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Prostate-specific markers are required to identify rare prostate cancer cells in liquid biopsies

EE van der Toom[3], **H Axelrod**[1,2], **JJMCH de la Rosette**[3], **ThM de Reijke**[3], **KJ Pienta**[1], and **KC Valkenburg**[1]

[1]The James Buchanan Brady Urological Institute, Johns Hopkins University School of Medicine, Baltimore, MD 21287 [2]Graduate Program in Cellular and Molecular Medicine, Johns Hopkins University School of Medicine, Baltimore, MD 21287 [3]Department of Urology, Academic Medical Center, Amsterdam, The Netherlands

Abstract

Despite early detection and treatment advancements, prostate cancer patients continue to succumb to their disease. Minimal residual disease may lead to relapse and distant metastases, and increasing evidence suggests that circulating and bone marrow disseminated tumor cells (CTCs and BM-DTCs) can offer clinically relevant biological insights into prostate cancer. In this review, we emphasize the pitfalls of using epithelial markers to accurately detect CTCs and BM-DTCs and discuss the pressing need for prostate-specific markers in the detection of these cells using rare cell assays. We have assembled a comprehensive list of published putative prostate-specific markers and posit an ideal strategy for staining rare cancer cells from liquid biopsies. The ideal prostate-specific marker is expressed on every CTC/BM-DTC throughout disease progression (high sensitivity), and is not expressed on non-prostate cancer cells in the sample (high specificity). We conclude that some markers are likely not specific enough to the prostate to be used as individual markers of prostate cancer cells, whereas other genes may be truly prostate-specific and would make ideal markers for rare cell assays. The goal of future studies is to utilize sensitive and specific prostate markers to consistently and reliably identify rare cancer cells.

Keywords

prostate-specific markers; prostate cancer; circulating tumor cells; disseminated tumor cells; rare cells; bone marrow

Corresponding author. KCV.

Author Contributions

EEvdT: Wrote approximately half of the manuscript; helped assemble figures and tables; edited manuscript, figures, and tables
HA: Edited manuscript, figures, and tables; added written sections
JJMCHdlR: Edited manuscript; provided input and funding
TMdR: Edited manuscript; provided input and funding
KJP: Edited manuscript; provided input and funding
KCV: Wrote approximately half of the manuscript; helped assemble figures and tables; edited manuscript, figures, and tables

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Introduction

Prostate cancer (PCa) is the most common cancer and the second most common cause of cancer-related deaths in men in the US[1]. Despite advances in PCa screening, surgery, hormone-related therapies, and chemotherapies, approximately 27,000 men still die of metastatic PCa each year in the U.S. Of the patients diagnosed with early-stage PCa, nearly half of them will not die of their disease without treatment. The other half of patients will undergo treatment, by either radical prostatectomy or radiation therapy, with the goal to cure their disease. Unfortunately, approximately 30% of these patients recur biochemically, based on rising prostate specific antigen (PSA) levels in blood (FIG. 1)[2]. Approximately 40% of men with biochemical recurrence will develop metastatic disease, and 100% of those patients will succumb to their disease[2]. Notably, 100% of men who died of PCa and who were autopsied had PCa present in their bones[3]. Metastases often appear years after primary treatment, indicating that tumor cells must have escaped the primary tumor prior to therapy and disseminated to distant sites[4–6]. Tumor cell dissemination and metastasis is a complicated multi-step process[7] that requires primary tumor cells to enter the vasculature, where they are referred to as circulating tumor cells (CTCs). Most CTCs are unable to withstand the shear stress, immune surveillance, and lack of cell-cell adhesion in the circulation and will die prior to reaching distant sites. CTCs that are able to exit the circulation and establish residence at a distant site, such as the bone marrow (BM), are called disseminated tumor cells (DTCs; we will refer to DTCs in the BM as BM-DTCs). The specific timing of this cellular dissemination process in the natural history of PCa progression prior to metastatic development is largely unknown but highly intriguing (FIG. 1). Metastatic PCa remains incurable, and current imaging modalities are not sensitive enough to detect individual cancer cells or small colonies of disseminated cells. If CTCs and BM-DTCs can be identified prior to the formation of overt metastatic lesions, treatments can be aimed at preventing metastasis altogether[8–10].

Fine needle biopsies are the standard for PCa diagnosis and prognosis, but they are invasive and can cause significant morbidity. Therefore, there is much appeal for investigating the clinical utility of minimally invasive liquid biopsies to use CTCs and BM-DTCs as biomarkers of disease[11–13]. Accurate detection of these cells will also allow for their biological characterization, in which therapies can be more precisely targeted to the mechanisms leading to recurrence. Although we will focus mostly on CTCs and BM-DTCs, liquid biopsies can also provide clinically relevant information in the form of cell-free circulating tumor DNA (ctDNA) and exosomes, both of which can also be present in urine (FIG. 2)[14–16]. Liquid biopsies can provide a real-time non-invasive snapshot of the total tumor burden of a patient and can furthermore provide important complementary information on therapeutic targets and mechanisms of drug resistance. De Bono *et al.* previously reported that the number of CTCs found in patients with castration-resistant prostate cancer (CRPC) can predict overall survival. Patients with ≤5 CTCs (per 7.5 mL of blood) survived 10.2 months longer than patients with >5 CTCs (using EpCAM-based purification methods)[17]. Other studies have correlated the number of CTCs in metastatic PCa to therapeutic response and survival, while limited, but emerging, studies have been paralleled in pre-metastatic PCa patients[18–23]. As such, CTC data from blood draws are

extremely clinically relevant, and will continue to be so. Clinical correlations have not been as rigorously assessed for BM-DTCs, as bone marrow is more difficult to obtain, and it is more difficult to identify BM-DTCs than CTCs due to decreased marker specificity. While CTCs will likely play a more important role in providing clinically relevant data real-time, BM-DTCs may represent a more important cell population, as they have successfully migrated from the primary tumor to a distal site. We propose that BM-DTC data will provide much-needed information about timing of dissemination, as well as the genetic and epigenetic qualities of a successfully disseminated and proliferating cancer cell. As such, our ultimate goal is to determine prostate-specific markers that sensitively and specifically identify BM-DTCs for downstream analysis.

It is important to understand the lethal characteristics and clinical application of CTCs and BM-DTCs after they are reliably detected. The two most commonly used methods for CTC detection are reverse transcription PCR (RT-PCR) and fluorescence-based immunostaining (referred to as immunofluorescence, or IF). FISH (fluorescence in-situ hybridization) can be used as a tool similar to IF and PCR to identify CTCs via RNA expression, thereby helping to define the different gene expression patterns within these cells[24]. Each of these methods has its own set of advantages and limitations (TABLE 1), but IF has certain advantages that allow for further biological characterization of functional activity at the time of detection. Many different assays exist for the detection of CTCs (very few exist for BM-DTCs), and most rely on positive selection of cancer cells or negative selection of leukocytes, though selection-free methods also exist[25–27]. Most also involve the separation of red blood cells from white blood cells and cancer cells, which is commonly done via microfluidics chips, red blood cell lysis buffers, and/or centrifugation-based separation[26–29]. The type of detection methodology will change the resulting cell population and molecular composition that is analyzed, as certain cell types may be enriched or lost based on the experimental conditions. For instance, analyzing whole blood RNA for a specific marker without including a selection step will not yield meaningful results about the specificity of that marker to cancer cells. Many studies have used selection methods (usually via epithelial selection based on EpCam expression or size-based selection using a microchip) to detect CTCs from blood using RT-PCR, multiplex PCR, or digital droplet PCR[30–37]. These studies show that RT-PCR is extremely sensitive for CTCs, but no such success has been found in BM-DTCs.

Current standard markers used for CTC detection via IF include a nuclear marker (usually DAPI), a marker for white blood cells (WBCs; usually CD45) and one or more epithelial markers (usually EpCAM and/or pan-cytokeratin)[17,38]. A major limitation of relying on epithelial markers for CTC identification is that several studies have shown previously that these markers are not always highly expressed on cancer cells, and have also been shown to be expressed on cells of hematopoietic lineage[39–44]. Furthermore, it is thought that CTCs lose their epithelial phenotype after undergoing epithelial to mesenchymal transition (EMT) to escape the primary tumor, and thus they may lose EpCAM and/or cytokeratin (CK) expression[45–50]. While EpCAM-based detection methods have been the most common method to identify CTCs, it is unknown how frequently this loss of epithelial characteristics occurs. In addition, the blood, and particularly the BM, contains a vast heterogeneity of cells, many of which are stem or other cells that can epigenetically alter their phenotype.

This can lead to false positive immunostaining, in which the detection marker is no longer specific for prostate cells. Once CTCs are isolated, further characterization can be performed by using different functional assays, such as EPISPOT, which detects specific proteins during the *in vitro* culturing of CTCs[51]. Another example is the cancer cell line-derived xenografts (CDXs), by which cancer cells from cell lines or patient-derived CTCs are injected into immune-compromised mice, after which metastases will develop[52,53], although this has not been successful in PCa. This can give important *in vivo* information for more individualized treatment of cancer patients.

We posit that the use of prostate-specific markers to identify prostate CTCs and BM-DTCs will allow for more sensitive and specific detection of these rare cancer cells. So far, the identification of these markers for rare tumor cells has been challenging, as some reported prostate-specific markers are not very sensitive (not expressed in all PCa cells) or specific (also expressed by other cells in the blood or BM) (SUPPL. TABLE 1). Many studies on these markers have only assessed expression of protein at the tissue level (e.g. IHC on formalin-fixed paraffin-embedded tissue) or RNA in whole blood (e.g. RT-PCR), neither of which represents true sensitivity or specificity at the rare cell level. Therefore, this manuscript makes clear that each of these markers should be assessed in rare cell assays in blood and BM samples before any conclusion can be made as to their utility in liquid biopsies. Also, dedifferentiation and loss of prostate-specific markers can occur in a significant proportion of poorly differentiated prostatic adenocarcinomas[47,54,55]. It is thus imperative that we find highly sensitive and specific prostate markers that are expressed during all the stages of a patients' disease, expressed on every tumor cell, and not expressed on any blood or BM cells. In this review, we will discuss what is known about the putative prostatic lineage markers and highlight their pros and cons in the detection of CTCs and BM-DTCs (TABLE 2).

Prostate Specific Markers

Prostate specific antigen and other kallikreins

Prostate specific antigen (PSA, also known as kallikrein related peptidase 3, or KLK3, and human glandular kallikrein 3, or hK3) is currently the most important and clinically useful marker in PCa screening. It is produced by secretory epithelial cells in the prostate[56] and is an androgen-regulated serine protease expressed in both benign and malignant prostatic tissue. PSA is one of the oldest prostatic markers used in immunohistochemistry (IHC) to confirm that a metastatic carcinoma is prostatic in origin[57]. It has been widely shown that PSA has a high specificity for PCa, but that its expression also tends to decrease with cancer progression. PSA expression may be absent in around 5% of patients with high-grade PCa and distant metastases, as well as in around 10% of lymph node metastases[47,58–61]. The staining pattern for PSA is cytoplasmic, which can present an issue for IHC because diffuse cytoplasmic staining can generate false positives during analysis and is known to occur in IHC[62]. While PSA expression has been reported in a variety of non-prostatic tissues and tumors, including breast and lung carcinomas[58,63–67], others have reported high sensitivity and specificity of PSA in PCa using monoclonal and polyclonal anti-PSA antibodies[68]. PSA expression from PCa patient blood has been correlated with cancer at the RNA level via RT-

PCR, but neither study used a selection protocol to ascertain which cells expressed PSA[32,69]. Overall, PSA is a promising marker for rare cell assays, as it seems to be sensitive for most PCa cells while its expression has not been reported in blood or BM cells (unlike AR expression), although this must be tested in rare cell assays. Coupled with evidence that PSA can be controlled in an AR-independent manner[70], addressing sensitivity issues, PSA could potentially be a more promising rare cell marker than AR. PSA is also a widely-used biomarker of primary prostate tumor growth as well as for biochemical recurrence following radical prostatectomy or radiation therapy. As with all of the proteins we will discuss in this article, its full utility as a rare cell marker in blood and BM has yet to be ascertained in PCa and non-cancer patients.

PSA belongs to the kallikrein serine protease family, which contains 15 family members. Besides PSA, two other kallikrein family members, KLK2 and KLK4 (also known as prostase and KLK-L1), also seem to be prostate specific[71–78]. There is less known about the clinical utility of these markers, but both have been found in PCa patient tissue and serum. Both KLK2 and KLK4 seem to have a proteolytic function in activating PSA from its precursor pro-PSA form to its active PSA form. These kallikreins should be assessed in rare cell assays in addition to PSA.

Androgen receptor

The androgen receptor (AR) is the most widely studied protein related to prostate development and PCa. AR is a powerful transcription co-factor that affects the development and growth of male sex organs, including the prostate[79]. Androgen-mediated nuclear localization and activation of AR is required for the development and growth of the prostate gland[80–83], and deprivation of androgens inhibits proper ductal development of the gland[84]. These phenotypes can be seen during embryonic development, where fetal testicular secretion of androgens promotes prostate development[82]. The adult prostate's structural maintenance and reproductive function also requires androgens and AR activity[85]. Binding of dihydrotestosterone to AR causes it to translocate to the nucleus and bind androgen response elements in genomic DNA to initiate[86,87] or down-regulate transcription of target genes[88,89]. AR also has non-transcription-related functions, but these are less well understood and have only been reported in cancer tissue[90]. Expression of many other prostate-specific genes that we will discuss in this article is transcriptionally regulated by AR. Due to its crucial roles in the development, growth, and maintenance of the prostate, it is not surprising that AR plays critical roles in PCa. Some groups have reported tumorigenic properties of AR in mouse models[91,92]. However, mice lacking AR specifically in the murine prostate had increased cellular proliferation, indicating that the role of AR in cancer initiation is still not fully understood[93]. Interestingly, while PCa is one of the most prevalent cancers in men, there are almost no cancers of the seminal vesicle or bulbourethral gland, both of which express AR[94]. AR is strongly expressed in most PCa tumors, and PCa maintenance seems to depend on AR signaling[95–98]. Androgen deprivation therapy (ADT) and AR targeting therapies have significant survival benefits in advanced PCa patients and are widely used in the clinical setting[99–101]. Importantly, AR expression can be lost in some PCa tumors, particularly those with neuroendocrine or small cell PCa pathology[102–105]. Of great interest and potential utility in rare cell assays are the AR splice variants. It has been

shown that expression of the AR-V7 variant increases in castration resistant PCa[106]. Moreover, expression of the full-length version of AR versus the AR-V7 variant in PCa CTCs can predict ADT response[21,47,107,108], and this has led to its use in guiding therapeutic strategy[19]. However, the use of AR solely as a CTC/BM-DTC marker for rare cell assays poses specificity issues because it is expressed on BM cells and platelets, as well as in other tissues[109–112] (SUPPL. TABLE 1). We believe that AR is not specific enough for prostate tumor cells to be used as an individual marker for rare PCa cells, but has potential as an adjuvant marker for clinical management. Furthermore, because AR is expressed in certain blood and BM cells, and AR regulates the expression of many other putative prostate-specific markers, each of these markers must be rigorously assessed for its expression in blood and BM to determine specificity. A non-androgen-regulated prostate-specific gene would be an ideal marker in prostate CTC and BM-DTC detection assays, but such markers are seemingly rare.

Prostate specific membrane antigen

Prostate specific membrane antigen (PSMA, also known as folate hydrolase 1, or FOLH1) is a membrane-bound glycoprotein with high specificity for both benign and malignant prostatic tissues. In contrast to other androgen-regulated prostate genes, PSMA is suppressed by androgens in an AR-dependent manner[113]. The initial cloning of the gene of PSMA was accomplished by Israeli *et al.* in 1993 using the LNCaP PCa cell line[114]. PSMA is currently being explored extensively as a promising target for molecular imaging as well as a therapeutic target in prostate and renal cancers. For PCa, it may be useful in the setting of biochemically recurrent disease, where PSMA-targeted radiotracers seem to be superior to conventional imaging for detection of metastatic PCa[115–117]. PSMA is expressed at low levels in benign prostatic epithelium and is strongly expressed in most prostate carcinomas[118]. PSMA is, in contrast with PSA, highly up-regulated in high-grade tumors and corresponding metastases[119]. Normal prostate epithelium often has a low level of diffuse cytoplasmic staining, while high-grade and metastatic tissues mostly have a very intense cytoplasmic and focal membrane staining[61,119]. Unfortunately, as it was originally thought to be strictly expressed in prostatic tissue, it is now known that PSMA is widely expressed in a variety of non-prostatic solid tumors and vasculature, including urothelial, renal, gastrointestinal, and breast carcinomas, in addition to bone diseases such as Paget's disease and healing bone fractures[120–130] (SUPPL. TABLE 1). PSMA expression in non-prostatic cancer cells is mostly restricted to the cytoplasm[61]. Furthermore, a study by Kinoshita *et al.* reported the detection of the PSMA protein in an exceptional variety of healthy tissues, including the urinary bladder and proximal tubules of the kidney[122]. Uhlén *et al.* demonstrated mRNA expression of PSMA in normal male and female BM, but no protein expression[112]. PSMA expression in PCa and non-PCa patient blood was ascertained in a selection-free way via RT-PCR of whole blood RNA, and its sensitivity and specificity were reported as 59% and 47%, respectively; however, due to lack of selection, there was no way to ascertain which cells expressed the marker[131]. While PSMA is a promising marker for overt prostate tumor detection, the application of PSMA as a marker for rare PCa cells needs further assessment, as its true specificity is still in question.

Prostate stem cell antigen

Prostate stem cell antigen (PSCA) is an androgen-regulated glycosylphosphatidylinositol-anchored membrane-bound glycoprotein, originally identified as a prostate-specific tumor-promoting antigen in 1998[132,133]. Its expression is restricted to the basal layer of the prostate, and it is the only protein in this article that is expressed by basal cells[132]. It is expressed in approximately 88–94% of primary PCa specimens[132,134], one study observed 100% (9/9) of bone metastatic lesions to be PSCA-positive[134]. Another study by Lam et al. found a PSCA protein expression in 87.2% (41/47) of cases of bone metastases[135]. PSCA may be a useful marker for PCa prognosis[135–137], as one study reported PSCA mRNA expression in the peripheral blood of 71% of PCa samples, 13% of benign prostatic hyperplasia samples, and 0% of non-prostate disease controls[138]. A similar study reported a sensitivity of 40% in patients with gastrointestinal tumors[139]. However, because there was no selection process in these studies, whole blood RNA was assessed, so it is unclear whether the PSCA-positive cells were actually prostate cells or another type of cell. As we have discussed, this is one significant drawback to RT-PCR compared to IF assays. Though there are several reports showing absence of PSCA expression in non-prostatic tissues[132,134], others have found expression in the normal epithelium of various tissues, including the urinary bladder, kidney, and intestine[112,134,140–142] (SUPPL. TABLE 1). PSCA is also overexpressed in various cancers, including urothelial, kidney, and lung[143–147]. In some cancers, it is down-regulated, indicating it may also play a tumor suppressive role, depending on the tissue[140,142,148–150]. Overall, data suggest that PSCA expression is not actually specific to the prostate, which makes it a less desirable marker for rare cells assays on its own. However, its expression in the basal cell compartment of the prostate indicates that it could potentially be used for certain subsets of PCa that are of basal cell origin.

Alpha-methylacyl-CoA racemase

Alpha-methylacyl-CoA racemase (AMACR, also known as P504S) is a peroxisomal and mitochondrial enzyme involved in bile acid biosynthesis and beta-oxidation of branched-chain fatty acids, and it is not androgen-regulated[151,152]. Its expression is granular and cytoplasmic. Apart from the prostate, AMACR is expressed in other normal tissues, including BM cells[112] (SUPPL. TABLE 1). AMACR is also overexpressed in almost every type of carcinoma assessed, including over 95% of PCa cases[112,153,154]. It is thus not useful in distinguishing PCa from other malignancies. However, it is still commonly used as a diagnostic biomarker for PCa due to its stronger expression in malignant relative to normal tissue, and it is often used in combination with a negative marker for PCa such as the basal cell marker p63[155–157]. In an RNA-based study from patient blood, AMACR expression was found in only 16/22 PCa patients, as well as 11/20 non-PCa patients, indicating poor sensitivity and specificity, although there was no selection process, so there is no way to assess which cells were expressing the marker[158]. AMACR can be detected (in tissue studies) in approximately 80% of atypical, non-hormonally-regulated PCa, such as small foci prostate adenocarcinomas and pseudohyperplastic carcinomas[157,159]. AMACR is also overexpressed in non-cancerous prostate diseases, such as adenosis, post-atrophic hyperplasia, partial atrophy, and prostatic intraepithelial neoplasia[160]. AMACR RNA is expressed in the BM[112]; therefore, it cannot be used for BM-DTC detection in PCR assays.

However, more work needs to be done to determine its sensitivity and specificity in rare cell assays.

Prostate specific acid phosphatase

Prostate specific acid phosphatase (PSAP, also known as prostatic acid phosphatase (PAP) and prosaposin) is a glycoprotein that hydrolyzes esters under acidic conditions to yield inorganic phosphates, and it is one of the major proteins that is secreted by the prostate[161,162]. It is an androgen-regulated protein that was first discovered in 1938 by Gutman *et al.* who showed that the level of PSAP was increased in the blood of patients with localized PCa, and was even more highly expressed in metastatic disease, relative to healthy individuals[163]. It thus became the first serum tumor marker for biochemical testing to diagnose and monitor progression of PCa. Later, PSA was found to be a more sensitive and specific biomarker and replaced PSAP in these assays. A study by Walsh *et al.* evaluated 460 localized PCa cases, and only 0.9% of cases were PSAP-positive and PSA-negative, indicating that PSAP detection would not capture additional cancer cells that would not already be detected by PSA[164]. PSAP is still occasionally used for the evaluation of PCa tissue by IHC, where it shows granular cytoplasmic staining. PSAP is expressed at moderate to high levels in normal prostate tissue and is strongly expressed in >95% of malignant prostatic tissue[165–167]. While these studies are tissue-based, and not cell-based, they suggest that PSAP may be a sensitive marker for PCa in general. However, a study by Perner *et al.* showed that PSAP was expressed in only 84% and 77% of lymph node and distal metastases, respectively, suggesting that express may be lost in a clonal fashion during metastasis[61]. It is also expressed in a variety of other cancers, including melanoma, lymphoma, cancer of the testis, and urothelial cancer[112] (SUPPL. TABLE 1). Several studies have reported expression of PSAP protein in normal non-prostatic tissues, including granulocytes[112,165,167–171]. Importantly, Uhlén *et al.* detected protein and mRNA in normal female and male BM tissues, indicating decreased specificity for BM-DTC detection[112]. Despite its high expression in most prostate carcinomas, the distribution of PSAP expression in other healthy tissues, particularly immune cells and other BM cells, indicates that PSAP is not as prostate-specific as was initially suggested, and may not be specific enough to be used alone as a detection marker for CTCs or BM-DTCs.

TMPRSS2-ERG

The transmembrane protease, serine 2 (TMPRSS2) gene is androgen-regulated and is located close to the erythroblastosis virus E26 transformation specific related gene (ERG) on chromosome 21. In about 50% of PCa patients a gene rearrangement occurs between *TMPRSS2* and *ERG*, which produces the androgen-regulated over-expressed fusion protein TMPRSS2-ERG, where ERG is the driving oncogene[172]. The TMPRSS2-ERG fusion is typically assessed via FISH, and is nearly 100% specific for prostate tissue (SUPPL. TABLE 1). ERG expression by IHC can also be used as a surrogate for expression of the fusion gene[173], and ERG staining has been associated with worse prognosis for PCa patients[174]. Even before the discovery of the TMPRSS2-ERG gene fusion, the presence of ERG in PCa was reported[175]. Similar to PCA3, TMPRSS2-ERG has utility as a biomarker in urine tests with 37% sensitivity and 93% specificity[176]. When TMPRSS2-ERG and PCA3 detection in urine samples was combined, sensitivity increased to 73%, which still falls short of the ideal

sensitivity for a rare cell assay. However, due to their high specificity for PCa cells, both of these markers have value moving forward, likely in combination with other markers. The biggest advantage of using TMPRSS2-ERG to detect PCa cells is that it is specific to cancer cells, and has not been found in normal prostate tissue. Most of the other candidate prostate-specific markers discussed in this article have been detected in benign tissue, making it difficult to differentiate cancer from benign. In rare cell assays, it is likely that only cancer cells will be present in blood or BM, but that has not been definitively proven. It is possible that non-cancer cells could slough into the blood and be identified as cancer cells based on expression of prostate-specific markers. In patients known to have TMPRSS2-ERG expression in their primary tumor, including TMPRSS2-ERG as an additional marker for CTC/BM-DTC detection would eliminate doubt about the origin of the rare cells in question. It is important to note that other gene fusions exist in PCa, including a prostein-ERG fusion[177], TMPRSS2 fusion with other ETS family genes such as TMPRSS2-ETV4[178], as well as many other fusions that have not been assessed for their sensitivity but could be useful in identifying cancer cells in a multiplex FISH staining strategy[179].

Prostate cancer antigen 3

Prostate cancer antigen 3 (PCA3, initially known as differential display clone 3, or DD3), is an androgen-regulated long non-coding RNA (lncRNA) that was discovered in 1999[180,181]. PCA3 down-regulates expression of the tumor suppressor PRUNE2, thereby promoting tumor progression[182,183]. PCA3 is overexpressed in around 95% of PCa cases and is thought to be prostate-specific, as it was not detected in 18 other normal tissues in a major study (although blood and BM were not assessed)[180] (SUPPL. TABLE 1). As a lncRNA, PCA3 cannot be detected by IHC or IF, and its detection is limited to RT-PCR or fluorescent *in situ* hybridization (FISH) assays[29,184]. PCA3 is currently being tested as a urinary biomarker for PCa, although its sensitivity is limited, even when combined with urinary biomarkers[176,185,186]. Overall, PCA3 holds some promise as a marker of rare PCa cells, but because the combination of IF with FISH is technically challenging, we are less enthusiastic about this marker for rare cell assays.

Homeobox protein NKX3.1

NKX3.1 is a homeobox-containing transcription factor. It is androgen-regulated and is therefore largely prostate-specific, although – like PSA – its expression can be regulated independent of AR. It is often used as an IHC marker of prostatic origin in metastatic tumors[187]. NKX3.1 is primarily detected in secretory prostatic epithelia, and its staining pattern is primarily nuclear, though it can also be seen in the cytoplasm[188]. It is one of the earliest known markers of prostate development[189]. It is a putative tumor suppressor in PCa, as it functions to inhibit prostate cell growth and proliferation in a context dependent manner, and one allele is frequently deleted in patients with PCa[189]. It has been reported that NKX3.1 expression is high in primary PCa tumors, but low in high-grade tumors and absent in metastatic PCa[190,191]. However, Gurel *et al.* assessed the performance of NKX3.1 as a marker of hormone naïve metastatic PCa and found that the sensitivity for NKX3.1 expression was 98.6%[187], as 68/69 of cases were positive. The same study showed the specificity of NKX3.1 was 99.7% as only 1/349 non-prostatic tumors was positive. This discrepancy with previous studies is most likely explained by the use of different antibodies,

where the latter study used an ostensibly better antibody[190,191]. NKX3.1 has been found in rare invasive lobular breast carcinomas and in benign testis[189,192,193] (SUPPL. TABLE 1). Uhlén *et al.* detected mRNA expression in a plethora of healthy tissues, including the salivary glands, kidney, testis, and importantly, the bone marrow, but did not assess protein expression[112]. Altogether, these data suggest that NKX3.1 is relatively sensitive for PCa cells, but potentially not specific enough to differentiate PCa cells from BM cells, although this has yet to be tested at the protein level.

Homeobox B13

Homeobox B13 (HOXB13) is a transcription factor that is involved in prostate development and is one of the few markers discussed here whose expression is androgen-independent[194,195]. HOXB13 may physically interact with AR in the nucleus of prostate cells, potentially in an inhibitory fashion[196,197]. It is expressed in normal prostatic tissue[198], and overexpressed in PCa[197,199]. It is used to identify metastatic prostate tissue[200]. The HOXB13 G84E variant mutation is associated with significantly increased risk of hereditary PCa[201]. The fact that there is a reported lack of any truncating mutations in HOXB13 and the recurrent nature of the G84E change, suggest a carcinogenic mechanism that is most likely of oncogenic nature (gain of function) than of tumor-suppressor nature (loss of function). The staining pattern of HOXB13 is primarily nuclear, but can also be seen in the cytoplasm. Weak to moderate cytoplasmic staining has been observed in some non-prostatic cancers, such as in liver and lung cancers[112] (SUPPL. TABLE 1). Furthermore, Uhlén *et al.* reported low expression of HOXB13 in patients with lymphoma[112]. A recent study by Barressi *et al.* compared the diagnostic value of HOXB13 and PSA protein expression to determine if metastatic tissue was of prostatic origin[202]. HOXB13 immunostaining was strong in >75% of the neoplastic cells in 100% (15/15) of the prostatic metastases, and weak staining was found in <25% of the neoplastic cells in 17% (2/12) of urothelial carcinoma metastases. The sensitivity and specificity of HOXB13 for metastatic PCa were 100% and 94%, respectively. Furthermore, the sensitivity and specificity of PSA for these metastatic PCa tissues were 53% and 100%, respectively[202]. A study by Varinot *et al.* also assessed HOXB13 sensitivity, and reported that while all 400 PCa tumors they assessed expressed some level of HOXB13, bone metastases had less frequent HOXB13 expression, although this could have been due to decalcification of the bone tissue[200]. Another group showed that HOXB13 expression was found in 52% of 10,216 PCa patient samples, and that stronger staining was associated with PCa cells relative to normal prostate cells, giving it prognostic relevance[197]. Interestingly, it appeared that HOXB13/AR interaction resulted in a reduction of PSA expression, indicating that HOXB13 and PSA could be used together in rare cell IF assays. Overall, these data suggest that HOXB13 is a promising candidate marker for the detection of prostate CTCs and BM-DTCs due to its specificity and androgen-independence in tissue-based assays, but work needs to be done in rare cell assays to fully ascertain its utility.

Prostatic secretory protein of 94 amino acids

Prostate secretory protein of 94 amino acids (PSP94, gene name *MSMB*) is one of the first three secretory proteins in the prostate to be identified, in addition to PSA and PSAP[203]. PSP94 was originally identified as beta-microseminoprotein (MSMB)[204], or beta-inhibin[205],

and is an androgen-regulated immunoglobulin-binding factor that is secreted into seminal plasma[206–209]. Its specific function is still uncertain, but it has been suggested that it increases sperm quality[210] and acts as a fungicidal agent in sperm[211]. PSP94 protein has been found in numerous additional secretions, including mucous gland secretions[212]. Its expression has also been detected in tonsil, skin, bronchus, stomach, testis, and seminal vesicle tissue[112] (SUPPL. TABLE 1). PSP94 expression in cancer is somewhat unclear. Overexpression of PSP94 has been observed in ovarian cancer[213], while several studies have shown that it acts as a tumor suppressor in PCa[214–218]. One study in PCa showed that while PSP94 expression was inversely correlated with Gleason score, its expression persisted after hormone therapy while PSA expression decreased, indicating that PSP94 expression can be up-regulated in the absence of androgens[219]. Support for its putative role as a tumor suppressor comes from the observed association of the loss of function of variant *MSMB* alleles with increasing PCa risk[220,221], as well as its antifungal, and therefore anti-inflammatory properties[221]. It has also been shown that a driver of PCa, EZH2, targets and silences PSP94[222]. Finally, a synthetic peptide corresponding to certain PSP94 amino acids has been shown to decrease vascular endothelial growth factor (VEGF) expression in endothelial cells, indicating PSP94 may have anti-angiogenic effects[223]. All in all, PSP94 is not likely a suitable candidate for CTC and BM-DTC detection due to its varied expression throughout PCa progression.

Prostein

Prostein (also known as p501s, and solute carrier family 45 member 3, or SLC45A3) is one of the latest prostate-specific markers to be discovered, having been found via a genome-based approach in 2001[224]. It is also the least published marker in this article, with only 86 results in PubMed, compared to 29,628 results for PSA (FIG. 3). Prostein is an androgen-regulated type IIIa transmembrane protein located in the Golgi apparatus with functions related to macromolecule transport[225]. Prostein is expressed in normal prostate tissue as well as PCa tissue[61,226], even when PSA is negative[225,227]. It has a unique granular staining pattern, which helps to distinguish it from other markers and increases confidence of true staining. Prostein has been used to differentiate PCa (prostein-positive, p63-negative) from urothelial cancers (prostein-negative, p63-positive) in tissue IHC[55]. Along with HOXB13, prostein is one of the most prostate-restricted proteins in tissue-based assays, though its expression has also been found in lung and bladder cancer[228] (SUPPL. TABLE 1). To date, prostein expression has been analyzed on different normal non-prostatic tissue, but none of these tissues expressed this marker, though it has not been extensively characterized. One study compared tissue expression of prostein to expression of PSA, PSAP, PSMA, AR, and ERG in primary PCa and metastatic tumors, and found that prostein sensitivity was decreased in metastatic tumors, although it was still expressed in 89% of tumors[61]. They also found that when PSA was absent in tumors, prostein and AR were present, indicating that more than one prostate-specific marker should be used to increase sensitivity in IHC and certainly in rare cell assays. Taken together, we believe that prostein is a promising marker for use in IF-based rare PCa cell assays, although this has not been directly tested.

Murine Prostate Markers

Mice are used extensively as *in vivo* models of prostate cancer metastasis, and rare cell assays have recently been developed for xenograft, syngeneic, and transgenic mouse models[29]. Xenograft models utilize human cancer cells, for which the markers we have thus far discussed are applicable. However, when using mouse models that develop murine prostate cancer (syngeneic models or genetically engineered mouse models (GEMMs)), one must consider the similarities and differences between rodent and human prostates at the anatomical and cellular expression levels. While the mouse prostate gland is histologically quite similar to the human prostate gland, there are significant differences. The human prostate surrounds the urethra at the base of the bladder. It is broken up into "zones" for grading and staging purposes, but anatomical zonation is not grossly apparent. The mouse prostate is broken up into several lobes: the anterior lobe, which is immediately next to the seminal vesicle; and the dorsolateral and ventral lobes, which are anatomically similar to the human at the base of the prostate[229,230]. In the mouse and human, all prostate glandular secretions go into the urethra and make up a significant portion of the ejaculate. Another significant difference between the human and mouse prostate is the ratio of luminal to basal cells. In the human, the ratio is approximately one luminal cell per basal cell, and in the mouse, the ratio is closer to 3:1[231].

In terms of gene expression, mice do not express PSA, KLK2, or PCA3 (Table 3). Of the kallikreins, only KLK4 has a murine ortholog[232]. Mice express a PSCA ortholog, which is 70% similar to human[132]. PSP94, PSMA, and PSAP are also expressed, and are specific to the mouse prostate[233–235]. Mice also express Hoxb13 independent of androgen, and this gene has been used to create a GEMM of PCa[236,237]. Nkx3.1 is another marker present in mice, and its role in prostate development and tumorigenesis has been studied extensively in mouse models[238–240]. Mice also express an AMACR ortholog, though its role in murine prostate biology is limited[241]. It is unclear based on published literature if prostein is expressed in the mouse prostate at the protein level, although RNA ISH has shown that the *Slc45a3* gene is expressed throughout developing tissue in mouse embryos[242]. Mice express both TMPRSS2 and ERG, although with no prostate specificity, and the TMPRSS2-ERG fusion does not occur in mice because they never develop *de novo* PCa[243]. Mice also express AR; in fact, many of the androgen signaling paradigms have been discovered by studying mouse or rat AR (see above section on AR). However, an important consideration is that AR activity in mice might differ from human due to the amount of testosterone in either species at any given time – it has been shown that a hormonally intact male mouse has approximately as much circulating testosterone as an androgen-ablated male human[85,244].

Perhaps the best way to use mice as an *in vivo* model for rare cell studies is to inject genetically labeled human or mouse cancer cells into the mice, harvest blood and/or BM at specific time points, and then use the genetic marker for CTC/BM-DTC detection[29]. It is inefficient and less desirable to conduct rare cancer cell research in most GEMMs due to the slow progression of the disease. However, some of the newer rapidly progressing PCa models, especially those marked with fluorescent molecules, may allow for further study of CTCs and DTCs in GEMMs[245]. Some of the mouse PCa marker orthologs that exist could be useful for detecting mouse CTCs/BM-DTCs with the intent to characterize and study

their roles. Ultimately, while mouse models have been invaluable to model prostate development and disease, there is no substitute for detecting human prostate-specific markers on prostate cancer cells in human blood or BM.

Discussion

Despite early detection and treatment advancements, PCa patients continue to have poor outcomes largely due to bone metastasis. CTCs and BM-DTCs are the source of overt bone metastases; therefore, these rare cells can offer important clinical insights, as well as a better understanding of the biology underlying successful dissemination[12,13,246]. Due to easier sample access (blood versus BM), CTCs represent a cell population that will likely be more clinically useful in real time. BM-DTCs, however, may represent a more biologically important cell population because they have successfully disseminated. However, as discussed, it is difficult to detect and accurately identify BM-DTCs due to their rarity and the lack of sensitive and specific protein markers. While putative CTCs can generally be found using epithelial markers in IF assays, BM-DTCs are more difficult to assess due to the complex cellular heterogeneity of the BM relative to the blood, which includes autofluorescent cell types and occasional cells that express certain epithelial markers[247,248]. While certain cancer-specific markers (e.g. Myc) might be expressed in rare cancer cells, they are often also expressed in a variety of other cells in blood and BM. Therefore, we propose that using prostate-specific markers could improve the accurate detection of rare PCa cells in liquid biopsies.

Due to the sensitivity requirement of rare cell assays (detection level of one single cancer cell in a field of millions of WBCs), new challenges have arisen with regard to the specificity of putative prostate-specific markers. Several of the prostate-specific markers described in this paper are used to help differentiate PCa tumors from other types of cancer, particularly in the metastatic tissue setting. In rare cell assays, the use of RT-PCR and IF (coupled with automated scanning microscopy)[29,249] allows for highly sensitive detection of RNA and protein, respectively. However, published reports about the specificity of these putative prostate-specific markers were not focused on rare cell detection but rather sectioned tissue, and thus were not as focused on confirming that every positively stained cell was indeed of prostate origin. A protein that is considered sensitive and specific in a tissue-based assay may not be considered as such in a rare cell assay. For example, if a BM liquid biopsy containing ten million WBCs were to be stained for a putative cancer-specific marker, and only 0.01% of WBCs expressed that marker, approximately 1,000 WBCs would incorrectly be identified as a cancer cell using highly sensitive scanning techniques. Therefore, putative PCa markers require rigorous testing in known control and patient samples using rare cell-based assays, rather than tissue-based assays[250,251]. RNA from formalin-fixed CTCs or cells obtained via fluorescence activated cell sorting (FACS) or via selection techniques and assess via RT-PCR for finite gene panels is one promising methodology[33]. New technologies, such as multiplexed ion beam imaging coupled with mass cytometry (CyTOF) to determine the expression of a panel of approximately 100 markers at one time could be extremely useful to ascertain sensitivity and specificity of marker in rare cells assays[252–254].

For IF-based assays, the selection of the detection antibody is particularly important, as staining patterns and positivity can vary widely. Polyclonal antibodies are in general more sensitive and have a higher probability of detection in a range of different conditions, but they are generally less specific than the monoclonal antibodies[255]. There are many other factors that can influence the staining of an antibody, such as tissue processing, fixation reagents and timing, antigen retrieval type and timing, microscope type, and automated scanning settings[256–258]. Proper training at each of these stages, as well as proper recording and communication of protocols, is of utmost importance during the process of identifying new markers for rare cell assays[259]. Even if an antibody has been rigorously tested, depending on the type of tissue and exact staining protocol involved, it can still result in false positivity or negativity. For instance, NKX3.1 is present in the nucleus of prostate cells, but can also stain in the cytoplasm of other tissues[187]. Markers that only stain in the cytoplasm, like PSA, might not be ideal markers for rare cell assays because diffuse false positive cytoplasmic staining is seen on occasion simply due to processing. Therefore, it would be ideal to combine markers that have different staining patterns using multiplex staining. For example, an ideal multiplex protocol might include a nuclear marker (e.g. HOXB13), cytoplasmic marker (e.g. PSA), and a marker with a unique staining pattern (e.g. prostein, which localizes to the Golgi apparatus) (FIG. 4). In this review, we have largely focused on protein expression because IF can provide more information than other techniques, such as RT-PCR. While RT-PCR is more sensitive in terms of its ability to detect small amounts of RNA, it does not provide information about cellular heterogeneity in terms of which cells express which RNA. IF can provide visual evidence of protein expression, and in multiplex assays can provide expression information about multiple proteins on a single cell. Given the fact that protein expression provides insight into function, IF-based assays also have the advantage of being able to understand the role and clinical application of detected cells. In addition, single cell picking techniques have improved to the point where genomic and proteomic analyses can be performed at the single cell level[26,260–262].

Each prostate marker we have discussed in this article has a varying degree of specificity to the prostate gland or PCa. Some, like PSA, prostein, HOXB13, and KLK2, appear to be highly specific for prostate tissue, based on tissue-based assays. Others, like AR, PSAP, PSCA, and PSMA are much less specific. In addition, some markers become aberrantly expressed in a variety of cancers, even if they were not expressed in the corresponding healthy tissue (e.g. PSA is occasionally found in lung cancer even though it is not expressed in healthy lung tissue). However, we postulate that a prostate-specific marker only needs to be specific to PCa cells in that any other cells that are present in a liquid biopsy do not express the marker. This includes blood and BM cells such as all immune cells, hematopoietic and mesenchymal stem cells, BM stromal cells, osteoclasts, and endothelial cells, among others. This is based on the high unlikelihood that a PCa patient will have cancer of another tissue, whereby even if a marker of interest is highly expressed in prostate cells but also expressed in pancreatic cells, it would still be acceptable for use in a liquid biopsy.

The sensitivity of the detection marker is also extremely important to ensure that every PCa cell that is present in a blood or BM sample from a patient is identified. Since CTCs and BM-DTCs are so rare, failing to detect only a few cells could have major clinical

implications. This means that every PCa cell that enters the bloodstream and/or BM would ideally express the detection marker. Unfortunately, information to this degree is severely lacking in the published literature. Most reports have determined the sensitivity of prostate markers via IHC, where sensitivity is discussed in terms of the percentage of patients where positive staining was observed. Instead, for rare cell assays, the number of PCa cells that are detected with the marker out of a known total number of PCa cells present should be determined. This may be impossible to assess in clinical samples, considering there is no perfectly sensitive marker to our knowledge that would provide the true number of cancer cells present in a sample. To overcome these obstacles, increasing the number of markers so as to "catch" every cell would be helpful, as long as they are each highly specific. Even so, for some less common types of PCa (e.g. neuroendocrine, small cell, or carcinoid), the classic prostate markers like PSA or NKX3.1 will not be helpful[104]. Instead, other markers such as synaptophysin or chromogranin might be required to identify these cells[263].

An important concept to consider is that a marker does not need to be as sensitive or specific if it is not being used for detection purposes. Once the CTC/BM-DTC is detected by highly sensitive and specific marker(s), it does not matter if a marker being used to study biological characteristics or to drive therapeutic decisions is also present on a non-PCa blood or BM cell. For example, we have discussed AR as being a relatively non-prostate-specific marker, as it is expressed in many other healthy tissues, including the BM. Therefore, we would not recommend using AR to detect or identify PCa CTCs or BM-DTCs. However, the expression of full-length AR or its variant form (AR-V7) has been shown to be clinically informative as to whether to treat metastatic PCa patients with either taxanes or second line hormonal therapy[19,21,107]. This is an excellent example of the importance and applicable range of using liquid biopsies and rare cell assays on liquid biopsies to directly impact patient care.

Concluding Remarks

The aims of this review article were to emphasize the difficulties in accurately identifying rare prostate CTCs or BM-DTCs with the commonly used epithelial markers, and the subsequent need for prostate-specific biomarkers in the detection of these cells. While much has been done to identify and quantify CTCs in the blood of cancer patients, much less has been done in bone marrow to identify BM-DTCs. BM-DTCs are likely the "important CTCs," meaning they are responsible for lethal bone metastases, and therefore contain biological characteristics required for successful dissemination. As rare cell assays need to be exceptionally sensitive, it is crucial that sensitive and specific markers are used to differentiate cancer cells from blood and BM cells, but unfortunately little is known about candidate marker expression on PCa cells at an individual cell level. We have attempted to compile an exhaustive list of published prostate-specific markers as a starting point for determining which markers should be investigated further to be used for CTC/BM-DTC detection in the future. Some markers, like AR and PSAP, are too non-specific to be used as individual markers of PCa cells, while others, such as PSA, prostein, and HOXB13, hold more promise as sensitive and specific markers. It is likely that multiple specific markers will have to be combined to increase overall sensitivity. The goal of future studies must be to consistently and reliably identify rare cancer cells using sensitive and specific markers.

Although this review has focused on PCa, the same strategies are applicable to rare cell assays in any type of cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Key points

- Liquid biopsies, particular from bone marrow, may allow for the detection of recurrent disease before overt lethal metastasis develops.
- Prostate cancer cells from liquid biopsies, particularly bone marrow, are rare and extremely difficult to identify accurately.
- Prostate-specific markers may help identify rare prostate cancer cells from liquid biopsies using rare cell immunofluorescence assays.
- Expression of putative prostate-specific markers is not always constrained to prostate cells. Expression of candidate markers for rare cell assays must be ascertained on an individual basis as to their sensitivity and specificity.
- Immune cells in the blood and bone marrow provide a significant source of non-specific staining, so measures must be taken to reduce this background staining.
- Combinatorial staining of multiple prostate-specific markers will increase accuracy in identifying rare prostate cancer cells in liquid biopsies to understand the role and clinical application of these important cells.

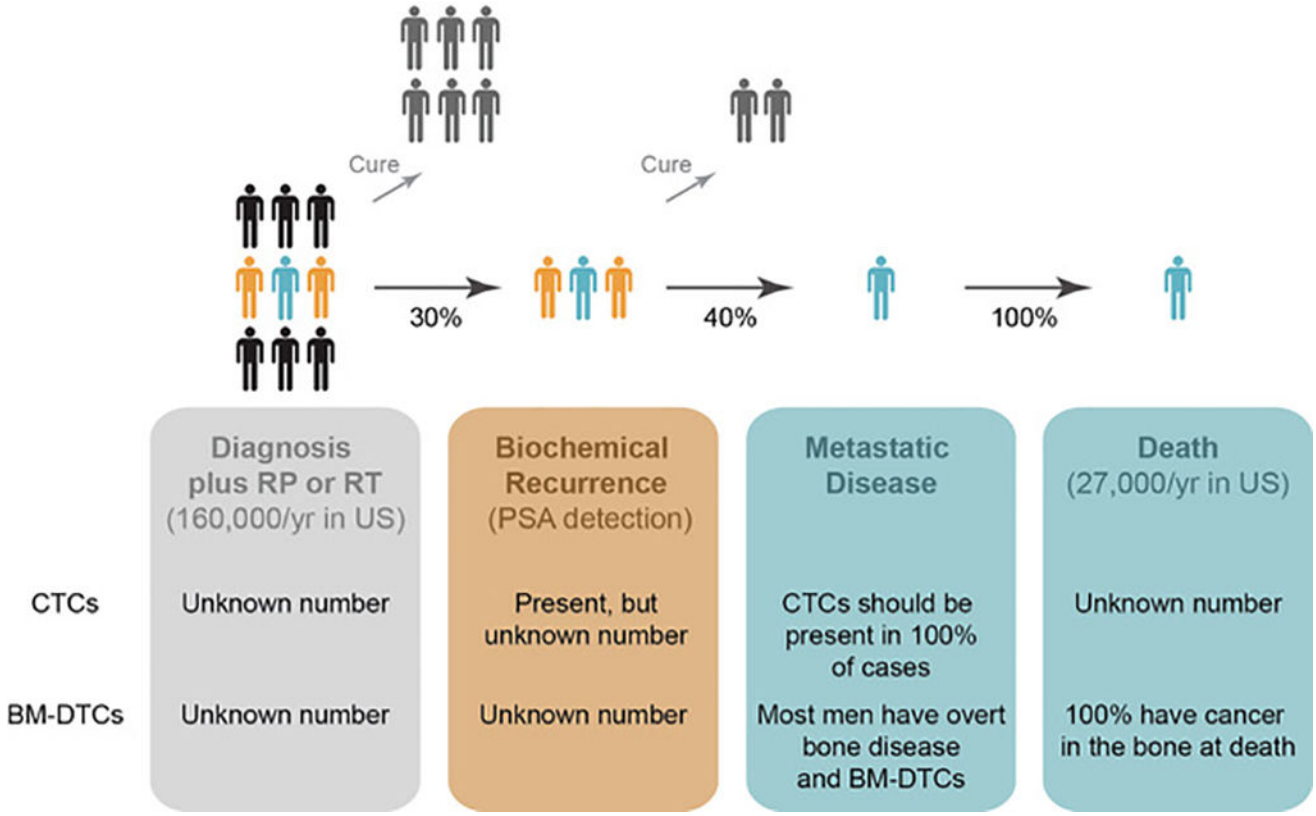

Figure 1. Timing of tumor dissemination through prostate cancer progression

Approximately 70% of men who are diagnosed with prostate cancer and treated with either radical prostatectomy (RP) or radiation therapy (RT) will be cured, but 30% (blue and orange stick figures) will develop biochemical recurrence based on the prostate specific antigen (PSA) blood test. Of these men, about 40% (blue stick figure) will fail treatment (hormone therapy and/or chemotherapy) and progress to castration resistant metastatic disease, for which there is no cure. Few experimental data exist regarding the timing of dissemination of cancer cells to the bone marrow. The detection and study of rare cancer cells throughout the natural history of prostate cancer could enable the earlier identification of high-risk patients for metastatic disease. This would, in turn, allow for earlier intervention and the design of therapies aimed at preventing metastasis. CTC: circulating tumor cell; BM-DTC: bone marrow disseminated tumor cell; and Rx: treatment.

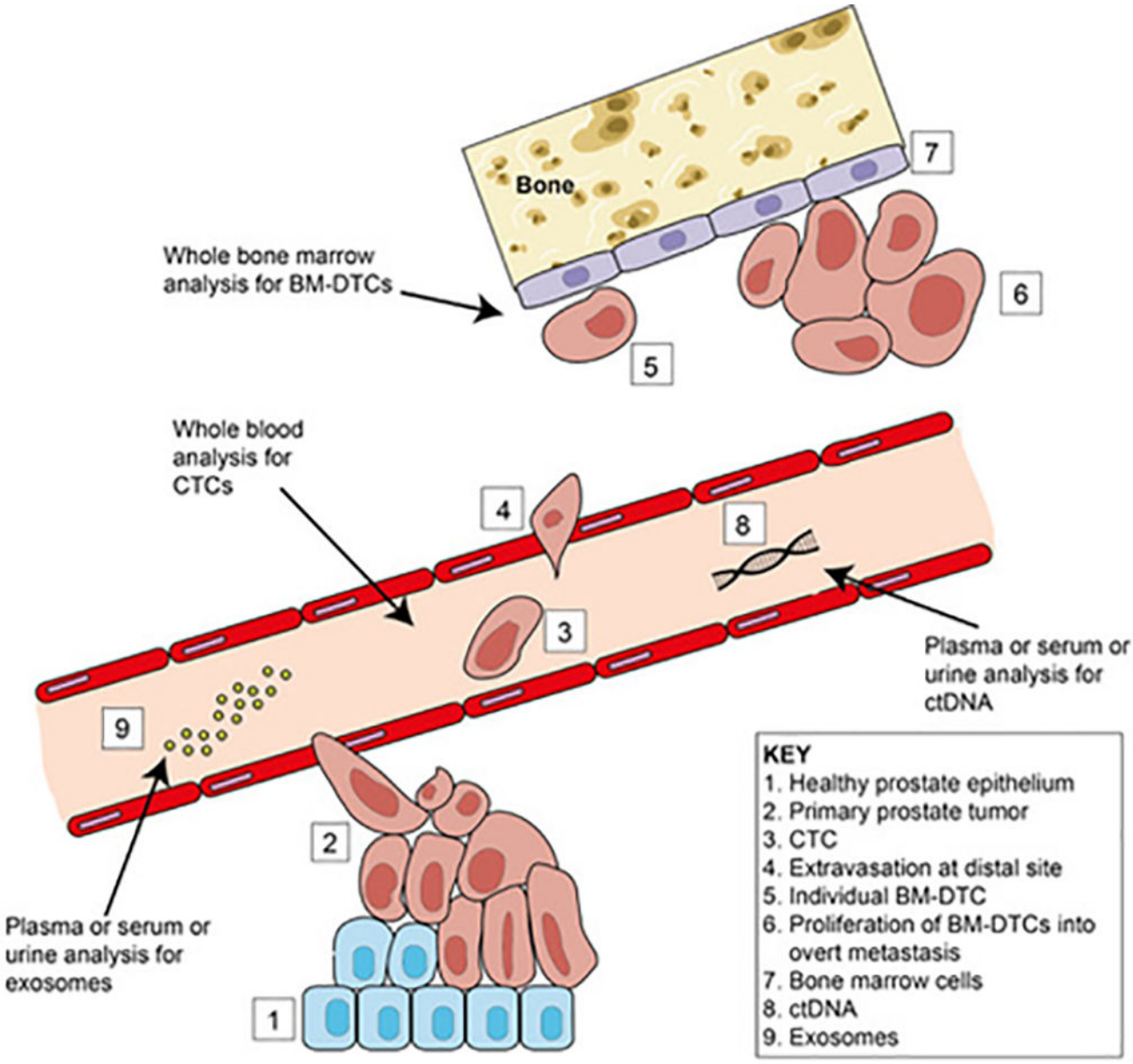

Figure 2. Liquid biopsies in cancer

Schematic overview of liquid biopsy sampling from blood or bone marrow in order to detect circulating tumor cells (CTCs), bone marrow disseminated tumor cells (BM-DTCs), circulating tumor DNA (ctDNA), and/or exosomes.

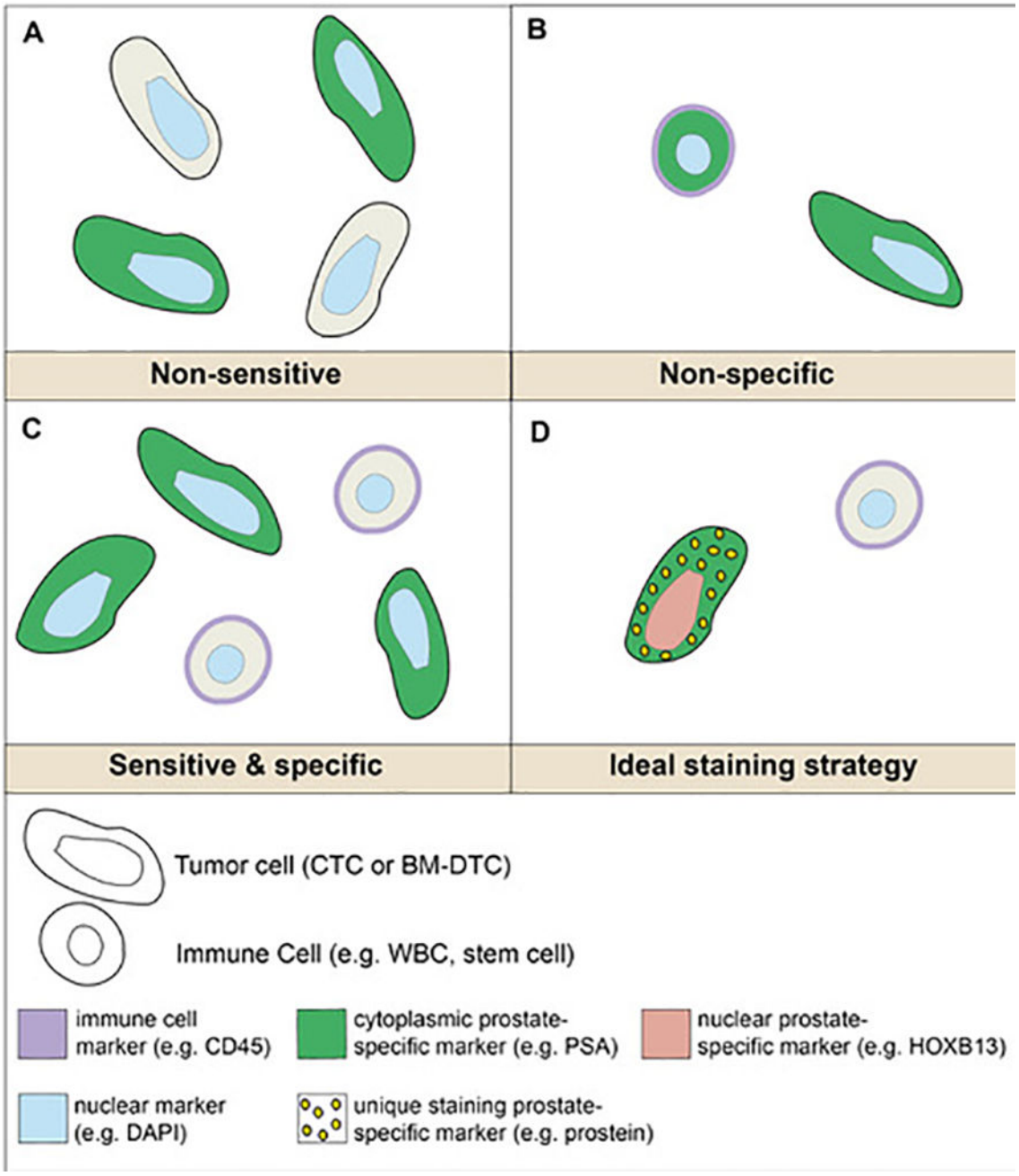

Figure 3. Ideal expression patterns of prostate-specific markers for identification of rare cancer cells

(A) Example of a non-sensitive marker, expressed on only 50% of the cells; (B) example of a non-specific marker, expressed on the tumor cell as well as the immune cell; (C) example of a sensitive and specific marker, expressed on all of the tumor cells present but none of the immune cells; and (D) the ideal strategy for detection includes multiple sensitive and specific markers, each with a different staining pattern. CTC: circulating tumor cell; BM-DTC: bone marrow disseminated tumor cell; WBC: white blood cell; DAPI: 4’,6-diamidino-2-phenylindole; and PSA: prostate specific antigen.

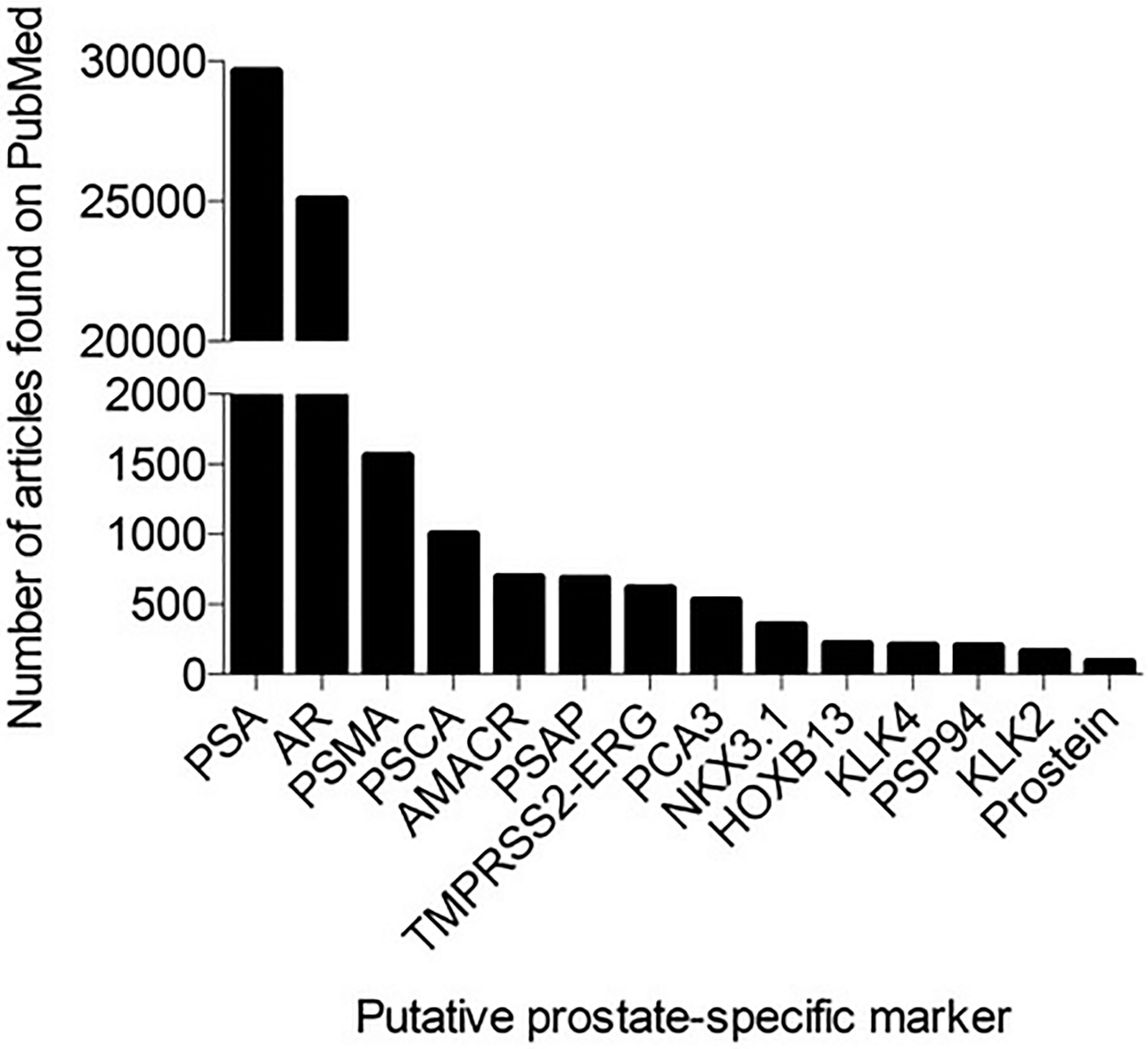

Figure 4. Prevalence of prostate-specific marker publications

We performed a search on PubMed (www.ncbi.nlm.nih.gov/pubmed) on August 17, 2017 for the following search terms in all fields. PSA: “prostate specific antigen or psa or klk3;” AR: “androgen receptor;” PSMA: “psma or folh1;” PSCA: “psca;” AMACR: “alpha-methylacyl-CoA racemase;” PSAP: “psap or prosaposin;” TMPRSS2-ERG: “tmprss2-erg;” PCA3: “pca3 or dd3;” NKX3.1: “nkx3.1 or nkx3-1;” HOXB13: “hoxb13 or hox-b13;” KLK4: “klk4;” PSP94: “prostatic secretory protein of 94 amino acids or psp94 or msmb;” KLK2: “klk2;” prostein: “prostein or p501s or slc45a3.”

Table 1

Comparison of RT-PCR and immunofluorescence methods for CTC/BM-DTC detection

	RT-PCR	**Immunofluorescence**
Analyte	RNA	Protein
Advantages	• Very high sensitivity • Optimization of primers is trivial • Rapid analysis	• Can determine number of cancer cells present in a given volume • Permits co-expression data on same cell by multiplexing • Permits biological characterization • Permits further downstream analysis through single cell picking
Limitations	• Does not indicate number of cancer cells present • Co-expression data per cell unavailable • RNA expression does not always correlate with protein expression • Does not permit biological characterization	• Not as sensitive as RT-PCR • Antibody performance dependent on many factors • Different staining protocols can give different results • Lengthier analysis

Table 2

Characteristics of putative prostate-specific markers

Marker	AR regulated	Expressed in healthy peripheral blood	Expressed in healthy bone marrow	Cellular staining pattern	References
PSA	Yes	No	No	Cytoplasmic	47, 56–70
AR	Yes	No	Yes	Nuclear/cytoplasmic	21, 47, 79–112
PSMA	Yes	No	Yes (mRNA)	Membranous in PCa/cytoplasmic in other tissues	61, 112–131
PSCA	Yes	No	No	Membranous	112, 132–150
AMACR	No	No	Yes (mRNA)	Granular cytoplasmic	112, 151–160
PSAP	Yes	No	Yes (mRNA and protein)	Granular cytoplasmic/membranous	112, 161–171
TMPRSS2-ERG	Yes	No	No	Nuclear	172–179
PCA3	Yes	No	No	Not applicable (RNA molecule)	29, 176, 180–186
NKX3.1	Yes	No	Yes (mRNA)	Nuclear	112, 187–193
HOXB13	No	No	No	Nuclear	112, 194–202
KLK4	Yes	No	No	Cytoplasmic	71–78
PSP94	Yes	No	No	Nuclear/cytoplasmic	112, 203–223
KLK2	Yes	No	No	Cytoplasmic	71–78
Prostein	Yes	No	No	Granular cytoplasmic	55, 61, 224–228

Table 3

Mouse orthologs of putative prostate-specific genes

Marker	Human Gene	Mouse Ortholog
PSA	*KLK3*	**Does not exist**
AR	*AR*	*Ar*
PSMA	*FOLH1*	*Folh1*
PSCA	*PSCA*	*Psca*
AMACR	*AMACR*	*Amacr*
PSAP	*PSAP*	*Psap*
TMPRSS2-ERG	*TMPRSS2 and ERG*	*Tmprss2* and *Erg* (separate genes; no fusion product)
PCA3	*PCA3*	**Does not exist**
NKX3.1	*NKX3-1*	*Nkx3-1*
HOXB13	*HOXB13*	*Hoxb13*
KLK4	*KLK4*	*Klk4*
PSP94	*MSMB*	*Msmb*
KLK2	*KLK2*	**Does not exist**
Prostein	*SLC45A3*	*Slc45a3*