EMERGING BIOMARKERS IN PERSONALIZED THERAPY OF UROLOGIC TUMORS

EDITED BY: Rodolfo Montironi, Matteo Santoni, Alessia Cimadamore, Antonio Lopez-Beltran and Liang Cheng

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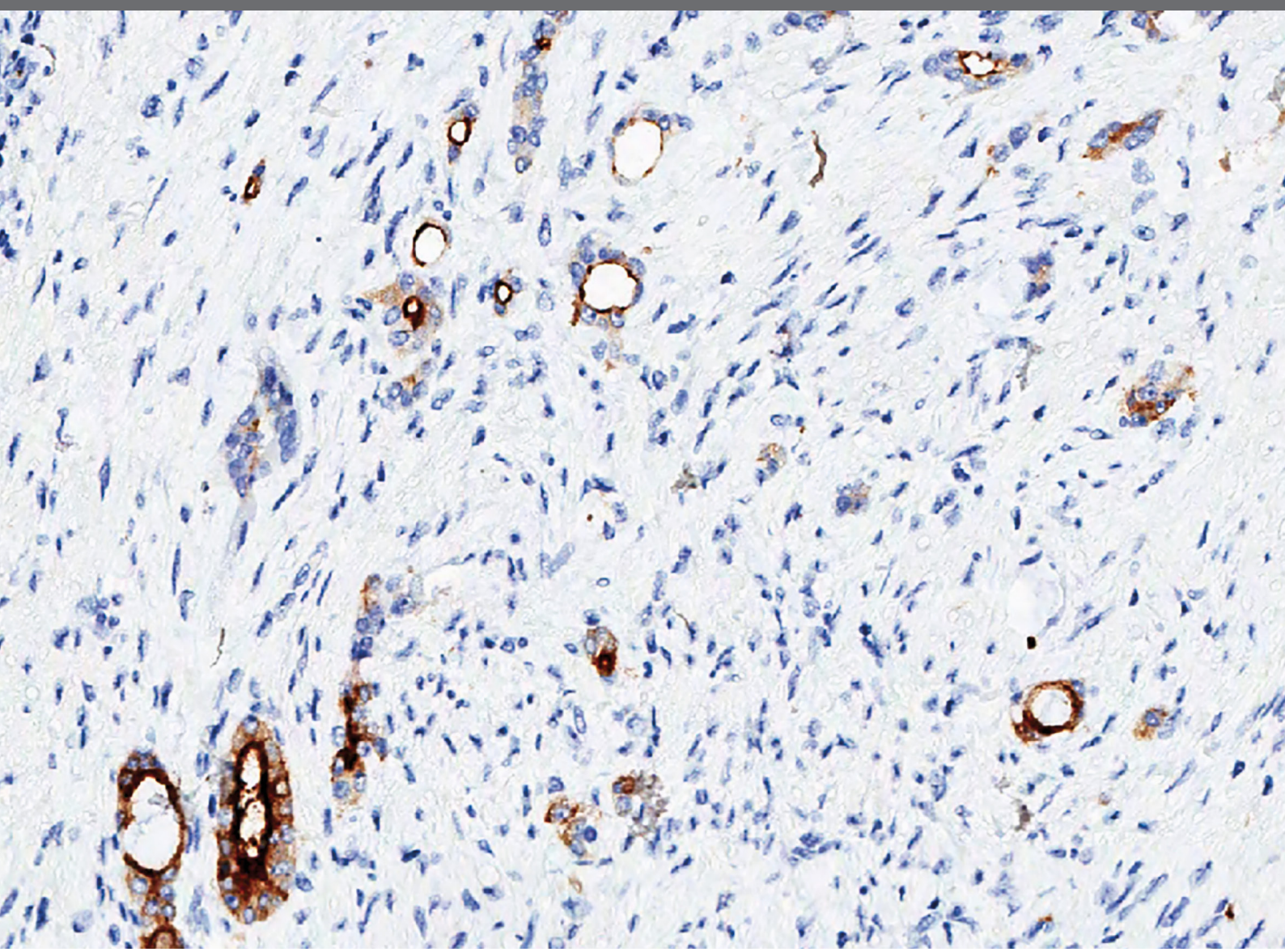

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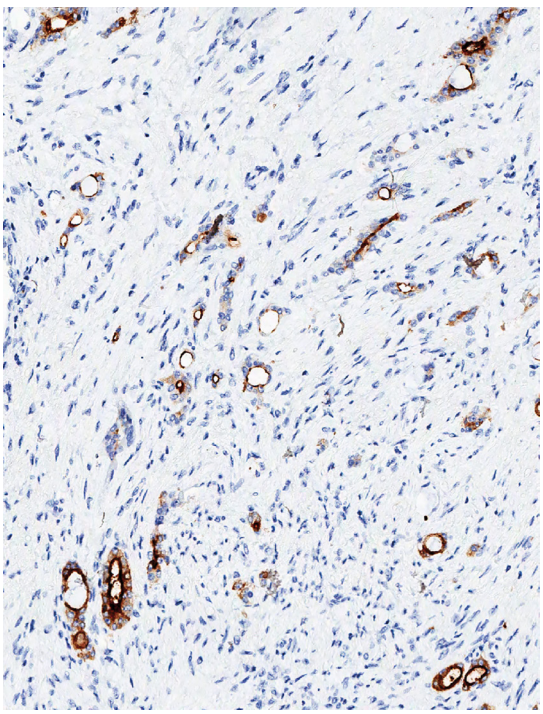
EMERGING BIOMARKERS IN PERSONALIZED THERAPY OF UROLOGIC TUMORS

Topic Editors:
Rodolfo Montironi, Polytechnic University of the Marche Region, Italy
Matteo Santoni, Macerata Hospital, Italy
Alessia Cimadamore, Polytechnic University of the Marche Region, Italy
Antonio Lopez-Beltran, Cordoba University, Spain
Liang Cheng, Indiana University School of Medicine, United States



PSMA expression in prostate cancer glands following radiotherapy.
Image by Dr. Alessia Cimadamore.

The identification of effective biomarkers has becoming a major focus in cancer research, mainly due to the necessity of selecting potentially responsive patients in order to improve their outcomes, as well as to reduce the toxicity and costs related to ineffective treatments. In genitourinary tumors, the lack of biomarkers does not allow for the development of personalized strategies for a single patient, thus representing a major goal in this field. This eBook includes the description of these emerging techniques and identify the most promising biomarkers in genitourinary tumors.

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EDITORIAL
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Editorial: Emerging Biomarkers in Genitourinary Tumors

Rodolfo Montironi[1]*, *Matteo Santoni*[2], *Alessia Cimadamore*[1], *Antonio Lopez-Beltran*[3] and *Liang Cheng*[4]

[1] Section of Pathological Anatomy, School of Medicine, United Hospitals, Polytechnic University of the Marche Region, Ancona, Italy, [2] Oncology Unit, Macerata Hospital, Macerata, Italy, [3] Department of Pathology and Surgery, Faculty of Medicine, Cordoba University, Cordoba, Spain, [4] Department of Pathology and Laboratory Medicine, Indiana University School of Medicine, Indianapolis, IN, United States

Keywords: genitourinary tumors, microbiome, renal cell carcinoma, prostate cancer, bladder cancer, liquid biopsy, PSMA, immunotherapy

Editorial on theResearch Topic

Emerging Biomarkers in Genitourinary Tumors

This is a contemporary update in the field of *Emerging Biomarkers in Genitourinary Tumors*. This series of papers, published in Frontiers in Oncology, section Genitourinary Oncology, by internationally renowned researchers, covers five major topics: (1). Identification of immunological biomarkers in genitourinary cancers; (2). New prostate cancer targets for imaging and therapy; (3) Liquid molecular biomarkers in genitourinary tumors; (4) Emerging biomarkers in testicular germ cell tumors; and (5) Future perspectives on molecular biomarkers in genitourinary tumors: toward a personalized approach to diagnosis, prognosis and prediction of response to therapy.

The first two papers are related to the Identification of immunological biomarkers in genitourinary cancers. In particular, the first, by Cimadamore et al., deals with the biological relationship between the gut microbiome and the immune system, in particular cancer development and treatment. As an example, Akkermansia muciniphila is a commensal associated with excellent clinical outcomes in renal cell carcinoma and non-small cell lung cancer. Interesting results have emerged on the microbiome in prostate cancer (PCa) patients, with specific bacteria as potential biomarkers in risk stratification. Abnormal gut microbiome composition could also have an influence on primary resistance to PD-1 blockade in mice xenografts and patients with cancer. The contribution by Lopez-Beltran et al. deals with the identification of novel immunological biomarkers in kidney cancers. Robust and reliable biomarkers are crucial for patient's selection for treatment with immunomodulatory drugs. PD-L1 expression is predictive of better response from both PD-1 and PD-L1 inhibitors in a variety of tumor types including RCC. A single biomarker for patient selection may not be feasible, given that immune responses are dynamic and evolve over time. A multidisciplinary approach is very much needed to fully develop the current and future value of immune checkpoint inhibitors in clinical practice.

The third paper of the whole series, by Cimadamore et al., deals with New PCa targets for imaging and therapy, focusing on Prostate-Specific Membrane Antigen (PSMA). This contribution reviews the current role of PSMA as a marker for PCa diagnosis, imaging and therapy. PSMA is expressed in the epithelial cells of the prostate and is strongly upregulated in PCa, with elevated expression correlating with androgen independence, metastasis and progression. PSMA has been found to be an active target of investigation by several approaches, including the successful use of small molecule inhibitors, RNA aptamer conjugates, PSMA-based immunotherapy, and PSMA-targeted prodrug therapy. The next three papers deals

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Edited and reviewed by:
Michael Kattan, Cleveland Clinic Lerner College of Medicine, United States

***Correspondence:**
Rodolfo Montironi
r.montironi@univpm.it

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with Liquid molecular biomarkers in genitourinary tumors. The first of the three, by Di Nunno et al., is related to recent advances in liquid biopsy in patients with castration resistant PCa. The selection of patients more likely to benefit from a specific therapeutic approach still remains a key issue as well as the early identification of patients with aggressive disease which could benefit from a more aggressive treatment strategy. They review the literature to explore current knowledge on liquid biopsy in PCa focusing on possible future applications. In particular they focus on circulating DNA and circulating tumor cells as a promising and attractive approach despite to date practical applications of these techniques are few and not validated. The paper by Santoni et al. is an updates on urine markers in superficial and non-superficial bladder cancer (BCa). There is a growing evidence toward the use of minimally invasive "liquid biopsy" to identify new biomarkers. DNA- and RNA-based markers in body fluids such as blood and urine are promising potential markers in diagnostic, prognostic, predictive and monitoring BCa. However, proteomic and genomic data must to be validated in well-designed multicenter clinical studies, before to be employed in clinic oncology. The paper by Santoni et al. deals with recent findings and future challenges of circulating tumor cells (CTCs) in renal cell carcinoma (Santoni et al.). Renal Cell Carcinoma (RCC) may absolutely benefit from the development of non-invasive and reliable biomarkers, allowing early and timely personalized treatment changes. The introduction of CTC analysis within daily clinical practice for patients with RCC seems still so far at the moment. However, the advances obtained in the last 5 years in isolating and analyzing CTCs bring optimism about the future therapeutic landscape in RCC patients.

The contribution by Chovanec et al. deals with Emerging biomarkers in testicular germ cell tumors (GCTs). The ability to predict prognosis and treatment response in GCTs did not improve for many years. Clinical trials with novel targeting agents that were conducted in refractory GCT patients have proven to have negative outcomes. Novel biomarkers have emerged in the field of GCT oncology. Since then, oncology has exploded with various molecular biomarkers to further refine the prognosis and treatment of malignancies. This review summarizes the current knowledge in the research of novel biomarkers in GCTs.

The remaining two papers deal with Future perspectives on molecular biomarkers in genitourinary tumors: toward a personalized approach to diagnosis, prognosis and prediction of response to therapy. The paper by Giulietti et al. is related to emerging biomarkers in BCa identified by network analysis of transcriptomic data. Such complex gene interaction networks can be revealed by a recently developed systems approach called Weighted Gene Co-expression Network Analysis (WGCNA). In this review, the authors focused on the studies where the WGCNA approach has been applied to analyze gene expression data deriving from BCa samples. The paper by Giunchi et al. is a perspective article on emerging molecular technologies in genitourinary tumors. In particular, they deal with wide spectrum mutational analyses using next generation sequencing (NGS) platforms that will soon represent the standard-of-care technologies for the assessment of genetic variants in genitourinary tumors. They also deal with genome-wide trascriptome analyses which include gene expression profiling, miRNA and non-coding RNA profiling and RNA sequencing. Toward the end of the contribution they refer to patient-derived xenografts (PDX), i.e., mouse models where disaggregated cells or little fragments of human tumors are implanted into immunodeficient mice. The establishment of a PDX allows treating and monitoring the response to treatment of the original tumor *in vivo* in the mouse, instead of the patient, providing the best therapeutic selection at the same time.

CONCLUSIONS

The identification of effective biomarkers has becoming a major focus in cancer research, mainly due to the necessity of selecting potentially responsive patients in order to improve their outcomes, as well as to reduce the toxicity and costs related to ineffective treatments. This Research Topic aims to include the description of these emerging techniques and identify the most promising biomarkers in genitourinary tumors.

AUTHOR CONTRIBUTIONS

RM and MS: Conception and design. AC: Drafting the manuscript. AL-B and LC: Critical revision of the manuscript and review of the literature.

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OPINION
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Microbiome and Cancers, With Focus on Genitourinary Tumors

Alessia Cimadamore[1], Matteo Santoni[2], Francesco Massari[3], Silvia Gasparrini[1], Liang Cheng[4], Antonio Lopez-Beltran[5], Rodolfo Montironi[1]* and Marina Scarpelli[1]*

[1] Section of Pathological Anatomy, School of Medicine, United Hospitals, Polytechnic University of the Marche Region, Ancona, Italy, [2] Oncology Unit, Macerata Hospital, Macerata, Italy, [3] Division of Oncology, S. Orsola-Malpighi Hospital, Bologna, Italy, [4] Department of Pathology and Laboratory Medicine, Indiana University School of Medicine, Indianapolis, IN, United States, [5] Department of Pathology and Surgery, Faculty of Medicine, Cordoba, Spain

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Edited by:
Ja Hyeon Ku,
Seoul National University, South Korea

Reviewed by:
Sergio Bracarda,
Azienda Ospedaliera S.Maria, Italy

****Correspondence:***
Alessia Cimadamore
alessiacimadamore@gmail.com
Rodolfo Montironi
r.montironi@univpm.it

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INTRODUCTION

Every individual is characterized by a specific "enterotype," based on the major components of her/his microbiome (i.e., collection of host and microorganism genomes and environmental conditions in an ecosystem) of the gut influenced by diet and geography. This is also influenced by the effects of the organisms present in the infancy as well as the type and pattern of the individual immune system (1).

In the last few years, a close biological relationship has emerged among the microbiome of the gut, the metabolism of the body, as well as the immune system including cancer development. With the increasing availability of high-throughput sequencing, single-cell transcriptomics, and mass spectrometry for a very precise characterization of single enteric, neoplastic, and immune cells, and more extensive databases of organisms already sequenced, experimental exploration of this network has become possible. These advances have also included "culturomics" to make an ever-expanding portion of the microbiota investigable, and sophisticated bioinformatics implements in order to achieve data deconvolution and combination. In 2008, the Human Microbiome Project (HMP) started to characterize the microbial communities from 300 healthy individuals, providing one of the broadest microbial genome databases targeting different body sites: nares, oral cavity, skin, gastrointestinal tract, breast, and urogenital tract (2, 3) (**Figure 1**).

MICROBIOME AND CANCER

In 2017, many experimental studies had been published to demonstrate the importance of single bacterial species on the intestine, the individual immune response and cancer progression, and response to therapy. One of the first unexpected pieces of evidence was that secondary tumor deposits in patients with colorectal cancers include bacteria, such as Fusobacterium species, including Bacteroides, Prevotella, and Selenomonas species, and its associated microbiome. These findings demonstrated the microbiome stability between paired primary and metastatic tumors. Antibiotic treatment of Fusobacterium-positive colon cancers mice-xenografts reduces tumor growth, cancer cell proliferation along with Fusobacterium load, which favors the hypothesis that Fusobacterium species is associated with neoplastic progression (5, 6).

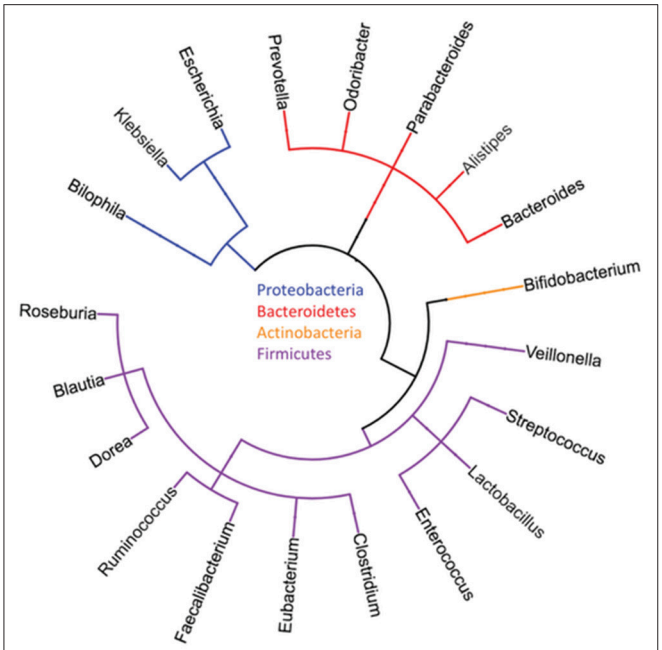

FIGURE 1 | Typical major phyla and genera of the human gut microbiome [reproduced with permission from Goodman and Gardner (4)].

In breast cancer, bacterium Methylobacterium radiotolerans was found relatively enriched compared to normal adjacent tissue from the same patient. Furthermore, bacterial DNA load was reduced in cancer samples vs. healthy tissue and correlated inversely with advanced disease (7, 8). In the distal esophagus, the impact of the microbiome in the pathogenesis of reflux-related disorders and in the development of intestinal metaplasia is well-demonstrated. Patients with esophagitis and Barrett's esophagus have a greater proportion of gram-negative anaerobes/microaerophiles with respect the normal controls. This altered microbiome may promote Barrett's metaplasia and progression to adenocarcinoma (9, 10). The compositions of bacteria community and the throat biodiversity in laryngeal carcinoma patients compared to a control population were different and might be a risk factor for laryngeal carcinoma (11).

The most clinical-affecting evidence regarding cancer microbiome is its contribution to therapy resistance. In pancreatic cancer the most common species identified belong to the Enterobacteriaceae and Pseudomonadaceae families. Enterobacteriaceae express a bacterial enzyme cytidine deaminase (CDD) isoform that confer resistance to gemcitabine. Supporting this, co-treatment with the antibiotic ciprofloxacin abrogate the gemcitabine resistance in colon cancer mouse models (12).

On the other side, there is evidence that corroborates the hypothesis of a protection role of microbiome toward neoplastic changes. Hence, results show that individuals with microbiota linked to a plant diet are the ones with a lower incidence of cancer of the colon (13). Such a diet stimulates bacteria to produce short-chain fatty acids (SCFAs), particularly butyrate, propionate, and acetate. These fatty acids show an anti-inflammatory property through the induction of T-regulatory cells of colonic tissues. Connections of microbiome, production of short-chain fatty acids, and the immune system become more interesting when researchers started to explore the influence of the microbiome in relation to immunotherapy drugs response.

MICROBIOME AND IMMUNOTHERAPY DRUGS RESPONSE

Immunotherapy based on and programmed death-ligand 1 (PD-L1)- and programmed death 1 (PD-1)-targeted antibodies has profoundly modified the prognostic and therapeutic landscape for many types of tumors, with demonstrated efficacy against renal cell carcinoma (RCC), non–small cell lung cancer (NSCLC), and melanoma. PD-L1 tissue expression is a poor prognostic factor as well as a predictor of good responses from both PD-1 and PD-L1 inhibitors in urothelial carcinoma (UC) and RCC (14). In a recent meta-analysis on the expression of PD-1 and PD-L1 in solid tumors, as a predictive biomarker of benefit from PD-1/PD-L1 axis inhibitors, odds ratios of objective response in PD-L1–positive patients compared with PD-L1–negative patients was 2.34 for RCC and 2.20 for bladder cancer (15). Liu et al. also confirmed that "patients with higher ratios of PD-L1-positive cells responded significantly better to both PD-1 and PD-L1 antibodies than those with lower ratios of PD-L1-positive cells" (16).

Each PD-1/PD-L1 drug approved by FDA is associated with a PD-L1, a immunohistochemistry (IHC)-based tissue assay. IHC-based PD-L1 assay is basically utilized to potentially predict the response to anti-PD-1 or/and anti-PD-L1 therapies. A fraction of patients with a negative IHC assay can show a response. This means that identification and utilization of other biomarkers is of great importance for a better selection of patients who might respond to such therapies.

Primary resistance to Immune checkpoint inhibitors (ICIs) has been linked to different factors, including poor intrinsic antigenicity of malignant cells, lack of priming by potentially immunogenic pretreatment with radio-, or/and chemotherapy (17), poor antigen presentation at the time of the priming phase (18), immunosuppression exerted locally by extracellular metabolites (19), and functional exhaustion of lymphocytes infiltrating the tumor (20, 21). On the contrary, high mutational burden and high immunogenic antigenicity of malignant cells are in favor of a better response to ICIs (22, 23). Recently, Routy et al. demonstrated that abnormal gut microbiome composition could have an influence on primary resistance to PD-1blockade in mice xenografts and patients with cancer. In particular, they showed that the clinical benefit of ICIs in patients with cancer at an advanced stage is inhibited by antibiotic therapy (ATB) (24). They tested the effect of ATB on patients with advanced UC, RCC, or NSCLC, who had received PD-1/PD-L1mAb following one or several previous therapies. Progression-free survival (PFS) and overall survival (OS) were significantly shorter in the ATB-treated cohort when either all patients were combined together or when individual cancer types were investigated. In univariate and multivariate analyses, ATB was a

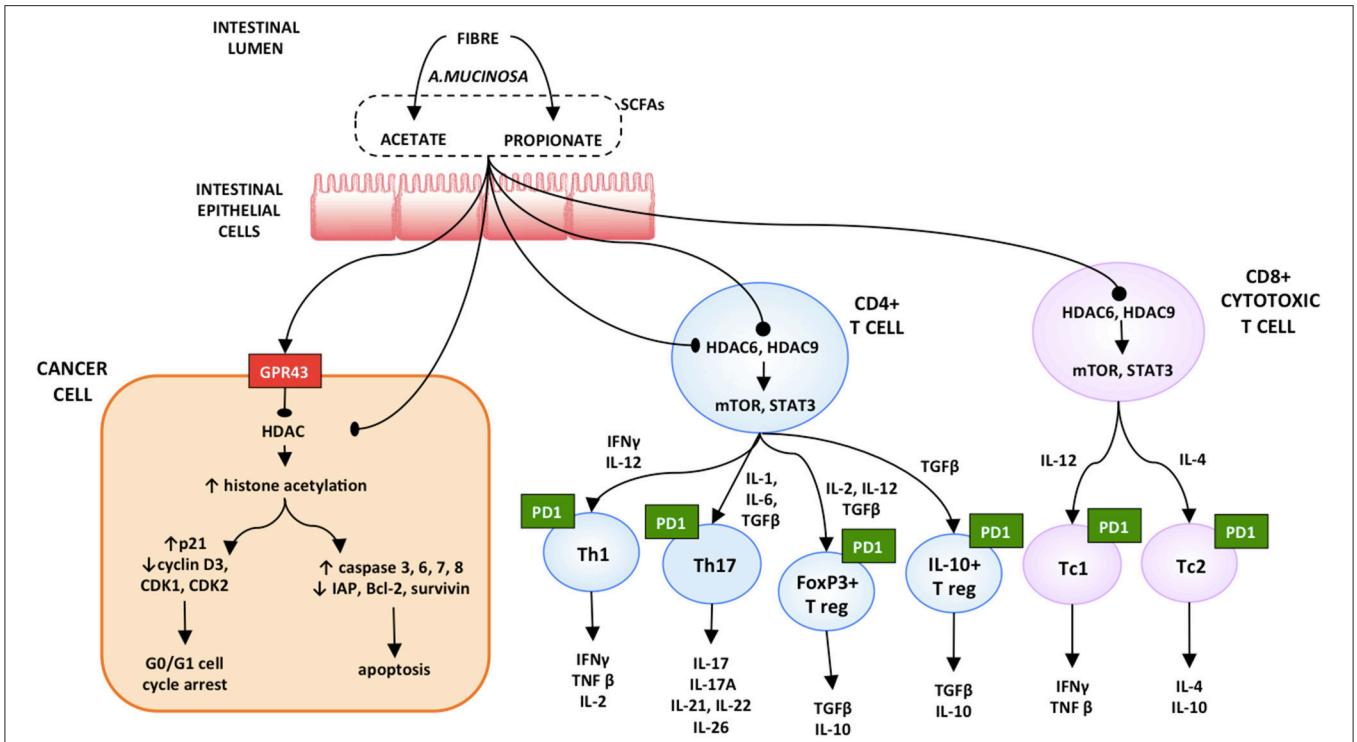

FIGURE 2 | Proposed model on the role of microbiota in resuming the response to immune checkpoint inhibitors [reproduced with permission from Santoni et al. (28)].

predictor factor for resistance to PD-1 blockade, not dependent from traditional prognostic markers in RCC and NSCLC. To evaluate the composition of the microbiota of the gut, they used quantitative metagenomics with analysis of the data in a reference catalog of 9.9 million genes. The greatest richness of the samples, analyzed at the levels of metagenomic species (MGS) and gene count, was correlated with the clinical response. This was defined by the lack of progression of disease 6 months following the initiation of ICIs.

Akkermansia muciniphila (A. muciniphila) was the commensal associated most significantly with excellent clinical outcomes in both RCC and NSCLC. When analyzing memory T cell responses from the peripheral blood of the patients, stimulated against microbiota following initiation of PD-1 blockade, the only immune response linked with the clinical benefit at the time of immunotherapy was the Th1 and Tc1 cell reactivity against A. muciniphila. This immunomodulatory effect might be explained by the production of SCFAs, such as propionate and acetate, by A. muciniphila. These short-chain fatty acids are ligands of the two orphan G-protein–coupled receptors 41 and 43 (GPR41 and GPR43). The former regulates the tumor cells apoptosis induced by SCFA and so exerts a tumor suppressor activity. Furthermore, propionate produced by the bacterium inhibits histone deacetylases and thus increases the histone hyperacetylation.

The inhibition of the expression of Histone Deacetylases (HDACs) has several effects, ranging from a pro-apoptotic activity to a pro-inflammatory response. By opening cell chromatin and thus increasing the DNA accessibility to transcription factors, the histone hyperacetylation induces overexpression of caspases 6, 7, and 8, including caspase 3, and reduces the inhibitor of apoptosis (IAP) family expression (25). The inhibition of expression the HDACs activates the mTOR-S6K and STAT3 pathways. All this stimulates Th17, Th1, FoxP3+, and IL-10+ T cells, as well as the production of IL-10, IFN-g, and IL-17 in CD8+ T cells in both Tc1- and Tc17-cell subsets (26). Moreover, propionate promotes T-cell migration by increasing the expression of intercellular adhesion molecule 1 (ICAM-1) and E-selectin on endothelial cells (27, 28) (**Figure 2**).

Derosa et al. demonstrated the effect of ATB in patients with RCC and NSCLC treated with anti-PDL1 mAb monotherapy or combination therapy. In patients with RCC, ATB compared with no ATB was linked with an increased risk of progressive disease, shorter PFS, and shorter OS. Similar rates were also obtained in the NSCLC cohort (29). Researchers are now planning to transfer fecal bacteria from patients who respond to treatment with checkpoint inhibitors into the intestine of non-responder patients. This process is currently called "fecal microbiome transplant." Microbiota composition might also be manipulated by the application of foods and prebiotics. The prevalence of a subspecies selected by diet rather than others could modify the population predisposition to a specific disease and the response to therapy of cancer patients (30). Fecal microbiota transplant, although not being probiotic, could be considered a fermented food, given the microbes, and nutrients present.

TABLE 1 | Metagenomic human studies identifying microbiota associated with cancer tissues [reproduced with permission from Goodman and Gardner (4)].

Tissue type	Species differential	References
Colorectal cancer	*Fusobacterium*, *Selenomonas,* and *Leptotrichia* species increased in cancer tissues	(5, 6)
Breast cancer	*Alistipes*, *Sphingomonas,* and *Methylbacterium* increased in cancer tissue	(7, 8)
Esophageal cancer	*Streptococcus*, *Prevotella,* and *Veillonella* species increased in cancer tissues	(9, 10)
Head and neck cancer	*Fusobacterium*, *Prevotella,* and *Gemella* species increased in cancer tissues; *Streptococcus* and *Rothia* species decreased in cancer tissues	(11)
Pancreatic cancer	Enterobacteriaceae, Pseudomonadaceae, Moraxellaceae, and Enterococcaceae increased in cancer tissues	(12)
Prostate cancer	*Propionobacterium acnes* increased in cancer tissues	(32–37)

MICROBIOME IN BLADDER AND PROSTATE CANCER

Of great interest is the urinary microbiota profile investigated by Wu et al. They analyzed DNA from urine pellet collected from male patients with urothelial carcinoma and non-neoplastic controls. They observed enrichment of some bacterial genera (such as Sphingobacterium, Anaerococcus, and Acinetobacter) and decrease of others (such as Roseomonas, Proteus, and Serratia) in the group with cancer in comparison with the control group. Patients with high risk of recurrence and progression had an enrichment of Herbaspirillum, Porphyrobacter, and Bacteroides. This means these bacteria can be considered as potential biomarkers in risk stratification (31).

In the last year, interesting results have emerged by investigations on the microbiome in PCa patients. The microflora of tumor, peri-tumor, and benign prostate tissue samples have recently been characterized by massive ultradeep pyrosequencing. Interestingly, differences in microbial populations among paired tumor/peri-tumor and non-tumor prostate tissues have been detected. This finding generates the hypothesis that the distribution of bacterial microbes varies according to the nature of tissue within the same gland. This suggests a pathophysiological association between the local microbial niche and composition, and the tumor itself (32, 33) (**Table 1**).

A case-control pilot study has been conducted by Golombos et al. to demonstrate the impact of the gut microbiota on PCa pathogenesis. They performed a computational genomics analysis on stool samples of men with benign prostatic conditions and men with intermediate or high risk clinically localized PCa. Biologically significant abundance differences of bacteria species and 23 metabolic differentially abundant pathways were identified between the two cohorts (34). Likewise, analyses on the urinary microbiome showed a prevalence of uropathogens and pro-inflammatory bacteria differentially abundant in PCa patients compared to healthy subjects in urine collected from men prior to biopsy for PCa (35).

Liss et al. developed a microbiome-derived risk profile for PCa, derived from altered metabolic pathways, comparing the taxonomic composition of samples (64 with PCa and 41 without) of rectal swab collected 2 weeks before prostate biopsy (36). Even though the differences between the two groups are not impressive, these results are hypothesis-generating and pave the way to further evaluate the manipulation of aberrant microbiomes to reduce PCa risk (3).

The composition of the microbiota in the gut is influenced by oral androgen receptor axis-targeted therapies (ATT) in prostate cancer patients. Results on fecal microbiota profile shows the abundance of species linked to response to anti-PD-1 immunotherapy, including Ruminococcaceae spp., and A. muciniphila, and an greater representation of bacterial gene pathways that are involved in steroid biosynthesis as well as steroid hormone biosynthesis in the fecal microbiota of men under ATT (4, 37). Additional studies are needed to evaluate whether the gut microbiota can influence clinical responses to ATT, and modulate the anticancer effects of future therapies, including immunotherapy.

As regards to genitourinary tumors, there are only few trials ongoing (38, 39). One to take into consideration is a prospective study on prostate cancer and breast cancer patients who are undergoing two different standards of care radiation regimens. Exposure to radiation can impact immune cells that are present in the blood as well as the underlying microbiota. The aim of this study is to study microbial changes and how these changes correlate with alteration in immune mediators (i.e., lymphocytes, cytokines) present in blood samples before, during, and after radiation, by collecting stool specimens at baseline, end of radiation therapy and during the follow up (ClinicalTrials.gov Identifier: NCT03383107).

CONCLUSIONS

The host and the microbiota share a complex balanced relationship that can be overthrown in a state of dysbiosis consequential to environmental changes. Alteration of this balance could lead to promotion of inflammatory diseases and cancer. There is evidence showing that the activity of microbiota in the restoration of response to immune checkpoint inhibitors involves both the immune and cancer cells. Stimulating recall Th1 responses against A. muciniphila improves immunosurveillance in cancer patients. Microbiome composition has the potential to be a novel biomarker of response to ICIs and a therapeutic opportunity for unresponsive patients. In patients with RCC, antibiotic therapy was linked to an increased risk of progressive disease, shorter PFS, and shorter

OS. Pioneer studies on bladder and prostate cancer patients' microbiome pave the way to the investigation of a possible novel prognostic, diagnostic, and therapeutic tool.

AUTHOR CONTRIBUTIONS

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The Identification of Immunological Biomarkers in Kidney Cancers

Antonio Lopez-Beltran[1], Vanessa Henriques[2], Alessia Cimadamore[3], Matteo Santoni[4], Liang Cheng[5], Thomas Gevaert[6,7], Ana Blanca[8], Francesco Massari[9], Marina Scarpelli[3] and Rodolfo Montironi[3]*

[1] Department of Pathology and Surgery, Faculty of Medicine, Cordoba University, Cordoba, Spain, [2] Pathology Service, Champalimaud Clinical Center, Lisbon, Portugal, [3] Section of Pathological Anatomy, United Hospital, School of Medicine, Polytechnic University of the Marche Region, Ancona, Italy, [4] Oncology Unit, Macerata Hospital, Macerata, Italy, [5] Department of Pathology and Laboratory Medicine, Indiana University School of Medicine, Indianapolis, IN, United States, [6] Laboratory of Experimental Urology, Organ Systems, KU Leuven, Leuven, Belgium, [7] Department of Pathology, AZ Klina, Brasschaat, Belgium, [8] Instituto Maimonides de Investigación Biomédica de Córdoba, Córdoba, Spain, [9] Division of Oncology, S. Orsola-Malpighi Hospital, Bologna, Italy

The recent approval of several agents have revolutionized the scenario of therapeutic management of metastatic renal cell carcinoma (RCC) allowing us to reach important clinical end points with extended patients' survival. Actually, every new drug approved has represented an important step forward to the improvement of patient's survival. On the other hand, we now understand that RCC includes a large group of tumor entities, each of them with different genetic and mutational alterations, but also showing different clinical behavior; a reason behind the needs of subtype specific personalized approach to therapy of RCC. Immunotherapy is gradually becoming a key factor in the therapeutic algorithm for patients with locally advanced or metastatic RCC. Due to the combination of potent treatment success and potentially deadly adverse effects from immune checkpoint inhibitors (ICI), gathering prognostic and predictive information about FDA-indicated tumors seems to be prudent. Robust and reliable biomarkers are crucial for patient's selection of treatments with immunomodulatory drugs. PD-L1 expression is a poor prognostic factor and predictive of better responses from both PD-1 and PD-L1 inhibitors in a variety of tumor types including RCC. Each FDA approved PD-1/PD-L1 drug is paired with a PD-L1 Immunohistochemistry (IHC) assay. Thus, there is need for improved knowledge and application of PD-1/PD-L1 IHC biomarkers in daily practice. IHC staining appears in membranous fashion. The atezolizumab approved IHC assay is unique in that only immune cell staining is quantified for the use of this assay in RCC. A single biomarker for patient selection may not be feasible, given that immune responses are dynamic and evolve over time. Biomarker development for ICI drugs will likely require integration of multiple biologic components like PD-L1 expression, TILs and mutational load. New methodological approaches based on digital pathology may be relevant since they will allow recognition of the biomarker and to objectively quantitate its expression, and therefore might produce objective and reproducible cut-off assessment. Multidisciplinary approach is very much needed to fully develop the current and future value of ICI in clinical practice.

Keywords: renal cell carcinoma, PD-L1, immunotherapy, RCC subtypes, immunological biomarker, predictive biomarker, tumor mutation load

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Fabio Grizzi,
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Elena Ranieri,
University of Foggia, Italy
Sanja Štifter,
University of Rijeka, Croatia

****Correspondence:***
Antonio Lopez-Beltran
em1lobea@uco.es

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INTRODUCTION

The recent approval of several agents have revolutionized the scenario of therapeutic management of metastatic renal cell carcinoma (RCC) allowing us to reach important clinical end points with extended patients' survival (1).

The first generation of immune checkpoint inhibitors (anti-CTLA-4 and anti-PD-1/PD-L1) targeted natural immune homeostasis pathways to drive anti-tumor immune responses. These agents led to unprecedented results in patients with previously incurable metastatic disease and therefore became first-line therapies for some advanced cancers (2–12). Since these agents are efficacious in only a minority of patients, however, newer strategies are becoming available that target additional immunomodulatory mechanisms to activate patients' own anti-tumor immune responses. Emerging targets include co-inhibitory and co-stimulatory markers of the innate and adaptive immune system.

In this review, we will discuss: (1) Pathologic and molecular subtypes of RCC; (2) Current landscape of targeted therapy in renal cell carcinoma; (3) Overview of immunotherapy in renal cell carcinoma; (4) Predictive immunological biomarkers in renal cell carcinoma; (5) Gene expression as predictive biomarkers in renal cell carcinoma; (6) The current status of PD-L1 immunohistochemistry; (7) MMR-deficiency and mutational load in RCC; and (8) Biomarkers of acquired resistance. Finally, we briefly highlight likely future perspectives of predictive biomarkers of immunotherapy in RCC.

PATHOLOGIC AND MOLECULAR SUBTYPES OF RENAL CELL CARCINOMA

Clear cell RCC (ccRCC) accounts for about 75% of kidney cancer while the other 25% are classified as non-clear cell renal cell carcinoma (nccRCC) (13). Over a dozen pathological subtypes are now recognized by the most recent World Health Organization classification of Tumors of the Urinary System and Male Genital Organs (13). These subtypes include papillary renal cell carcinoma (pRCC) (20%) and chromophobe renal cell carcinoma (chRCC) (5%), which are the most frequent nccRCC subtypes; hereditary leiomyomatosis and renal cell associated -carcinoma, collecting duct carcinoma, renal medullary carcinoma, MiT family translocation carcinoma, succinate dehydrogenase-deficient RCC, mucinous tubular and spindle cell carcinoma, tubulocystic RCC, Acquired cystic disease-associated RCC, clear cell papillary RCC, and RCC unclassified represent less common subtypes (13).

Several genomic changes have been found in ccRCC, mostly epigenetic reprogramming and oncogenic metabolism pathways alterations (13–18) with other common genetic changes in genes controlling cellular oxygen pathway (e.g., VHL) and the maintenance of chromatin structure (e.g., PBRM1) (19–22). TCGA analysis of a ccRCC cohort found similar genomic changes and reported recurrent alterations in the PI(3)K/AKT pathway and several epigenetic changes in DNA methylation (22). Molecular stratification of ccRCC revealed 2 different subtypes: clear cell type A (ccA) and B (ccB), with ccA patients having a markedly better prognosis (23, 24). A second TCGA study focussed on papillary RCC (pRCC) and found that type 1 and type 2 pRCC are distinctly different diseases based on molecular features and that type 2 pRCC is a heterogeneous disease with at least three different subgroups (25). A third TCGA project focussed on the chromophobe RCC (ChRCC) and found gene expression changes related to mitochondrial function and recurrent structural breakpoints within TERT promoter region (26). Recently, a multilevel molecular characterization of the 3 TCGA RCC databases revealed nine major genomic RCC subtypes, each one being distinct in terms of altered pathways and patient survival (16). Overlapping and subtype-specific genomic changes were observed, and good correlation with histologic subtypes was noticed. These molecular classes show substantial molecular diversity represented within each major histologic type, but importantly, actionable alterations also included PI3K and immune checkpoint pathways (16).

CURRENT LANDSCAPE OF TARGETED THERAPY IN RENAL CELL CARCINOMA

The better knowledge of molecularly altered pathways of RCC has led to the development of new classes of drugs rising the targeting therapy era (1, 12, 16–23, 27). Angiogenesis, the hallmark of RCC, is the final target of several TKi (Sunitinib, Axitinib, Sorafenib and pazopanib) (1–12). After angiogenesis, the finding that, the deregulation of the PI3K–Akt–mTOR pathway, activated at different levels of the signaling cascade, drives RCC progression has led to the development of the mTOR inhibitors everolimus and temsirolimus. The association between everolimus and lenvatinib (a VEGFR1, VEGFR2 and VEGFR3 FGFR1, FGFR2, FGFR3, FGFR4, PDGFR, RET and KIT inhibitor) has been recently explored in a phase II clinical trial which demonstrated a better progression free survival (PFS) for patients receiving the combination of these two drugs compared to those who received everolimus monotherapy (10). Recently, also the mesenchymal-epithelial transition and multi-tyrosine kinases inhibitor cabozantinib has been included in clinical practice (1–12).

These drugs have led to an improvement in overall survival (OS) (sunitinib, pazopanib, cabozantinib, temsirolimus) and PFS (sunitinib, axitinib, cabozantinib, sorafenib, pazopanib, everolimus and temsirolimus) showing a safety profile with a remarkable clinical activity in a disease which has always been poor of active treatments (12, 15).

OVERVIEW OF IMMUNOTHERAPY IN RENAL CELL CARCINOMA

Targeting drugs have significantly changed the course of RCC, but it's likely that a new classes of agents, the immune-checkpoint inhibitors (ICI), are destined to feed this new paradigm in RCC treatment (12, 28–51).

Programmed Death Receptor 1/Programmed Death Receptor Ligand 1 (PD 1/PD-L1) and Cytotoxic T Lymphocytes Antigen 4

(CTLA-4) inhibitors are agents able to target specific pathways related to immune-response which are often hyper-activated by tumor cell interaction (46). By inhibition of these targets, ICI could reactivate a specific immune response against tumor cells (**Figure 1**) (52). The observation that, RCC is related to a high mutation burden and so maybe to a high antigens expression, has led to test these drugs in different stages of the disease. Checkmate 025 was the first large phase III clinical trials comparing the PD-1 inhibitor nivolumab to everolimus in patients with locally advanced or metastatic RCC progressed to at least one VEGF/VEGFR inhibitor (11). This study met its primary endpoints showing an OS benefit in patients receiving nivolumab. Furthermore, patients treated with immunotherapy showed a higher overall response rate (ORR) compared to everolimus with an important percentage of patients achieving long lasting response (11). It is not surprising that the important results achieved in this trial have move to explore immunotherapy in other setting, such as adjuvant/neo-adjuvant stage and as first line therapy (12). Two different strategies been adopted: (1) the combination between an immune-checkpoint inhibitor and a VEGF inhibitor has been evaluated in a phase II trials. Indeed, in Immotion150 305 patients with locally advanced/mRCC and untreated RCC were randomized to receive: atezolizumab (an anti PD-L1 inhibitor) and bevacizumab, atezolizumab alone or sunitinib (41). The association arm resulted in a longer PFS compared to atezolizumab (6.1 months) and sunitinib arms with a higher percentage of ORR in combination arm (41). Of note, patients with PD-L1 positive expression ($\geq$1%) showed a longer PFS (14.7 months) and higher ORR (46%) in atezolizumab arm; (14) and (2) the combination between two immune-checkpoint inhibitors have been recently tested in a large phase III trial: The Checkmate 214. In this study patients were randomized to receive the nivolumab (anti PD-1) and Ipilimumab (Anti CTLA 4) combination or sunitinib as first line therapy (42). In ESMO 2017, Escudier et al. (42) presented primary results after 17.5 months of follow up showing that the combination between ipilimumab-nivolumab resulted in higher ORR and complete response rate in intermediate/poor risk patients. Of note, patients with intermediate/poor risk disease and PD-L1 expression $\geq$1% showed higher ORR and PFS compared to sunitinib, while patients with favorable category of risk (showing lower PD-L1 expression) displayed a longer PFS and a higher ORR with sunitinib (42) (**Table 1**).

These encouraging results suggest that we are about to enter a new era for the management of metastatic RCC since the data provided from these trials might represent only the tip of the iceberg, and therefore, we could expect more therapeutic novelties to come. Nonetheless, even if immunotherapy provides a new hope for patients with metastatic RCC, the "old" targeting therapy is far from being abandoned. Indeed, Checkmate 025 showed that nivolumab is better than everolimus, but there are other agents showing to be extremely effective after VEGF/VEGFR inhibitors progression, thus, the decision of second line treatment should be weighted on the basis of the clinical outcome pursued as well as patient preference and toxicity profile (53). Though in first line setting immunotherapy showed interesting results, it's probably that the positive effect could be restricted to patients with specific clinical features, such as intermediate/poor risk disease while patients with favorable profile could benefit from a standard therapeutic approach (46). It is probably that the worst clinical profile of the disease could be related to a high mutational burden of tumor cells, therefore resulting in a higher antigens expression. Preliminary data seems to indicate that these patients with high mutational load present a higher percentage of tumors with positive PD-L1 expression. Future studies will help us to better understand the role of PD-L1 as prognostic and predictive response factor since to date we have highly diverging information. Indeed, a meta-analysis of six published studies revealed that a higher level of PD-L1 expression increased the risk of death by representing therefore a negative prognostic factor (44). Differently to what was expected, the improved OS with nivolumab was not correlated with PD-L1 expression in Checkmate 025 while patients with positive PD-L1 expression seems to show more clinical benefit from immune-checkpoint inhibitors in Immotion150 and Checkmate 214 (**Table 2**).

PREDICITVE IMMULOGICAL BIOMARKERS IN RENAL CELL CARCINOMA

Due to the increasing role of immunotherapy in clinical practice, the research of predictive response factors remains a critical but still unmet issue. A PD-L1 assessment on tumor cells through Dako PD-L1 IHC 28-8 pharmDx test was performed in both the Checkmate 025 and 214 trials. In Checkmate 025 nivolumab efficacy was not influenced by PD-L1 expression. However, patients expressing PD-L1 more than 1% ($n = 181$) showed a worse OS in both treatment arms thus suggesting a prognostic role more than a predictive one. On the other hand, an exploratory analysis of the Checkmate214 showed a PFS benefit favoring combination only in patients expressing PD-L1 (1% or greater). Survival and ORR advantages were maintained in all PD-L1 categories. However, patients with higher PD-L1 expression showed greater benefit with the immune-combination. Taking together, these results seem to confirm that PD-L1 IHC expression does not act as predictor of response in patients with metastatic ccRCC receiving ICI immunotherapy (12, 15, 28, 46, 54). Furthermore, intratumoral eterogeneity of PD-L1 expression is another issue to take into consideration. As demonstrated by López et al. a multisite tumor sampling strategy identified a greater number of positive cases compared to current tumor sampling protocols and a different pattern of PD-L1 expression with positive and negative regions in the same tumor (55).

As seen in other neoplastic diseases in which immunotherapy has been successfully tested, tumor mutational burden and non-synonymous mutation expression have been related to higher neo-antigens tumor expression and to favorable immunotherapy response. A rationale supporting additional research of this variable in RCC derives from the evidence that immunotherapy is associated to higher clinical benefit in worst risk categories of

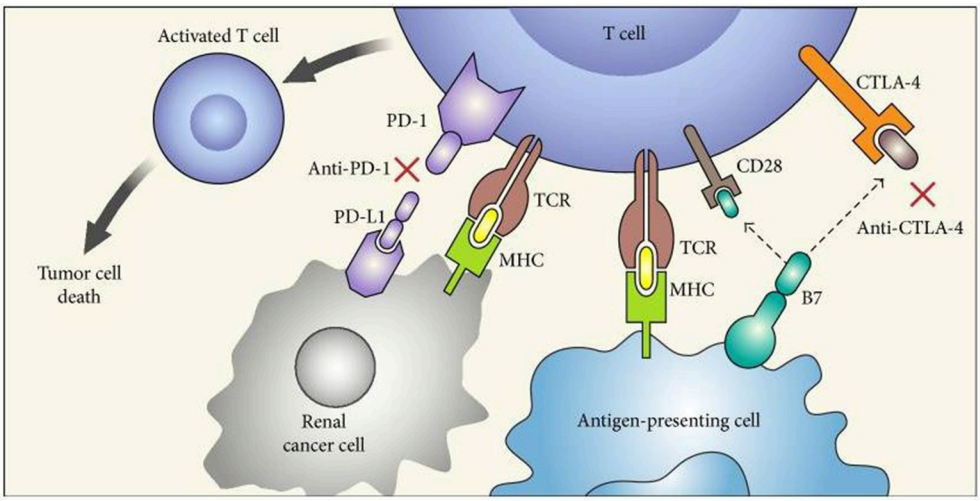

FIGURE 1 | "Mechanism of action of immune checkpoint inhibitors. PD-1 is expressed on activated T cells and when it binds to its ligand PD-L1 on tumor cells leads to T cell exhaustion. CTLA-4 competes with CD28 (costimulatory T cell molecule) for B7 ligands (CD80 and CD86 that are not shown in the figure) and upon activation decreases T cell proliferation as well as activity. Blockade of CTLA-4 (by anti-CTLA-4) and PD-1 (anti-PD-1) or PD-L1 stimulates effector T cells to produce antitumor responses. PD-1, programmed death-1; PD-L1, programmed death-ligand 1; MHC, major histocompatibility complex; TCR, T cell receptor; and CTLA-4, cytotoxic T lymphocyte antigen." Reproduced from Raman and Vaena (52). Available via license: CC BY 3.0.

TABLE 1 | Results obtained in selected trials exploring immune check point inhibitors in metastatic/locally advanced RCC using different combination of drugs.

Study name with experimental and comparator arms	Setting	N ITT	N PD-L1+	OS ITT	HR	OS PD-L1+	PFS ITT	HR	PFS PD-L1+	HR	ORR ITT	ORR PD-L1+	CR
IMMOTION150													
Atezolizumab + Bevacizumab	Untreated patients with locally advanced or metastatic renal cell carcinoma	101	164	NR	NR	NR	NR	NR	NR	NR	32%	46%	NR
Atezolizumab		103		NR		NR	NR		NR		25%	28%	NR
Sunitinib		101		NR		NR	NR		NR		29%	27%	NR
CHECKMATE 214													
Ipilimumab + Nivolumab	Untreated patients with locally advanced or metastatic renal cell carcinoma	550	204	NR	NR	NR	11.6	0.82	22.8*	0.48	NR	58%*	9.4%*
Sunitinib		546	224	NR	NR	NR	8.4		5.9*		NR	25%*	1.2%*

*NR, not reported; PFS: progression free survival; OS, overall survival; ORR, overall response rate; CR, complete response. *Intermediate/poor risk patients with PD-L1 expression ≥1%.*

RCC, a clinical category of RCC in which high mutational load is present (30–32). Indeed, considering the subgroup analysis of the Checkmate 025 study and the significantly better results of nivolumab-ipilimumab combination in intermediate/poor risk patients in the Checkmate 214, it seems likely that tumors with worst clinical features are those that better respond to immune-checkpoint inhibitors and this may be due to a higher mutational load resulting in higher neo-antigen content. Unfortunately, differently than expected, mutational load does not seem to correlate with MSKCC or IMDC prognostic criteria (31). Moreover, no difference has been observed between clear cell and sarcomatoid components of different tumor samples, suggesting that the level of mutational load is not a variable associated to worst clinical features of the disease, hypothesis that clearly needs further investigation (33).

Concerning the correlation between mutational burden and response to ICI immunotherapy in ccRCC, de Velasco et al. carried out a whole exomes and transcriptomes sequencing of 9 patients with metastatic RCC receiving nivolumab. They discovered that RCC had relatively few non-synonymous mutations and neo-antigens and, surprisingly, that among patients receiving nivolumab non-synonymous mutations were significantly higher in non-responder patients ($n = 6$) compared to responder patients ($n = 3$) (34). Of note, they found a

TABLE 2 | Summary of assays and response rates in immune checkpoint inhibitor trials.

Drug		Antiboy for PD-L1 IHC assay	Definition of PD-L1 positivity
Nivolumab (SA)	PD-1	Rabbit 28-8 (Dako)	PD-L1 ≥5% (TC)
Atezolizumab (SA)	PD-L1	Rabbit SP142 (Ventana)	IHC 1/2/3 (IC)
Nivolumab/ Ipilimumab(C)	PD-1/CTLA-4	Rabbit 28-8 (Dako)	PD-L1 ≥1% (TC)
Atezolizumab/ Bevacizumab (C)	PD-L1/antiVEGF	Rabbit SP142 (Ventana)	IHC 1/2/3 (IC)

Locally advanced or in metastatic renal cell carcinoma.
TC, tumor cells; IC, immuno cells in the microenvironment; SA, single agent; C, combination of agents; IHC 1/2/3: IHC1 is ≥1%, IHC2 is ≥5%, IHC3 is ≥10%.

very impressive expression of immune-mediated genes (PD-L1, PD-L2, CTLA4, PD-1, PRF1, GZMA, BTLA, CD8A) in a single patient with PD-L1 expression less of 5% but >1% who showed an impressive complete response to nivolumab. Although no final conclusion could be resumed from this study due to the small number of patients explored it is probably that tumors mutational burden and non-synonymous mutations play a different role in ccRCC as compared to other disease, however, further large prospective trials might be necessary to confirm this hypothesis.

It is worth mentioning the IMmotion 151 trial, a randomized Phase III study of Atezolizumab plus Bevacizumab vs. Sunitinib in untreated metastatic RCC (35). Primary endpoints included PFS in PD-L1 positive patients and OS in an intention to treat analysis. The IMmotion 151 trial met its primary PFS endpoint in the PD-L1 positive patients with atezolizumab + bevacizumab compared to sunitinib with fewer high grade adverse reactions. This data does support atezolizumab + bevacizumab as first line therapy in metastatic clear cell renal cell (35, 56).

GENE EXPRESSION AS PREDICITVE IMMUNOLOGICAL BIOMARKERS IN RENAL CELL CARCINOMA

Regarding gene expression, several data seem to correlate the expression of specific classes of genes (especially DNA repair genes) to immunotherapy outcomes (30). Reportedly, the most frequent event involved in ccRCC is the loss of chromosome 3p, which is associated with the development of VHL, PBRM1, BAP1 and SETD2 alterations in about 90% of ccRCC cases. Together with KDM5C, PTEN, MTOR and TP53, these represent the eight most frequently altered genes in ccRCC (22, 23, 36). However, second most frequently mutated sub-network included AID1A, SMARCA4, and PBAF SWI/SNF chromatin remodeling complex. When mutations occur in chromatin regulators PBRM1, BAP1, and SETD2, several related genes showed altered expression as compared to VHL mutation (22, 23, 36). In particular, chromatin modification pathways interact with several genes involved in hormonal activity (*ESR1*), *RAS oncogene*, transcriptional output (*HIF1A, JUN, FOS, and SP1*), TGF-beta and especially DNA repair (*BAP1*) and immune-mediated signaling (*NFKB1 and IL-6*) (22, 23, 36). To date, no data about the correlation between gene expressions (especially DNA repair gene alterations and immune-related genes) are available, but this appears to be an attractive hypothesis to test mainly focused on the detection of predictive markers and the better understanding of mechanisms related to immune response in ccRCC (22, 23, 36).

The fundamental role of the gene alterations of PBMR1, BAP1 and SETD2 has been recently enforced by the findings presented at ASCO Annual Meeting 2018. The Spanish Oncology Genitourinary Group (SOGUG) presented the results of an observational prospective study collecting samples from 77 RCC patients treated with mTOR inhibitors everolimus or temsirolimus (79 and 21% of cases, respectively) (37). The study analysis included both IHC for p-S6, p-S6K1, p-AKT, p21, BAP1, and PBRM1 and NGS (next generation sequencing) for mutational analysis on key genes of mTOR pathway in RCC. Among enrolled patients, 87% had ccRCC histology; 60% had intermediate, 39% good prognosis, and 1% poor prognosis (MSKCC). No association between p-S6, p-S6K1, p-AKT, and p21 staining and response to temsirolimus/everolimus was reported. However, negative IHC expression for BAP1 and PBRM1 was associated with better mTOR inhibitor response (OR = 4.0, 95%CI = 1.4–11.9, $p = 0.011$ and OR = 3.9, 95%CI = 1.2–12.8, $p = 0.025$).

On the other hand, Bossé et al. reported on the prognostic value of genetic alterations resulting in loss of function (defined by the presence of pathogenic gene variant or 2 copy deletion) of VHL, PBRM1, BAP1, SETD2, TP53, and KDM5C, which are frequently mutated in metastatic RCC, in patients with ccRCC stratified by IMDC risk classification and treated with 1st line VEGFR tyrosine kinase inhibitors (38). Tumor samples were analyzed by NGS or whole exome sequencing (TCGA). Three hundred and eight patients were included; 21% of them with IMDC good risk features, 54% intermediate and 17% poor risk (8% unknown). The presence of gene alterations in VHL, PBRM1, SETD2, BAP1, TP53, and KDM5C was, respectively, 77, 43, 29, 19, 11, and 11%. Gene alterations in BAP1 were associated with worst OS (HR 1.7; 95%CI 1.1–2.5, $p = 0.01$), while alterations in PBRM1 and KDM5C were correlated with longer OS. Patients with tumors PBRM1 wild type and harboring gene alterations in BAP1 had worse OS (37 vs. 50 months, HR 1.9, 95% CI 1.2–2.8, $p = 0.004$). Interestingly, when IMDC stratified criteria were applied the genomic profile was prognostic only in patients with intermediate risk.

The advances in understanding the molecular landscape of RCC parallel with the progresses in the histopathological characterization of this neoplasm. The 2016 WHO classification of the tumors of the kidney (13) has identified new renal entities including hereditary leiomyomatosis and renal cell carcinoma syndrome–associated RCC, succinate dehydrogenase–deficient RCC, tubulocystic RCC, acquired cystic disease–associated RCC, and clear cell papillary RCC. The list of histologic categories includes also emerging entities, such as RCC associated with ALK gene rearrangements and thyroid-like follicular RCC (13). A more accurate identification of the different histological tumor

categories represents another fundamental step forward for the selection of molecularly targetable approaches for patients with RCC thus enabling the possibility to selectively target the gene drivers of specific tumor variants (13). More recently, Chen et al. (16) surveyed 894 RCC cases for expression of genes involved in immune checkpoint pathways, including PD1 and PDL1 genes. Clear cell RCC subtypes had relatively high expression of several genes representing targets for immunotherapy, including PDCD1 (PD1), CD247 (CD3), PDCD1LG2 (PDL2), CTLA4 (CD152), TNFRSF9 (CD137), and TNFRSF4 (CD134). In addition, analysis of gene expression signatures and of DNA methylation signatures suggested greater levels of immune cell infiltrates, including T cells, within clear cell RCC relative to other RCC subtypes (16, 17).

Within clear cell-enriched (CC-e) RCC genomic subtypes, differential expression of specific checkpoint-related genes was observed mostly involving differences between CC-e.3 and CC-e.2 groups (more aggressive and less aggressive ccRCC categories, respectively). Compared to CC-e.2, CC-e.3 showed increased promoter methylation of miR-21 (MIR21) with corresponding decreased levels of the miR-21 target PTEN. In cancer, PTEN has an established role in intrinsic cellular control of PD-L1 (16, 17). Some other genes—including PDCD1, CTLA4, and TLR9—were associated with worse patient survival within ccRCC-associated cases; PDL1 expression was correlated with better patient survival, though this association was confounded by copy loss of 9p region associated with aggressive clear cell RCC and worse prognosis (18). In summary, better understanding the predictive and prognostic significance of PD1/PD-L1 expression and the identification of molecularly defined subtypes correlated with survival and response to therapy, represent quick steps toward implementing precision medicine in RCC via reducing the distance to the goal of identifying the best approach for a single RCC patient (28, 29, 40) (**Table 3**).

PD-L1 IMMUNOHISTOCHEMISTRY IN RCC

Imunocheckpoint inhibitors (ICI) have marked a new paradigm in the treatment of RCC. The anti-PD1 drug nivolumab has been the first ICI drug to obtain approval by the FDA and European Commission for the treatment of RCC, and showed a significant OS benefit in patients with RCC that progressed following antiangiogenic therapy compared with everolimus (mTOR inhibitor) (26). Several other ICI compounds are currently under investigation for the treatment of RCC, alone or in combination with TKIs or other drugs (57–59).

Predictive biomarker research to select RCC patients eligible for ICI has mainly focussed on the PD1-PD-L1 axis detected by means of IHC. Low-to-no expression of PD-L1 on IC (immune cells) and TC (tumor cells) correlated with a trend toward lower response (PFS and OS) to the anti-PD-L1 drug atezolizumab compared with moderate to high PD-L1 expression levels (60). Updated analysis further confirmed the association between high PD-L1 expression and improved OS with atezolizumab treatment (57). For the anti-PD1 drug nivolumab, early data suggested a positive correlation between PD-L1 expression on TC and ORR (61–64). Data from the Checkmate 025 trial showed that higher levels of PD-L1 expression are associated with poorer survival in RCC, but did not support PD-L1 as a marker predictive of treatment benefit in RCC; a benefit was observed, however, with nivolumab irrespective of PD-L1 expression (62). Furthermore, PD-L1 seems to be a dynamic biomarker since prior exposure to VEGF and mTOR inhibitors modulates its expression which can be largely variable after therapy (64, 65). Notably, a significant number of patients with PD-L1+ RCC do not respond to PD-1 pathway blockade, suggesting that additional intra-tumoral factors may influence treatment outcome (64, 65). Based on recent data, PD-L1 could be a prognostic biomarker for the adverse clinic-pathologic features of RCC but may not be discriminant enough to be a predictive biomarker (64, 66, 67). Furthermore, it was found that PD-L1 staining is almost exclusively observed in the high-grade component of a tumor and additionally a discordant expression of PD-L1 between primary tumors and their metastases was detected in ∼20% of cases (68). Similar heterogeneity has been observed between primary and metastatic tumor based on molecular analysis (69).

Other possible biomarkers like PD-L2 and CTLA4 are reported in literature, thus far without straightforward predictive value (57). Increased amounts of $CD3^+/CD8^+$ tumor-infiltrating T-cells have been reported after nivolumab treatment, but further research is needed to determine the biomarker-potential of this observations (70).

Recent data from a gene expression study on a small cohort of PDL1+ RCC patients treated with nivolumab identified a metabolic gene profile in the non-responding subgroup and overexpression of immunologic factors in the responding subgroup (71). Increasing mutational burden and neo-antigen formation have been associated with increased responsiveness to ICI in several other malignancies and recent data showed increased frequency of genomic alterations in RCC post-VEGFR therapy (72). These findings might explain the observed benefit of nivolumab post-VEGFR therapy and seem to correlate with the observation of lower response rates to nivolumab monotherapy in front line studies (70). A recent multilevel molecular analysis on the integrated TCGA RCC database showed relatively high expression of several genes representing targets for immunotherapy in ccRCC-associated molecular subtypes compared to other RCC subtypes, with additional differences within the several clear cell-enriched RCC genomic subtypes (16). These data also suggested greater levels of IC infiltrates within ccRCC relative to other RCC types (16). TCGA data suggest the hypothesis that clear cell-enriched RCC genomic subtypes would be most responsive to targeted immune checkpoints, hypothesis that awaits validation in prospective cohort series (16).

Several technical and biochemical issues are involved to explain the observed ambiguity of PD-L1 expression as predictor of response to ICI therapy in RCC. Differences in anti-PD-L1 antibody-clones, staining assays, tissue characteristics and scoring systems are amongst the major technical obstacles to overcome. The knowledge that PD-L1 expression is not binary, but instead shows a continuum with significant intratumour heterogeneity and therapy-induced changes, might

TABLE 3 | Prognostic and predictive biomarkers in Renal Cell Carcinoma.

Biomarker	Results	Association with	References
IHC expression of p-S6, p-S6K1, p-AKT, and p21	NA	No association with response to temsirolimus/everolimus	(37)
Negative IHC expression for BAP1	OR = 4.0, 95% CI = 1.4–11.9, $p = 0.011$	Better mTOR inhibitor response	(37)
Negative IHC expression for PBRM1	OR = 3.9, 95% CI = 1.2–12.8, $p = 0.025$	Better mTOR inhibitor response	(37)
Gene alterations in BAP1	HR 1.7; 95% CI 1.1–2.5, $p = 0.01$	Worse OS	(38)
Gene alterations in PBRM1	HR = 0.6; 95%CI 0.4–0.8, $p = 0.001$	Better OS	(38)
Gene alterations in KDM5C	HR = 0.4; 95%CI 0.2–0.8, $p = 0.007$	Better OS	(38)
SETD2, TP53, and VHL	NA ($p > 0.4$)	Not associated with prognosis	(38)
PBRM1 wild type + gene alterations BAP1	37 vs. 50 months, HR 1.9, 95% CI 1.2–2.8, $p = 0.004$	Worse OS	(38)
PDCD1, CTLA4, and TLR9	NA	Worse OS	(16)
9p deletion	HR 4.323; $p = 0.021$ HR 4.603; $p = 0.007$	High risk of recurrence and RCC-specific mortality	(18)

even represent a bigger challenge for being an ideal biomarker (73, 74). The recent report on the presence of compensatory inhibitory pathways (VISTA) in the setting of immunotherapy in metastatic prostate further underlines the complexity to predict the therapeutic response based on a single biomarker like PD-L1 (74). Recent concordance studies on non-small cell lung cancer have shown only minimal differences in staining patterns between most of the different validated and commercially available anti-PD-L1 antibody clones (73, 75–78). These findings are encouraging, although clinical cross-validation data between the different assays are not available at this moment. High concordances between the different assays and between the pathologists within a single assay were only found for PD-L1 scoring TC and not in immune cells IC (75, 76). Concerning RCC this could be a critical point since PD-L1 expression in IC is used as a companion biomarker for some FDA-approved anti-PD-L1 drugs.

MMR-DEFICIENCY AND MUTATIONAL LOAD IN RCC

Renal cell carcinoma are not considered to belong to the HNPCC (hereditary non-polyposis colon cancer) spectrum, but in sporadic RCC loss of MMR proteins is frequently observed, especially of MLH1 and MSH2 (79–81). Variable MMR gene alterations have been reported as underlying mechanisms, but others did not detect microsatellite instability (MSI) caused by either promoter hypermethylation or alteration of the coding region of MMR studied genes (81–83). The reduced MMR protein expression by IHC has been linked to RCC subtypes and might contribute to the respective different biological behavior (84). As addressed earlier in this review, MMR-deficiency is more and more recognized as an important biologic event in genitourinary cancers. MMR deficiency can occur in patients with Lynch syndrome (HNPCC) and in patients with sporadic MMR-deficient tumors (84). MMR-deficient tumors exhibit a higher rate of mutations (high mutational burden), which can result in the formation of neo-antigens to enhance the antitumor immune response (85). Furthermore, MMR-deficient tumors express different immune checkpoint ligands indicating that their active immune microenvironment is counterbalanced by immune inhibitory signals that resist tumor destruction (86). Recently reported data showing a better clinical response to the anti-PD-1 drug pembrolizumab in MMR-deficient patients support the hypothesis that MMR-deficient tumors respond better to anti-PD-1 therapy than do MMR-proficient tumors (87).

In RCC cancers, data on the relation between MMR-status and response to immunotherapy are still emerging (88, 89). Based on the promising results in patients with MMR-deficient cancers, FDA has recently approved pembrolizumab for the treatment of adult and pediatric patients with un-resectable or metastatic MMR-deficient solid tumors, irrespective of the tumor origin. In this context, MMR-deficient/MSI-H solid genitourinary tumors could be important candidates for anti PD-1 treatment. The reality might however be much more complex; for instance, several clinical trials have shown that some MMR-deficient tumors do not respond to immunotherapy, while mutations in other genes have also been linked to high mutational burden and upregulation of immune checkpoints (85). From a methodological point of view, there is an ongoing discussion and evolution in literature concerning the methodology to get reliable data on mutational load and MSI in a context of cost-efficiency and optimal logistics. Whole exome sequencing, T-cell receptor sequencing and targeted NGS can be used to assess mutational load (90) and promising data on novel platforms to detect MSI (e.g., MSI-Sensor and MANTIS) have recently been published (91). The detection of MMR-deficient tumors and the selection of those patients that will really benefit from immunotherapy remains an ongoing and challenging task.

BIOMARKERS OF ACQUIRED RESISTANCE

Despite the durable responses observed with immune checkpoint inhibition, nearly all patients will progress. A number of mechanisms have been identified including neo-antigen loss, upregulation of alternative immune checkpoints, loss of antigen presentation, and defective interferon signaling (92–94). A recent whole exome sequencing study on paired tumor samples prior to treatment with ICI and at the time of progression ($n = 4$, 2 treated with nivolumab/ipilimumab, 2 treated with nivolumab) was reported by Anagnostou et al. (92). Although they found an increase in total number of candidate neo-antigens, a subset of them was actually eliminated at the time of acquired resistance. In the four patients, there were 18, 10, 7, and 6 neo-antigens lost, and all of them had higher predicted MHC binding affinity. There were no copy number alterations of CD274 which encodes for PD-L1, PDCD1 encoding for PD-1, CTLA-4, JAK1, or JAK2. There were no genetic alterations in HLA or β2-microglobulin. They also evaluated clonal T-cell reactivity in three of these patients using peripheral blood mononuclear cells loaded with predicted neo-antigens cultured with purified T-cells. All patients showed clonal T-cell expansion to lost peptides and either no affinity or lower affinity for the wild type of the predicted neo-antigen (92). Neo-antigen loss and growth of a subclone lacking the neo-antigen eliciting the immune response are both potential explanations of this resistance mechanism, although the power of available information is limited. This mechanism of resistance underscores the rationale for using neo-antigen profiling as a predictive biomarker of benefit and also underscores the dynamic nature of these biomarkers.

Defects in the interferon-γ signaling pathway have also been identified as a major mechanism of resistance. Interferon-γ signaling plays a crucial role in the anticancer immune response. It has been shown to upregulate PD-L1 expression on TC and IC, to increase MHC Class I expression and promote antigen presentation, and recruit effector cells (92–97). It results in the downstream stimulation of JAK/STAT signaling pathway and expression of a number of anti-cancer genes (98). Mutations in JAK1/2 render cells insensitive to interferon-γ signaling, which results in escape from PD-L1 pathway inhibition and impairs the antitumor immune response. This has been identified as a mechanism of both primary and secondary resistance (99–101). Interferon-γ signaling has been demonstrated to increase expression of immune inhibitory molecules, such as indolaimine-2,3-deoxygenase (IDO) that can limit the anti-tumor response (101). Inhibition of IDO production is the subject of an ongoing clinical trial in combination with PD-1 immune checkpoint inhibition. Defects in antigen presentation, such as mutations in the β-2 microglobulin gene, have also been identified as a mechanism of resistance (100). Beta-2 microglobulin is essential for MHC class I molecule surface expression and a defect can block CD8-Tcell recognition. HLA loss is another potential mechanism of immune evasion and determining copy number alterations have been difficult due to the polymorphic nature of the locus. McGrahan et al. developed a computational tool using NGS data to determine HLA loss of heterozygosity in 100 early stage NSCLC patients. Interestingly, 40% of patients displayed HLA loss of heterozygosity and phylogenetic analysis shows that this is likely a later evolutionary event (102). TIM-3, LAG-3, and TIGIT are known alternative immune checkpoints that play a role in T-cell exhaustion and are expressed on tumor infiltrating lymphocytes (93). Koyama and colleagues identified TIM-3 to be upregulated in a murine model of NSCLC at the time of resistance to anti-PD-1 therapy and demonstrated a survival advantage with treatment using a TIM-3 blocking antibody. The authors additionally identified two patients with biopsies performed at the time of progression to anti- PD-1 therapy with increased TIM-3 expression (103). Novel therapeutic approach directed at these alternative immune checkpoints are the subject of ongoing clinical trials and are of potential relevance in RCC.

FUTURE PERSPECTIVE

The complex interplay of signaling pathways and inflammatory mediators seems to be crucial for RCC development and response to therapy (43, 44, 53). Immune cells including neutrophils, lymphocytes and macrophages have been implicated in promoting metastatic spread, tumor angiogenesis, in primary and acquired drug resistance, as well as in the formation of pre-metastatic niches (43, 44, 53). On this scenario, the checkpoint molecules have gained wide interest since the introduction of anti-CTLA-4 and anti-PD-1/PD-L1 agents into daily oncology practice (45). Beyond PD-1 and CTLA-4, a variety of molecules are emerging as potentially future therapeutic immunotargets in RCC (46). This list includes the V-domain immunoglobulin containing suppressor of T-cell activation (VISTA), which has been recently shown to exert its inhibitory activity by acting as a ligand on antigen presenting cells and as a receptor on T cells (104–106), chemokine receptors (45), the soluble lymphocyte-activation gene-3 (LAG-3), 4-1BB, B and T lymphocyte attenuator, and OX40 (CD134) (47).

Nowadays, there is not a clear-cut knowledge of the underlying mechanisms of immuno-checkpoint inhibitors-induced tumor response. To address this issue, Wei et al. investigated the effects of anti-PD-1 and anti-CTLA-4 inhibitors in human melanoma and murine tumor models (48). They first revealed that these agents are able to target distinct tumor-infiltrating T cell subpopulations. In particular, PD-1 blockade promotes the expansion of specific exhausted-like CD8-T cell population, while CTLA-4 blockade induces both an $ICOS^+$ Th1-like CD4 effector subset and exhausted-like CD8-T cells (48). This evidence favors the combined use of current and probably future checkpoint inhibitors in cancer patients. These combinations, seems to be characterized by a tolerable safety profile (107). Tumor responsiveness may vary according to the mutational load and the expression of immunotargets in the tumor environment, which is variable in the different phases of RCC development and progression (49, 108–110). Based on this evidence, assessing the expression of PD-1/PD-L1 or other emerging immunotargets only at the diagnosis of metastatic disease may not reflect tumor dynamicity.

To improve the feasibility and reduce the clinical impact of re-biopsy, assessing biomarkers on circulating tumor cells (CTCs) or exosomes (111) may represent a not invasive strategy that can be performed several times during cancer therapy in order to reflect the changes occurred in the tumor environment. An early identification of validated biomarkers would be crucial to definitively place immunotherapy into the era of precision medicine and to optimize the cost-effectiveness of ICI agents in cancer patients (50, 112). In addition, the recent paper by Routy et al. showed that primary resistance to ICI can be correlated with abnormal gut microbiome composition (51). In this study, the effectiveness of PD-1 blockade resulted enhanced by transplanting fecal microbiota from responder cancer patients into germ-free or antibiotic-treated mice (51), thus representing another step forward on the way to personalized and precision immunotherapy in cancer patients.

Another factor that results of great relevance to improve the efficacy of ICI in RCC patients is the comprehension of the immunological effects of TKIs and mTOR inhibitors (53, 113). Actually, these agents can indirectly exert their anti-tumor activity by targeting immune cells in the RCC microenvironment (53), and this should be considered in order to combine or sequence them with currently available and probably future immunotherapies. For instance, sunitinib has been shown to inhibit the colony forming units driven by GM-CSF and FLT3 ligand FLT3L (114) as well as dendritic cell antigen-presentation (115) (by decreasing the secretion of cytokines and the expression of MHC and CD1a molecules), to suppress the myeloid-derived suppressor cells (MDSCs are involved in RCC progression and drug resistance), to enhance tumor cell sensitivity to NK cell killing (116) and to reduce the total count of CD3 and CD4 T cells and regulatory T cells (117, 118). On the other hand, pazopanib showed lower inhibitory potency and affinity against FLT3 and c-kit compared to sunitinib (119). Interestingly, we previously showed that axitinib can increase the surface NKG2D ligand expression, thus promoting NK cell recognition and degranulation in A-498 RCC cells in a ROS-dependent manner (120). At present, few evidences are available on the immunomodulatory effects of cabozantinib and lenvatinib, recently introduced into RCC clinical practice.

EXPERT OPINION AND CONCLUSIONS

Optimizing the combination between immunotherapy and target agents as well as the possible favorable sequence of treatment between these two classes of drugs remain open questions at this moment but ongoing studies support this as of great future potential. On this way we have only limited data provided from Immotion150 which demonstrated that association between a PD-L1 inhibitor and bevacizumab is feasible, well-tolerated, and results in an effective clinical benefit from our patients. Of relevance, is to note that most studies explored immunotherapy in patients with ccRCC and the role of ICI still remains unknown in mccRCC. Though, there are several questions that need to be answered, current data support that immunotherapy represents a revolution for the management of RCC resulting in a dynamic and evolving scenario in which more novelties will be shortly made available. Because the potentially deadly adverse effects from immune checkpoint inhibitors, gathering predictive information in RCC seems to be prudent. However, recent scientific insights indicate that a single biomarker for patient selection may not be feasible, given that immune responses are dynamic and evolve over time (121). Biomarker development for ICI drugs will require integration of multiple biologic components like PD-L1 expression, TILs, mutational load, and probably many others now considered emergent biomarkers.

EXECUTIVE SUMMARY

- Immunotherapy is gradually becoming a key factor in the therapeutic algorithm for patients with renal cell cancers at different stages of disease.
- The increasing knowledge on the genomic landscape of renal cell carcinoma supports stratification of patients for targeted therapies.
- A single biomarker for patient selection may not be feasible, given that immune responses are complex, dynamic and evolve over time.
- Biomarker development for ICI drugs will require integration of multiple biologic components like PD-L1 expression, TILs and mutational load.

NEXT STEPS

- New methodological approaches likely based on digital pathology may be relevant since they allow objectively recognizing and quantitation of the biomarker and therefore might produce objective and reproducible cut-offs useful in patient's therapeutic stratification.
- Radiologic derived biomarkers, such as artificial intelligence derived, radiopharmaceutic, and liquid biopsy derived biomarkers, are likely to enter the biomarker-field in the next coming years.
- Large-scale biomarker-driven prospective trials with consensus methodologies on biomarker assessment and scoring are needed to reach clinical validation of different biomarkers, needed for a reliable single-patient appointment to the appropriate immunotherapy.
- Multidisciplinary approaches are needed to fully develop the current and future value of ICI in clinical practice.
- Better understanding of solid tumor genomics shows that also for RCC, combining targeted therapy with ICI has the potential to improve cancer outcomes, and that reliable biomarkers will be crucial for a stringent patient selection in trials of targeted and checkpoint inhibitor drugs and to apply novel therapeutic strategies aimed at restoring effective antitumor immunity in patients with cancer.

AUTHOR CONTRIBUTIONS

AL-B, RM, MSc, and LC: conception and design; AL-B and VH: drafting the manuscript; AB, AC, and VH: review of the literature; AL-B, LC, RM, MSc, MSa, FM, VH, and TG: critical revision of the manuscript.

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MINI REVIEW
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New Prostate Cancer Targets for Diagnosis, Imaging, and Therapy: Focus on Prostate-Specific Membrane Antigen

Alessia Cimadamore[1], Monica Cheng[2], Matteo Santoni[3], Antonio Lopez-Beltran[4], Nicola Battelli[3], Francesco Massari[5], Andrea B. Galosi[6], Marina Scarpelli[1] and Rodolfo Montironi[1]**

[1] Section of Pathological Anatomy, School of Medicine, United Hospitals, Polytechnic University of the Marche Region, Ancona, Italy, [2] Department of Radiology and Imaging Sciences, Indiana University School of Medicine, Indianapolis, IN, United States, [3] Oncology Unit, Macerata Hospital, Macerata, Italy, [4] Department of Pathology and Surgery, Faculty of Medicine, Cordoba, Spain, [5] Division of Oncology, S. Orsola-Malpighi Hospital, Bologna, Italy, [6] Institute of Urology, School of Medicine, United Hospitals, Marche Polytechnic University, Ancona, Italy

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***Correspondence:**
Alessia Cimadamore
alessiacimadamore@gmail.com
Rodolfo Montironi
r.montironi@univpm.it

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The rising incidence rate of the cancer in the prostate gland has increased the demand for improved diagnostic, imaging, and therapeutic approaches. Prostate-specific membrane antigen (PSMA), with folate hydrolase and carboxypeptidase and, internalization activities, is highly expressed in the epithelial cells of the prostate gland and is strongly upregulated in prostatic adenocarcinoma, with elevated expression correlating with, metastasis, progression, and androgen independence. Recently, PSMA has been an active target of investigation by several approaches, including the successful utilization of small molecule inhibitors, RNA aptamer conjugates, PSMA-based immunotherapy, and PSMA-targeted prodrug therapy. Future investigations of PSMA in prostate cancer (PCa) should focus in particular on its intracellular activities and functions. The objective of this contribution is to review the current role of PSMA as a marker for PCa diagnosis, imaging, and therapy.

Keywords: prostate cancer, prostate-specific membrane antigen, PSMA, small molecule inhibitors, RNA aptamer conjugates, PSMA-based immunotherapy, PSMA-targeted prodrug therapy, positron emission tomography

INTRODUCTION

Prostate-specific membrane antigen (PSMA) is a type 2 integral membrane glycoprotein with folate hydrolase and carboxypeptidase, and internalization activities. This internalization capability is increased up to 3-fold when PSMA is linked to anti-PSMA antibodies. PSMA expression is highest in prostate tissue (secretory acinar epithelium), but detectable levels of PSMA protein are also found in the kidney (proximal tubules), the small bowel (i.e., jejunal brush border), neuroglia (Schwann cells and astrocytes), and salivary glands (1, 2). Notably, PSMA is highly expressed in prostate cancer cells and the vessels of various non-prostatic solid tumors (it is not expressed in the normal vasculature) (3).

With the rise and evolution of several targeted approaches to examine prostate cancer using PSMA, the aim of this contribution is to review the current role of PSMA as a marker for PCa diagnosis, imaging, and therapy.

EXPRESSION AND ROLE OF PSMA IN PCA

PSMA was originally discovered using the monoclonal antibody 7E11 obtained from the cell membrane of the LNCaP cell line (4). It has been shown by immunohistochemistry that expression of PSMA at the tissue level increases through the progression from normal prostate cells to high-grade prostatic intraepithelial neoplasia (HGPIN) and to PCa (3) (**Figure 1**). There exists a strong positive correlation between PSMA expression and Gleason score. Elevated PSMA expression is strongly correlated with a high serum PSA. These indications are associated with increased tumor angiogenesis and lack of ets-related gene (ERG) expression which leads to reduced vitamin D and androgen receptor expression (5). PSMA expression is regulated by the androgen receptor (AR). PSMA expression increases dramatically during androgen-deprivation therapy (6).

Downregulation of PSMA expression by AR may be associated to the presence of an enhancer region although no androgen response elements have been identified (7).

PSMA expression is significantly correlated with prostate growth and differentiation (8). In particular, *in vitro* expression of PSMA is associated with an increased cellular folate content. This induces a proliferative property to cells expressing PSMA (9, 10). In addition, PSMA stimulates PCa cell proliferation, migration and survival through the phospho-p38 (P-p38) MAPK pathway in LNCaP cancer cells (11). Guo et al. demonstrated that PSMA knockdown in a LNCaP cell line was associated with not only the inhibition of the pathway of phosphatidylinositol 3-kinase/Akt signaling but also decreased cell proliferation, migration and survival (12).

PSMA is involved in the development of PCa metastases. Xu et al. evaluated four prostate cancer cell lines (i.e., DU145, LNCap, PC-3, and 22RV1) for metastasis-related genes potentially involved in PCa metastasis regulated by PSMA. In their study, *CDH6, MMP3,* and *MTSS1* were seen as PSMA-related genes. Their expression was inversely related with the stage of cancer, thus suggesting their possible involvement in the suppression of PCa metastasis by PSMA (13).

PSMA-BASED IMAGING IN PATIENTS WITH PCA

Conventional imaging techniques, such as ultrasound, CT, bone scintigraphy and Magnetic Resonance Imaging (MRI), are at present utilized to detect primary PCa and its metastatic deposits. However, the limitation of such traditional imaging techniques and modalities is their low sensitivity in the detection of recurrent or/and metastatic PCa. Improved imaging modalities are needed to optimize the management of the patients with PCa.

Positron Emission Tomography (PET) and single photon emission computed tomography (SPECT) with emerging radiopharmaceuticals provide more accurate staging for primary cancer, detection of metastatic disease, and restaging of tumor recurrence. PSMA has received considerable attention as a useful marker for imaging purposes in patients with PCa (14, 15). Several PSMA-based approaches have been developed, including antibodies, nanobodies, and small molecule inhibitors.

Antibodies and Nanobodies

Indium-111 capromab pendetide (^{111}In-capromab, ProstaScint®) was the first monoclonal antibody against PSMA used in PCa immunoscintigraphy. Correlation of scan results with tissue specimens showed that ^{111}In-capromab detected soft tissue metastases, with an average negative predictive value of 70%, sensitivity of 60%, and positive predictive value of 60% (16–18). However, ^{111}In-capromab lacks sensitivity because it recognizes an intracellular epitope of PSMA, thereby targeting only apoptotic/necrotic or damaged cells.

Unlike ^{111}In-capromab, J591 is an antibody against the extracellular domain of PSMA. ^{111}In-labeled J591 has been evaluated against conventional imaging techniques in the evaluation of bone metastases. ^{111}In-labeled J591 identifies 93.7% of skeletal lesions detected by a conventional imaging technique. Thirteen out of Eighteen bone deposits detected only with ^{111}In-labeled J591 were successively confirmed to be metastases (19). In a more recent study, J591 has been radiolabeled with 89Zr (20) and 64Cu (21) for PET imaging and demonstrate robust targeting of skeletal, nodal and soft tissue metastasis (22).

A new strategy in the development of high-contrast nuclear imaging is the utilization of specific antibody fragments, called nanobodies. Nanobodies contain antibody-derived smaller fragments (typically the variable domain alone of heavy chain antibodies) that largely retain the specific antigen binding properties of the original antibodies, but with more rapid pharmacokinetics and lower immunogenic potential. Evazalipour et al. compared the properties of different nanobodies radiolabeled with 99 m-Technetium (99 mTc) in $PSMA^+$ LNCaP and $PSMA^-$ PC3 cell lines and in $PSMA^-$ and $PSMA^+$ tumor-bearing xenografts through SPECT/micro-CT imaging and tissue analysis. Among the evaluated molecules, nanobody PSMA30 resulted in an important compound for future applications in PCa imaging trials (23).

Interesting results were also obtained with minibodies, i.e., IAB2M, an 80-kDa minibody genetically engineered from the parent antibody J591 that targets the extracellular domain of PSMA. A phase I dose-escalation study in patients with metastatic prostate cancer demonstrated PET imaging with 89Zr-Df-IAB2M is feasible and well tolerated, and targets both bone and soft-tissue disease (24).

Small Molecules

The identification of the functional (25) and structural (26) homology between N-acetylaspartylglutamate peptidase or NAAALDASE (for which a number of enzymatic inhibitors had been identified) (27, 28) and PSMA has been a major step forward for the development of PSMA-targeted radiotracers. Generally, small molecule PSMA inhibitors consist of zinc binding compounds linked to a glutamate isostere or glutamate. Phosphonate-, phosphate-, and phosphoramidates (1) and ureas (2) constitute the two main families of compounds. Based on NAALADASE homology, several compounds have been

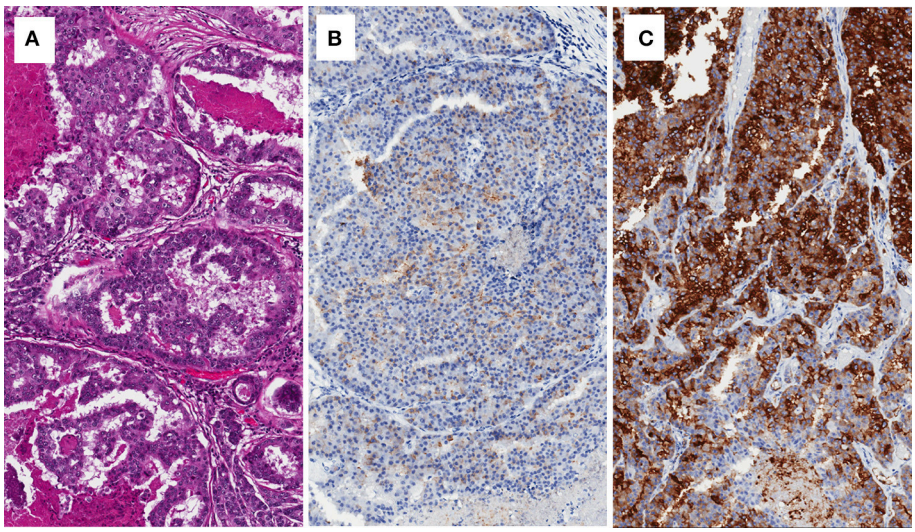

FIGURE 1 | Brain metastasis of prostate cancer with cribriform pattern **(A)**, showing low expression of PSA **(B)**, and intense expression of PSMA **(C)**.

developed and labeled with 123I (20, 29, 30), 99mTc (21, 31), 18F (32), 111In (33), and 68Ga (34).

123I-MIP-1072 and 123I-MIP-1095 were the first small molecule inhibitors of PSMA adopted in the clinic. SPECT/CT using these compounds showed a rapid detection of PCa deposits in the bone, soft tissue, and prostate gland of men with metastatic PCa (35). A phase I trial on 131I-MIP-1095 in men with mCRPC is now active (NCT03030885).

Among the emerging PSMA small molecule inhibitors, *N*-(*N*-((*S*)-1,3-dicarboxypropyl) carbamoyl)-4-(18F)fluorobenzyl-L-cysteine (18F-DCFBC) is under evaluation in several ongoing studies. Using 18F-DCFBC, $PSMA^{+}$ PC-3 PIP xenografts were early visualized with little radioactivity in the $PSMA^{-}$ isogenic PC-3 flu xenografts. After 2 h, the PC-3 PIP xenografts remained visible, with clearance of background radioactivity from kidneys, liver and blood (36, 37).

The use of 18F-DCFBC has been investigated in a few patients with Gleason scores between 7 and 9 and with radiological evidence of metastatic PCa. Bone scans or CT identified 21 lesions (5 bone and 16 lymph node lesions), while 32 lesions were visible with 18F-DCFBC PET. Ten of Eleven additional lesions were located in the bone and were suggestive of early bone deposits, indicating the potential of 18F-DCFBC PET in this subpopulation (38). Currently, the use of 18F-DCFBC PET/CT is under evaluation in a study enrolling patients scheduled for surgical prostate (Group 1), or with biochemical recurrence after surgery or radiotherapy (Group 2), or in metastatic PCa patients (Group 3) (NCT02190279). In addition, another ongoing phase I/II study is assessing the potential of 18F-DCFBC PET in the detection of primary PCa, nodal and bone metastases in men at initial diagnosis (NCT01496157) (**Table 1**).

As for the PSMA inhibitor 18F-DCFPyL (2-(3-{1-carboxy-5-((6-((18)F)fluoro-pyridine-3-carbonyl)-amino)-pentyl}-ureido)-pentanedioic acid), Chen et al. evaluated its use in immunocompromised mice utilizing isogenic PSMA PC3 PIP and PSMA- PC3 flu xenografts, suggesting that this agent could be viable and effective in this setting (32). A phase I study is now assessing the biodistribution and pharmacokinetic of 18F-DCFPyL in patients with advanced PCa (NCT02151760).

The early distinction between local disease and metastasis is crucial in the management of patients with PCa. 18F-choline can distinguish lesions with moderate to good sensitivity, but its activity is limited to patients with a PSA >1 ng/mL (39). The results obtained by ^{68}Ga-labeled PSMA inhibitors showed a high potential in the detection of small recurrent PCa lesions in patients with low levels of serum PSA (40–42). Indeed, ^{68}Ga-labeled PSMA inhibitors are characterized by accumulation in small metastatic deposits and a rapid clearance from the tissue in the background (43). Recently, a comparison between PET/CT and PET/MRI hybrid systems using a ^{68}Ga-labeled PSMA compound for the detection of recurrent PCa has been performed. The results showed that Ga-PSMA PET/MRI was far more accurate in the detection of PCa and, at the same time, associated with lower radiation exposure (34).

Beyond ^{68}Ga-labeled compounds, 99mTc-labeled inhibitors of PSMA have shown great promise in the detection of PCa lesions. Presently, a phase II study is testing 99mTc-MIP-1404 PSMA inhibitor in patients with high-risk PCa scheduled for radical prostatectomy (RP) surgery including extended pelvic lymph node (LN) dissection compared to histopathology (NCT01667536). Results are expected from the completed phase 3 trial proSPECT-AS (NCT02615067). Primary outcome measures of the study are sensitivity and specificity of 99mTc-MIP-1404 SPECT/CT image assessments to correctly detect clinically significant prostate cancer when compared to histopathology following either RP or prostate biopsy in men with newly diagnosed PCa whose biopsy indicates a histopathologic Gleason Score of $\leq 3+4$.

Furthermore, BAY1075553 [2-PMPA analogs (2*S*, 4*S*)-2-18F-fluoro-4-(phosphonomethyl) pentanedioid acid] has

TABLE 1 | Current completed trial on PSMA-based imaging.

NCT Identifier	Study phase	Tracer and technique	Study outcomes
NCT01496157	Phase 1 Phase 2	18F-DCFBC PET	PET detection of primary and sextant localization of PCa and detection of metastatic disease at initial staging
NCT02048150	Phase 1	MDX1201-A488	Assess the best dose given before robotic assisted laparoscopic RP to aid in visualization of the prostate
NCT02151760	Phase 1	18F-DCFPyL	Compare diagnostic accuracy of 18-DCFPyL to CT and bone scintigraphy for the detection of metastatic PCa
NCT01667536	Phase 2	99mTc-MIP-1404	Assessment of the diagnostic accuracy in detection PCa within the prostate and metastatic PCa
NCT03558711	Phase 1	18F-PSMA	Safety of administration
NCT02611882	Phase 1 Phase 2	Ga-68 HBED-CC PSMA	SE and SP for the detection of nodal metastasis in high-risk pre-RP patients, of metastatic disease in patients with BCR after RP or radiation therapy, comparison to conventional imaging in CRPC patients.
NCT03486886	Not Applicable	PSMA -PET/CT	Evaluation of detection Yield performance and reproducibility in mPCa Patients
NCT02796807	Phase 2	68Ga-HBED-CC-PSMA PET/CT	Correlation Between SUV on 68Ga-HBED-CC-PSMA and GS in PCa
NCT02488070	Phase 1 Phase 2	68Ga-PSMA PET/CT	Average SUVmax of Ga68 PSMA Uptake Outside the Expected Normal Biodistribution
NCT01359189	Phase 1	ProxiScan	Evaluation of a Transrectal Scintigraphic Detector (ProxiScanTM) for Detection of Primary PCa
NCT02918357	Phase 2 Phase 3	Ga-68 labeled PSMA-11 PET	Sensitivity and PPV of for the detection of metastases a per-patient and per-region-basis confirmed by histopathology
NCT02916537	Phase 1	CTT1057 PET/MR	Evaluation of the safety, pharmacokinetics, and [18F] radiation dosimetry
NCT00712829	Phase 1	123-I-MIP-1072 123-I-MIP-1095	Evaluating the safety, pharmacokinetics, tissue distribution
NCT01615406	Phase 1	99mTc MIP 1404	Comparison study of 99mTc-MIP-1404 (SPECT)/CT imaging to histology
NCT01261754	Phase 1	99mTc-MIP-1404 and MIP-1405	Safety, pharmacokinetics, biodistribution in mPCa patients; newly diagnosed, high-risk PCa and healthy subjects
NCT01572701	Phase 1	99mTc-MIP-1404	Measure activity counts in tissue samples post-surgery, Intensity of 99mTc-MIP-1404 Uptake with Respect to PSMA expression in Men With PCa Undergoing RP and/or PLND
NCT02190279	Early phase 1	18F-DCFBC PET/CT	Assess the ability to identify sites of localized, recurrent and metastatic PCa
NCT00992745	Phase 1	123-I-MIP-1072	Estimate the imaging SE and SP of 123I MIP 1072 compared to 111In capromab pendetide in mPCa
NCT02349022	Phase 2	[89Zr]Df-IAB2M	Compare SE/SP/PPV/NPV/Accuracy of [111In] capromab pendetide SPECT/CT to [89Zr]-Df-IAB2M PET/CT as confirmed by pathology
NCT02615067	Phase 3	99mTc-MIP-1404 SPECT/CT	Safety and Efficacy of 99mTc-MIP-1404 SPECT/CT Imaging to Detect Clinically Significant PCa in Men With Biopsy Proven Low-Grade PCa Candidates for AS (proSPECT-AS)
ACTRN12617000005358	Phase 2	Ga-68 PSMA-PET/CT	Compare the diagnostic accuracy of Ga-68 PSMA-PET/CT to that of conventional imaging for detecting nodal or distant metastatic disease.

Source: https://clinicaltrials.gov.

PSMA, Prostate specific membrane antigen; RP, radical prostatectomy; MDX1201-A488, Anti-PSMA monoclonal antibody; PCa, prostate cancer; SE, sensitivity; SP, specificity; BCR, biochemical recurrence; CRPC, castration resistant prostate cancer; GS, Gleason score; PPV, positive predictive value; 123-I-MIP-1072, MIP 1404, MIP-1405, small molecule inhibitor of PSMA; PLND, Pelvic Lymph Node Dissection; NPV, negative predictive value; AS, active surveillance.

demonstrated high uptake in $PSMA^+$ LNCaP tumor xenografts (44). The phase I study showed that BAY1075553 was able to detect primary PCa, lymph node and bone metastases, although its high uptake with degenerative bone lesions may limit its use in assessing bone disease (45).

Worth mentioning is the registrational phase II/III OSPREY study (NCT02981368) that evaluated the diagnostic accuracy of 18F-DCFPyL PET/CT relative to histopathology, for detecting PCa in pelvic lymph nodes in patients with high risk localized prostate cancer who are planned for RP with lymphadenectomy, and in patients with locally recurrent or metastatic disease willing to undergo biopsy.

Imaging at Diagnosis of PCa

A number of recent studies has dealt with the use of PSMA-based imaging for the purpose of diagnosing primary PCa (**Figure 2**).

Fendler et al. assessed the accuracy of ^{68}Ga-PSMA-11 PET/CT in identifying PCa at the initial diagnosis in men with

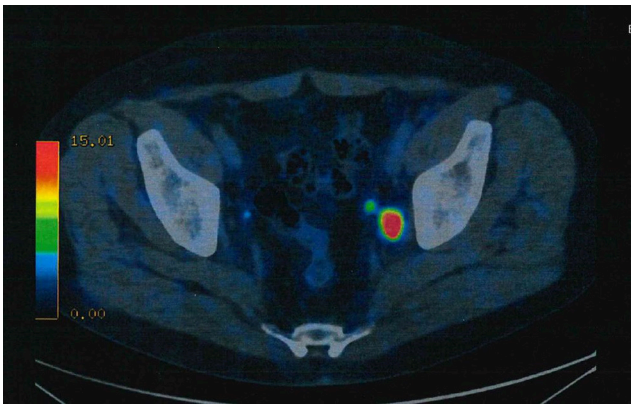

FIGURE 2 | ^{68}Ga-PSMA ligand PET/CT exhibits solitary left iliac radiotracer-positive lymph node.

biopsy-proven PCa (46). They found that the optimal SUV_{max} cutoff for distinction of histopathology-positive segments from histopathology-negative segments is of 6.5. With this approach they obtained 67% sensitivity, 92% specificity, 97% positive predictive value, and 72% accuracy.

Woythal et al. (47) evaluated the association of intraprostatic ^{68}Ga-PSMA PET/CT features and PSMA immunohistochemical expression in 31 patients who underwent RP and preoperative ^{68}Ga-PSMA-11 PET/ CT. ^{68}Ga-PSMA-11 PET/CT demonstrated sensitivity and specificity of 87 and 97%, respectively, in the detection of PCa. However, there was no correlation between Gleason Score (GS) and the SUV_{max} of PCa.

On the other hand, Uprimny et al. (48) found that PCa with a GS of 6, 7a (3 + 4) and 7b (4 + 3) showed lower ^{68}Ga-PSMA-11 uptake, with SUV_{max} of 5.9, 8.3, and 8.2, respectively, compared to men with a GS greater than 7 (median SUV_{max}: 21.2). In addition, men with a PSA of 10.0 ng/mL or above it showed a greater uptake than those patients with PSA levels below 10.0 ng/mL.

The correlation of intraprostatic PSMA uptake with clinical parameter, such as PSA value, GS and d'Amico risk score, was analyzed by Koerber et al. in 104 patients with newly diagnosed PCa (49). Results of this study indicated that men with higher PSA, higher d'Amico risk score and higher GS had greater intensity of PSMA uptake on PET/CT.

The comparison between the multiparametric Magnetic Resonance Imaging (mpMRI) and ^{68}Ga-PSMA-11 PET/CT findings showed a concordance in the detection of intraprostatic tumor lesions, with the highest GS of 89.55%. By giving additional molecular imaging information to the mpMRI features, this method can be improved to avoid false-negative results or understaging tumors, in particular the detection of those with the highest GSs. In addition, PSMA PET/ MRI may prove useful in finding lower rates of indolent cancer detection and a great number of intermediate- and high-risk tumors.

Imaging at Staging of PCa

For pre-operatory staging, current guidelines recommend at least abdomino-pelvic cross-sectional imaging (MRI or CT) and a bone scan, for intermediate- and high-risk PCa (50) only. In a prospective study 30 patients with intermediate- and high-risk PCa underwent preoperative 68Ga-PSMA PET/CT followed by RP and extended pelvic LN dissection. Using pathology as reference, 68Ga-PSMA PET/CT showed a sensitivity of 64% for the evaluation of LN metastasis, with a 95% specificity, 88%, positive predictive value, and 82% negative predictive value (51).

In a prospective, phase II, single center study, Gorin et al. analyzed the diagnostic value of PSMA targeted ^{18}F-DCFPyL PET/TC in the preoperative staging of 25 patients considered to be at high risk for having metastatic PCa, despite a negative conventional staging result. With this technique, they obtained a sensitivity and specificity of 71 and 88%, respectively, per patient analysis and 66 and 92% per LN packet analysis (52).

The retrospective study conducted by Maurer et al. (53) involved a 130 men with intermediate and high risk PCa staged with ^{68}Ga-PSMA-PET/magnetic resonance tomography or PET/CT. The sensitivity, specificity and accuracy of ^{68}Ga-PSMA-PET were 65.9, 98.9, and 88.5%, and those of morphological imaging were 43.9, 85.4, and 72.3%, respectively. Such figures are higher than those for traditional imaging techniques and other alternative PET tracers. Hence, the addition of ^{68}Ga-PSMA PET to traditional approaches has the potential to replace current standard imaging, enabling more complete and accurate primary staging.

Imaging at Biochemical Recurrence of PCa

In men with biochemical recurrence (BCR) after RP or radiotherapy the detection rate of ^{68}Ga-PSMA PET/CT increases with higher pre-scan PSA value. In the post-RP patients the rate of ^{68} Ga-PSMA PET/CT was 11.3, 26.6, 53.3, 79.1, and 95.5% for serum PSA levels of 0.01 to <0.2 ng/mL, 0.2 to <0.5 ng/mL, 0.5 to <1 ng/mL, 1 to <2 ng/mL, and ≥ 2 ng/mL, respectively. In the post-radiotherapy patients, the rate was 33.3% for PSA 0.01 to < 0.5 ng/mL, 71.4% for PSA 0.5 to <1 ng/mL, 93.3% for PSA 1 to <2 ng/mL, and 100% for PSA ≥ 2 ng/mL (54). Such figures are in agreement with the meta-analysis data by Perera et al. (55). In that study, on per-patient analysis, the sensitivity and specificity of 68Ga-PSMA-11 PET were both 86%. On per-lesion analysis, the sensitivity and specificity were 80 and 97%. ^{68}Ga-PSMA PET positivity increased with a shorter PSA doubling time.

Higher rates have been reported by Raucher et al. (56) in a cohort of men with PSA value between 0.2 and 1 ng/ml after RP. The rate of detection was 55% in men with "very low" serum PSA (0.2–0.5 ng/ml) and of 74% in patients with "low" PSA (0.5–1.0 ng<ml). In such investigation the most relevant predictors for 68GaPSMA-ligand PET/CT positivity in multivariable analysis were concurrent androgen deprivation therapy and serum PSA value. Identification of the sites of recurrent disease is of great importance, thus avoiding unnecessary localized treatments in patients of systemic recurrence and avoid the side effects of systemic treatments in men with localized recurrence (57, 58).

Table 1 summarizes the completed trials on PSMA and imaging. For additional trials please visit: https://clinicaltrials.gov.

^{18}F-fluciclovine (Axumin®) (18F-FACBC) is an amino-acid targeting radiotracer and not a PSMA based PET/CT agent (59).

The sensitivity of 18F-fluciclovine PET for identifying recurrent disease changes with PSA levels, with reported detection rates in the post-prostatectomy biochemical failure setting of 72.0% (for PSA values of less than 1 ng/mL) 83.3% (for PSA 1-2 ng/mL), and 100% for PSA levels of 2 or more ng/mL (60). In patients with pathologically enlarged lymph nodes, presence of true-positive lesions was noted in 29% patients with 18F-fluciclovine vs. 7% patients with CT (61, 62). A prospective study compared overall detection rate of 18F-FACBC and 11C-Choline PET/CT on 28 patients with biochemical relapse after RP. Anti-3-18F-FACBC PET/CT detected 60% additional tumor lesions including 5 (17.8%) additional patients (63).

PSMA-Targeting Strategies for PCa Therapy

PSMA has been widely utilized as a target antigen due to its constitutive or induced internalization property as well as to its high expression in PCa. Several strategies, including peptides, monoclonal antibodies and aptamers, have been utilized as nanoparticles or prodrugs to improve targeting efficiency in PCa cells. The discovery and development of anticancer aptamers may prove to be relevant contribution to PCa molecular imaging.

Aptamers are short DNA, RNA or peptide oligomers able to assume a specific and stable three-dimensional shape *in vivo* (64). Their high affinity and specificity, similar to antibodies, is achieved by a three-dimensional conformation complementary to the target surface. At this regard, Lupold et al. identified two RNA aptamers (A9 and A10) characterized with high binding affinity to PSMA, leading to the inhibition of its NAALADase/glutamate carboxypeptidase II activity (65). Successively, Xu et al. conjugated A10 aptamer on the surface of micelles, showing high drug uptake in $PSMA^+$ cancer cells both *in vitro* and *in vivo* investigations (66).

PSMA can be used as target for delivery of therapeutic agents such as in antibody-drug conjugated (ADC) therapy. PSMA ADC is a fully human anti-PSMA monoclonal antibody conjugated to monomethyl auristatin E through a valine-citrulline linker.

Wang and his group assessed the antitumor activity of PSMA ADC in PCa cell lines *in vitro* and in a novel *in vivo* model of taxane-refractory human PCa. They observed that *in vitro* cytotoxic activity was efficient for PCa cells with increased PSMA expression (>105 molecules/cell; IC50 0.022 nmol/L). In addition, PSMA ADC showed high *in vivo* activity in treating xenograft tumors that have progressed on previous docetaxel therapy (67).

Petrylak et al. (68) reported data from a phase II trial based on PSMA-ADC at 2.5 mg/kg in patients with taxane-refractory metastatic castration-resistant PCa (CRPCa). Thirty-Nine Percent of the patients had been treated with both cabazitaxel and docetaxel, while 58% had received both enzalutamide and abiraterone. Dosing was started at 2.5 mg/kg and adjusted at 2.3 mg/kg for tolerability. The study demonstrated that PSA decline of 30% or more was observed in 36% (2.3 mg/kg) and 16% (2.5 mg/kg). Circulating tumor cell (CTC) decline of ≥50% was seen in 74% patients in both 2.3 and 2.5 mg/kg. Duration of therapy on 2.3 mg/kg was far longer than on 2.5 mg/kg, as well as the rate of

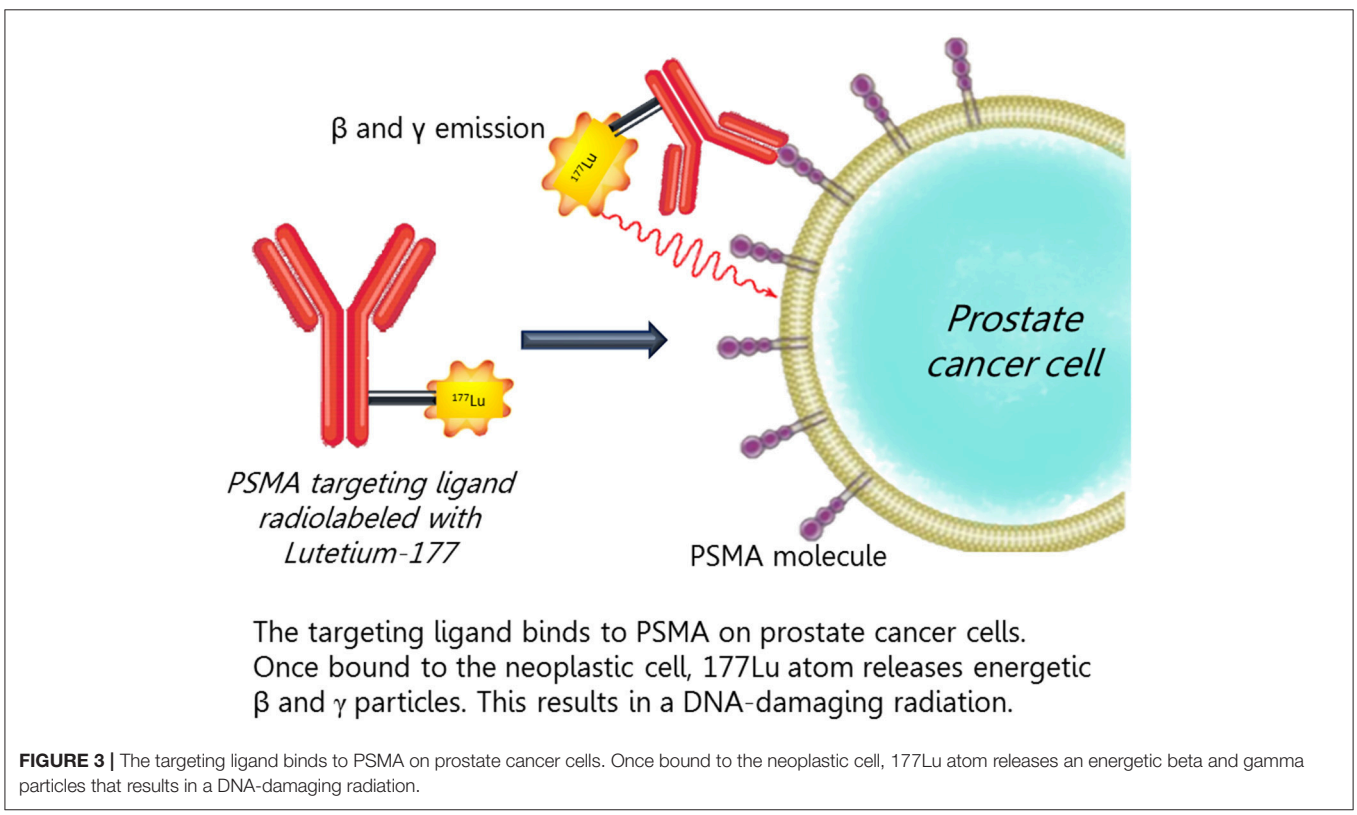

FIGURE 3 | The targeting ligand binds to PSMA on prostate cancer cells. Once bound to the neoplastic cell, 177Lu atom releases an energetic beta and gamma particles that results in a DNA-damaging radiation.

serious adverse events (37 vs. 59%). Notably, PSA and CTC decline was associated with higher PSMA expression + CTC level, while PSA responses alone were correlated with lower neuroendocrine (NE) marker expression, thus suggesting that NE differentiation may have a role in this context. On the basis of such results, this study has been further extended (see NCT02020135).

Phage display technology has been used by researchers in the identification of peptide sequences, which can bind to PSMA and, at the same time, inhibit its enzymatic activity. Denmeade et al. conjugated a PSMA-specific peptide to an inhibitor (i.e., Thapsigargin) of the sarcoplasmic/endoplasmic reticulum calcium adenosine triphosphate (SERCA) pump. The type of pump shares the catalytic properties of ion-motive ATPases of the P-type family. It transports calcium ions from the cytoplasm into the sarco-endoplasmic reticulum. Its activity is needed for viability by all types of cells. The conjugate remains inactive until the PSMA-specific peptide is cleaved, thereby starting SERCA inhibition. In xenograft models, thapsigargin induced tumor regression at doses that appeared to be minimally toxic

TABLE 2 | Selection of trials of PSMA-based therapy (Selection based on active and completed trials).

NCT identifier	Study phase	Drug	Study objectives (Number of patients)	Study results
NCT01695044 NCT02020135	Phase 2 (Extension Study)	PSMA ADC	Assess total serum PSA response, CTC response, overall radiologic response in mCRPC pts (119 pts- completed 17 pts) in two groups: (1) CHT-experienced and (2) CHT naïve.	-PSA response: >30% Decrease in PSA: 29% (1); 32% (2). >50% Decrease in PSA: 11% (1); 21% (2). -CTC response >30% Decrease in CTC: 81% (1), 92% (2). >50% Decrease in CTC: 74%(1),85%(2). -Overall radiologic response Stable disease 61% (1),69% (2); Progressive disease: 13% (1), 9% (2); Partial response: 0 (1), 6% (2)
NCT01414283	Phase 1	PSMA ADC	Determine the maximum tolerated dose of PSMA ADC (13 weeks) (52 pts)	No results posted
NCT01414296	Phase 1 Extended 39-Week	PSMA ADC	Safety and tolerability of PSMA ADC as measured by all adverse events in mCRPC patients (10 pts)	No results posted
NCT00705835	Phase 1	Rs-PSMA	Safety, tolerability, and immune response of vaccine therapy with increasing dose levels of rsPSMA protein (14 pts)	No results posted
NCT00015977	Phase 2	PSMA peptide vaccine	Immunization with PSMA peptide vaccine followed by injection of Interleukin-12 in Metastatic PCa patients, determinate disease response (13 pts)	No results posted
NCT01140373	Phase 1	Autologous T cells targeted to PSMA	Safety and tolerability using increasing doses of engineered autologous T cells targeted to PSMA after cyclophosphamide in CMPC patients (13 pts)	No results posted
NCT02202447	Phase 1	EC1169	Safety, pharmacokinetic profile and preliminary efficacy of PSMA Targeting-Tubulysin Conjugate EC1169 in Patients With Recurrent Metastatic CRPC (40 pts)	No results posted
NCT00694551	Not Applicable	Polypeptide vaccines: PSMA27-35-PSMA687-701	Pilot immunotherapy study of combination PSMA and TARP peptide with Poly IC-LC adjuvant in patients with elevated PSA after initial definitive treatment (29 pts)	Adverse events (Grade 3 or higher): 0 pts PSA doubling: 19/29 pts; No PSA doubling: 10/29 pts
NCT00916123	Phase 1	177Lu-J591	Effectiveness of 177Lu-J591 antibody in combination with docetaxel chemotherapy against metastatic CRPC (15 pts)	No results posted

For a full list of trials please visit: https://clinicaltrials.gov PSMA ADC, Prostate Specific Membrane Antigen Antibody Drug Conjugate; CHT, chemotherapy; PSA, prostatic specific antigen; CTC, Circulating tumor cells; 177Lu- J591, Anti-prostate-specific Membrane Antigen Monoclonal Antibody; CMPC, Castrate Metastatic Prostate Cancer; TARP, T-cell receptor γ alternate reading frame protein; CRPC, castrate-resistant prostate cancer; Rs-PSMA, Recombinant Soluble PSMA.

(69). Based on such findings, a phase I study is evaluating the Thapsigargin prodrug G-202 in patients with advanced PCa and other solid tumors (NCT01056029).

PSMA can be used in immunotherapy and radiotherapy approaches. "Adoptive immunotherapy based on infusion of designer T cells engineered with chimeric antigen receptors (CARs)" to potentiate their antitumor activity could serve as a highly specific modality for the treatment of cancer. Thus, PSMA×CD3 diabody is able to retarget human $CD4^+$ and $CD8^+$ lymphocytes to lyse PSMA-expressing C4-2 PCa cells. Other 1st and 2nd generation anti-PSMA designer T cells have shown their activity in both *in vitro* and *in vivo* studies (70). More recently, mouse-human chimeric IgG1 of 2C9 (KM2777) has been fused with C-terminus interleukin-2 (IL-2). In a xenograft tumor model using PSMA-expressing PCa cells, this fusion, KM2812, showed evident antitumor activity, with complete regression in some cases (71). Bispecific antibodies have been utilized in human clinical trials. A phase I trial has studying the safety of adoptive transfer of autologous T cells targeted to PSMA for the treatment of castrate metastatic PCa patients (NCT01140373). Vaccine is another very important area that utilizes PSMA as a target to increase cellular and humoral immune responses against tumor cells (72).

Concerning the potential role of PSMA targeted antivascular radiotherapy, Bandekar et al. has evaluated liposomes loaded with the α-particle generator 225Ac to kill in a selective manner PSMA positive PCa cells. In such study, anti-PSMA–targeted liposomes have been able to kill PSMA positive cells, including the endothelial cells expressing PSMA, thus suggesting their use for selective antivascular radiotherapy (73).

Therapy of Metastatic Castration Resistant PCa (CRPCa) With Radiopharmaceuticals

Recently, studies have explored the role of PSMA-based treatments with radiopharmaceuticals of metastatic castration resistant PCa (**Figure 3**). The first antibody-based radiotherapeutic was Yttrium-90 (90Y) capromab. Phase 1 (74) and Phase 2 (75) studies were unsuccessful for significant toxicity and lack of efficacy.

J591 was the first humanized monoclonal antibody directed against the extracellular domain of PSMA (76). The PSMA antibody–based radiotherapeutic Lutetium-177 J591 (177Lu-J591) showed acceptable toxicity with evident targeting of known metastatic sites in a phase 1 trial. In phase II, almost 60% of men showed a decline in serum PSA levels, 10.6% of them experiencing a >50% decline in PSA (77). Myelosuppression associated with treatment was reversible. "Fractioned dosing allowed for higher cumulative doses with less toxicity" (78). However, there are limitations to the use of PSMA antibody—based radiotherapeutic. Lutetium-177 J591, i.e., slow diffusion of the antibodies into solid tumor lesions and hematotoxicity caused by a long circulation time in the blood (79). Retrospective studies have caveats such as treatments that have been done outside of clinical trials, unrecognized and unmeasured covariates that might influence final results.

A recent systematic review showed that 43% of the men showed a maximum decline of serum PSA of ≥50% following treatment with 177Lu-PSMA radioligand therapy (RLT). In particular, 177Lu-PSMA RLT gave objective remission and decline of PSA of ≥50%, more often than with third-line treatments (enzalutamide and cabazitaxel). Median survival was longer after 177Lu-PSMA RLT than after third-line treatment, the difference being not significant from statistically point of view (80).

A promising available option for patients with mCRPC is the recently investigated compound PSMA–DKFZ-617, a small molecule peptide, rather than an antibody, chemically conjugated with 177Lutetium that binds with high affinity to PSMA. Unlike antibodies such as J591, it shows more rapid plasma clearance, higher affinity binding to PSMA and lower toxicity. Interesting outcomes came up from a single-arm, single-center, phase 2 trial (ACTRN12615000912583) recently published by Hofman et al. (81, 82). Patients with mCRPC and progressive disease after standard treatments underwent screening PSMA and FDG-PET/CT to confirm high PSMA-expression. After four cycles of intravenous [^{177}Lu]-PSMA-617, 17 (57%) of 30 patients (95% CI 37–75) obtained a PSA decrease of 50% or more, objective response in nodal or visceral disease in 14 (82%) of 17 patients with measurable disease and reported minor toxic effects and improvement in pain severity. Phase 3 trial of 177Lu-PSMA-617 (NCT03511664) is currently recruiting. A multicenter randomized trial comparing LuPSMA with cabazitaxel chemotherapy (NCT03392428) is ongoing.

Table 2 summarizes a selection of active and completed trials on PSMA and therapy. For a full list of trials please visit: https://clinicaltrials.gov.

CONCLUSION

PSMA represents an attractive target for the detection and treatment of patients with PCa. PSMA immunohistochemical evaluation should be further investigated as a predictive marker in men with metastatic PCa, to guide clinicians in the selection of the most appropriate imaging technique and therapy in individual patients. The choice of emerging PSMA-targeted tracers and therapeutic agents requires further investigation in order to identify the most specific compound for the distinct sites and phases of the disease (83–85). As our understanding of the role of PSMA in prostate carcinogenesis advances and molecular techniques become more refined, PSMA-based strategies will have a crucial role in the evolving diagnostic and therapeutic landscape of patients with PCa.

AUTHOR CONTRIBUTIONS

RM and MSc conception and design. AC and MSa drafting the manuscript. MC, FM, and AG review of the literature. NB and AL-B critical revision of the manuscript.

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Recent Advances in Liquid Biopsy in Patients With Castration Resistant Prostate Cancer

Vincenzo Di Nunno[1], Lidia Gatto[1], Matteo Santoni[2], Alessia Cimadamore[3], Antonio Lopez-Beltran[4], Liang Cheng[5], Marina Scarpelli[3], Rodolfo Montironi[3] and Francesco Massari[1]**

[1] Division of Oncology, S.Orsola-Malpighi Hospital, Bologna, Italy, [2] Oncology Unit, Macerata Hospital, Macerata, Italy, [3] Section of Pathological Anatomy, School of Medicine, United Hospital, Polytechnic University of the Marche Region, Ancona, Italy, [4] Department of Pathology and Surgery, Faculty of Medicine, Cordoba, Spain, [5] Department of Pathology and Laboratory Medicine, Indiana University School of Medicine, Indianapolis, IN, United States

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Edited by:
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Memorial Sloan Kettering Cancer Center, United States

***Correspondence:**
Alessia Cimadamore
alessiacimadamore@gmail.com
Francesco Massari
fmassari79@gmail.com

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Management of localized and advanced prostate cancer benefits from several therapeutic options with a surprising improvement in terms of clinical outcome. The selection of patients more likely to benefit from a specific approach still remains a key issue as well as the early identification of patients with aggressive disease which could benefit from a more aggressive treatment strategy. The lack of reliable bio-marker in castration resistant setting able to monitor response to treatment and early inform about tumor progression is an emerging issue. Accordingly, circulating DNA and circulating tumor cells appears a promising and attractive approach despite to date practical applications of these techniques are few and not validated. The aim of this review of the literature is to explore current knowledge on liquid biopsy in prostate cancer focusing on possible future applications.

Keywords: prostate cancer, metastatic castration resistant prostate cancer, CTCs, liquid biopsy, circulating DNA

INTRODUCTION

Prostate Cancer (PCa) represents the most common adult malignancies ranking as one of the major cause of cancer related death in men (1). Management of the disease accounts various options in both localized and advanced stages. Each options are generally evaluated according to different variables related to patients (performance status, comorbidities, disease related symptoms, and patients' preferences) and tumor features (biological aggressiveness and site and number of metastases). Thus, the management of localized stages could range from a first instance no invasive approach (watchful waiting or active surveillance approach) to a radical approach by surgery, external radiation treatment, a combination of both of them (radiation treatment in case of positive surgical margins) or also brachytherapy (which consists on the prostate implantation of sealed radiotherapy sources) with or without an adjuvant androgen deprivation therapy (ADT) (2–8).

Similarly, advanced stages of the disease count different therapeutic options. As first approach ADT represents the cornerstone of advanced prostate cancer due to the high sensitivity of tumor cells to hormone deprivation. The addiction of further treatment including anti-androgens abiraterone acetate or docetaxel can improve the outcome of patients with metastatic castration sensitive prostate cancer (mCSPC) (9–15).

After a first period of hormone deprivation sensitivity, tumor cells develop several mechanisms which lead to overcome the hormone inhibition leading to metastatic castration resistant prostate cancer (mCRPC). In this setting, several different agents have demonstrated to be effective treatment: new hormonal agents (abiraterone, enzalutamide, apalutamide), chemotherapy (docetaxel, cabazitaxel), radiometabolic drugs (Radium 223), and Sipuleucel-T immunotherapy (16–25).

RATIONAL FOR LIQUID BIOPSY IN PROSTATE CANCER

The availability of several active therapeutic options has led to different emerging needs in clinical practice requiring the development of reliable markers able to monitor response to treatment and help clinicians to select patients more likely to benefit from one approach rather than another.

Prostate-Specific Antigen (PSA) represents a reliable and useful biomarker adopted for early detection and early diagnosis of disease recurrence progression. However, it does not give information about biological features of the disease and it loses its predictive rule in mCRPC setting (26).

Liquid biopsy is an emerging technique which purposes is the detection of tumor cells/tumor DNA from patients' peripheral blood.

There are several issues which make the development of liquid biopsies in prostate cancer an attractive approach: (1) the low invasiveness; (2) the early detection of more aggressive tumors since early phases;(3) the early diagnosis of residual tumors or micro-metastases after surgery. (4) the monitoring of tumor response/progression to systemic treatment in advanced setting of the disease and especially in mCRPC; (5) the prediction of tumor sensitivity/resistance to systemic treatments; (6) the acquisition of an accurate genetic assessment of the disease focusing on key alterations which are related to tumor resistance. In particular, several genomic alterations seem to be attractive target due to their correlation to treatment resistance and/or sensitivity to specific treatments (27–30). Some of the more attractive targets are:

- **Phosphate and tensin homolog (PTEN) loss**. PTEN loss results in PI3K/AKT activation which has been associated to worst survival due to higher tumor proliferation and resistance to hormonal treatment. The inhibition of the PI3K/AKT/mTOR pathway could be an interesting target in this subgroup of patients which could be associated to an Androgen Receptor (AR) inhibition (31, 32).
- **MYC** amplification is generally acquired in metastatic phases of the disease and is correlated to poor prognosis and higher Gleason score. Furthermore, more than one evidences seem to correlate the combination of MYC amplification and PTEN loos to worst prognosis and increase risk of tumor related death (33, 34).
- **Androgen Receptor (AR) mutations** and in particular AR splice variant 7 (AR-V7) is known to be related to resistance to hormonal treatments including also new hormonal agents abiraterone and enzalutamide (35).
- **TMPRSS2-ERG** gene fusion leads to ETS-related gene (ERG) and steroidogenic enzyme AKR1C3 co-overexpression which promotes AR signaling and represents a promising target in prostate cancer (36, 37).
- **DNA repair genes deficiency** and in particular genes related to the identification of single strand breaks (such as PARP1 and PARP2) as well as the identification of the alterations of non-homologous recombination system genes (such as BRCA1, BRCA2, PALBB2, MRE11, Check2, RAD51, XRCC2/3) appears an attractive approach for two reason. First, tumors with repair genes deficiency are related to more aggressive features and poorer survival. Second, therapeutic implications related to these genomic assessments involve a possible sensitivity to platinum cytotoxic therapy. The development of PARP inhibitors represents another possible target for the management of advanced prostate cancer which has already been evaluated in small trials and is currently under clinical investigation (38–43).

Due to these issues, the development of reliable techniques able to perform liquid biopsy appears a promising and suggestive approach (**Table 1**). Here we performed a review of the main techniques adopted or under investigation focusing our attention on approaches based on circulating tumor cell (CTC) and circulating DNA (ct-DNA) detection. **Table 2** summarizes the current methods available for CTC detection as well as the percentage of detection (See also below).

CIRCULATING TUMOR CELLS IN PROSTATE CANCER

To date, the CellSearch system assay is the only FDA approved method for the detection of CTCs in prostate cancer (**Table 2**). This device consists of different components including a CellPrep system which is a semi-automated sample preparation system and a CellSearch Epithelial Cell Kit. This last component involves ferro-fluids coated with epithelial cell-specific EpCAM antibodies and a mixture of antibodies directed against cytokeratins 8, 18, 19, CD45 conjugated to allophycocyanin and DAPI (nuclear dye 4', 6 diamidino-2-phenylindole for fluorescent cells label). After an incubation period in which CTC are isolated from peripheral blood and enriched in EpCAM composed ferro-fluids the MagNest Cell Presentation Device (a device composed of a chamber with two magnets) orients labeled cells for analysis in a CellSpotter Analyzer (a four-color semi-automated fluorescence microscope) for the CTCs enumeration (44).

Initial studies carried out on patients with different solid tumor demonstrated a promising activity with this method and regarding patients with PCa detection of CTCs was possible in 57% of patients (44).

Further study aimed to investigate the clinical value of CTCs detected by CellSearch assay showed that CTCs baseline levels were an independent prognostic factor for overall survival (OS) (49).

In 2008, de Bono et al. identified a correlation between CTCs number and median overall survival. In this study carried out on 231mCRPC two distinct subgroup of patients were identified:

TABLE 1 | An overview of ongoing clinical studies evaluating CTCs/ctDNA in prostate cancer patients.

Trial	Patients enrolled	Study description/outcomes
NCT03284684	Patients undergoing surgery for non-metastatic solid tumors: colon, breast and prostate.	Change in concentration of total mutant circulating DNA. Change in proportion of mutant circulating DNA. Change in integrity index of circulating DNA for ACTB gene. Change in integrity index of circulating DNA for KRAS gene.
NCT02449837	Patients undergoing radiation treatments for one of six cancer types including PC.	To measure CTCs levels to evaluate the change pre- and post-treatment. Change in CTC levels from Baseline to Post-RT treatment and the correlation with local tumor response or pathological evaluation
NCT01961713	Subjects with prostate cancer diagnosed on prostate biopsy who undergo radical prostatectomy	To evaluate the relationship between pre-operative CTC quantity and pathologic stage in men with early stage prostate cancer undergoing prostatectomy. To examine the relationship between persistent CTCs and biochemical recurrence after radical prostatectomy for localized prostate cancer
NCT02997709	Men with intermediate to high risk prostate cancer who are candidates for radiotherapy (RT)	Comparison of Pre- and Post-Treatment Quantitative Imaging Parameters to Changes in Circulating Tumor Cells Over Time in Prostate Cancer Patients Receiving Radiation Therapy (RT) with or without Androgen Deprivation Therapy per standard of care
NCT02853097	Prostate cancer patients at various points throughout androgen deprivation therapy and at the initiation of androgen deprivation therapy, enzalutamide, abiraterone and docetaxel.	To document the appearance of androgen receptor isoform splice variant 7 (AR-V7) expression over the course of therapy in castration-resistant prostate cancer (CRPC). To determine whether detectable AR-V7 is associated with a shortened duration of treatment benefit of abiraterone or enzalutamide.
NCT03089099	mCRPC	To determine whether sequentially analyzing the expression of molecular markers in high volume circulating tumor cells in metastatic castration-resistant prostate cancer patients can predict the therapeutic effects and outcomes of these patients.
NCT03488706	Prostate cancer screening with PSA is plagued by high rate of unnecessary prostate biopsies, especially in the "gray zone" (PSA levels: 4.00 ng/ml e 10.99 ng/ml)	Circulating tumor cells detection Using a circulating-tumor-cell (CTC) test to detect prostate cancer in patients in the PSA "gray zone" level
NCT03236688	mCRPC	Demonstrate detection of ARv7 splice variant transcripts from exosomes in the circulation of MCRPC patients pre and post treatment with selective Androgen pathway inhibitors (i.e., abiraterone and enzalutamide)
NCT02771769	Patients with planned prostate biopsy	Multi-center prospective study in which blood samples will be taken from 1500 male patients aged between 21–80 scheduled for prostate biopsy. Analysis of cell-free cancer DNA extracted from these samples will be undertaken to determine whether copy number instability scores derived from the cfDNA correlates with PSA screening levels and prostate biopsy results (i.e., Gleason score) in these patients
NCT02723526	Patients with newly Diagnosed Metastatic Hormone-Sensitive Prostate Cancer	To determine whether sequentially analyzing the expression of tumor markers in circulating tumor cells in newly diagnosed metastatic hormone-sensitive prostate cancer patients can predict the outcome of these patients.
NCT02742259	Metastatic prostate cancer to the bone	Confirmation of the clinical utility of the cutoff level for the Prostate Cancer Assay for prognosis of progression free survival (PFS) in comparison to the predicate device, CellSearch CTC Assay
NCT02456571	Metastatic PC	To explore the prevalence of expression of four immune checkpoint biomarkers on circulating tumor cells (CTCs) from men with metastatic prostate cancer
NCT02735252	Metastatic PCa.	Develop a first-in-man CTC-based molecular taxonomy of CRPC. Comparison of median PFS to CTC-based AR-v7 status.
NCT02099864	Advanced PCa patients receiv- ing enzalutamide therapy.	Correlation between PSA response and gene expression signatures, DNA copy number alterations, mutations. Assess the association for changes in CTC counts from baseline and maximal PSA observed while on study.

one (Unfavorable group) which showed a CTCs number of 5 or more and the other (favorable) with < 5 CTCs per 7.5 mL of blood. Overall survival was significantly better in favorable group (21.7 vs. 11.5 months). Moreover, patients who presented a significant decrease of CTCs number during or after treatment (moving from unfavorable to favorable groups) significantly improved their survival compared to patients who continued to present a CTCs number of 5 or more CTCs. According to the results of de Bono et al, a meta-analysis of 10 studies confirmed the prognostic rule of CTCs in patients with prostate cancer (50).

Furthermore, pre-planned analyses of large phase III trials: SWOG 20421 (docetaxel with or without atrasentain in mCRPC patients), COU-AA-301 (in which a score composed by LDH levels and CTCs divided patients in 3 different subgroups with favorable, intermediate and poor prognosis) and AFFIRM (enzalutamide in patients with mCRPC progressed to chemotherapy) confirmed the prognostic rule of CTCs as independent factor related to OS (51–53).

Unfortunately, none of these studies demonstrated an association between CTCs number and response to treatment and so the role of CTCs in this setting still remains unclear.

TABLE 2 | An overview of CTCs detection techniques.

Method	Mechanism	CTCs detection rate/ other outcomes	Limitations
CELLSEARCH System (44)	A 7.5 mL sample of blood is placed in a special tube, centrifuged to separate solid blood components from plasma, then placed in the CELLTRACKS® AUTOPREP® System. Cells binds ferro-fluid nanoparticles presenting antibodies targeting epithelial adhesion molecules, then CTCs are magnetically separated from other blood cells. CTCs are stained with cytokeratin monoclonal antibodies, DAPI (a DNA stain) and leukocytes which may have contaminated the sample are marked by antibody targeting CD45. Stained CTCs are then placed onto Cell-Spotter Analyzer (a four-color semi-automated fluorescence microscope) for the CTCs enumeration CTCs+: DAPI+, cytokeratine +, CD45−cells	CTCs detected in 57% of patients with prostate cancer	- Low CTCs detection rate in non-metastatic prostate cancer - Conflicting results about correlation between CTCs number and treatment response.
CELLCOLLECTOR EPISPOT (45)	Cell-Collector is based on a sterile stainless steel medical wire, covered with 2 μm gold and a hydrogel layer which is covalently coupled with antibodies against the EpCAM protein and pan-keratins. CD45 staining (performed to exclude unspecific leucocytes) CTCs +: CTCs identified as pan-keratin positive, leukocyte marker CD45 negative. EPISPOT on an EpCAM-independent enrichment method (i.e., leukocyte depletion) and enables the identification of viable PSA-secreting tumor cells CTCs+: PSA+ cells.	Combining Cellsearch, CellCollector and Epispot assay, detection rate of CTCs was 81.3%	- Experimental approach. - This approach does not offer a characterization of CTCs. - Impact on prognosis and predictive value under investigation.
Microfluidic capture of CTCs (46).	Considering the expression of PSA (up-regulated by AR) and PSMA (down regulated by AR) they classified CTCs in AR on, AR mixed and AR positive according to the expression of PSA (+ in AR on and mixed) and PSMA (+ in AR off and mixed).	CTCs detection rate: 80%	- Experimental approach - Under investigation for detection of anti-androgen resistance mechanisms.
EPCAM cells enrichment and sequencing (47).	The recovered cells, enriched with CTCs, were deposited into dense arrays of subnanoliter wells and imaged by automated epifluorescence imaging. Enrichment was obtained through Illumina MagSweeper CTCs expressing EpCAM. Individual EpCAM (+) CD45 (–) CTCs were recovered by robotic micromanipulation for whole genome amplification using multiple displacement amplification.	Mutation concordance between CTCs and primary or metastatic tumor tissue: 86%	- Experimental approach. - High cost. - Loss of concordance between CTCs mutation and primary/metastatic tumor tissues.
ADNAtest (48).	Is a device able to isolate MUC1-negative and EpCAM positive CTCs. After CTCs isolation, cells are lysed and RNA is extracted for downstream analyses with RT-PCR. Of note this device adopts primers against EGFR, PSA and PSMA making a sample positive if one of these genes are expressed.	CTCs detection rate: 62%	- Experimental approach - High cost - Few data about the application of this approach in localized/ non metastatic prostate cancer.

Moreover, another possible issue which could partially explain the failure of this approach in clinical practice is the low detection rate of CTCs in non metastatic patients which ranges only from 5 to 27% (54). To avoid this problem, Kuske et al combined three different methods for the detection of CTCs before and after prostatectomy in non metastatic patients with PC, CellSearch system assay, CellCollector (a system capturing EpCAM-positive CTCs by an antibody-coated needle introduced in arm vein) and EPISPOT (a system able to enrich CTCs by negative depletion of leukocytes and detects circulating prostate cancer cells thanks to their active secretion of PSA) (45). CTCs were detected in 37, 54.9 and 58.7% of patients using CellSearch, CellCollector, and EPISPOT, respectively. The cumulative positivity rate of the three CTC assay was 81.3% and despite it is not a validated approach, it represents an attractive early method able to estimate the risk of tumor recurrence or persistence after surgery.

A combined analysis of COU-AA-301 and IMMC-38 trials showed that an increase of 30% in CTCs count from baseline was independently associated to worst OS in patients treated with abiraterone and chemotherapy (53). To sustain the correlation between CTCs count changes and survival, another analysis performed on 119 patients with CRPC treated at the Royal Marsden Hospital suggested that a decrease of 30% in CTC counts from baseline was associated to improved survival (55).

The only CTCs enumeration resulted in an independent prognostic factor with an unclear role in terms of early diagnosis of disease recurrence/persistence after surgery as well as a predictive response factor. Another interesting approach consists in a characterization of CTCs resulting in a genetic assessment and in a detection of target altered pathways.

AR protein has been extensively investigated in prostate cancer CTCs. Through a FISH based assay AR gene amplification detection in CTCs was possible in 40% of cases, a percentage comparable to the AR amplification described in bone metastases biopsy analyses (47). Further investigations demonstrated that patients with higher cytoplasm expression of AR resulting in a reduction of nuclear translocation was significantly associated to better response to docetaxel (56). By a microfluidic capture

of CTCs Miyamoto et al. evaluated dynamic changes in CTCs AR expression. In particular, considering the expression of PSA (up-regulated by AR) and PSMA (down regulated by AR) they classified CTCs in AR on, AR mixed and AR positive according to the expression of PSA (+ in AR on and mixed) and PSMA (+ in AR off and mixed). Moreover, Authors identified that AR status changed from "on" to "off" during ADT while patients treated with abiraterone acetate with an increase of AR-on CTCs or baseline level of AR-mixed more than 10% were significantly associated to worse overall survival (46). The technology developed by Myamoto et al was also adopted for the detection of anti-androgen resistance mechanisms in CTCs demonstrating higher activation of Wnt signaling and considerable heterogeneity in signaling pathways, expression of AR gene mutations and splicing variants (57).

Due to the important role of AR-V7 in mCRPC (35), several studies have focused on the detection of this splice variants on CTCs. An EpCAM assay demonstrated that CTCs-ARV7+ detection was associated to resistance to enzalutamide and abiraterone but no to docetaxel and cabazitaxel and that the detection of these CTCs was independently associated to worse clinical outcome compared to patients with CTCs-ARV7- cells (58–60). Other studies modified the CTCs and ARV7 detection method in order to evaluate the AR-V7 cellular localization (61) and the presence of other splice variants of AR (62). Particularly, not only AR-V7 but also other slice variants of the AR protein were significantly associated to worse progression free survival. Moreover 6 of 17 poor responders to treatment were AR-V7 negative, but carried other AR perturbations (62).

About other pathways detected in CTCs, the PTEN loss assessed by FISH and Epic Sciences test (an assay which adopted a fibreoptic array scanning techniques for the detection of DAPI, CD45, cytokeratins stained cells) has been associated to worse clinical outcomes (63, 64) while the detection of TMPRSS2-ERG fusion gene performed by microfluidic device and by the use of a RT-PCR analysis failed to show a predictive response value to abiraterone acetate in mCRPC patients (47).

Next Generation Sequencing (NGS) involves a series of different techniques able to perform a whole genome sequencing of tumor cells. The possibility to obtain a complete genomic assessment from CTCs appears a novel and promising approach investigated in different studies.

In 2014, Lohr et al evaluated a method able to perform a CTCs isolation, enrichment (throuEp-CAM expressing CTCs), genomic amplification and sequencing in metastatic PC (65). They demonstrated that a complete mapping of the standard exome was possible in CTCs. NGS analysis of CTCs and tumor sample of a single patient with advanced prostate cancer showed a concordance of 86% from the mutations isolated in CTCs and genomic anomalies identified in primary or metastatic tumors (66). Despite NGS performed to CTCs represents an attractive approach, to date no validated or prospective studies have been carried out and so this method is still under investigation.

Another interesting issue is the detection of whole blood RNA, without enriching for CTC. In 2012, Ross et al assessed a whole blood RNA transcript based model as prognostic factor in patients with PC. After the analysis of blood collected from 62 men with mCRPC, they identified a six gene model (genes considered were: ABL2, SEMA4D, ITGAL, C1QA, TIMP1, CDKN1A which are genes involved mainly in immunity regulation) able to divide patients in two risk groups with different mOS (67). In the same year, Olmos et al carried out a validation study of a nine-gene signature as prognostic factor (68). Design of the study consisted in a derivation set in which patients with mCRPC and patients in Active Surveillance were used as case and control groups respectively. After genomic assessment 94 patients were divided in four distinct prognostic groups. Thus nine altered genes HMBS, TMCC2, SLC4A1, STOM, GABARAPL2, RIOK3, TERF2IP, TFDP1) isolated in prognostic groups with worst survival (composed of only mCRPC patients) were validated in a validation set of patients with mCRPC. More recently, an assessment of 5 key genes (KLK3, KLK2, HOXB13, GRHL2, FOXA1) obtained after reverse transcription polymerase chain reaction (RT-PCR) demonstrated to be a reliable prognostic marker compared to CellSearch system count (48). Isolation of two or more of the selected genes were possible in 53% (51 /97) patients with mCRPC. AdnaTest is a technique adopted for CTCs enrichment and consists of a device able to isolate MUC1-negative and EpCAM positive CTCs. After CTCs isolation, cells are lysed and RNA is extracted for downstream analyses with RT-PCR. Of note this device adopts primers against EGFR, PSA, and PSMA making a sample positive if one of these genes are expressed. Sensitivity of KLK2, KLK3, HOXB13, GRHL2, and FOXA1 genes detection by this method is similar to DDPCR (direct detection PCR) and both of these techniques showed a higher sensitivity compared to CellSearch system (69).

Concerning the several devices utilized for CTCs detection, enrichment and evaluation, only CellSearch has been approved from FDA. However, despite a large range of potential applications (such as diagnosis, evaluation of treatment response, early detection of tumor relapse, and progression) CTCs detection by CellSearch is not commonly adopted in clinical practice. This mainly due to a low sensitivity of the method as well as a conflicting relationship between CTCs and treatment response evaluation. Several other approaches are under investigation. It is likely that CTCs evaluation will be an important factor able to improve our decisions in clinical practice (48, 69).

CIRCULATING DNA IN PROSTATE CANCER

The evidence that cell-free DNA could be detected in peripheral blood is a well known issue, and its application in clinical practice has been investigated only in last years. Regarding cancer patients, the unique composition of tumors' ctDNA presenting several genomic mutations (especially single base-pair substitution) which are not detectable in ctDNA originating from normal cells make tumor ctDNA an ideal markers of the disease. Moreover, the possible correlation between mutations detected on ctDNA and genomic mutations of primary or metastatic

tumors make cDNA a unique markers able to provide key information by a no-invasive approach.

Regarding PC, ctDNA could be detected in peripheral blood and detection of known driver aberrations can be obtained in more than 97% of cases. Moreover, changings in ctDNA genomic mutations could be detected by repeated analyses of ctDNA with high grade of concordance with genomic assessment of primary tumors or metastases (70, 71).

The quantitative assessment of ctDNA has been related to prognosis of patients with PC in different studies (72, 73). In particular, Romanel A et al exanimated AR status of mCRPC patients starting abiraterone acetate. They detected a 45% of patients (tot number 97) with AR point mutations (T878A/L702H) before the first administration of abiraterone who showed a significant worse overall survival (73). Similarly, other studies confirmed the prognostic role of AR genomic alterations as prognostic markers raising the acquisition of ctDNA examination as a possible to monitor response to hormonal agents and to achieve an early diagnosis of progressive disease (74–77).

As known, mutation in DNA repair genes is acquiring an increasing interest in PC due to the association by these mutation and more aggressive tumor features and to the possible benefit derived from a PARP targeted treatment. Mutations in repair genes are common in prostate cancer. In a DNA assessment of 692 men with metastatic PC a total of 84 germiline DNA repair gene mutations (BRCA2, ATM, CHEK2, BRCA1, RAD51D, and PALB2) were found in 82 men (78). This study demonstrated that incidence of germline mutations of DNA-repair gene were common (as detected in 11.8% of all patients) in metastatic patients regardless to age and family history of prostate cancer.

PARP inhibition is one of the important strategy currently under investigation in patients with metastatic prostate cancer. In a phase II trial 50 mCRPC patients received the PARP inhibitor Olaparib (41). 17 (33%) patients showed an objective response while NGS sequencing showed that 16 patients presented homozygous delections, deleterious mutations, or both in DNA repair genes (BRCA1/2, ATM, Fanconi's anemia genes and CHECK2). A subsequent analysis of tumors DNA highlighted that patients with an overall reduction of 50% or more of ctDNA were associatied with better OS and PFS (79). In ASCO 2018, Clarke et al presented the results of a phase II study comparing the administration of Abiraterone with Olaparib or placebo in 171 patients with mCRPC (80). This trial met its primary endopoint showing a better radiological PFS in patients receiving olaparib. Of note Authors researched homologous recombination repair mutations by a NGS approach in tumor samples and plasma. Sequencing was possible on 91 of 136 patients and positive results (defined as discovery mutated patients) was obtained in 13 patients. By germline analysis and tumor sample analyses detection of homologous recombination repair mutations were identified in 3 patients (38 tumor samples analyzed of 68 total samples) and 7 patients (by a germline analysis of 102 patients). Results of this study raising the dosage and analyses of cDNA as possible approach for the detection of keys DNA-repair gene mutations. Other larger prospective trials are needed to explore the role of ctDNA in this setting.

Another promising target gene is represented by PTEN loss which has show to be a predictive biomarker of response to treatments targeting PI3K/AKT pathway. Hyper-activation of the PI3K/Akt/mTOR resulting from PTEN loss is related to decreased AR transcription output and stability and vice versa. The addiction of ipatasertib (an Akt inhibitor) to abiraterone acetate increased radiological PFS of patients with mCRPC and PTEN loss previously treated with docetaxel based therapy and progressed during at last one previous hormonal therapy (81).

Extracellular vesicles are membrane-enclosed structures that are released from all cells in the body. These vesicles contain several substances such as proteins, lipids, RNA, and DNA and are considered a very promising tumor-related biomarkers. Recently, it has been demonstrated that large extracellular vesicles isolated from plasma of patients with prostate cancer cells are an important source of chromosomal DNA which reflects faithfully genetic aberration of the cell of origin, including copy number variations of genes frequently altered in metastatic prostate cancer (such as MYC and PTEN) (82). The study of extracellular vesicles represents a novel and promising approach for biomarkers development in prostate cancer however further studies are needed to explore the effective value of this method.

CONCLUSION

The surprising potential of CTCs or tumors' ctDNA detection, characterization and genomic assessment have start a revolution which probably will give important results in next years. Despite to date application of these techniques are few probably that better knowledge of genomic anomalies of PC and their correlation with the clinical course of the disease as well as their potential relationship with specific targeted treatments will increase the attention on this issue.

AUTHOR CONTRIBUTIONS

RM and FM: conception and design; VD and LG: drafting the manuscript; MSa and AC: review of the literature; LC, MSc, and AL-B: critical revision of the manuscript.

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Urinary Markers in Bladder Cancer: An Update

Giorgio Santoni[1]*, *Maria B. Morelli*[1,2], *Consuelo Amantini*[2] and *Nicola Battelli*[3]

[1] Immunopathology Laboratory, School of Pharmacy, University of Camerino, Camerino, Italy, [2] Immunopathology Laboratory, School of Biosciences, Biotechnology and Veterinary Medicine, University of Camerino, Camerino, Italy, [3] Oncology Unit, Macerata Hospital, Macerata, Italy

Bladder cancer (BC) is ones of the most common cancer worldwide. It is classified in muscle invasive (MIBC) and muscle non-invasive (NMIBC) BC. NMIBCs frequently recur and progress to MIBCs with a reduced survival rate and frequent distant metastasis. BC detection require unpleasant and expensive cystoscopy and biopsy, which are often accompanied by several adverse effects. Thus, there is an urgent need to develop novel diagnostic methods for initial detection and surveillance in both MIBCs and NMIBCs. Multiple urine-based tests approved by FDA for BC detection and surveillance are commercially available. However, at present, sensitivity, specificity and diagnostic accuracy of these urine-based assays are still suboptimal and, in the attend to improve them, novel molecular markers as well as multiple-assays must to be translated in clinic. Now there are growing evidence toward the use of minimally invasive "liquid biopsy" to identify biomarkers in urologic malignancy. DNA- and RNA-based markers in body fluids such as blood and urine are promising potential markers in diagnostic, prognostic, predictive and monitoring urological malignancies. Thus, circulating cell-free DNA, DNA methylation and mutations, circulating tumor cells, miRNA, lncRNA and mRNAs, cell-free proteins and peptides, and exosomes have been assessed in urine specimens. However, proteomic and genomic data must to be validated in well-designed multicenter clinical studies, before to be employed in clinic oncology.

Keywords: urinary biomarkers, bladder cancer, liquid biopsy, microRNA, exosomes

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***Correspondence:**
Giorgio Santoni
giorgio.santoni@unicam.it

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INTRODUCTION

Bladder cancer (BC) represents the 9th and 4th most common cancer worldwide and in men in the USA, respectively (1, 2). Its main histological type is urothelial carcinoma (UC). About 70–80% of BC is diagnosed as non-muscle invasive BC (NMIBC) and 20–30% as muscle invasive (MIBC). Because 10–30% of patients with NMIBC progress to invasive disease (3–8), early diagnosis and early detection of recurrence are very important. BC diagnosis requires cystoscopy and biopsy, which are unpleasant and costly procedures (9). It is necessary to develop new diagnostic methods less invasive and expensive for BC diagnosis and surveillance. The Food and Drug Administration (FDA) has approved the use of multiple urine-based tests that are commercially available. However, none of these tests has been routinely used and incorporated in the American Urological Association or in the European Association of Urology clinical guidelines for BC treatment (10). In this mini-review we discuss the clinical implementation by the use of novel molecular approaches and liquid biopsy in BC.

At present, the gold standard methods for BC diagnosis are urine cytology and cystoscopy. Cytopathology of urine specimens is the widely used non-invasive test for detection and surveillance of BC (11–13). Cytology is very specific (about 86%), but it is low sensitive (48%) limiting its use in low-grade BC (14–16). Diagnostic accuracy of urinary cytology is subjective, depending on cytopathologist expertise (17). Thus, new molecular-based urinary tests for reducing or substituting, the endoscopy frequency in BC recurrence patients, are required (18, 19).

Advanced technology utilizes patients' urine as samples instead of primary BC tissues to identify novel predictive biomarkers. At present, the major problem is to translate the extensive proteomic and genomic data in clinical practice and to validate the expression of these biomarkers in well-designed multicenter clinical studies (20).

PROTEOMIC AND PEPTIDOMIC ANALYSIS

Proteomic analyses have opened a new horizon for cancer biomarker discovery (21). At present, seven tests are available: FDA approved six on seven of these tests, and the last one is in agree with the Clinical Laboratory Improvement Act standards. NMP22, NMP22 BladderChek, and UroVysion have FDA approval for BC diagnosis and surveillance; immunocytology (uCyt+), BTA-TRAK, and BTA-STAT have been approved only for surveillance (22–26).

In order to improve sensitivity, specificity and diagnostic accuracy in BC diagnosis, novel protein markers, waiting to be approved, are used experimentally. BCLA-1 and BCLA-4 are nuclear matrix proteins specifically targeting BC tissues, with no interference with infection, smoking, catheterization or cystitis (27). In patients with hematuria, aurora A kinase (AURKA) discriminates between low-grade BC vs. normal patients (28). The Aura Tek FDP Test™ in urine can detect BC recurrence (29). The activated leukocyte cell adhesion molecule (ALCAM), a cell adhesion molecule (30), positively correlates with tumor stage and overall survival (OS), after adjusting for patients, clinical features and Bacillus Calmette-Guerin treatment (31). Nicotinamide N-methyltransferase is high in BC patients and correlate with histological grade (32). Apurinic/apyrimidinic endonuclease 1/redox factor-1 (APE/Ref-1) levels are higher in BC, respect to non-BC, and correlate with tumor grade and stage; moreover it is high also in patients with recurrence history of BC (33). The cytokeratin-20 (CK20) urine RT-PCR assay shows 78–87% sensitivity and 56–80% specificity for urothelial BC detection, with improved diagnostic accuracy in tumor progression (34) but it has poor performance for low-grade tumors. Higher levels of CK8 and CK18 was detected in the urine by UBC Rapid test in high- vs low-grade BC (35).

As multiple markers for BC detection, increased urinary levels of apolipoprotein A1, A2, B, C2, C3, *E* (APOA1, APOA2, APOB, APOC2, APOC3, APOE) were found in BC relative to healthy controls (36, 37). A signature of 4 urinary fragments of uromodulin, collagen α-1 (I), collagen α-1 (III), and membrane-associated progesterone receptor component 1 seems to discriminate MIBCs from NMIBCs (38). Other panel employs IL-8, MMP-9/10, ANG, APOE, SDC-1, α1AT, PAI-1, VEGFA, and CA9 to diagnose BC starting from urine samples (39). The advantage of these multi-urinary protein biomarkers was evident in high- and low-grade and high- and low-stage disease (39). The combination of urinary markers such as midkine (MDK) and synuclein G or MDK, ZAG2 and CEACAM1 (40), angiogenin and clusterin (41) evaluated by immunoassay and urine cytology increases the sensitivity and specificity in NMIBC diagnosis (40). Increased CK20 and Insulin Like Growth Factor II (IGFII) levels were detected in the urine sediments of NMIBC patients compared to controls (42). Increased levels of urinary HAI-1 and Epcam evaluated by ELISA, are prognostic biomarkers in high-risk NMIBC patients (43). Urinary survivin evaluated by chemiluminescence enzyme immunoassay correlates with tumor stage, lymph node and distant metastases and represents a potential marker for preliminary BC diagnosis (44). Snail overexpression represents an independent prognostic factor for tumor recurrence in NMIBC (45). Finally, specific glycoproteins were identified by glycan-affinity glycoproteomics nanoplatforms in the urine of low- and high-grade NMIBC; among these, increased urinary CD44 levels were evidenced in high-grade MIBC (46).

Urinary metabolomics signature could also be useful in early BC. By ultra-performance liquid chromatography time and mass spectrometry, imidazole-acetic acid was evidenced in BC (47). Moreover, acid trehalose, nicotinuric acid, AspAspGlyTrp peptide were upregulated; inosinic acid, ureidosuccinic acid and GlyCysAlaLys peptide were downregulated in BC, but not in normal cohort (48). A metabolite panel with indolylacryloylglycine, N2-galacturonyl-L-lysine and aspartyl-glutamate permits to discriminate high- vs. low-grade BC (49). In addition, the alteration of phenylalanine, arginine, proline and tryptophan metabolisms was evidenced by UPLC-MS in NMBIC (50).

CIRCULATING TUMOR AND CELL-FREE DNA

Tumors release DNA fragments into circulation, called circulating tumor DNA (ctDNA) containing tumor-specific mutations, variations of copy number and alterations in DNA methylation status. This ctDNA reflects the heterogeneity of tumor subclones. In BC patients, ctDNA is detectable in over 70% of urine samples (51) and it allows to discriminate between BC patients and control subjects (52). CtDNA measures about 180 and 200 base pairs. It is easily accessible, but it is rapidly cleared from circulation following systemic therapy (53). PCR-based approaches, and more recently, digital-PCR and genome sequencing, represent the methods of choice for cell-free DNA (cfDNA) analysis.

DNA Methylation

The methylation status of tumor-related genes represents a very important epigenetic alteration affecting cancer initiation and progression. Hyper- and hypo-methylated regions are

identified in BC and in premalignant lesions. Alterations in DNA methylation status are chemically stable, develop early during tumorigenesis and can be assessed in circulating cfDNA fragments and in cells shed into the urine (54). A significant prevalence of methylated genes, for example APC and cyclin D2, was found in the urine from malignant vs. benign cases (55). Hyper-methylation in GSTP1 and RARβ2 and APC genes has been identified in the urine from BC patients (56). The evaluation of Twist Family BHLH Transcription Factor 1 (TWIST1) and NID2 genes methylation status in urine permits to differentiate primary BC patients from controls with 90% sensitivity and 93% specificity (57). In addition, the evaluation of the methylation status of NID2 and TWIST1 or CFTR, SALL3 and TWIST1 genes in urinary cells in combination with cytology, has been found to increase sensitivity and high negative predictive value in BC patients (58, 59). The analysis of 1,370 loci specific DNA methylation patterns seem to permit to distinguish NMIBC from MIBC (60). Sun and coworkers demonstrated higher recurrence predictivity than urine cytology and cystoscopy (80 vs. 35 vs. 15%) by using SOX-1, IRAK3, and Li-MET genes methylation status from urine sediments of BC patients (54). POU4F2 and PCDH17 methylation levels in urine distinguish BC from normal controls with 90% sensitivity and 94% specificity (61). Promoter hyper-methylation of HS3ST2, SEPTIN9 and SLIT2 genes combined with FGFR3 mutation showed 97.6% sensitivity and 84.8% specificity for diagnosis, surveillance and risk stratification in low- or high-risk NMIBC patients (62). Finally, the methylation status of p14ARF, p16INK4A, RASSF1A, DAPK, and APC tumor suppressor genes has been found to correlate with BC grade and stage (63).

Altogether, although promising results were obtained, accuracy of urinary methylated DNA is variable and results still await validation studies and complementary markers for clinical implementation (64, 65). In this regard, the recent introduction of the methylation-sensitive High Resolution Melting and Methylated CpG Island Recovery methods could further increases the sensitivity for the detection of methylome in BC urine (**Table 1**) (72, 73).

cfDNA, Mutation and Microsatellite Alterations

Since tumor-derived DNA can be released into circulation and mutations in cfDNA can be detected in various biological fluids, their use as non-invasive cancer biomarkers has been proposed. Urinary TERT promoter mutations, that occur early in urothelial neoplasia, FGFR3 mutation and telomere length correlate with high-risk BC recurrence (66, 67). TERT, evaluated by telomeric repeat amplification protocol, in combination with FGF3 and OTX1 shows high sensitivity in NMIBCs as well as in pT1 tumors and in high-grade BC (68). In addition, increased FGFR3 and PIK3CA mutated DNA levels in urine has been found to be indicative of progression and metastasis in NMIBC (69). Microsatellite analysis in circulating DNA of BC patients targets highly polymorphic, short tandem repeats. Loss of heterozygosity (LOH) analysis is more sensitive than urine cytology (97 vs. 79%), particularly for low-grade BC diagnosis. It also significantly improves the detection of low-grade and low-stage BC, with 95% sensitivity for G1-G2 grades and 100% for pTis and pTa tumors (**Table 1**) (74).

Histone Tail Modifications

The levels of histone methylation are lower in advanced tumors respect to controls and correlated to poor survival. Thus, increased levels of HAK20me3 were evidenced in a MIBC subset (70); furthermore high H3K27me3 levels correlate with worse survival after cystectomy in pT1-3 and pN- BC patients (71). H2AFX1 gene methylation was detected in paraffin-embedded BC and its expression correlated with increased recurrence rates (**Table 1**) (75).

URINARY TUMOR RNA

Several RNA classes, messenger RNAs (mRNAs), microRNAs (miRs) and long non-coding RNAs (lncRNAs), have been recognized as potential non-invasive cancer biomarkers (76). Altered levels of circulating RNAs in cancer, which returned to normal following surgery have been reported (77), suggesting release of RNA molecules from tumors.

miRNAs (miRNAs)

miRNAs are short (21–23 nucleotides length) non-coding RNAs regulating gene expression by pairing to the 3′untranslated region (UTR) of their target mRNA. Several miRNAs have been found to play an important role in tumorigenesis, progression and metastasis of cancer cells (78, 79). Urine seems to be a good source for miRNA detection for its content of cell-free nucleic acid in supernatant or sediments (80). However, the diagnostic significance in the detection of miRs in urine as respect to blood of BC patients is controversial (81). MiR-126 urinary levels were found to be enhanced in BC compared to healthy controls (82). Urine miR-146a-5p is significantly increased in high-grade BC (77). Low miR-200c expression correlates with tumor progression in NMIBCs (83). Chen et al. detected 74 miRNAs, of which 33 upregulated and 41 downregulated in BC compared to healthy patients (84). The most interesting are let-7miR, mir-1268, miR-196a, miR-1, miR-100, miR-101, and miR-143 (84). MiR-200 was identified as epithelial–mesenchymal transition regulator in BC cells by targeting Zinc Finger E-Box Binding Homeobox 1 (ZEB1), ZEB2 and Epidermal growth factor receptor (EGFR) (85). Some miRNAs have been associated with hemolysis including miR-451a, miR-16, miR-486-5p, and miR-92a (86). Eissa et al. by screening BC patients with negative cystoscopy, identified miR-96 and miR-210 in BC (87). Sapre et al., by using a panel of 12 miRNA, reduced the cystoscopy rates by 30% by increasing sensitivity and specificity (88). MiR-125b, miR-30b, miR-204, miR-99a, and miR-532-3p were downregulated in BC patient's urine supernatant, with miR-125 levels (95.7% specificity, 59.3% sensitivity) (89). MiR-9, miR-182 and miR-200b correlated with MIBC aggressiveness, recurrence-free and OS (90). MiR-145 distinguishes NMIBCs from non-BCs (91). MiR-144-5p inhibited BC proliferation, affecting CCNE1, CCNE2, CDC25A, PKMYT1 target genes (92). Cell-free urinary miR-99a and miRNA-125b were found to be downregulated

TABLE 1 | Urinary tumor-derived DNAs as biomarkers in BCs.

Urinary tumor-derived DNA	Gene	Application	References
CfDNA	TERT and FGFR3	Recurrence	(66, 67)
	TERT, FGFR3/OTX1	BC diagnosis	(68)
	FGFR3 and PIK3CA	Progression/metastasis	(69)
Histone modifications	HAK20me3	Poor survival	(70)
	H3K27me3	Poor survival	(71)
DNA methylation status	GSTP1 and RARb2 and APC	BC diagnosis	(56)
	TWIST1 and NID2	BC diagnosis	(57)
	SOX-1, IRAK3, and Li-MET	Recurrence	(54)
	POU4F2 and PCDH17	BC diagnosis	(61)
	HS3ST2, SEPTIN9, SLIT2/FGFR3	surveillance, low vs. high risk	(62)
	NID2 and TWIST1	BC diagnosis	(58)
	CFTR, SALL3/TWIST1	BC diagnosis	(59)
	p14ARF, p16INK4A, RASSF1A	BC grade and stage	(63)
	DAPK and APC		

BC, Bladder cancer; CfDNA, circulating-free DNA.

in the urine supernatants of BC patients (sensitivity 86.7%; specificity 81.1%) (93). Urinary levels of miR-618 and miR-1255b-5p in MIBC patients were increased in comparison to controls (94). Multiple miRNA assay shows higher diagnostic performance than single RNA assay (95). By whole genome analysis increased miR-31-5p, miR-191-5p and miR-93-5p levels were identified in the urine of BC patients as compared to controls (96).

Recently, a miRNA profile, identified in urine by next-generation sequencing (NGS) analysis, has been capable to stratify different BC subtypes (97). In NMIBC G1/G2 patients a miR-205-5p upregulation compared to controls was observed. Among NMIBC G3, upregulation of miR-21-5p, miR-106b-3p, mir-486-5p, miR-151a-3p, miR-200c-3p, miR-185-5p, miR-185-5p and miR-224-5p and downregulation of miR-30c-2-5p and miR-10b-5p were observed. In MIBCs, miR-205-5p, miR-451a, miR-25-3p and miR-7-1-5p were upregulated, while miR-30a-5p was downregulated compared to controls (97). The application of NGS have increased the diagnostic accuracy. However results obtained in NGS were only partially overlapping with that obtained by qRT-PCR (98) (**Table 2**).

Long Non Coding RNAs (lncRNAs)

Long non coding RNAs (lncRNAs) regulate gene expression or epigenetic levels. Several findings show lncRNA changes in cancers suggesting a role in the promotion of tumor development and progression (105, 106). The use of lncRNAs as non-invasive BC marker has recently interested (107). Circulating urothelial carcinoma antigen 1 (UCA1) levels in urinary sediments represents a potential diagnostic marker for UC, with 81% sensitivity and 92% specificity (108). Du et al. describe high uc004cox.4 lncRNA level association with poor recurrence-free survival in NMIBCs (102). The retrotrasposome, long interspaced element-1 (LINE-1) has been found to be hypo-methylated and its expression was associated with long recurrence-free and tumor specific survival in BC (109) (**Table 2**).

Messenger RNAs (mRNAs)

Circulating messenger RNAs (mRNAs) were detected in cancer patients, although the majority of circulating mRNAs are degraded by RNases (110). Given their role in intracellular protein translation, their presence reflects the status of intracellular processes and they are potential cancer biomarkers. Urine Ubiquitin Conjugating Enzyme E2 C (UBE2C) mRNA levels were higher in BC patients, compared to normal and hematuria specimens (111). The expression of isoleucine glutamine motif-containing GTAase-activating proteins (IQGAP3) mRNA in urine was found higher in BC than in controls (112). Further analysis of IQGAP3, with respect to tumor invasiveness and grade also yielded a high diagnostic accuracy, suggesting that IQGAP3 can be used to discriminate BC from non-BC patients with hematuria (112).

In regard to mRNAs extracted by exfoliated urinary cells, the Xpert BC Monitor measuring ABL1, corticotropin releasing hormone (CRH), IGF2, uroplakin 1B (UPK1B), annexin A10 (ANXA10) mRNAs by RT-PCR, increased the overall sensitivity over urinary cytology in low-grade and pTa disease (113).

In addition, the presence of carbonic anhydrase 9 (CAIX) splice variant mRNA in the urine, increased the diagnostic performance for BC (90% sensitivity and 72% specificity) (114). The downregulation of N-Myc downstream-regulated gene 2 (NDRG2) mRNA levels in the urine of BC patients correlated with tumor grade and stage (99) (**Table 2**).

Transfer RNA Fragments (tRFs)

Elevated levels of transfer RNA fragments (tRF) are found in cancer (115). tRF are 14-32 base long single-stranded RNA derived from mature o precursor tRNA. They are grouped into 3 classes (tRF-1, −3, and −5) and, depending of their cleavage site within a mature RNA, they are further divided in 5 subclasses. The first identified tRF in NMIBCs was miR720/3007a (101) (**Table 2**).

TABLE 2 | Urinary tumor-derived RNAs as biomarkers in BCs.

Urinary tumor-derived RNAs	RNA/Protein	Application	References
mRNA	CK20, IGF-II	BC diagnosis	(42)
	ABL1, CRH, IGF2, UPK1B and ANXA10	BC diagnosis	(78)
	NDRG2	Tumor grade and stage	(99)
miRNA	miR-146a	BC diagnosis	(77)
	miR-126	BC diagnosis	(82)
	miR-200c	Tumor progression	(83)
	let-7,miR-1268,−196a,−1,−101,−143	BC diagnosis	(84)
	miR-451a,−16,−486,−92a	Hemolysis	(86)
	miR-96,−210	BC diagnosis	(87)
	miR-125b,−30b,−204a,−99a,−532	BC diagnosis	(89)
	miR-9,−182,−200b	aggressiveness, recurrence	(90)
	miR-145	BC diagnosis	(91)
	miR-99a,−125b	BC diagnosis	(93)
	miR-618,−1255b	BC diagnosis	(94)
	miR-21,−106b,−486,−151a,−200c	NMIBC diagnosis	(97)
	−185,−224, 30c-2,−10b		
	miR-205,−451a,−25,−7-1,−30a	MIBC diagnosis	(97)
	miR-31,−191,−93	BC diagnosis	(96)
miRNA/EVs and Exosomes	miR-375,−146a	BC diagnosis	(100)
miRNA/tRF	miR720/3007a	BC diagnosis	(101)
lncRNA	uc004cox.4	Recurrence	(102)
lncRNA/exosomes	HOX-AS, ANRIL, and linc-RoR	BC diagnosis	(103, 104)

BC, Bladder cancer; NMIBC, muscle non-invasive BC; MIBC, muscle invasive; mRNA, messenger RNA; miR, microRNA; EVs, extracellular vesicles; tRF, transfer RNA fragments; lncRNA, Long non-coding RNA.

EXTRACELLULAR VESICLES (EVS) AND EXOSOMES

Extracellular Vesicles (EVs) enrichment was found in BC patient urine. EVs, analyzed by MS based proteomics, demonstrated specific protein and miRNAs pattern in BC patients (116). By using a microarray platform and RT-PCR analysis, miR-375, and miR146a have been found to specifically identify high-grade and low-grade BC, respectively (100). The application of nanowires anchored into a microfluidic substrate will enable the efficiency of EV collection, thus permitting to identify EV harboring miRNAs (117).

Exosomes are membrane vesicles secreted in nearly all body fluids at elevated levels in cancer patients relative to healthy subjects (118, 119). They realize intercellular communication through transferring distinct biologically active molecules (RNAs, DNA, and proteins), thus influencing the therapeutic responses. The HOX transcript antisense RNA (HOTAIR) together with other IncRNA, such as HOX-AS-2, ANRIL, and linc-RoR, were augmented in urinary exosomes from high-grade MIBC patients (103). Loss of HOTAIR expression in BC cells alters the expression of SNA1, TWIST1, ZEB1, ZO1, MMP-1, Laminin Subunit Beta 3 (LAMB3), and Laminin Subunit Gamma 2 (LAMC2) epithelial-to mesenchymal transition genes. Moreover, the tumor-associated calcium-signal transducer 2 (TACSTD2*)* was found in BC exosomes by proteomic analysis (104). EVs can also promote BC progression by delivering the protein EGF-like repeat and discoidin I-like domain-containing protein-3 (120).

Exosomes in urine also contain miRNAs, in particular miR-1224-3p, miR-135b, and miR15b; in particular, miR-126/miR-152 ratio correlated with positive BC diagnosis (121) (**Table 2**).

Although EVs and exosomes represent an interesting source of cancer biomarkers, the lack of accurate isolation and detection methods affects their utilization in practice. In the next future, the development of sensitive capture platforms for exosomes, likely increases their introduction into clinic.

URINARY MICROBIOME

Dysbiosis of urinary microbiome has been suggested to be involved in bladder tumorigenesis. Recently, Wu et al. by analyzing DNA extracted by urine pellets, observed specific enrichment of *Acinetobacter*, *Anaerococcus*, and *Sphingobacterium* in BC cohort as respect to controls (122). Moreover, the increase of *Herbaspirillum*, *Porphyrobacter*, and *Bacteroides* in high-risk BC patients suggested that these genera may represent new potential biomarkers (122).

CONCLUSIONS AND PERSPECTIVES

We provide the state of art into the use of urinary biomarkers as tool to aid diagnosis of BC. Urine cytology, utilized for

decades, shows poor sensitivity, particularly for low-grade tumors. The addition of immunoassay and FISH analysis has provided an additional diagnostic armamentarium to determine which patients may need further evaluation. At present, there are growing evidence toward the use of "Liquid Biopsy" to identify urinary biomarkers such as circulating cell-free DNA, DNA methylation, miRNA, cell-free proteins/peptides and exosomes, useful for discriminating NMIBC from MIBC (123). The potential introduction of "smart toilets" working with a more advanced "nano-sensor" able to detect RNA and proteins in urine is close to reality, more that we think (124). However, now in clinical reality, there is an urgent need to validate the recently discovered extensive proteomic and genomic, epigenomic, transcriptomic and metabolomic data as urinary biomarkers in well-designed multicenter clinical studies (125, 126).

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OPINION
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Circulating Tumor Cells in Renal Cell Carcinoma: Recent Findings and Future Challenges

Matteo Santoni[1†], Alessia Cimadamore[2†], Liang Cheng[3], Antonio Lopez-Beltran[4], Nicola Battelli[1], Francesco Massari[5], Marina Scarpelli[1], Andrea Benedetto Galosi[6], Sergio Bracarda[7] and Rodolfo Montironi[1]*

[1] Oncology Unit, Macerata Hospital, Macerata, Italy, [2] Section of Pathological Anatomy, School of Medicine, United Hospitals, Polytechnic University of the Marche Region, Ancona, Italy, [3] Department of Pathology and Laboratory Medicine, Indiana University School of Medicine, Indianapolis, IN, United States, [4] Department of Pathology and Surgery, Faculty of Medicine, Córdoba, Spain, [5] Division of Oncology, S. Orsola-Malpighi Hospital, Bologna, Italy, [6] Department of Urology, School of Medicine, United Hospitals, Marche Polytechnic University, Ancona, Italy, [7] Medical Oncology, Department of Oncology, Azienda Ospedaliera S. Maria, Terni, Italy

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INTRODUCTION

Renal cell carcinoma (RCC) is the most common tumor of the kidney. After diagnosis, 20–30% of patients will relapse, with a high probability of death from cancer-related causes. The development of non-invasive biomarkers will allow the identification of patients with a high risk of recurrence after radical or partial nephrectomy and will improve the assessment of tumor response to targeted therapy or immunotherapy. The search for non-invasive diagnostic techniques represents one of the most difficult challenges for cancer researchers. The contemporary scenario includes a variety of strategies that share the aim of maximally reducing the impact of the diagnosis on patients' quality of life (QoL). In this context, liquid biopsy offers a promising perspective for cancer diagnosis and monitoring, with several advantages compared to traditional diagnostic procedures (1). Indeed, it can be more frequently performed and allows for better tracking of tumors and mutations over a period of time (1). Moreover, it has been showed that genomic profiles of liquid biopsies can match very closely with the corresponding tumors (2).

The liquid biopsy of circulating tumor cells (CTCs), which belong to the larger family of circulating rare cells (CRC), has been validated and approved by the US Food and Drug Administration (FDA) as a useful prognostic tool in a variety of cancer types (3). This is based not only on the ability of CTCs to be a mirror of tumor heterogeneity but also on the possibility to combine the genetic and transcriptomic status of single CTCs (4) with epigenome analyses (5). Although CTCs have received great attention based on their potential in evaluating the status of localized and metastatic diseases, their clinical implementation is not yet widespread.

In the last decade, several studies have investigated the clinical and pathological significance of CTC numbers and characteristics in patients with urogenital cancers (6, 7). In view of the increasing number of dedicated clinical trials, genitourinary tumors represent the next urgent field of application of molecular diagnostics and drug discovery after gastro-intestinal and thoracic oncology. Among these tumors, the absence of reliable predictive biomarkers in RCC prevents the proper selection of patients who will benefit from any one of the three main drug categories approved for treating this disease: (1) anti-vascular endothelial growth factor (VEGF) monoclonal antibodies (i.e., bevacizumab) or tyrosine kinase inhibitors (i.e., sunitinib, sorafenib, pazopanib, axitinib, cabozantinib, tivozanib, and lenvatinib); (2) immune checkpoint inhibitors [i.e., anti-programmed death(PD)-1 Nivolumab alone or in combination with anti-cytotoxic T-lymphocyte antigen (CTLA)-4]; and (3) Mammalian target of rapamycin (mTOR) inhibitors

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****Correspondence:***
Rodolfo Montironi
r.montironi@univpm.it

† These authors have contributed equally to this work

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(i.e., everolimus and temsirolimus). In this manuscript we describe the emerging data on the role of CTCs in the diagnosis and treatment of RCC, focusing on their future applicability in daily clinical practice.

INTRA- AND INTER-TUMOR HETEROGENEITY IN RCC

Intratumoral heterogeneity—in terms of somatic mutations, chromosome aberrations, and tumor gene expression—is a characteristic feature of clear cell RCC (8–11). These alterations are primarily centered around the *Von Hippel–Lindau (VHL)* gene and include LOH at 3p and epigenetic silencing. *VHL* inactivation is a crucial event in the majority of clear cell RCC and represents the only ubiquitous event, in contrast from the other genetic and/or epigenetic aberrations reported which are just subclonal (12–14). In this setting, Xu et al. firstly revealed by single-cell exome sequencing in a single patient that <30% of gene alterations are common to multiple cells within tumor tissue, whilst the majority are only cell-specific (15). In the same view, activated drug target pathways have been shown to be considerably variable within the same tumor and between primary RCC and lung metastases (16).

The rate of patients with late-relapsing disease (>5 y after radical or partial nephrectomy) (17–19) and the common intravenous tumor embolization (20) are just two of a series of clear signs suggesting that CTCs may provide fundamental information to optimize RCC diagnosis and evaluate tumor response to therapy and progression.

CTC ISOLATION AND CHARACTERIZATION IN RCC

The collection, identification, enrichment, and analysis of CTC require the use of different methods, including the following: (1) Epithelial or non-epithelial marker-dependent isolation; (2) RT-PCR-based methods; (3) and morphological- and cell size-based detection (21).

The first method consists of the detection of CTCs through epithelial markers, such as the epithelial cell adhesion molecule (EpCAM, **Figure 1**). EpCAM is a transmembrane glycoprotein involved in cell signaling, migration, proliferation, and differentiation (22). The number of CTCs that can be isolated by EpCAM is usually low (23). This is based on the biological behavior of clear cell RCC, which often transdifferentiates through a process named "epithelial-to-mesenchymal transition (EMT)," a morphological transformation that is phenotypic of RCC cells (24, 25) and that leads to the loss of their epithelial antigens and the acquisition of mesenchymal features (i.e., vimentin expression). Detecting EMT markers on CTCs provides fundamental information on the status of the disease, considering the straight association between EMT and the prognosis of RCC patients (24) as well as its role in the acquisition of invasive properties and resistance to anti-VEGF TKIs (24). More recently, antibodies directed against membrane carbonic anhydrase 9 (CA9/CAIX) and CD147 [a widely expressed membrane glycoprotein involved in matrix metalloproteinase induction, cell adhesion and T cell activation (26)] have been developed to increase the number of selected CTCs in RCC patients (**Figure 1**). Indeed, Liu et al. reported that while EpCAM was found only in about 18% of clear cell RCC tumors, CAIX, and CD147 were present in more than 97% of samples (27).

The second method is based on the RT-PCR approach. The three main targets of this technique are *CAIX*, *VHL* and *Cadherin-6 (CDH-6)* (**Figure 1**). *VHL* gene alterations detected in tumor samples have reported concordance with those identified on peripheral blood in about 75% of cases (28). On the other hand, *CDH-6* gene expression by RT-PCR has been observed in 45% of clear cell RCC blood samples (29).

The third method is built upon size-based blood filtration combined with morphological and genetic analyses. The need to associate different techniques derives from the evidence that cytomorphological classification alone is not sufficient to detect CTCs in RCC patients (30). Interestingly, the use of these combined methods has enabled the detection of the presence of circulating clusters with a core of cancer cells surrounded by an external coating of endothelial cells (31).

The survival mechanisms underlying the circulation and migration of CTCs depend on multiple factors. Their biological characteristics, genetic alterations, epithelial mesenchymal transition, and cancer stem cell properties are internal factors that influence their survival. Great importance is now also being attributed to the external factors in the bloodstream microenvironment, consisting of platelets, immune cells, cytokines, and circulating tumor microemboli (CTM) (32).

CTM are composed by cell clusters, from two to more than 50 CTCs, together with leukocytes, cancer-associated fibroblasts, endothelial cells, and platelets (33). CTM demonstrated high metastatic potential by inhibiting apoptosis, promoting cell clonal proliferation, conferring resistance to shear stress, and protecting the innermost cells from immune surveillance from the identification of NK cells and their cytolytic activity (34). Testing the expression of Ki-67 on CTCs and CTMs has revealed a sharp contrast between CTCs (Ki-67 positive) and CTMs (all negative for Ki-67), even in patients with Ki67 (+) CTCs (35). This gives rise to the hypothesis that CTMs, in comparison to CTCs, may have the capability to remain inactive and "dormant" for long time periods, inhibiting apoptosis and cell destruction in the bloodstream. They may also have the capacity to confer resistance to cytotoxic drugs for cells within CTM, as observed by Hou et al. in patients with non-small–cell lung cancer (36). Colorectal cancer patients with CTMs in their blood have a shorter survival period than patients with only CTCs detected (37). Similar results were obtained in blood samples from gastric cancer patients, in which the presence of CTMs was an independent predictor of shorter PFS and OS in stage IV patients in multivariate analysis (38). Detection of CTM has emerged as a valuable tool to improve the prognostic significance of liquid biopsy (39) (**Figure 2**). Comparing the epigenomes of CTC clusters and single CTCs, Gkountela et al. demonstrated that CTC clusters are enriched with binding sites for several key transcription factors (TF)—such as OCT4, NANOG, and SOX2—that confer to clusters stem cell features, whereas single

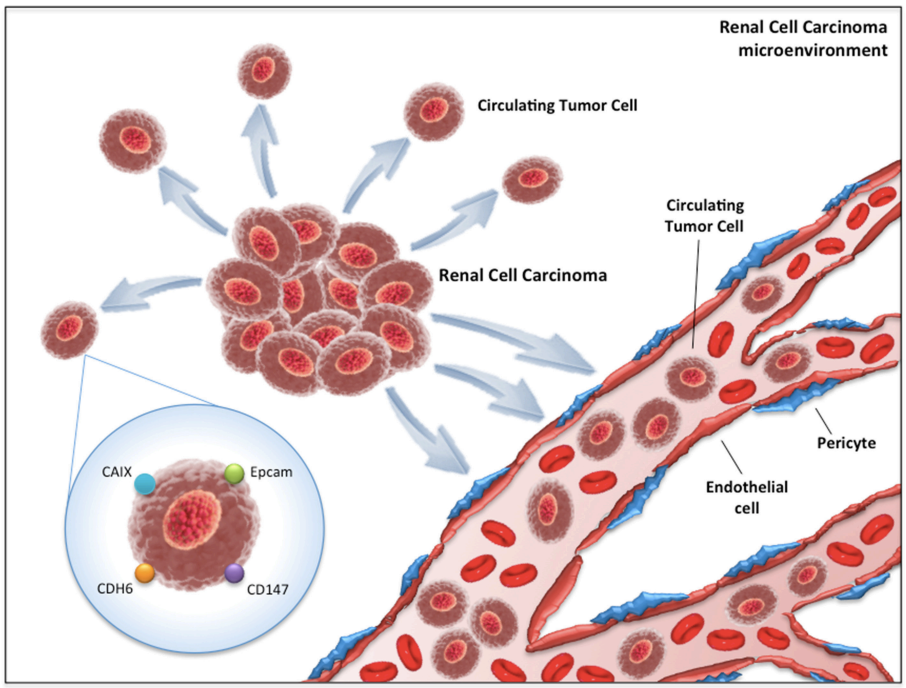

FIGURE 1 | Circulating tumor cells (CTCs) in renal cell carcinoma microenvironment. CAIX, Carbonic anhydrase 9; CDH6, Cadherin-6; EpCAM, Epithelial cell adhesion molecule.

CTC have enrichment in different sets of TF. Moreover, stem cell TF binding is lost when clusters are dissociated into single cells via Na+/K+ ATPase inhibitors such as digitoxin. In xenograft mouse models generated using *ex vivo* expanded CTC lines, administration of digitoxin significantly inhibited the capacity of CTC clusters to generate metastases (40, 41). These results open an area of new research and new therapeutic targets.

NEW METHODS OF DETECTION

New methods of isolation and analysis such as subtraction enrichment (SE) combined with immunostaining-fluorescence *in situ* hybridization (iFISH) are being developed to better characterize CTCs. With this method, independent of cell size variation, and free of hypotonic damage as well as anti-EpCAM perturbing, it is possible to karyotype chromosome ploidy of CTCs and phenotype multi-protein expression. Among different cancers, an aneuploid chromosome 8 (tetraploid or polyploid) identified a positive CTC (42–44). This allows for efficient enrichment, identification, and characterization of both large and small size CTCs as well as CTM in various biofluid samples including cerebrospinal fluid (CSF). Unlike conventional methodologies, SE-iFISH enables the characterization of different heterogeneous CTC subtypes classified by both chromosome ploidy and the expression of biomarkers (44). Broncy et al. recently applied single-cell genetic analysis after isolation by SizE of Tumor/Trophoblastic cells (ISET®) in order to assess the specificity and sensitivity of cytopathology. They performed single cell analysis targeted to VHL mutations in all the 205 CRCs identified by cytopathology in the blood of 29 ccRCC patients after ISET filtration. They found a complete (100%) specificity of the cytopathological approach in the identification of circulating cancer cells (CCC) with a low sensitivity (35%) compared to genetic analysis (72%) (45, 46).

A novel straight microfluidic chip technology to focus and capture CTCs has been applied in head and neck cancer patients. The microchip is designed based on inertial migration of cells in a straight microchannel and allow to isolate single CTCs, CTCs clusters, and CTM by a size-based method, with high recovery efficiencies and low background cell contamination (47, 48).

FUTURE PERSPECTIVES

In future years, technical advances should aim to isolate a greater number of CTCs in metastatic patients than in patients with localized disease and to find the same mutations present in the correspondent histologic sample or, especially, in the metastatic cohort. In our opinion, this could be realized by improving the connection between cytomorphological and genetic analyses, thus overcoming the limits of present techniques, which can be challenging and time-consuming. Indeed, the specificity of CAIX and CDH-6 for CTCs in RCC patients is poor and could be increased only by the design of studies focused on comparing CTCs isolated from patients with clear cell and from patients with benign kidney diseases or healthy volunteers.

We hope that in future years whole genome, transcriptome, and proteome analyses of single cells could lead to an increase in our knowledge of tumor heterogeneity and acquired drug resistance. In the localized RCC setting, CTCs could have potential as a surveillance biomarker for disease recurrence. Earlier detection of metastatic RCC, prior to the onset of symptoms, may lead to improved clinical outcomes. In patients

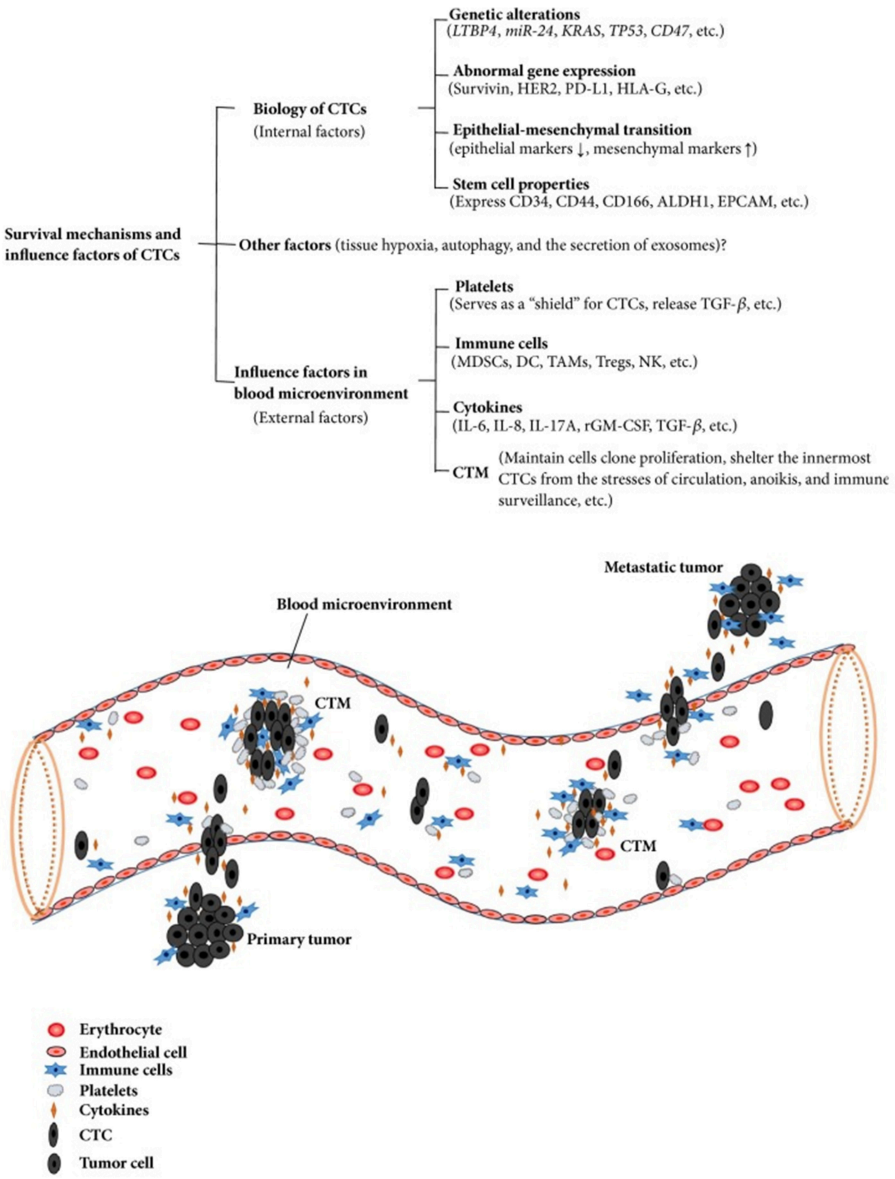

FIGURE 2 | Survival mechanisms and influence factors of CTCs. Copyright © 2018 Wang et al. (32). This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

with metastatic disease, CTC analysis could be used to select patients for biomarker-guided clinical trials. As in colorectal cancers, the mutational profile of metastatic RCC could evolve after treatment progression, developing an acquired resistance to therapy potentially investigable in a non-invasive way with CTCs. Moreover, the introduction of data on CTCs within the TNM classification represents another step forward on the route of personalized medicine for RCC patients.

CONCLUSIONS

RCC may benefit from the development of non-invasive and reliable biomarkers, enabling early and timely personalized treatment changes. The introduction of CTC analysis within daily clinical practice for patients with RCC seems still far at the moment. However, the advances obtained in the last 5 years in isolating and analyzing CTCs bring optimism about the future therapeutic landscape in RCC patients.

AUTHOR CONTRIBUTIONS

RM and MSc: conception and design; MSa, AC, and FM: drafting the manuscript; NB and ABG: review of the literature; LC, SB, and AL-B: critical revision of the manuscript.

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Emerging Prognostic Biomarkers in Testicular Germ Cell Tumors: Looking Beyond Established Practice

Michal Chovanec[1,2], *Costantine Albany*[2], *Michal Mego*[1], *Rodolfo Montironi*[3], *Alessia Cimadamore*[3] *and Liang Cheng*[4,5]*

[1] 2nd Department of Oncology, Faculty of Medicine, Comenius University and National Cancer Institute, Bratislava, Slovakia, [2] Division of Hematology and Oncology, Indiana University Melvin and Bren Simon Cancer Center, Indianapolis, IN, United States, [3] Section of Pathological Anatomy, Polytechnic University of the Marche Region, School of Medicine, United Hospitals, Ancona, Italy, [4] Department of Pathology and Laboratory Medicine, Indiana University School of Medicine, Indianapolis, IN, United States, [5] Department of Urology, Indiana University School of Medicine, Indianapolis, IN, United States

Testicular germ cell tumors are unique among solid cancers. Historically, this disease was deadly if progressed beyond the stage I. The implementation of cisplatin-based chemotherapy regimens has drastically changed the clinical outcome of metastatic testicular cancer. Several biomarkers were established to refine the prognosis by International Germ Cell Collaborative Group in 1997. Among these, the most significant were primary tumor site; metastatic sites, such as non-pulmonary visceral metastases; and the amplitude of serum tumor markers α-fetoprotein, β-chorionic gonadotropin, and lactate dehydrogenase. Since then, oncology has experienced discoveries of various molecular biomarkers to further refine the prognosis and treatment of malignancies. However, the ability to predict the prognosis and treatment response in germ cell tumors did not improve for many years. Clinical trials with novel targeting agents that were conducted in refractory germ cell tumor patients have proven to have negative outcomes. With the recent advances and developments, novel biomarkers emerge in the field of germ cell tumor oncology. This review article aims to summarize the current knowledge in the research of novel prognostic biomarkers in testicular germ cell tumors.

Keywords: testis, testicular germ cell tumors, molecular genetics, biomarkers, liquid biopsy

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***Correspondence:**
Liang Cheng
liang_cheng@yahoo.com

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INTRODUCTION

Testicular germ cell tumors (GCT) are unique in terms of molecular landscape, pathogenesis, clinical presentation, and response to chemotherapy (1). The exceptional position of GCT among the solid cancers can be perhaps attributed to their developmental origin in primordial germ cells. While the cure rate of patients with metastatic disease exceeds 80% (2), the ones failing the initial and salvage chemotherapy die of their disease in young age. About 40–80% of patients with relapsed GCT fail the salvage chemotherapy, resulting in the loss of 35 years of life on average (3–5). The utility of biomarkers to risk-stratify the treatment is well-established in GCT. Markers of the risk of relapse in the stage I disease, such as tumor size of >4 cm and rete testis invasion for seminoma, and lymphovascular invasion and predominance of embryonal carcinoma for non-seminoma, are currently used to risk-stratify the patients for surveillance or adjuvant treatment (6–9). International Germ Cell Cancer Collaborative Group (IGCCCG) presented the risk-stratification model for metastatic disease in 1997 using biomarkers such as primary tumor

site, metastatic sites, the amplitude of serum α-fetoprotein (AFP), β-chorionic gonadotropin (HCG), and lactate dehydrogenase (LDH) (10). These criteria are based on patient series collected retrospectively between 1975 and 1990. Since then, the treatment strategy was optimized, and outcomes improved as reported from high volume centers (2, 11, 12). Further refining of IGCCCG criteria is expected soon in the updated version of the IGCCCG classification (**Figure 1**).

New reports on novel biomarkers are scarce since the introduction of the commonly used GCT biomarkers over three decades ago. The utility of novel molecular biomarkers in numerous solid cancers has significantly moved the advancement of oncology. Malignancies, such as lung cancer, melanoma, and kidney cancer, were previously considered untreatable, but now the array of molecular markers renders these diseases treatable with targeting agents ultimately prolonging lives of patients with incurable cancer (13, 14). Such advancement seemingly evades testicular GCT due to lack of known drugable targets. While the overall cure rate of GCT patients is excellent, ones refractory to standard chemotherapy lack the possibility to receive novel effective treatments and their prognosis is dismal. The biology of GCTs is unique, therefore translational research to uncover the biological implications is essential in the pursuit of treatment targets that may improve the prognosis of platinum refractory GCT patients. This article aims to summarize the current knowledge on the emerging biomarkers in GCT.

BRIEF OVERVIEW OF MOLECULAR LANDSCAPE IN TESTICULAR GERM CELL TUMORS

Understanding why we lack a significant predictive biomarker in GCT requires a look into their molecular landscape and developmental origins. The origin of GCT particularly show how different their biology is compared to other solid cancers. The data from The Cancer Genome Atlas (TCGA) show a rather quiet mutational landscape in GCT compared to other solid tumors (15).

Several genomewide studies suggested driver mutations in only three genes (*KIT*, *KRAS,* and *NRAS*) in 4–31% of seminoma, and up to 14% of non-seminoma patients (16–19). Since these mutations were discovered in a minority of patients, a single universal mutational driver is not a feasible explanation in the development of GCT. Rather, a polygenic nature of testicular cancer was proposed, where the number of low frequency susceptibility genes (up to 50 risk loci reported until present) seems to produce an increased risk for the GCT (20). A recent paper by Shen et al. conducted a comprehensive molecular characterization of available tissue from 137 GCT patients. The authors confirmed findings of previously known mutated genes (*KIT, KRAS,* and *NRAS*) and provided yet additional evidence of low mutational burden with frequency of 0.5 mutations per megabase (15).

Despite the unimpressive mutational characteristics, GCT share a unique epigenetic landscape. GCT subtypes are an example of developmental processes from pluripotent embryonic stem cells toward certain degrees of differentiation to somatic tissues. The mapping of GCT methylome is perhaps the most comprehensively assessed to this date. The global DNA-methylation status clearly correlates with the state of differentiation in the histological GCT subtypes. Seminomas, which show the lowest degree of differentiation are typically unmethylated or severely hypomethylated tumors. Embryonal carcinomas show low to intermediate levels of global DNA methylation and well-differentiated yolk sac tumors, and teratomas show high levels of DNA methylation. Thus, the significant histological variability complies with the epigenetic heterogeneity. These findings also comply with the epigenetic landscape of healthy tissues where differentiated somatic tissues show hypermethylated pattern (21–23). Non-CpG methylation, acetylation, and methylation of histones are also mechanisms likely involved in the biology of GCT. They are, however, poorly understood in present time. microRNA (miR) signaling research on the other hand seems to provide promising results toward increasing the knowledge about molecular biology of GCT. While the miR signaling is generally complex and is a subject of innumerous interactions, the clusters of miR discussed later in this paper provide a significant biomarker potency to further refine the management of GCT.

The unique germline origin of GCT is underlined with the overexpression of markers of pluripotency such as NANOG, OCT3/4 or a tissue stem cell factor KIT and its' ligand (24–30). The expressions of these markers have been linked to epigenetic regulation with DNA methylation and histone acetylation (30–34).

EMERGING BIOMARKERS IN GERM CELL TUMORS

Clinical Biomarkers

IGCCCG vol. 2 will bring a long-awaited update for risk stratification of treatment of GCT based on clinical characteristics. The advent of clinical biomarkers is rather slow since the original publication of the IGCCCG criteria. Several other risk assessment criteria were proposed that considered a more detailed look into clinical characteristics in GCT patients. Adra et al. published results of their retrospective analysis of 273 patients with a poor risk disease treated at Indiana University (35). Primary mediastinal non-seminoma (PMNSGCT), brain metastases and increasing age were significant predictors of mortality (HR = 4.63, 3.30, and 1.06, respectively). Multiple criteria for a poor risk disease carried a significantly worse prognosis compared to a single criterion (35).

Necchi et al. proposed an improved model for intermediate risk patients in the two-institutional initiative using PMNSGCT, brain metastases, pulmonary metastases, and age at diagnosis as risk factors. According to the results, a number of intermediate risk patients would suffice from treatment with BEPx3, whereas the current standard remains BEPx4 (11). While the refining of prognosis based on clinical criteria may have reached its limits, authors from Memorial Sloan Kettering Cancer

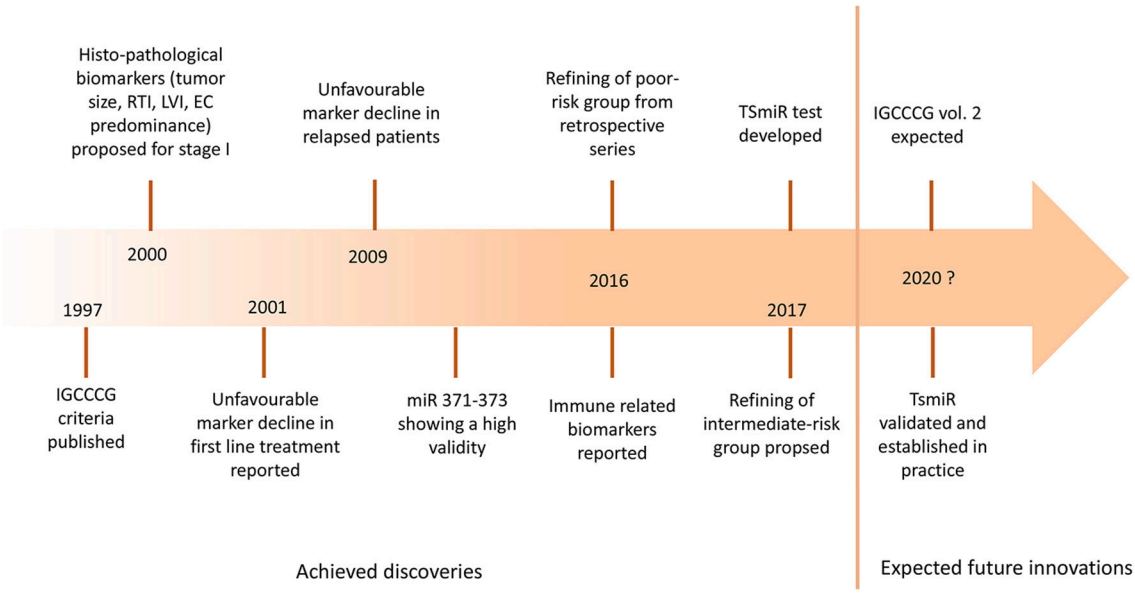

FIGURE 1 | The landmarks of prognostic biomarkers in germ cell tumors. IGCCCG, International Germ Cell Cancer Consensus Group; RTI, rete testis invasion; LVI, lymphovascular invasion; EC, embryonal carcinoma; miR, microRNA; TSmiR, targeted serum microRNA test.

Center have proposed a novel prognostic marker based on a marker decline after the first course of chemotherapy (36). Patients who had unfavorable (slower) marker decline after the initiation if chemotherapy had reportedly worse outcomes compared to patients with favorable marker decline (72 vs. 95% for 2-year overall survival; $P < 0.01$) (36). These findings were subsequently replicated in independent studies (37, 38).

Furthermore, the prognostic significance of tumor marker decline was reported also in patients with relapse (39–41). Fizazi et al. conducted a randomized phase III study in poor risk GCT. Patients receiving first cycle of BEP had an assessment of serum markers prior to second cycle and ones with an unfavorable decline were randomized to receive either remaining three cycles of standard BEP or dose-intensified chemotherapy regimen. Based on this biomarker-based strategy, a significant advantage was reported for 5-year progression-free survival (PFS) (60 vs. 48%, $P = 0.037$), but not for 5-year overall survival (OS) (70 vs. 61%, $P = 0.012$) (42, 43). Interestingly, in cases of progression, patients from this study relapsed predominantly in brain (54% of all relapses) (44).

Molecular Biomarkers From Immunohistochemistry Studies

Immunohistochemistry studies have started to emerge in recent years to supplement the clinical biomarkers in predicting the prognosis of GCT. The higher expression of DNA repair enzyme poly (ADP-ribose) polymerase (PARP) was reported in GCT tissue compared to normal testicular tissue. However, no association with clinical characteristics nor the survival difference was reported in regard to levels of expression (45).

Kalavska et al. published two studies examining the prognostic value of carbonic anhydrase nine assessed from plasma and from tumor tissue (46, 47). Levels of this marker of hypoxia and aggressive tumor behavior correlated in plasma and in tumor tissue. High expression in tumor was associated with shorter PFS; however, the clinically more useful utility of plasmatic assessment failed to be prognostic in GCT (46, 47). The hepatocyte growth factor (HGF) and its receptor c-MET were investigated by immunohistochemistry in tumors and in cell-line culture. c-MET is a known proto-oncogene involved in tumor progression and metastasis. Authors of this study reported an abundant immunohistochemical expression in both seminomas and non-seminomas, particularly in epithelial structures of well-differentiated subtypes such as teratomas, yolk sac tumors, and choriocarcinomas. Upon the activation of c-MET in an NT2 cell line (embryonal carcinoma), the cells acquired a more robust ability to proliferate, migrate, and invade. This may create the rationale for further research; however, the clinical significance of this finding is currently unknown (48).

Immune-Related Biomarkers

The discovery of novel immune-related biomarkers, programmed-death receptor and its ligand (PD-1 and PD-L1) in various cancers, led to a confirmation of active PD-1/PD-L1 signaling also in GCT by Fankhauser et al. (49). The authors conducted an immunohistochemistry study and showed a frequent PD-L1 expression in 479 GCT tissue samples. Both seminomas and non-seminomas exhibited a significant expression of PD-L1 (in 73% and 64% of patients, respectively) (49).

Another research team led by Mardiak et al. performed a similar study and scored the PD-L1 expression semi-quantitatively with multiplicative quick score. The scores were correlated with clinical outcome. Patients with low levels of PD-L1 expression had significantly better PFS (HR = 0.40; $P = 0.008$) and OS (HR = 0.43; $P = 0.040$) (50). Furthermore, the expression of PD-L1 on tumor infiltrating lymphocytes (TIL) proved to be highly predictive of outcome in a reverse manner. Patients with high PD-L1 expression on TIL had significantly better prognosis than patients with low PD-L1 TIL (51). The prognostic significance of TIL was earlier reported by Bols et al., who also performed the phenotyping of immune-cell infiltrates (52). However, the abundant expression of PD-L1 does not seem to be predictive of response to treatment with immune-checkpoint inhibitors.

A phase II study with anti-PD1 agent pembrolizumab provided data about insufficient anti-tumor activity in refractory patients with GCT (53). While several case reports documented possible responses to immune-check point inhibitor, these are likely due to concomitant treatment with chemotherapy (54–56). Another phase II study with anti-PD-L1 agent avelumab is currently ongoing, which will shed more light on single agent immunotherapy in refractory GCT (NCT03403777).

Currently, there is a level of uncertainty in predicting response according to PD-L1 expression levels. While several cancer types have proven to be sensitive to PD-1/PD-L1 blockade based on PD-L1 expression, PD-L1 negative tumors were described to respond to such treatment as well. On the other hand, the expression of PD-L1 in tumor and TIL in GCT signifies a vivid immunogenic microenvironment but fails to respond to immunotherapy according to our present knowledge. As such, PD-1/PD-L1 axis seems to be only a part of the involved immune machinery and we are lacking a deeper understanding. Shen et al. recently published findings of comprehensive molecular characterization of GCT and did not discover a significant neoantigen signal in GCT, thus the insufficient activity of immune check-point inhibitors in GCT may be partly explained by this fact and the presence of very low mutational load (15).

Two independent studies published simultaneously examined the role of a simple marker of proinflammatory macroenvironment, a systemic-immune infiltration index (SII) (57, 58). SII is calculated from total counts of neutrophils, lymphocytes, and platelets. Fankhauser et al. reported numerous markers associated with poor prognosis in GCT, including low hemoglobin and albumin, high leukocytes, neutrophils, CRP, neutrophil to lymphocyte ratio, and SII (58). At the same time, our study showed that high SII was associated with poor prognosis in two independent cohorts of GCT patients. We also evaluated a combined prognostic value of SII and PD-L1 expression on TIL. As a result, we identified patients who never experienced death nor a relapse if they exhibited low SII and high PD-L1 on TIL (57). Both studies reported the prognostic significance of SII being independent from the standard IGCCCG risk criteria. SII can be easily calculated from complete blood count performed prior to treatment and offers a simple tool to predict outcome in metastatic GCT. Poor prognosis in patients exhibiting high levels of SII also suggests that proinflammatory pathways likely unleashed by an aggressive tumor microenvironment may point to an unsuccessful struggle of the host immune system to overcome the tumor growth. Furthermore, signaling of proinflammatory cytokines, such as IFN-α2, IL-2Rα, or IL-16, was reported to be associated with poor risk clinical characteristics and inferior survival in GCT patients (59).

Nilius et al. recently reported that high expression of β-1,4-galactosyltransferase-I (B4GALT1) in peripheral T-lymphocytes is a marker of lower risk of relapse in GCT patients treated with salvage high-dose chemotherapy and peripheral stem cell transplant (HR = 0.66; 95% CI 0.45–0.97; $P = 0.02$) (60). T-cells were collected before the high-dose chemotherapy using the non-myeloablative chemotherapy and granulocyte growth factor (60). B4GALT1 is important for interaction and adhesion of immune cells and its role in disease control in stage I lung cancer has been established (61). This study supported their hypothesis of the importance of activated peripheral T cells in *in vitro* experiments by lectin stimulation of mononuclear cells with Concavalin A. As a result, B4GALT1 was upregulated, particularly in $CD4^+$ cells and an antiinflammatory cytokine IL10 was significantly expressed. Interestingly, higher levels of IL10 from patient T cells were also associated with better outcome in GCT (60). Activated T cells, thus, seem to play an important role in cancer control.

Liquid Biopsies and Epigenetic Biomarkers

Sensitive and specific biomarkers indicating the presence of cancer that are assessed from peripheral blood represent an attractive and convenient approach in the diagnosis malignancies. Researchers recently published an array of articles showing that certain clusters of miR are highly informative of the presence of viable cancer in GCT patients (62–70). Serum examination for miR371-373 showed sensitivity of 98–100%, exceeding the sensitivity of the commonly used serum tumor markers AFP and HCG (71, 72). The targeted serum miRNA test (TSmiR) was developed and it seems to be very effective in predicting viable GCT after orchiectomy in clinical stage I patients or after chemotherapy in metastatic disease (72). The clinical utility of the TSmiR test is therefore very promising and clinicians may be expecting this novel biomarker to be implemented in the common practice in the near future (73). One possible utility of these highly sensitive miRNAs seems to be predicting the presence of a microscopic disease in clinical stage I GCTs. As such, these are likely to change the outlook over adjuvant treatment vs. surveillance. Another valuable input would be predicting the presence of viable cancer in post-chemotherapy residual masses, thus refining the need to perform often difficult surgeries in this setting. However, TSmiR does not identify teratoma components which still represent a diagnostic dilemma in the residual disease. Establishing the novel clinical practice stems from our ability to validate the utility of TSmiR in larger prospective cohorts of patients.

Majewski et al. assessed five patients with stage I seminoma and evaluated a possible role of liquid biopsy in identifying the presence of the tumor. The study showed promising

results and identified candidate genes in whole blood prior to orchiectomy. This series is, however, too small to draw any conclusions and a larger study is suggested for validation (74).

A global DNA hypermethylation was proposed as one of the acting mechanisms in cisplatin resistance, the most frustrating challenge for oncologists treating GCT patients. *In vitro* epigenetic studies suggested that treatment with DNA demethylating agents may restore the sensitivity to cisplatin (75–77). In a study by Beyrouthy et al., a GCT cell-line treated with decitabine was resensitized to cisplatin (78). Based on these findings, Albany et al. performed a series of experiments in cell-line culture and patient-derived xenograft mouse model using a second-generation inhibitor of DNA-methyltransferase guadecitabine. Upon treatment of platinum resistant xenografts, a significant growth inhibition and even complete tumor regression was registered (79). An ongoing phase I trial using guadecitabine in combination with cisplatin in refractory GCT will shed more light on clinical significance of these promising findings (NCT02429466).

CONCLUSION

The investigation for biomarkers in testicular cancer has been insufficient in the past, but with emerging data our knowledge it is built up with an increasing consistency. Such consistency is essential to generate experimental data and perform laboratory research which will ultimately lead to development of novel drugs with a promise to overcome the resistance to cisplatin.

AUTHOR CONTRIBUTIONS

MC and LC contributed to conception and design. MC drafted the manuscript. CA, MM, RM, and AC contributed critical revision of the manuscript.

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Emerging Biomarkers in Bladder Cancer Identified by Network Analysis of Transcriptomic Data

Matteo Giulietti[1]*, *Giulia Occhipinti*[1], *Alessandra Righetti*[1], *Massimo Bracci*[2], *Alessandro Conti*[3], *Annamaria Ruzzo*[4], *Elisabetta Cerigioni*[5], *Tiziana Cacciamani*[6], *Giovanni Principato*[1] and *Francesco Piva*[1]

[1] Department of Specialistic Clinical and Odontostomatological Sciences, Polytechnic University of Marche, Ancona, Italy, [2] Department of Clinical and Molecular Sciences, Polytechnic University of Marche, Ancona, Italy, [3] Department of Urology, Bressanone/Brixen Hospital, Bressanone, Italy, [4] Department of Biomolecular Sciences, University of Urbino "Carlo Bo", Fano, Italy, [5] Unit of Pediatric and Specialistic Surgery, United Hospitals, "G.Salesi", Ancona, Italy, [6] Department of Life and Environmental Science, Polytechnic University of Marche, Ancona, Italy

Bladder cancer is a very common malignancy. Although new treatment strategies have been developed, the identification of new therapeutic targets and reliable diagnostic/prognostic biomarkers for bladder cancer remains a priority. Generally, they are found among differentially expressed genes between patients and healthy subjects or among patients with different tumor stages. However, the classical approach includes processing these data taking into consideration only the expression of each single gene regardless of the expression of other genes. These complex gene interaction networks can be revealed by a recently developed systems biology approach called Weighted Gene Co-expression Network Analysis (WGCNA). It takes into account the expression of all genes assessed in an experiment in order to reveal the clusters of co-expressed genes (modules) that, very probably, are also co-regulated. If some genes are co-expressed in controls but not in pathological samples, it can be hypothesized that a regulatory mechanism was altered and that it could be the cause or the effect of the disease. Therefore, genes within these modules could play a role in cancer and thus be considered as potential therapeutic targets or diagnostic/prognostic biomarkers. Here, we have reviewed all the studies where WGCNA has been applied to gene expression data from bladder cancer patients. We have shown the importance of this new approach in identifying candidate biomarkers and therapeutic targets. They include both genes and miRNAs and some of them have already been identified in the literature to have a role in bladder cancer initiation, progression, metastasis, and patient survival.

Keywords: WGCNA, bladder cancer, tumor biomarkers, gene expression, heterogeneity

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Simona Di Francesco,
Independent Researcher, Chieti, Italy
Francesco Massari,
Azienda Ospedaliera Universitaria Integrata Verona, Italy

***Correspondence:**
Matteo Giulietti
m.giulietti@univpm.it

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INTRODUCTION

Bladder cancer (BCa) is the ninth most prevalent malignant disease globally, with more than 400,000 new cases diagnosed each year, especially in males and elderly, and the thirteenth most common cause of cancer death. Five-year survival rate for early stage BCa patients reaches 95.7%, while for metastatic patients it is just 5% (1). Recent advancements in therapy include immunotherapy with PD-1 antibody pembrolizumab and the PD-L1 antibody atezolizumab

which have yielded better results compared to chemotherapy (2). The most common BCa type is transitional cell carcinoma, also called urothelial carcinoma, while squamous cell carcinoma and adenocarcinomas are rare. For the majority of new BCa patients, non-muscle-invasive BCa (NMIBC) is diagnosed. This low-grade tumor often recurs and in about 20% of cases it progresses to high grade, i.e., to the muscle-invasive BCa (MIBC) which is more likely to develop metastases (3). In addition, molecular data, including chromosomal aberrations, mutation rates, presence of mutated tumor suppressor genes, gene, and miRNA expression levels have led to the definition of specific molecular subtypes. This depicts BCa as a molecularly and clinicopathologically heterogeneous disease. Diagnosis can be based on cystoscopy, microscopy, voided urinary cytology, blood detection in the urine and assessment of urine-based tumor markers, such as complement factor H-related protein (BTA test) and nuclear matrix protein 22 (NMP22) (4). However, the sensitivity and specificity of these markers decrease in the presence of inflammatory cells and other contaminating cells in the sample (5). Therefore, the identification of new biomarkers could improve diagnostic or prognostic performance of BCa tests. Also small extracellular vesicles released by BCa are currently being investigated, since they are known to be involved in cancer growth, progression and metastatic spread and the molecules contained in these vesicles may be potential BCa biomarkers (6, 7).

An effective method to discover biomarkers is transcriptome profiling, by microarrays or more recently by RNA-seq, that allows the determination of differentially expressed genes and non-coding RNAs under different conditions. For example, the comparison of the expression levels of thousands of genes in healthy vs. cancer tissue samples, or among different tumor stages or tumors under different treatments, has led to the identification of several differentially expressed genes. They could represent candidate therapeutic targets or biomarkers for tumor onset, progression, or prognosis. However, over the past few years, a major drawback has emerged regarding differential expression analysis. In particular, differentially expressed genes are treated individually and this does not allow the identification of co-regulation mechanisms among them. Highlighting these gene interactions is important, because they are often altered in complex genetic disorders like cancer (8–10). This emerging systems biology method focuses on the analysis of gene regulation alterations allowing a better understanding of cancer onset and its progression and the identification of critical cancer driver genes. Moreover, these clusters of co-expressed genes, that constitute the regions of a complex gene network, often correspond to cellular pathways (8–10).

Currently, a widely used approach to process gene expression data and investigate network alterations is the Weighted Gene Co-expression Network Analysis (WGCNA), that draws gene networks where the connections among pairs of genes are identified and weighted based on their correlated expression levels across multiple samples (11). Briefly, after processing the expression profiles into weighted connections, WGCNA can identify the network topology and, by using the topological overlap dissimilarity as the measure of distance among genes, it allows the identification of sub-networks, called modules [for further details see (12)]. Therefore, only highly co-expressed genes (i.e., connected with strong weights in the network) can compose the gene modules. It is also possible to relate these modules to clinical traits of interest. For example, regarding the comparison of two distinct networks deriving from tumor and normal gene expression data, WGCNA can identify the modules and genes belonging to them that reflect the regulatory alterations related to transcriptional changes. In particular, the most interconnected genes in a module (hub genes) are often functionally important and thus they could play a key role in cancer and represent candidate diagnostic and prognostic biomarkers or potential therapeutic targets (13–15).

EMERGING BCA BIOMARKERS IDENTIFIED BY THE WGCNA METHOD

In this review, we focused on all studies where the WGCNA method was applied to analyze gene expression data deriving from BCa samples (**Table 1**).

Recently, WGCNA was applied on publicly available microarray gene expression data deriving from 93 BCa patients in order to identify genes related to different tumor stages (Ta, T1, T2, T3, and T4) and therefore to suggest potential prognostic biomarkers (16). A network module was highly correlated with tumor progression and further processing was performed in order to identify which cellular pathways were represented by the module genes. In general, since not all genes of a pathway are present in a module, the term "enrichment" is used to indicate how significantly a pathway overlaps with a module. A functional enrichment analysis is usually performed on a list of genes in order to reveal these enriched pathways. The identified module was significantly enriched in genes belonging to important biological processes (**Table 1**). Within this module, four hub genes were identified: COL3A1, COL5A2, FBN1, and POSTN and, by using independent expression datasets, the COL3A1 (Collagen type III α1 chain) gene was validated as both a diagnostic and prognostic biomarker. In fact, it was upregulated in BCa tissues compared to normal ones and its high expression strongly correlated with tumor progression, shorter overall survival (OS) and disease-free survival (DFS) times. While there are few literature data about COL5A2 and FBN1 genes, the role of POSTN (periostin) in BCa has been widely investigated. In particular, high grade BCa showed very low levels of POSTN gene and the artificial restoration of its expression suppressed cell invasiveness and metastasis (22). It was also shown that the decrease of invasiveness and the inhibition of the epithelial-to-mesenchymal transition (EMT) were due to bladder-specific upregulation of the E-cadherin expression by periostin (23).

Lately, microarray gene expression data of 165 primary BCa samples were analyzed by WGCNA in order to identify genes correlated with TNM staging and OS (17). In total, 11 modules correlated with TNM staging and they were enriched in genes belonging to cell proliferation associated pathways (**Table 1**). A filtering step using protein-protein interaction

TABLE 1 | Hub genes and miRNAs detected by different authors using WGCNA method.

Comparison	Number and source of samples	Expression data source	Genes	Involved pathways	References
Different BCa stages	93 BCa tissue samples	NCBI GEO microarray dataset GSE31684	COL3A1, COL5A2, FBN1, POSTN	Extracellular matrix (ECM) organization, ECM-receptor interaction, regulation of actin cytoskeleton, cell adhesion, focal adhesion	(16)
Different BCa stages	165 primary BCa tissue samples	NCBI GEO microarray dataset GSE13507	AURKB, BUB1B, CCNB2, CDC45, CENPA, CEP55, KIF2C, KIF4A, KIF15, NUSAP1, PRC1, UBE2C (only diagnostic); AEBP1, CDC25B, COL5A2, MMP11, TK1, TPX2 (only prognostic); CDCA3, CDCA8, CENPF, FOXM1, TOP2A (both diagnostic and prognostic)	Cell cycle, nuclear division, chromosome segregation, organelle fission	(17)
SCCB vs. normal	75 tumor cells and 18 normal cells from the tissue of one patient	NCBI SRA single cell RNA-seq SRP078083	ARHGAP15, BCAR3, CACNA2D3, CENPH, CTNND2, DOHH, GCC2, HERC2, LINC00189, NLK, OR9Q1, PCSK6, POU2F3, SCN2A, TUBGCP2	Spliceosome complex, VEGF, MAPK, neurotrophic signaling, and cell cycle pathways	(18)
BCa vs. normal	9 BCa and 9 normal tissue samples *	NCBI GEO microarray dataset GSE3167	ATF7, CER1, CYP1A2, GDF9, KCNIP1, LRRC15, PTPRJ, TRPM3	Response to stimulus, regulation of localization, gamma-hexachlorocyclohexane degradation, fatty acid metabolism, adherens junction, tryptophan metabolism, and Wnt signaling pathways	(19)
NMIBC vs. MIBC	8 NMIBC and 11 MIBC tissues*	NCBI GEO microarray dataset GSE37317	ATP2A2, BCAP31, BRD2, CYP3A5, DCAF8, LRRC37A2, MEIS3P1, POLR2A, PURA, SRPK2, TRAK1, UBE2I, UPF3A, VPS13D, WDFY3, ZZEF1	Regulation of mitotic cell cycle, cell cycle phase, organelle organization, negative regulation of programmed cell death, DNA replication, DNA recombination, mRNA splicing, cellular localization, B cell receptor signaling pathway, Ras pathway	(20)
BCa vs. normal	418 BCa and 19 normal tissue samples	TCGA miRNA-Seq dataset BLCA	miR-1-1, miR-1-2, miR-28, miR-133a-1, miR-133a-2, miR-133b, miR-139, miR-143, miR-145, miR-195, miR-548ba, miR-3199-2, miR-6507	Cell proliferation, regulation of cell growth, regulation of actin cytoskeleton, proteoglycans in cancer, focal adhesion, Wnt, PI3K-Akt, MAPK, and p53 signaling pathways	(21)

*BCa, bladder cancer; SCCB, squamous cell carcinoma of bladder; MIBC, muscle-invasive BCa; NMIBC, non-muscle-invasive BCa. * Note that WGCNA has been performed using too few samples, indeed at least 20 samples are required for a robust WGCNA analysis and to obtain reliable results.*

network information collected in STRING tool (https://string-db.org/) resulted in 17 genes with a potential role in BCa (**Table 1**). Moreover, 11 hub genes could be considered as prognostic biomarkers, since their lower expression was associated with better OS of BCa patients. Some identified hub genes had been previously investigated. In particular, the high expression of AURKB (Aurora Kinase B), implicated in cancer through development of aneuploidy and chromosomal instability, correlated with advanced BCa stages (24). Similarly, the mitotic checkpoint protein BUB1B, known to contribute to chromosomal instability, was over-expressed in advanced BCa stages and correlated with high cell proliferation (25). High expression levels of CDC25B (Cell division cycle 25B) were associated with advanced stages, recurrence and poor prognosis in BCa patients (26). Cyclin B2 (CCNB2) expression level was higher in cancer than in normal bladder mucosa and its downregulation inhibited cell migration, invasion, and metastatic abilities (27). Also the role of FOXM1 (Forkhead box M1) has been widely investigated, indeed it was found to be a reliable prognostic biomarker for MIBC (28) and its high expression level correlated with TNM stage, histological grade, metastases, and poor prognosis in BCa patients, whereas its down-regulation through miR-24-1 inhibited cell proliferation, migration, and invasion (29, 30). Since CCNB2 and FOXM1 suppression resulted in BCa inhibition, they can be considered as reliable therapeutic targets and deserve further exploration. Moreover, MMP11 (Matrix metalloproteinase-11) overexpression correlated with very aggressive phenotypes (advanced pT status, nodal metastasis, high histological grade) and with unfavorable clinical outcomes (31). Serum concentration of TK1 (Thymidine kinase 1) gene was associated with tumor stage, degree of invasion, and metastasis (32). TOP2A

(Topoisomerase-IIA) free DNA in urine has been identified as a diagnostic biomarker and its levels can also distinguish NMIBC from MIBC (33). Its over-expression has also been associated with high-grade and high-stage BCa and with high rates of recurrence in NMIBC (34). Moreover, TOP2A protein levels have been identified as a predictor of DFS times (35). TPX2 (microtubule nucleation factor) gene was upregulated in tumor tissues compared to normal bladder samples and it was strongly associated with pT status, high histological grade, lymph node metastasis, and shorter OS time. Indeed its overexpression promoted proliferation and tumorigenicity and suppressed apoptosis (36). UBE2C (Ubiquitin conjugating enzyme E2) upregulation was associated with high BCa stages, presence of lymphovascular invasion, progression to MIBC, and it has been suggested as a biomarker of unfavorable prognosis (37, 38).

In an experiment where single-cell transcriptomics was applied to squamous cell carcinoma of urinary bladder (SCCB), 75 tumor cells and 18 normal cells were isolated from cancer and normal control fresh resected tissues from one patient (18). Then, gene expression analysis was performed by single-cell RNA-seq. WGCNA analysis identified five large modules enriched in genes belonging to several cancer-associated pathways (**Table 1**). While some of the identified hub genes (**Table 1**) have rarely been reported in previous cancer studies, some of them have already been suggested as BCa biomarkers. For example, CTNND2 (Catenin delta 2) gene is frequently amplified in BCa, with high copy numbers (39). Moreover, copy number variations of PCSK6 (Proprotein convertase subtilisin/kexin type 6) gene have been reported to be a prognostic marker for NMIBC progression (40). Intriguingly, POU2F3 (POU class 2 homeobox 3) is a transcription factor expressed in stratified squamous epithelia and related to squamous epithelial stratification (41), so it could play a role in squamous cell BCa (SCCB).

Microarray gene expression profiles of 9 normal bladder and 9 transitional cell carcinoma tissue samples have been analyzed by the WGCNA method (19). A differential co-expression network analysis was carried out and eight hub genes were identified (**Table 1**). Interestingly, among the identified hub genes, the rs762551 polymorphism in CYP1A2 (a cytochrome P450 family member) gene was associated with decreased BCa risk (42). Molecular mechanisms explaining this association are still undetermined, however since it lies in the first intron of the gene, it could alter pre-mRNA splicing processing at 5′UTR level, transcription regulation, or protein folding (43–51).

Recently, different gene expression profiles between NMIBC and MIBC have been investigated using public microarray expression data from 8 and 11 snap frozen cancer tissues, respectively (20). WGCNA analysis showed significant correlations between three modules and the tumor stage. In particular, these modules were enriched in genes mainly involved in cell cycle (**Table 1**). Among them, 16 hub genes have been identified (**Table 1**). Therefore, they can be involved in BCa progression from NMIBC to MIBC phenotype. Many hub genes have been previously suggested as candidate diagnostic or prognostic BCa biomarkers. For example, TRAK1 (trafficking kinesin protein 1) gene has been identified as a favorable prognostic marker, since its low level expression was associated with poorer survival (52). Polymorphisms in CYP3A5 (a cytochrome P450 family member) can define a subset of BCa patients who better respond to cabazitaxel and temsirolimus, in terms of lower toxicity and higher efficacy (53, 54).

However, regarding the last two studies (19, 20), it should be noted that WGCNA was performed using sample sizes that were too small, that is 8, 9, or 11 samples for each condition. Indeed, at least 20 samples are required for a robust WGCNA analysis and to obtain reliable results.

Recently, also miRNA expression data of 418 BCa and 19 normal tissue samples collected in The Cancer Genome Atlas (TCGA) have been investigated (21). After the selection of differentially expressed miRNAs, WGCNA analysis allowed the identification of a module closely related to BCa progression. Thirteen downregulated miRNAs (**Table 1**) also had a prognostic value since their low expression levels were associated with poorer OS in BCa patients. Interestingly, the predicted targets of these miRNAs were found to be significantly enriched in cancer-related pathways (**Table 1**). It has been shown that miR-1-1 and miR-133a are under-expressed in BCa cells and act as tumor suppressive miRNAs since, when overexpressed, they inhibited cell proliferation and invasion and increased apoptosis (55). In particular, miR-1 can exert its tumor suppressive function by targeting both coding genes, for example CCL2 (56), and non-coding RNAs, including UCA1 (57). MiR-133a can also induce apoptosis through silencing of GSTP1 in BCa cell lines (58) and, along with miR-1, it can inhibit BCa cell proliferation and increase apoptosis by targeting TAGLN2 mRNA (59). Moreover, miR-133a and miR-145 suppress cancer cell proliferation by directly regulating FSCN1 expression (60). MiR-133b plays a key role in proliferation and apoptosis by silencing Bcl-W and AKT1 genes (61) and its downregulation is associated with BCa progression and poor prognosis (62). Also miR-139 is a tumor suppressive miRNA since it can inhibit BCa cell proliferation by targeting the BMI1 oncogene (63) and cell migration and invasion by silencing matrix metalloprotease 11 (MMP11) (64). It has been observed that miR-143 can inhibit tumor cell proliferation, it is associated with BCa resistance to gemcitabine (65) and, along with miR-145, is a good prognostic biomarker for BCa patient survival (66). Interestingly, the polymorphism rs353293 in the common promoter of miR-143 and miR-145 is associated with BCa risk (67). Finally, miR-195 suppressed cancer cell proliferation by silencing GLUT3 (68), CDK4 (69), and CDC42 (70) genes. Therefore, these tumor suppressive miRNAs can be considered for *in vivo* delivery of therapeutics in bladder cancer, even though there are still challenges to the development of miRNA delivery strategies without toxicity induction.

FURTHER ANALYSES ON IDENTIFIED HUB GENES AND MIRNAS

We performed a Gene Ontology analysis using all hub genes identified by WGCNA in the reviewed studies. Overall, they were found to belong to related biological processes. In particular, we highlighted pathways such as cell cycle, mitosis, mitotic spindle organization, kinetochore assembly, and nuclear division. All

these processes are involved in the uncontrolled cell proliferation in cancers. In addition, by using the transcription factor binding data generated by the ENCODE project consortium (www.encodeproject.org), we identified the E2F4 and FOXM1 transcription factors as the master regulators of hub gene expression, since they resulted as being significantly over-represented in the promoters of hub genes ($p = 5.892e\text{-}9$ and $p = 3.220e\text{-}8$, respectively). Moreover, in order to investigate whether relationships exist between these miRNAs and the hub genes listed in **Table 1**, we performed a comprehensive literature search and identified which hub genes are targets of the hub miRNAs. In order to increase the analysis stringency, we considered only the experimentally assessed miRNA targets. Results reported in **Table 2** show that nearly all hub miRNAs target at least one hub gene, therefore these miRNAs could be involved in the deregulation of hub genes. Alternatively, hub miRNAs and genes could be deregulated due to the alteration of master regulators, such as transcription factors.

CONCLUSIONS

WGCNA is a recently developed method for the analysis of gene expression data able to propose candidate therapeutic targets or diagnostic/prognostic biomarkers. Here, we reviewed all studies where WGCNA has been applied for the analysis of expression data from BCa. They include analyses of gene and miRNA expression data. Notably, neither lncRNA expression or splicing isoform-specific RNA expression have yet been investigated in BCa by WGCNA, as recently carried out, for example, in pancreatic cancer (15) and in clear cell renal cell carcinoma (ccRCC) (86), respectively. In addition, expression data of circular RNAs and RNAs in exosomes have not been analyzed by WGCNA in any cancer type. Recently, this network strategy has also been applied to proteomic and metabolomic data, although, due to the low coverage of proteomic and metabolomic analytical methods, WGCNA needed to be modified (87). Moreover, since RNA-seq data simultaneously allow the gene expression measure and mutation analysis, it would be interesting to perform an

TABLE 2 | Experimentally validated targets (among BCa hub genes previously identified by WGCNA) of hub miRNAs recently identified in BCa (21).

Hub miRNAs	Targeted hub genes	Validation assay	Cell lines	References
miR-1-1/2	CENPF	Proteomics (pSILAC)	HeLa (human cervical cancer)	(71)
	FBN1			
	HERC2			
	KIF2C			
	KIF4A			
	UBE2I			
miR-28	PTPRJ	PAR-CLIP	HCT116 (human colon cancer)	(72)
miR-133a-1/2	CDCA8	PAR-CLIP	C8166 (HIV-1 infected human T cells) and TZM-bl (HIV-1 infected human epithelial cells)	(73)
	FBN1	Microarray	T24 and KK47 (human bladder cancer)	(58)
miR-139	MMP11	Luciferase reporter assay, Microarray, qRT-PCR, Western blot	T24 and BOY (human bladder cancer)	(64)
miR-143	BRD2	PAR-CLIP	HCT116 (human colon cancer)	(72)
	CDC25B			
	COL3A1	*In situ* hybridization, qRT-PCR, Western blot	NF-38 (human normal gastric fibroblasts) and CaF-38 (human cancer-associated fibroblasts)	(74)
	COL5A2	Microarray	USSCs (human unrestricted somatic stem cells)	(75)
miR-195	CEP55	PAR-CLIP	HEK293S (human embryonic kidney) and MCF7 (breast cancer)	(76–79)
	PTPRJ	HITS-CLIP	Jijoye (EBV-transformed B cells)	(80)
	PURA			
	TRAK1	HITS-CLIP	HEK293S (human embryonic kidney) and fresh-frozen human heart tissues	(81, 82)
	UBE2I	Microarray, qRT-PCR	PBMCs (peripheral blood mononuclear blood cells)	(83)
miR-6507	CDC25B	PAR-CLIP	HCT116 (human colon cancer)	(72)
	CDCA8			
	DCAF8	PAR-CLIP	HEK293S (human embryonic kidney)	(76)
	PRC1			
	CENPA	PAR-CLIP	hESCs (human embryonic stem cells)	(84)
	CEP55	PAR-CLIP	BC-1 and BC-3 (KSHV-infected primary effusion lymphoma)	(85)

pSILAC, pulsed Stable Isotope Labeling with Aminoacids in Cell culture; PAR-CLIP, PhotoActivatable Ribonucleoside-enhanced CrossLinking and ImmunoPrecipitation; HITS-CLIP, HIgh-Throughput Sequencing of RNA isolated by CrossLinking and ImmunoPrecipitation.

evaluation of the mutation effects on the gene co-expression module assignment of a gene.

Although WGCNA requires expression data from at least 20 samples in order to obtain reliable results, only 8, 9, or 11 samples for each condition were analyzed in two reviewed studies (19, 20). Generally, in order to overcome the problem of a small sample size, researchers use more than one expression dataset, particularly during analysis of microarray data. However, systematic and technical differences between different microarray platforms and datasets (called batch effects) could emerge. The correct data pre-processing is needed since WGCNA is sensitive to batch effects. Moreover, the presence of outliers (samples with very different expression profiles form the bulk) may affect WGCNA results, thus their removal is a critical step (12). Unfortunately, sometimes researchers tend to reject few outliers because of the small sample size. A further critical element for WGNCA analysis is the highly variable gene expression among samples due to tumor heterogeneity. To overcome this problem, large sample size expression datasets should be used and, in particular, datasets that include samples isolated from different points of a single cancer tissue should be preferred. Moreover, we suggest performing sample clustering based on expression data before WGCNA analysis, in order to process less heterogeneous samples, as recently carried out for ccRCC (88).

High BCa heterogeneity, different microarray platforms, specific setting of WGCNA algorithm and different patient therapies, lifestyle, stages, age, and sex could explain the little overlap between key genes identified among the studies. However, since these hub genes are highly co-expressed, they could highlight a common mechanism of transcriptional and post-transcriptional regulation, thus revealing mechanistic insights into cancer development. In particular, we identified the hub gene FOXM1 as the master regulator of the other genes identified by WGCNA. Hub genes could also be related at a functional level, since they belong to similar pathways and are regulated by a few common hub miRNAs. Furthermore, these miRNAs are known to be involved in the same pathways of hub genes, thus supporting the role in BCa of genes and miRNAs identified by WGCNA. Finally, although currently no hub gene has sufficient validity for clinical practice, many of them are already known to serve as biomarkers in BCa. Therefore, these genes are worth being further explored, since they can shed light into the molecular mechanisms of BCa, thus leading to the definition of novel personalized therapies. For example, regarding biomarkers for chemotherapy efficacy and toxicity, WGCNA identified the cytochrome P450 family member CYP3A5, already found to be useful in defining BCa patients with better responses to treatments.

AUTHOR CONTRIBUTIONS

MG and FP conception and design. MG, GO, ARi, and FP drafting the manuscript. GO, ARi, MB, and AC review of the literature. ARu, EC, TC, and GP critical revision of the manuscript.

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Emerging Molecular Technologies in Genitourinary Tumors

Francesca Giunchi[1], *Alessia Cimadamore*[2] *and Michelangelo Fiorentino*[1]*

[1] *Laboratory of Oncologic Molecular Pathology, S. Orsola-Malpighi Teaching Hospital University of Bologna, Bologna, Italy,*
[2] *Section of Pathological Anatomy, Polytechnic University of the Marche Region, School of Medicine, United Hospitals, Ancona, Italy*

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INTRODUCTION

Diagnostic molecular pathology of genito-urinary (GU) tumors is facing new technological challenges in the era of genome-wide analyses and patient-derived animal tumor models. In view of the increasing number of dedicated clinical trials, GU tumors represent the next urgent field of application of molecular diagnostics and drug discovery after gastro-intestinal and thoracic oncology.

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***Correspondence:**
Michelangelo Fiorentino
fiorentinomichelangelo@gmail.com

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DNA-BASED GENOME-WIDE ANALYSES

Wide spectrum mutational analyses using next generation sequencing (NGS) platforms will soon represent the standard-of-care technologies for the assessment of genetic variants in solid tumors (1). These technologies apply successfully to archival pathology specimens, cytological samples and even liquid biopsies (blood or pleural effusions) (2). Mutational analyses can be wider (whole exome sequencing, WES) or restricted to selected genes or amplicons (targeted gene sequencing TGS). Both approaches are used to identify single or multiple genetic variants as predictive biomarkers of response to targeted oncologic therapies. At least the following three genome-wide mutational analyses will become routine diagnostic tests for GU tumors in the immediate future. Analysis of *BRCA1* and *BRCA2* germ-line mutations will be required to assess inherited prostate cancer risk and to predict response to treatment with poly(ADP-ribose) polymerase (PARP) inhibitors and even next-generation anti-androgens (3, 4). Given the complexity of the *BRCA1* and *BRCA2* mutations the NGS sequencing is the ideal method for their assessment. Similarly, deep sequencing of the DNA mismatch repair genes will be required in patients with familiar prostate and colorectal cancer for suspected Lynch syndrome (3). Mutations in homologous recombination repair genes (ATM/BRCA1/2 specifically) is enriched in men with advanced clinical stage ($\geq$ cT3) and higher Gleason grade groups ($\geq$ 3) (5). Patients with metastatic castration-resistant prostate cancer whose tumors harbor homologous recombination DNA repair gene alterations, experience a different response to PARP inhibitor therapy. In particular, patients with cancer harboring DNA repair alterations in genes other than BRCA2 are often non-responders (6). The assessment of tumor mutation burden defined as the number of mutations per mega-base of tumor cell DNA is becoming the most relevant candidate biological predictor of response to immunotherapies targeting the PD-1/PD-L1 axis (7). Tumor mutation load can be achieved either by WES or by TGS using NGS dedicated panels covering at least 2 mega-bases of tumor DNA. Assessment of tumor mutation load is also prognostically relevant in metastatic renal cell cancer and in muscle-invasive bladder cancer (8–10). Finally, epigenetic changes, including CpG island hypermethylation can be investigated using genome-wide methylation NGS panels in the attempt to better stratify high-grade and low-grade disease (11).

RNA-BASED GENOME-WIDE ANALYSES

Genome-wide trascriptome analyses include gene expression profiling, miRNA and non-coding RNA profiling and RNA sequencing. In particular, RNA sequencing with high-throughput NGS platforms starting from RNA libraries allows simultaneous analysis of differential gene expression, allele-specific expression, splicing variants, and gene rearrangements (12). These analyses can also be done on RNA and DNA contained in small extracellular vesicles (EVs) that could be found in blood, urine, and other biological fluids (13). RNA abundance and sequence can be also investigated by array hybridization using platforms such as the NanoString System (14). Immediate clinical application of RNA sequencing to GU tumor include primarily the following fields of interest. The study of tumor immune micro-environment through the expression analysis of immune response genes is becoming important to assess tumor response to immune check-point inhibitors and BCG in bladder cancer (15, 16). The new molecular classification of muscle-invasive bladder cancer is largely based on gene expression profiling (17). Recognition of the molecular subtypes has prognostic and therapeutic implications for patients with advanced urothelial cancer. The assessment in the tumor tissue of the AR-V7 splicing variant of the androgen receptor (AR) gene is a predictor of poor response to anti-androgens and good response to chemo-therapy in castration-resistant prostate cancer (CRPC). The presence of AR splicing variants can be successfully investigated by RNA sequencing in prostate cancer tissue samples (18).

PATIENT-DERIVED ANIMAL MODELS

Patient-derived xenografts (PDX) are mouse models where disaggregated cells or little fragments of human tumors are implanted into immunodeficient mice. The establishment of a PDX allows treating and monitoring the response to treatment of the original tumor *in vivo* in the mouse, instead of the patient, providing the best therapeutic selection at the same time (19). This procedure is ethically and commercially valuable since it spares pointless drug toxicity to the patient while saving money for oncological treatments that would be ineffective. Successful PDX establishment for monitoring response to treatment has been described in GU tumors (20). In CRPC there are available examples of PDX for treatment with abiraterone and enzalutamide as well as for a number of drugs in pre-clinical phase of development (21). In papillary type kidney cancer harboring *MET* mutations, there is evidence of successful treatment of PDX with Cabozantinib and other MET inhibitors (22, 23). PDX created using human bladder tumor tissues have been utilized to assess response rates to cisplatin or PI3K inhibitors (24). The success of PDX establishment is highly variable and depends on several tumor-related or animal-related factors. For instance, in a meta-analysis on bladder cancer, the tumor engraftment rate varied between 20 and 100% (24). In addition, several flaws can affect the reliability of PDX as surrogate models of original patients' tumors. Tumor histological appearance may change in the PDX frequently toward squamous or sarcomatoid or neuroendocrine differentiation. Cancer cell proliferative rates in PDX may increase as well as cancer mutations may turn out enriched or underestimated (25). On the other hand, host mice for PDX can be selected to be totally immunodeficient or "humanized" by forcing in the animals the expression of cytokines or injecting in the mouse bloodstream human bone marrow stem cells to re-create the tumor inflammatory microenvironment. Humanized PDX have been established for several tumor types but not yet for GU cancers (26).

Organoids are 3D cell-cultures recapitulating the natural complex environmental organization of a normal or a cancer tissue. They differ from the cell-lines that grow flat in 2D and lack the signal trafficking and the organization of a tissue (27). Organoids can be constructed from human cancer cells or tissues and can be utilized for testing the response to drugs (28). Compared to PDx, organoids are more amenable to grow but they are transient in nature and represent a methodological choice in-between cell-lines and animal xenografts. Organoid models have been created to trait rare phenotypes or genotypes of prostate cancer and to test their potential response to drugs, or to track evolution of bladder cancer (29, 30).

Patient-derived models are increasingly used to address questions in GU oncology. There are still limitations to the reliability of these models to actually guide patients' therapy. In addition, these model technologies require dedicated infrastructures (such as bio-banks, laboratories, and animal facilities) and experienced professionals. There are also several ethical restrictions to the use of model systems in different countries. Notwithstanding, PDX and organoids represent a fascinating opportunity to enhance cancer drug discovery and to provide more therapeutic options to cancer patients.

AUTHOR CONTRIBUTIONS

MF: Conception and design; FG: Drafting the manuscript and review of the literature; AC: Critical revision of the manuscript.

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