PSA levels to increase the detection of potentially lethal tumors still in the window of curability. However, Vickers et al. reported that performing biopsies in men with low PSA but elevated PSAV led to a large increase in unnecessary biopsies while missing a significant portion of clinically significant disease [55]. Also, it has been reported that, although relevant to prognosis, baseline total PSA levels and relative PSAV in the first two years following diagnosis of localized prostate cancer could not accurately predict which patients would have a lethal cancer-specific outcome [56]. In addition, several other studies have found that PSAV does not provide additive data in the prediction of prognosis following treatments [57, 58]. Potential reasons for such lack of accuracy may be that the observation period necessary for obtaining a valid calculation of PSAV that is not disturbed by considerable short-term fluctuations is too long or that the number of PSA measurements is too high for use in clinical practice [33]. And it just may be that PSAV does not correlate with early tumor progression but could be a mere indicator of aggressive disease which would not be considered curable even with early detection. Furthermore, a rapid elevation of PSA level is certainly a phenomenon more common in men with a high starting PSA level [59]. However, such situation will not be common scenario for men in a screened cohort. Despite the reported findings indicating the usefulness of PSAV and the suggestions that PSAV should be a part of guidelines and inclusion criteria for clinical trials, more evidences are necessary to confirm clinical utility of PSAV [14]. It should be taken into account that most of relevant studies, in reality, did not include PSAV with PSA in the multivariate predictive models [60].

Another method developed to enhance the predictive value of PSA is PSA doubling-time (PSADT), which is defined as the time it takes for serum PSA level to double. Initial studies on PSADT showed its potential utility in discriminating the biological significance of tumor recurrence after treatment [18]. Pound et al. reported that a postoperative PSADT <10 months was associated with worse metastasis-free survival [61]. Subsequent studies demonstrated that lower PSADT was associated with increased risk of cancer-specific mortality in men with PSA recurrence after surgery and radiation therapy [62, 63]. Still, controversy continues regarding the utility of PSADT as well as PSAV. Although some researchers have mentioned that PSA kinetics in the forms of PSAV and PSADT has improved the prognostic value of PSA measurements, a systematic review by Vickers et al. indicated that PSAV or PSADT provided little additive information over that offered by PSA level alone [18, 60]. Also the EAU guidelines state that PSAV and PSADT have limited use in the diagnosis of prostate cancer due to BPH, variations in interval between PSA measurements, and acceleration/deceleration of PSAV and PSADT over time [64].

Another modality devised to enhance the diagnostic accuracy of PSA was PSA density (PSAD), the concept of which was first described by Benson et al. as the ratio of serum PSA level to prostate volume [65]. This initial study identified the potential utility of PSAD in differentiating prostate cancer from benign disease with subsequent studies demonstrating a modest improvement in diagnostic accuracy with application of PSAD in addition to PSA [66]. Since the transition zone is primarily involved in BPH, several investigators studied the value of adjusting PSA to transition zone volume rather than total prostate volume [67]. Despite some positive findings reported, PSAD failed to receive wide acceptance in screening setting [68]. Other studies have demonstrated the utility of PSAD in predicting clinicopathological features of disease, such as Gleason score and total cancer volume at radical prostatectomy [69]. Overall as some reported that PSAD may contribute to predicting the risk of progression in men on active surveillance, PSAD may be most effective when used in conjunction with other clinical factors to stratify risk and weigh treatment options [70]. A significant problem with PSAD is the need for transrectal ultrasound, which is not a routine part of screening process and often not done as a separate procedure prior to biopsy. Also transrectal ultrasound measurement of prostate volume is prone to be affected by interexaminer variability. Meanwhile, a recent study demonstrated that a new ERSPC risk calculator incorporating prostate volume based upon DRE provided comparable predictive accuracy in predicting significant prostate cancer with transrectal ultrasound-based risk calculator (AUC 0.85 versus 0.86) [71]. Such replacement of transrectal ultrasound measurements with DRE estimates may well enhance the implementation of PSAD as well as prostate volume into risk stratification in clinical setting.

6. PSA Subforms and Human Kallikrein 2 (hK2)

In 1991, Lilja et al. reported that the major immune-detected fraction of PSA in serum exists in complex with protease inhibitor α1-antichymotrypsin [72]. Also, Lilja et al. identified a distinct PSA-epitope present only on the free, noncomplexed, minor fraction of total PSA in circulation. PSA that is catalytically inactive does not form complexes and circulates as free PSA (fPSA). Distinct isoforms of fPSA will be discussed separately in the following section. Levels of fPSA can be detected and compared to total PSA level, yielding the proportion of fPSA (%fPSA). Studies have shown that men with the highest proportions of complexed PSA are more likely to have prostate cancer and that %fPSA is lower in men with prostate cancer as compared to BPH [18, 73, 74]. In 1995, Luderer et al. reported from performing a comparative study that %fPSA outperformed PSA in the "diagnostic gray zone" (PSA: 4.0–10.0 ng/mL) [75]. In a study of men with intermediate PSA levels, a fPSA threshold of 25% yielded 95% sensitivity and 20% specificity for prostate cancer diagnosis [76]. Meanwhile, the fPSA threshold of 20% improved specificity to 38% among men with PSA in the range of 3.0–7.0 ng/mL. In 1998, a prospective study demonstrated that %fPSA decreased the rate of unnecessary biopsies by 20% when using a threshold fPSA of 25% and that, in general,

cancers detected at fPSA > 25% were of smaller volume and lower grade [77]. Such findings were also observed in other studies most commonly implementing %fPSA thresholds in the range of 20 to 27% [78]. In consideration of these published findings, %fPSA was approved by the FDA as an adjunct to PSA for use in the screening and diagnosis of prostate cancer in men with PSA levels between 4.0 and 10.0 ng/mL. Some studies indicated that measuring %fPSA may increase diagnostic accuracy in cases presenting with either low or high PSA values. A randomized, population-based study demonstrated that low %fPSA in men with PSA levels < 3 ng/mL was associated with a 5- to10-fold increased risk of prostate cancer [79]. Also the %fPSA was observed to increase the accuracy of prediction of biopsy results in men with PSA in the range of 10–20 ng/mL [80]. Subsequent studies identified potential shortcomings in using fPSA, such as higher %fPSA in larger prostates, conditions at the time of sample obtainment, in vitro instability, and interassay variability, which may help explain the inconsistencies in the performance of fPSA [18]. Also, published data on the usefulness of fPSA in predicting clinical and pathological outcomes following surgical treatment have shown inconclusive results [81, 82]. However, a meta-analysis of 66 published studies concluded that the %fPSA outperforms PSA in the detection of prostate cancer [83]. Despite some limitations, overall fPSA appears to be a useful modality for diagnosis of prostate cancer, particularly in men with intermediate PSA levels [18].

Human kallikrein 2 (hK2: also known as human kallikrein-related peptidase 2), a secreted serine protease sharing an 80% sequence homology with PSA, is responsible for the cleavage of pPSA to active mature PSA [84, 85]. Both hK2 and PSA are primarily expressed in the prostate gland [16]. Despite these similarities, hK2 and PSA differ in their enzymatic activity [33]. Since the covariance of hK2 and PSA is less than 60% and both markers demonstrate different expression patterns on an immunohistochemistry level, hK2 is considered a marker independent of PSA [86, 87]. The levels of hK2 in prostate, semen, and serum are less than 2% compared with PSA. Similar to PSA, serum hK2 is present in two forms in the blood: one bound to various protease inhibitors and the other (preponderant) free in the circulation [33]. Initial reports showed that although prostate cancer and BPH patients showed no significant difference in hK2 levels, the ratio of hK2 to fPSA (%hK2) increased the accuracy over %fPSA in differentiating prostate cancer from BPH in men with PSA in the range of 4–10 ng/mL [88]. Subsequently, several groups reported that %hK2 contributed to an enhanced discrimination between prostate cancer and noncancer patients [89, 90]. However, others reported that predictive value of %hK2 may not be higher than %fPSA [91, 92]. Meanwhile, it has been suggested that hK2 could also be useful in predicting pathologic stage and grade along with biochemical outcome in patients treated with radical prostatectomy [93, 94]. However, this finding is yet to be validated as some observed that hK2 was unable to discriminate pathologic stage or failed to demonstrate additional value over existing variables [95, 96]. Overall most relevant studies concluded that hK2 has additive role in the detection of prostate cancer. More investigation is warranted on the potential role of hK2 as a prognostic marker.

There are three distinct cleavage isoforms of free PSA in the serum: BPH-associated PSA (BPSA), intact free PSA, and pro-PSA which has been associated with prostate cancer [97]. The precursor of PSA is a 261-amino acid preproprotein, and subsequent processing by human glandular kallikrein2 (hK2) produces active 237-amino acid mature PSA [33]. In men with PSA levels between 6.0 and 24.0 ng/mL, the [−2]pro-PSA fraction was found to be significantly higher in men with prostate cancer [98]. Furthermore, the same group later reported on the value of the pro-PSA to fPSA ratio for screening patients with PSA levels between 2.5 and 4.0 ng/mL and between 4.0 and 10.0 ng/mL [99]. For proper measurement of [−2]pro-PSA, blood samples should be centrifuged within 3 hours of sample collection [30]. Serum may be stored at room temperature or refrigerated (4°C) for a maximum of 48 hours and should be frozen, if stored for a longer period [33]. Two freeze-thaw cycles have no effect on [−2]pro-PSA stability [100]. Catalona et al. reported that elevated pro-PSA to fPSA ratios were associated with aggressive pathological features and decreased biochemical disease free survival after radical prostatectomy [101]. Stephan et al. demonstrated that a new automated tool using [−2]pro-PSA assay with a %fPSA based artificial neural network was capable of detecting prostate cancer and more aggressive disease with higher accuracy than PSA or %fPSA alone [102].

7. Combination of Kallikrein Markers for Improved Cancer Detection

Since it is unlikely that a single biomarker will be enough to make a clear-cut decision regarding the diagnosis and/or prognosis of prostate cancer, efforts have been made to also evaluate the combination of a panel of complimentary biomarkers. A group led by Lilja and Vickers devised a statistical model for predicting prostate biopsy outcomes based on age, DRE, and a panel of four kallikrein markers which were total PSA, fPSA, intact PSA, and hK2. Analyzing the data from the randomized prostate cancer screening trial in Göteborg, Sweden, which was also a part of ERSPC trial, they reported that for every 1000 previously unscreened men with elevated PSA, the use of the model to determine whether to perform biopsy would reduce biopsy rates by 573, while missing only 31 of 152 low grade cancers and 3 of 40 high grade cancers [103]. Such results were also replicated via an independent cohort in which the use of model resulted in the reduction of biopsy by 513 per 1000 men with elevated PSA, whereas 54 of 177 low grade cancers and 12 of 100 high grade cancers were missed [104]. In men who recently have undergone previous screening, the use of the model demonstrated improvements in predictive accuracy [105, 106]. Recently, the panel of four kallikrein markers was shown to predict the outcome of prostate biopsy in men who had previously undergone prostate biopsy during previous screening [107]. In this study, utilizing the dataset obtained from ERSPC trial, the application of four-kallikrein panel significantly enhanced the predictive accuracy of a base

model, incorporating age, DRE, and PSA. As the panel of four kallikrein markers is in the phase of being commercialized (OPKO 4Kscore Prostate Cancer Test), it may soon be available in clinical setting. Meanwhile, published studies have shown that the Beckman Coulter Prostate Health Index (phi), which is a mathematical combination of total PSA, fPSA, and p2PSA (aforementioned as [−2]pro-PSA), as well as p2PSA and its derivatives, namely, %p2PSA, defined as (p2PSA/fPSA) × 100, may significantly improve the accuracy in the detection of prostate cancer over the PSA and the %fPSA [15, 108, 109]. Recently, US FDA has approved the phi test as a new way to test for risk of prostate cancer in men with PSA values in the 4–10 ng/mL range. A head-to-head comparison of phi and PCA3 recently showed that phi was more accurate than PCA3 in both initial and repeat biopsy setting although no statistical significance was noted [110]. In this study, PCA3 did not increase the accuracy of predicting prostate cancer when phi was assessed.

8. Other Kallikreins

As they are also expressed in prostate tissue and are closely related to PSA and hK2, the other members of the tissue kallikrein family have also been investigated upon for their potential role in the detection and prognosis of prostate cancer. Some have reported that higher kallikrein-related peptidase 4 (*KLK4*) mRNA levels of the prostate tissue obtained via biopsy are correlated with higher Gleason score and stage [4]. Kallikrein-related peptidase 5 (*KLK5*) is overexpressed in normal versus cancerous prostatic tissue, and an inverse relationship has been reported between *KLK5* levels and pathologic tumor stage and grade [111, 112]. Also, some studies reported severe overexpression of *KLK5* gene transcription levels with treatment of the androgen-independent prostate cancer cell lines PC3 and DU145 with chemotherapeutic agents widely used in clinical setting [113, 114]. The observation that modulation of the expression levels of these genes was triggered by anticancer agents showed the potential value of *KLKs* in monitoring and evaluating therapeutic responses to chemotherapy in androgen-independent prostate cancer [4]. Meanwhile, elevated kallikrein-related peptidase 11 (*KLK11*) mRNA expressions have been found to be associated with a less advanced stage, lower Gleason score, and an optimistic disease course for prostate cancer [4]. Expression of kallikrein-related peptidase 14 (*KLK14*), which is likely to have a major role in seminal clot liquefaction, indicates an adverse clinical outcome of prostate cancer patients as elevated *KLK14* mRNA and protein levels have been associated with more aggressive tumors [4, 115]. The overexpression of kallikrein-related peptidase 15 (*KLK15*) transcript variants, including alternatively spliced ones, has been observed to be associated with more aggressive prostate cancer [116, 117]. Overall, despite the promising findings reported, further research would be needed to elucidate potential roles for these kallikreins as biomarkers for prostate cancer. Emerging and ongoing efforts on studying *KLK*-mediated pathways will provide further information to support evaluations of *KLKs* as potential biomarkers for prostate cancer. Although *KLKs* may individually lack sufficient specificity and/or sensitivity to be used as a useful biomarker, groups of *KLKs*, possibly with other markers, may offer enhanced accuracy.

9. Conclusions

Interests in kallikreins as biomarkers for cancer began with the advent of PSA which certainly opened up a new era in the management of prostate cancer. Decades following the introduction of PSA, controversy persists regarding the appropriate use of the biomarker. Continuous efforts have been made to improve the accuracy of PSA and/or develop new biomarkers for prostate cancer. Various forms of PSA dynamics failed to significantly enhance the predictive value of PSA. Meanwhile, an integrated approach of applying a panel of different molecular markers, namely, PSA and other *KLKs*, may hold the promise of improving the screening, diagnosis, and monitoring of prostate cancer. Despite the substantial advances in the understanding of *KLKs*, their role in the pathophysiology of prostate cancer is just beginning to be understood. With advances in genomics, proteomics, and other biotechnology, the actual roles of *KLKs* in prostate cancer will likely be elucidated in the near future to help provide novel biomarker for improving screening, diagnosis, prognostication, and eventually patients' survival.

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Conflict of Interests

The author declares that there is no conflict of interests regarding the publication of this paper.

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