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Novel Biomarkers in Determining Prostate Cancer Diagnosis and Prognosis

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Abstract: The clinical behavior and molecular pathology of prostate cancer is highly variable. Current "traditional" prognostic markers cannot reliably distinguish the potentially life-threatening cancer from indolent cancer. Identification of additional new predictors of cancer aggressiveness is therefore urgently required. This communication is aimed at a brief review of new biomarkers in prostate cancer diagnostics and prognostics. Pubmed systematic search was performed to collect both original and review articles addressing prostate cancer prognostic biomarkers using key words genetics, prostate cancer, biomarkers and prognosis. The development of molecular and immunohistochemical methods enabled the identification of potential biomarkers in relation to diagnosis and prognosis. Numerous promising markers and approaches have been identified. Some of these markers may be translated into clinical practice after verification in larger prospective trials in future and can help to determine diagnosis and prognosis of CaP more accurately.

Keywords: Prostate Cancer, Biomarkers, Diagnosis, Prognosis

Introduction

Prostate Cancer (PCa) is most common nondermatologic malignancy of men in Western Europe (Jemal *et al*., 2009; Ziaran *et al*., 2009). The clinical behavior and molecular pathology of PCa is highly variable. Identifying patient subgroups that require less treatment from those that should be targeted with more aggressive therapy is therefore a key goal. Because of the highly variable natural history of PCa, additional new predictors of cancer aggressiveness are urgently required. Over-treatment of PCs is a particular concern leading to substantial cardiovascular and skeletal morbidity (Ziaran *et al*., 2013a; 2013b). This is especially true for many Prostrate-Specific Antigen (PSA) screen-detected cancers, which in the absence of treatment, may never become life threatening. Conversely, more conservative approaches to disease detection and management can leave potentially aggressive cancers untreated. Therefore, improved biomarkers are required to allow radical therapies to be targeted to men with potentially lethal cancers, so that the others, with more benign-behaving indolent cancers, are spared inappropriate treatment.

Current "traditional" clinicopathologic prognostic markers predictive of outcome in men with CaP after Radical Retropubic Prostatectomy (RRP) consist of Gleason score, TNM stage, surgical margin status and preoperative serum Prostate-Specific Antigen (PSA) (Zummerova *et al*., 2010; Epstein *et al*., 2005; Repiska *et al*., 2005). Beyond the current clinicopathologic parameters, there have been other biomarkers and approaches proposed to: (i) Distinguish between indolent and potentially life threatening disease (ii) aid the decision for rebiopsy in previous negative biopsies with rising PSA, (iii) monitor the disease progression and its responsivness to therapy These approaches and markers include Genome-Wide Association Studies (GWAS), chromosomal aberrations, DNA-based markers, RNA-based biomarkers and protein markers (tissue, serum, urine biomarkers) (Manolio, 2010). Available methods to identify potential biomarkers include genomics, proteomics and tissue based immunohistochemical staining. Quantitation of cancer biomarker transcripts using Real-Time quantitative Polymerase Chain Reaction (qRT-PCR) of large samples may help in the search for clinically useful cancer biomarkers that can be integrated into clinical trial design (Jiang *et al*., 2007). Gene expression array technology applied to PCa has resulted in the identification of a number of genes that have been associated with outcome. More recently, Next-Generation Sequencing (NGS) have been described, which could bring promising information in

our understanding of the cancer genome of several tumor types (Macconaill and Garraway, 2010).

This article provides review of current most promising molecular and immunohistochemical biomarkers in CaP diagnosis, prognosis and clinical behavior.

Materials and Methods

Pubmed systematic search was performed to collect both original and review articles addressing CaP prognostic biomarkers using key words genetics, prostate cancer, biomarkers and prognosis.

Results and Discussion

Genetics of Prostate Cancer

Initiation of prostate cancer is a process resulting from the progressive accumulation of genetic disorders. On the basis of the constellation of polymorphisms of germ cell cancer the risk of developing cancer is individual. The cumulative effect of polymorphism genome may lead to one or more disorders and/or may "offer" an environment for further genetic alterations in prostate epithelial cells. Over time, additional somatic genetic disorders alter the behavior of prostate epithelial cells until it represents signs of malignancy (Zheng *et al*., 2008). Epigenetic alterations are other common events in carcinogenesis, including CaP, which may lead to aberrant expression of critical genes such as tumor suppressors and oncogenes. Although most CaP are classified into a single group of adenocarcinomas, there is no universal molecular path of CaP development. It is also unlikely that two prostate adenocarcinomas share the same genetic path in the development of cancer. Genetic and genomic technologies have helped to clarify the changes in genes that lead to the development of CaP. These changes provide a molecular basis from which diagnostic, prognostic and predictive biomarkers can be developed (Febbo, 2009). For genetically determined diseases, genetic alterations can be identified via methods of molecular genetics (e.g., polymerase chain reaction, fluorescence in situ hybridisation, genome sequention). These alterations, when identified, can be in turn used as biomarkers for prognosis of CaP and other cancers.

Criteria for a Candidate Biomarker

The National Cancer Institute defines a biomarker as a biological molecule found in blood, other body fluids, or tissues that is a sign of a normal or abnormal process or of a condition or disease. A biomarker may be objectively measured and evaluated as an indication of normal biologic processes, pathogenic processes, or pharmacologic responses to a particular treatment or condition.

Biomarker is an analyte that signifies the presence or degree of a biological process, which in itself is frequently directly linked to the clinical expressions and result of a particular disease. The selection of a cancer biomarker should have a biological or therapeutic basis or, at minimum, the biomarker should indicate a reliable correlation with the presence, characteristics, or aggressiveness of the cancer. Also, there should be an evaluation of the strength of the marker in relation to the outcome of the disease, which, together with other factors, should be carried out as an independent predictor in a multivariable assay in the general population (Hartwell *et al*., 2006). Biomarkers for the diagnosis and prognosis of PCa include DNA-based markers, RNA-based biomarkers and protein markers. They may be useful for prognostic purposes in the outcome of diseases, with particular attention on the quantitative biomarkers that demonstrate a relationship with the clinical manifestation of the disease and that have an effect on quality of life, risk of complications, or survival. Surrogate biomarkers have a significant function in disease monitoring after accepted treatments are introduced. Surrogates are particularly important for those treatments that are uncommon, such as cases in which the direct study has proved to be very difficult because of the limited number of patients and varying expression of their primary illness or in which the efficiency of the treatment must justify the high cost.

Identifying Discriminating Markers

With the completion of the Human Genome Project, the publication of the International Haplotype Map Project (a catalog of millions of common single nucleotide polymorphisms, or SNPs, in the human population) and a decrease in the cost of high-throughput genotyping, an unbiased genomewide search for inherited variants associated with PCa risk has become feasible. This approach, called a Genome-Wide Association Study (GWAS), scans the entire genome, evaluating common inherited variants (minor allele frequency >1-5% in the population) in large numbers of cases and controls (Manolio, 2010).

GWAS indicate genetic heterogeneity for the onset of disease with numerous low risk loci described along with two notable high-risk loci at 8q24 and 7q31. The linked loci on 8q24 are located immediately downstream of the MYC gene that is upregulated in PCa (Beuten *et al*., 2009; Robbins *et al*., 2007).

There are several potential mechanisms by which a genetic variant may be associated with altered cancer risk, including: (i) Genetic linkage to a coding variant in a cancer-relevant gene (i.e., the risk SNP is merely a proxy for the true causal exonic variant that was not tested in the GWAS), (ii) alteration in promoter/enhancer binding sites or chromatin structure affecting expression

of adjacent or distant genes, or (iii) change in the expression of noncoding RNAs. There is also a high probability that PCa genes/alleles act cooperatively in the aetiopathogenesis of the disease supporting the notion that it is unlikely that any one biomarker alone is likely to be conclusive in detecting and predicting outcome of cancer (Clarke *et al*., 2010).

Novel Biomarkers for CaP Diagnosis

The PCA3 and TMPRSS2: ERG Fusion

PCA3 is a noncoding RNA with expression confined to the prostate and which is highly overexpressed in 95% of PCas compared with normal or benign hyperplastic prostate tissue (Salagierski and Schalken, 2012). PCA3 has been assayed from urine following prostatic massage in 11 separate clinical studies totalling 2737 men from Western countries (Tosoian *et al*., 2010; Van Gils *et al*., 2007; Marks *et al*., 2007) with an overall sensitivity of 69% and specificity of 70% for men with PCa. The role of PCA3 in clinical practice as a commercially-available test remains uncertain with most advocates indicating a place in patients who have already had TRUS biopsies with a negative result for cancer but in whom PCa remains suspected.

Detection of the TMPRSS2: ERG fusion in urine has been reported to yield >90% specificity and 94% positive predictive value for PCa detection (Hessels *et al*., 2007), although a clinical diagnostic test is not yet available. The combination of urinary PCA3 and TMPRSS2-ERG with serum PSA levels has been reported to improve screening performance compared to PSA alone (Salami *et al*., 2013). Moreover, the recent study indicates that integration of levels TMPRSS2: ERG transcripts in urine, with PCA3-score androgenic status, genetic status and traditional clinical variables could significantly increase detection of high risk localized PCa (Cornu *et al*., 2013).

Early Prostate Cancer Antigen

Leman *et al*. (2007) reported results on a serum biomarker called Early Prostate Cancer Antigen (EPCA) using an antibody assay against the EPCA-2.22 epitope. The study involved 385 men and reported a 92% specificity for healthy men and men with benign prostatic hyperplasia and a 94% sensitivity for overall PCa detection. In addition, the authors indicated that EPCA-2.22 was highly accurate in differentiating between localized and extracapsular disease (Witt *et al*., 2000).

SPINK1

SPINK1 (also referred to as TAT1) is a biomarker for PCa that can be detected in prostatic massage urine. SPINK1, a trypsin inhibitor secreted from pancreatic acinar cells, is thought to function in the prevention of trypsin-catalyzed premature activation of zymogens within the pancreas and the pancreatic duct. Mutations of this gene are associated with hereditary pancreatitis and tropical calcific pancreatitis (Bhatia *et al*., 2002).

Laxman *et al*. (2008) showed that a multiplexed qPCR assay including SPINK1 on sedimented urine from patients presenting for prostate biopsy or prostatectomy outperformed serum PSA or PCA3 alone. SPINK1 expression in urine is also an independent predictor of biochemical recurrence after resection. On the other hand, recent study concludes that SPINK1 protein expression (evaluated by immunochemistry) may not be a predictor of recurrence or lethal PCa amongst men treated by radical prostatectomy (Flavin *et al*., 2014).

α-Methylacyl Coenzyme A Racemase (AMACR)

AMACR is an enzyme localized to the peroxisome and involved in fat metabolism and has been identified to function as a growth promoter, independent of androgens, in prostate cancer (Zha *et al*., 2003). By using various experimental methods and different PCa specimens, the AMACR gene has been shown to be overexpressed in PCa tissue at the mRNA and protein levels and making it a highly specific tissue biomarker currently used to aid in the pathological diagnosis (Jiang *et al*., 2004).

When PCa tissues were compared with normal controls, a 9-fold increase in mRNA levels of AMACR was discovered in 88% of the sample PCa tissues (Rogers *et al*., 2004). Immunodetectable serum autoantibodies generated in response to the AMACR tumor-associated antigen may also be useful in preliminary diagnosis, especially if combined with PSA screening. A considerably more enhanced sensitivity and specificity in PCa patients with mid-range PSA levels have been observed with AMACR antibodies than that with PSA. This demonstrates that AMACR can be useful in discriminating control subjects from those with PCa (Sreekumar *et al*., 2004). Interestingly, it has been described, that trifluoroibuprofen, an AMACR inhibitor, reduces cancer cell proliferation and Inhibits in vivo tumor growth in aggressive PCa models (Festuccia *et al*., 2014). This makes AMACR one of possible therapeutical targets in future.

Glutathione S-transferase P1 (GSTP1)

GSTs are aubiquitous family of multifunctional enzymes that conjugate reactive substrates with reduced Glutathione (GSH) and are involved in detoxification. Their role is in protecting the cells from oxidative attack. The GSTP1 gene has been observed to be unmethylated in all normal human tissues and BPH, but hypermethylated in specimens of PCa tissues (Harden *et al*., 2003). GSTP1 has been shown to be acutely sensitive in detecting the presence of prostatic intraepithelial neoplasia and PCa,

thereby distinguishing patients with these diseases from patients with BPH (Lee *et al*., 1994).

Biomarkers for Determining CaP Prognosis and Progression

Loss of PTEN

The PTEN gene on 10q23 is mutated in up to 1/3 of hormone refractory PCa and homozygous deletions and mutations have been identified in a subset of primary PCa. Loss of PTEN protein in primary PCa, as determined by immunohistochemistry, correlates with high Gleason score and advanced stage (McMenamin *et al*., 1999). PTEN is a dual protein and lipid phosphatase that is responsible for dephosphorylation and inactivation of phosphatidylinositol 3,4,5-trisphosphate (PIP3), a second messenger that is produced after activation of PIP3 kinase in response to ligation of several growth factor receptors, including IGF-1. PIP3 activates the protein kinase AKT. AKT signalling results in inhibition of apoptosis in response to a variety of signals and to increased cell proliferation (Vivanco and Sawyers, 2002).
In assessing the relationship of PTEN deletion with the TMPRSS2-ERG fusion, two independent groups found that patients with neither lesion had a favorable prognosis (Reid *et al*., 2010).

Other markers tested in combination with PTEN loss for prognostic information include tumor protein p27 gene loss (Halvorsen *et al*., 2003), hemoxygenase-1 overexpression (Li *et al*., 2011) and HER2/3 overexpression (Ahmad *et al*., 2011). A four-protein signature, as assessed by immunohistochemical staining for PTEN in combination with a subset of proteins involved in tumor growth factor-b signaling: SMAD4, cyclin D1 and SPP1, was found to predict biochemical recurrence significantly better than Gleason score alone (Ding *et al*., 2011). The most promising pathway in which this is likely to be employed in the near future is the PTEN/PI3K pathway as a number of clinical trials using inhibitors of this pathway are in development or underway in PCa (Thomas *et al*., 2004).

Thus, the measurement of PTEN protein levels and downstream targets of AKT in prostate needle biopsies may have value in the future if these trials show promise (Thomas *et al*., 2004). It remains to be determined which combinations of events will provide the most reliable prognostic information to guide clinical decision making. Moreover, it has been found that PI3K and Androgen Receptor (AR) pathway crosstalk plays an intortant role in castrate resistant PCa development, with potentially important implications for PCa etiology and therapy (Mulholland *et al*., 2011).

C-MYC

The C-MYC protein is a nuclear transcription factor that regulates a number of cellular processes including cell cycle progression, metabolism, ribosome biogenesis, protein synthesis and mitochondrial function (Dang *et al*., 2006). In PCa, there is evidence that C-MYC is involved in PCa progression since a region encompassing the MYC locus (8q24) is somatically amplified at low levels in a subset of patients and the presence of amplification in this region correlates with both high histological grade and worse prognosis (Ribeiro *et al*., 2007).

It has been long known that a subset of PCa lesions express elevated levels of MYC mRNA, often in parallel with increased expression of PIM-1, a gene known to cooperate with MYC in other malignancies (Tomlins *et al*., 2006) and that is often overexpressed in PCa (Cibull *et al*., 2006). Targeted overexpression of the human MYC gene in the mouse prostate results in early invasive prostate adenocarcinoma and rare metastatic adenocarcinoma (Ellwood-Yen *et al*., 2003). These findings provide evidence that C-MYC overexpression can drive neoplastic transformation in the mouse prostate and thus may play a role in initiation and progression of human PCa.

AZGP1 and hCAP-D3

Zinc-Alpha2-Glycoprotein (AZGP1) is present in high concentration in human seminal plasma and considered to be a soluble homologue of MHC-I (Hassan *et al*., 2008). Some studies reported on the highly predictive value of AZGP1 expression after Radical Prostatectomy (RP) specimens as as a predictor of metastatic PCa (Henshall *et al*., 2006). In addition, urine detected AZGP1 showed promising results in the prediction of PCa, making him a potentional urine biomarker (Katafigiotis *et al*., 2012). Lapointe *et al*. (2008) reported a combination of immunohistology for AZGP1 and RNA in situ hybridisation for hCAP-D3 expression in tissues from RP specimens which distinguished even more clearly those patients whose tumours would reccur.

Annexin A3 (ANXA3)

ANXA3 has an inverse relationship to cancer and the immunhistochemical staining in prostatic tissue correlates with disease progression, Gleason score and malignancy. ANXA3 belongs to a family of calcium and phospholipid binding proteins that are implicated in cell differentiation and migration, immunomodulation, bone formation and mineralization in PCa metastasis (Gerke *et al*., 2005). ANXA3 represents a promising candidate tissue marker and when combined with the standard prognostic parameters, may provide a more precise prediction of prognosis in the individual patient (Köllermann *et al*., 2008).

Forkhead Box Protein A1 (FOXA1)

FOXA transcription factors are potent, context-specific mediators of development that hold specialized

functions in hormone-dependent tissues. Over the last several years, FOXA1 has emerged as a critical mediator of nuclear steroid receptor signalling, manifest at least in part through regulation of androgen receptor and oestrogen receptor activity. Recent findings point towards a major role for FOXA1 in modulating nuclear steroid receptor activity in breast and PCa and suggest that FOXA1 may significantly contribute to pro-tumourigenic phenotypes (Augello *et al*., 2011).

Jain *et al*. (2011) examined the expression of forkhead box protein A1 (FOXA1). Their findings suggest that increased expression of FOXA 1 is associated with the development of metastatic CaP. Metastatic PCa specimens demonstrated high nuclear FOXA1 staining in 89% of tissues as compared with 19% of patient-matched primary tumour samples. FOXA1 colocalized with androgen receptor in all samples and FOXA1 levels were positively correlated with tumour size, extraprostatic extension and lymph node metastasis. Such data implicate that FOXA1 is strongly associated with metastatic disease in PCa.

Epigenetic Alterations and Prostate Carcinogenesis

Epigenetic alterations represent important contributing factors in prostate carcinogenesis and may provide useful biomarkers for disease progression (Nelson *et al*., 2009). For example, DNA methylation has been implicated in silencing genes involved in signal transduction, hormonal response, cell cycle control and oxidative damage response, such as GSTP1 and others (Harden *et al*., 2003).

One key modification associated with prostate carcinogenesis is trimethylation of lysine residue 27 of his tone H3 (H3K27-me3), which is mediated by the his tone methyltransferase enzyme Ezh2, a key oncogenic driver of advanced disease and metastasis (Varambally *et al*., 2002). Since the H3K27-me3 mark is associated with transcriptional repression, increased levels in PCa are associated with repression of tumor suppressor genes such as DAB2IP, a member of the Ras GTPase family (Chen *et al*., 2005).

A number of other genes have also been found to be hypermethylated in PCa. Using quantitative real-time methylation specific PCR (Real Time-MSP), Yegnasubramanian *et al*. (2004) assessed the extent of hypermethylation in 16 different genes in PCa and found strikingly high frequencies of hypermethylation in the CpG islands associated with APC, RASSF1a, PTGS2 and MDR1, but virtually no methylation in normal prostate tissues.

It is clear that epigenetic regulation plays an important role in the development and progression of CaP, but the significance of identified genes still remains hypothetical (Febbo, 2009). There is mounting evidence, however, that methylation of genes (e.g., GSTP1, APC, PTGS2, EDNRB and T1G1) plays an important role in the development and prognosis of CaP, that makes then promising biomarkers in near future (Tomlins *et al*., 2005).

Androgen Receptor and Prostate Cancer

Although androgen deprivation therapy is the gold standard for the treatment of metastatic CaP, patients gradually become resistant to castration levels of androgens and the disease progresses. It is now clear that even though CaP progression in a state of low levels of androgens, most cancers is still dependent on stimulation of Androgen Receptor (AR) (Ziaran *et al*., 2011).

In the vast majority of PCa, it is clear that AR function is essential for tumor development and progression, that these activities are supported by FOXA1 and that resurgent AR activity after hormone therapy (a hallmark of the transition to lethal disease) requires FOXA1 activity (Linja *et al*., 2001).

At present, it is impossible to distinguish carcinoma, which is really independent of AR stimulation and cancer which maintains the dependence. Therefore, there are currently being developed predictors based on AR transcriptional activity, which could predict the activity of AR during treatment (Pulukuri *et al*., 2007).

In normal prostate epithelium, AR suppresses cellular proliferation, as probasin-Cre-mediated conditional deletion of AR leads to increased proliferation accompanied by decreased expression of differentiation markers (Kawamoto *et al*., 2007). In PCa, however, AR suppresses proliferation of basal cells, supports survival of luminal cells and promotes metastasis (Phé *et al*., 2010).

When PCa progresses to castration resistance, AR activation and signaling remains sustained through a variety of mechanisms. Several molecular mechanisms have been described for the ability of AR to retain signaling activity in castration resistant PCa. These mechanisms include the amplification of AR gene copy number in approximately one-third of castration-resistant carcinomas (Tomlins *et al*., 2005). Another 10-30% of tumors have gain-of-function mutations of AR that may confer increased protein stability, greater sensitivity to androgens, novel responses to other steroid hormones, ligand-independent activity, or increased recruitment of AR coactivator proteins (Demichelis *et al*., 2007). In addition, recent studies have shown that expression of alternative splice isoforms encoding constitutively active AR variants also occurs in castration-resistant cancer (Maher *et al*., 2009). Finally, an unusual mechanism for increased AR signaling activity is the endogenous expression of androgen synthetic enzymes by tumor tissue, which can lead to de novo androgen synthesis or conversion of weaker adrenal androgens into testosterone and dihydrotestosterone (Varambally *et al*., 2005). Ligand-independent activation of AR activity can

also take place through activation of growth factor signaling pathways. Notably, up-regulation of the PI3K pathway through Pten deletion appears to be particularly effective, as PIN lesions in Nkx3.1; Pten double-mutant mice display castration resistance prior to carcinoma formation (Tomlins *et al*., 2007).

Conclusion

There is an urgent need for novel biomarkers for assessing CaP diagnosis and prognosis, due to the highly variable natural history of CaP. "Traditional" markers cannot reliably distingish the potientially life-threatenig cancer from insignificant cancer. The development of molecular and immunohistochemical methods enabled the identification of potential biomarkers in relation to prognosis. Numerous promising markers and approaches have been identified and used (expression of FOXA1, loss of PTEN, fusion of genes TMPRSS2 ERG and ETV1, C-MYC, ANXA3, AR and FOXA1, AMACR, GWAS, epigenetic modifications, next generation sequencing, combination of "traditional" markers with novel biomarkers). In addition, attempts to identify cancers with different response to hormonal therapy have been used. The common feature of most current studies is their lack of prospectivity, limited mumber of patients and have to be verified in larger prospective studies. However, some of these markers may be translated into clinical practice in future and can help to assess prognosis of CaP more accurately.

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Author's Contributions

All authors equally contributed in this work.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and no ethical issues involved.

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