ANOTHER SET OF BIOMARKERS

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There is a steady flow of PCa markers appearing. This short note discusses a recent set from Spain. It includes HIST1H2BG, SPP1, ELF3 and PCA3. We examine this for potential usage.

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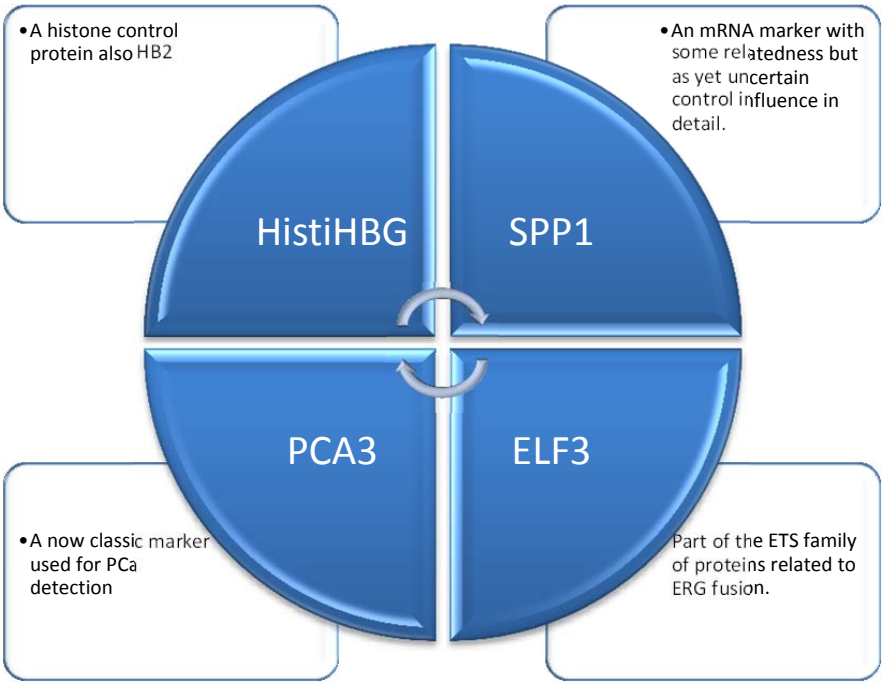
1 INTRODUCTION

As we have noted there seems to be a never ending progression of biomarkers for PCa as well as other cancers. In this most recent one a Spanish research group (see Mengual et al) makes the following proposition:

Seven of the 42 genes evaluated (PCA3, ELF3, HIST1H2BG, MYO6, GALNT3, PHF12 and GDF15) were found to be independent predictors for discriminating patients with PCa from controls. We developed a four-gene expression signature (HIST1H2BG, SPP1, ELF3 and PCA3) with a sensitivity of 77 % and a specificity of 67 % (AUC = 0.763) for discriminating between tumor and control urines. The accuracy of PCA3 and previously reported panels of biomarkers is roughly maintained in our cohort. Our four-gene expression signature outperforms PCA3 as well as previously reported panels of biomarkers to predict PCa risk. This study suggests that a urinary biomarker panel could improve PCa detection. However, the accuracy of the panels of urinary transcripts developed to date, including our signature, is not high enough to warrant using them routinely in a clinical setting.

Admittedly we have a set of such non-invasive markers, including the 4K, which have been approved for use to ascertain patients who may have PCa versus those who do not. Let us consider the four proposed as an interesting case.

The authors conclude on the four markers shown below:



2 MRNA SPECIFICS

The following Table and details discuss the four genes which they use.

Gene	***Description (NCBI)***
HIST1H2BG[1] (also H2B/a; H2BFA; H2B.1A)	Histones are basic nuclear proteins that are responsible for the nucleosome structure of the chromosomal fiber in eukaryotes. Nucleosomes consist of approximately 146 bp of DNA wrapped around a histone octamer composed of pairs of each of the four core histones (H2A, H2B, H3, and H4). The chromatin fiber is further compacted through the interaction of a linker histone, H1, with the DNA between the nucleosomes to form higher order chromatin structures. The protein has antibacterial and antifungal antimicrobial activity. This gene is intronless and encodes a replication-dependent histone that is a member of the histone H2B family. Transcripts from this gene lack polyA tails; instead, they contain a palindromic termination element. This gene is found in the large histone gene cluster on chromosome 6p22-p21.3
SPP1[2]	The protein encoded by this gene is involved in the attachment of osteoclasts to the mineralized bone matrix. The encoded protein is secreted and binds hydroxyapatite with high affinity. The osteoclast vitronectin receptor is found in the cell membrane and may be involved in the binding to this protein. This protein is also a cytokine that upregulates expression of interferon-gamma and interleukin-12.
ELF3[3] (also see Wang et al)	Aberrant regulation of the Wnt/β-catenin pathway plays important roles in colorectal carcinogenesis, with over 90% of cases of sporadic colon cancer featuring β-catenin accumulation. While ubiquitination-mediated degradation is widely accepted as a major route for β-catenin protein turnover, little is known about the regulation of β-catenin in transcriptional level. …Elf3, a member of the E-twenty-six family of transcription factors, drives β-catenin transactivation and associates with poor survival of colorectal cancer (CRC) patients. … first found recurrent amplification and upregulation of Elf3 in CRC tissues, and further Gene Set Enrichment Analysis identified significant association between Elf3 expression and activity of WNT/β-catenin pathway. Chromatin immunoprecipitation and electrophoretic mobility shift assay consistently revealed that Elf3 binds to and transactivates β-catenin promoter. Ectopic expression of Elf3 induces accumulation of β-catenin in both nucleus and cytoplasm, causing subsequent upregulation of several effector genes including c-Myc, VEGF, CCND1, MMP-7 and c-Jun. Suppressing Elf3 in CRC cells attenuates β-catenin signaling and decreases cell proliferation, migration and survival. Targeting Elf3 in xenograft tumors suppressed tumor progression in vivo. Taken together, our data identify Elf3 as a pivotal driver for β-catenin signaling in CRC, and highlight potential prognostic and therapeutic significance of Elf3 in CRC.

[1] http://www.ncbi.nlm.nih.gov/gene/8339

[2] http://www.ncbi.nlm.nih.gov/gene/6696

[3] http://www.ncbi.nlm.nih.gov/gene/1999

Gene	*Description (NCBI)*
PCA3[4]	This gene produces a spliced, long non-coding RNA that is highly overexpressed in most types of prostate cancer cells and is used as a specific biomarker for this type of cancer. This gene is embedded in an intronic region of the prune2 gene on the opposite DNA strand. The transcript regulates prune2 levels through formation of a double-stranded RNA that undergoes adenosine deaminase actin on RNA-dependent adenosine-to-inosine RNA editing. In prostate cancer derived cells, overexpression of PCA induced downregulation of prune2, leading to decreased cell proliferation. Conversely, silencing in prostate cancer cells resulted in increased proliferation. Regulation of this gene appears to be sensitive to androgen-receptor activation, a molecular signature of prostate cancer. Alternative splicing results in multiple transcript variants.

Some additional comments are worth note.

2.1 HIST12BG

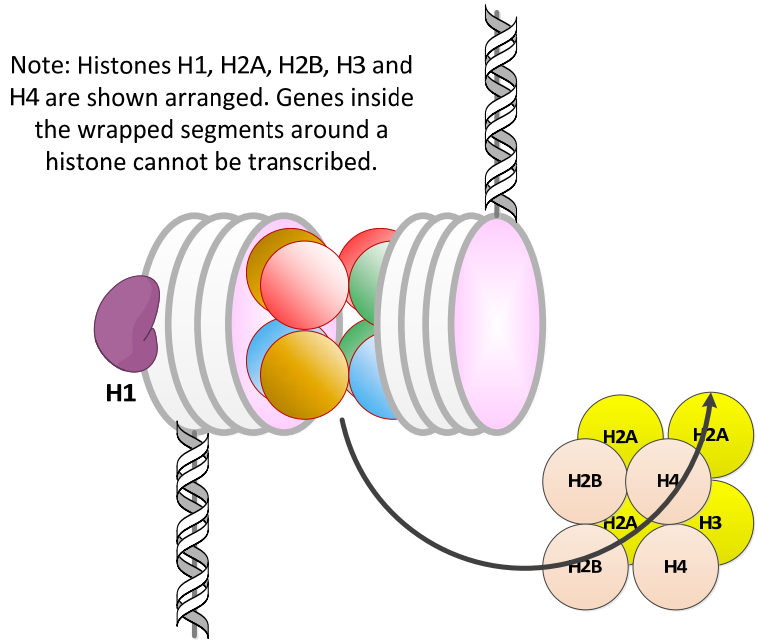
Histones are proteins that assist the structuring of the DNA into nucleosomes. As noted by Stankiewicz et al:

Results…. argue for the significance of epigenetic mechanisms in the regulation of chronic stress. Microarray studies have revealed alterations in mRNA expression levels of seven factors involved in chromatin modification in adult male Swiss-Webster mice subjected to various stressors for five weeks.

Three transcripts that encode histones were found to be upregulated (H2afj, Hist1h2bm, and Hist1h2bg), and four were down-regulated. These four down-regulated genes encoded histones (Hist1h2bn, Hist1h2bh), a silencing factor known to recruit histone methyltransferases and deacetylases (Satb1, and a protein involved in histone acetylation (Hmgn2.

We show the relationship of H2B to the histone and nucleosome structure below:

[4] http://www.ncbi.nlm.nih.gov/gene/50652



2.2 SPP1

As we have noted previously, SSP1 is secreted phosphoprotein 1, also commonly known as Osteopontin (OPN), also known as bone sialoprotein I (BSP-1 or BNSP), early T-lymphocyte activation (ETA-1), 2ar and Rickettsia resistance (Ric), is a human gene product which is also conserved in other species[5].

From Hendig et al, they state that it is a secreted, highly acidic phosphoprotein that is involved in immune cell activation, wound healing, and bone morphogenesis and plays a major role in regulating mineralization processes in various tissues. Increased expression is often associated with pathological calcification. Furthermore, is a constitutive component of human skin and aorta, where it is localized to the elastic fiber and hypothesized to prevent calcification in the fibers?

SPP1 *is* a predominantly transcriptional regulated gene, and the promoter is highly conserved among different species *(22)*. Several polymorphisms in the gene affect expression and have been associated with various disorders, e.g., systemic lupus erythematosus and arteriosclerosis.

SPP1 is a SIBLING glycoprotein that was first identified in osteoblasts. OPN is an important anti-apoptotic factor in many circumstances. OPN blocks the activation-induced cell death of macrophages and T cells as well as fibroblasts and endothelial cells exposed to harmful stimuli. OPN prevents non-programmed cell death in inflammatory colitis. It has been shown that OPN

[5] Also see http://www.ncbi.nlm.nih.gov/gene/6696 also see http://www.wikigenes.org/e/gene/e/6696.html

drives IL-17 production; OPN is overexpressed in a variety of cancers, including lung cancer, breast cancer, colorectal cancer, stomach cancer, ovarian cancer, melanoma and mesothelioma; OPN contributes both glomerulonephritis and tubulointerstitial nephritis; and OPN is found in atheromatous plaques within arteries. Thus, manipulation of plasma OPN levels may be useful in the treatment of autoimmune diseases, cancer metastasis, osteoporosis and some forms of stress. Research has implicated osteopontin in excessive scar-forming and a gel has been developed to inhibit its effect.

2.3 ELF3

ELF3 is part of the ETS family. The ETS family of genes is positive or negative regulators of gene expression. They can up or down regulate expression. They are named for the initial gene discovered, the E26 Transforming Sequence, where E26 was the oncogene v-ets characterized in 1986 of an avian transforming virus called E26. It is also called the erythroblast transforming specific family, as discussed by Zong et al. As Watson et al note regarding this gene:

Four genes, ESE3 (EHF), ESE1 (ELF3), ESE2 (ELF5) and PDEF, were expressed at higher levels in breast cancer cells than normal epithelial cells. The expression of ELK3, ETS1 and FLI1 were reported to be reduced in breast cancer cells [114]. This pattern defined in cell lines does not absolutely correlate to that observed in tissue specimens. As noted above, ETS1 is over-expressed and PDEF protein is often reduced or lost in human breast cancer. While further studies are needed, ESE3 protein was absent in one breast cancer sample examined by IHC.

2.4 PCA3

PCA3 has received a great deal of attention of late. It is a non-coding RNA and the controlling gene is located at 9q21-q22[6]. It is also called prostate cancer antigen 3 (non-protein coding). The presence of PCA3 is generally now believed to be a marker for PCa. Testing is now underway on many patients to determine if they have PCa using the PCA3 assay. Thus there is a great deal of interest in better understanding what the full networks are for PCA3 generation as well as looking at those pathways as a possible means to control PCa. We examine two recent studies in this area.

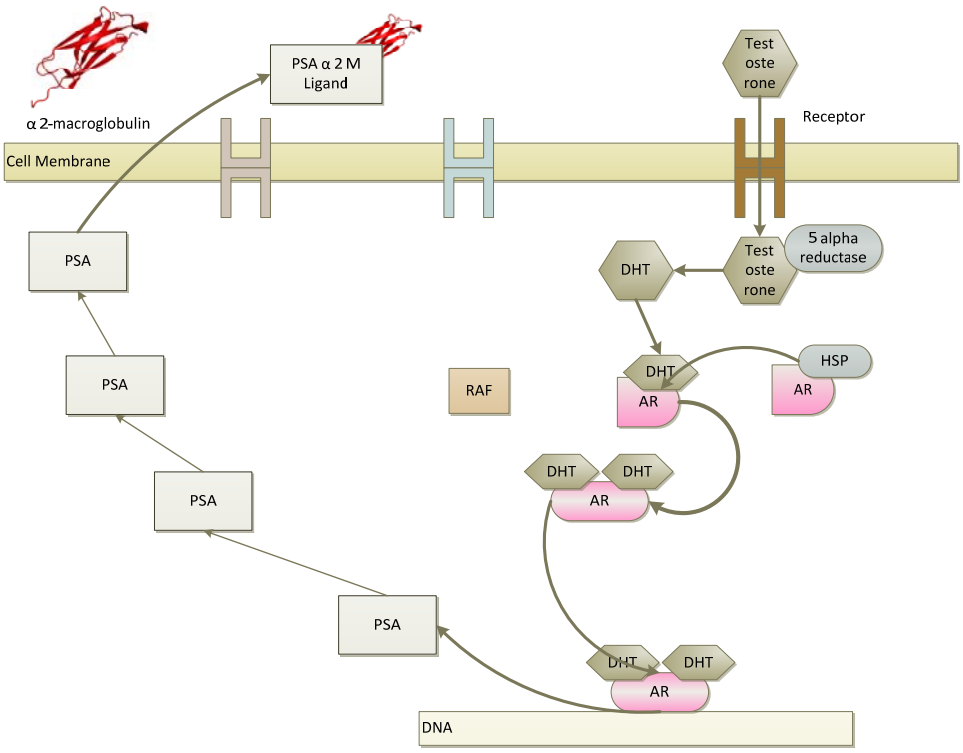
In the recent paper by Ferreira et al, they state:

Our findings suggest that the ncRNA PCA3 is involved in the control of PCa cell survival, in part through modulating AR signaling, which may raise new possibilities of using PCA3 knockdown as an additional therapeutic strategy for PCa control.

This may be of significant merit as a new potentially useful therapeutic. Now it should be recalled that the AR pathway and the PSA generation is known as shown below[7].

[6] http://www.ncbi.nlm.nih.gov/gene/50652

[7] Note we use the reference, Prostate Cancer Genomics, McGarty (2012, DRAFT, http://www.telmarc.com/Documents/Books/Prostate%20Cancer%20Systems%20Approach%2003.pdf) as the source for some of this information. From this source one may obtain the initial sources.



Now Ferreira et al continue:

Due to the increased PCA3 expression in androgen-responsive cells compared with androgen-insensitive cells, and because AR signaling is an important pathway controlling PCa survival, we tested whether PCA3 expression was modulated by the androgen-active metabolite DHT and whether this expression pattern involved the activated AR.

Upregulation of PCA3 expression in response to LNCaP stimulation with DHT was significantly counteracted by the AR antagonist flutamide, indicating that PCA3 expression was induced by the activated AR. AR activation was further confirmed by the observation that LNCaP cells stimulated with DHT also showed AR transcriptional activity. Consistently, the entire AR target genes tested that contains canonical AR response elements (AREs) in their promoter sequences was upregulated upon DHT treatment. Although eight of the genes showed at least a 1.5-fold increase after AR activation, only two of them showed a significant increase in their expression levels. Interestingly, PCA3 upregulation upon DHT treatment has been observed previously, but no study has demonstrated the involvement of activated AR in PCA3 expression by using AR antagonists. Although our data also suggest that PCA3 is an androgen-responsive gene, the precise molecular mechanism by which PCA3 expression responds to this activation is still unknown.

One hypothesis is that activated AR can directly activate the PCA3 promoter, as has been demonstrated for the miR-101 and miR- 21 regulatory regions, which are also modulated by the activated AR. However, no consensus AREs has been identified in the 500-bp PCA3 promoter region. We further screened for consensus ARE elements in the entire PCA3 genomic region at the 5 Kb region upstream from the PCA3 transcription start site, and have so far identified no canonical element (data not shown). Nevertheless, we cannot exclude the possibility that other, noncanonical ARE elements could also promote AR binding and directly activate PCA3 expression, as has been previously described for other genes modulated by the AR activation. PCA3-upregulated expression in response to DHT treatment could also be a result of activated AR binding to the regulatory regions of other AR-responsive genes, which in turn could induce PCA3 expression. Further experiments should investigate direct AR binding to different PCA3 genomic regions, in order to answer these open questions.

Now they examined genes which are known pathway controllers of PCa. The CDKs especially control cell cycle flow.

As an approach to investigate the signal by which PCA3 controls PCa cell survival, we analyzed the transcript expression of PSA, AR, TMPRSS2, NDRG1, GREB1, FGF8, CDK1, CDK2, and PMEPA1 genes, all of which have key roles in PCa growth and progression, and are classical AR target genes.

Also highly regulated by androgens, fibroblast growth factor 8 (FGF8), cyclin-dependent kinase 1 (CDK1), cyclin-dependent kinase 2 (CDK2), and the gene regulated in breast cancer 1 (GREB1) gene products have classical stimulating roles in prostate growth and proliferation. Conversely, the PMEPA1 gene, although a direct transcriptional target of the AR, has been described as a negative regulator of cell growth in the prostate epithelium, as well as negatively regulating AR protein levels in different cell-culture models. We also observed that the AR transcription level was downregulated after PCA3 knockdown. These results accord with previously published data, which demonstrated that the AR gene is transcriptionally regulated by AR through binding to AR regulatory elements (autoregulation). However, differently from the other AR-responsive genes tested here, the ARE elements required for this process have not been found in the AR promoter or in the 5'-flanking region, but rather in AR coding sequences.

The observation that PCA3 is involved in the control by modulation of the AR target genes is a key observation. As we have shown, based upon various prior works, the change in AR is critical to the loss of any control over the PCa cells. They state:

Here we demonstrate for the first time that PCA3 is involved in the control of PCa cell survival, at least in part by modulating the transcriptional activity of AR target genes. To our knowledge, this is the first characterization of the functional role of PCA3 in PCa cells, and will not only improve the understanding of key roles of this transcript in prostate carcinogenesis, but also suggests an alternative strategy to use PCA3 as a putative specific target for PCa treatment approaches. Because PCA3 seems to be a regulator of the expression of AR target genes and PCa cell survival, treatment options aiming to downregulate PCA3, in combination with other androgen-depletion-based strategies, could potentially circumvent androgen-ablation resistance mechanisms.

In an earlier paper by Ferreira et al, they state:

The prostate cancer antigen 3 (DD3/PCA3) is a non-coding RNA (ncRNA) specifically expressed in prostate tissues and overexpressed in prostate cancer (PCa) tumors. Although widely applied as a diagnostic marker for PCa, to date nothing has described about its role in PCa biology. We used herein small interfering RNA (siRNA) in order to knockdown DD3 mRNA message as an approach to elucidate DD3 functional roles in PCa cells.

LNCaP cell line was been used herein as an in vitro model for DD3 functional assays. siRNA sequences were specifically designed for DD3 exon 4 mRNA sequences (siDD3), as well as scrambled siRNA (siScr), as negative control. LNCaP cells were transiently transfected with siDD3 or siScr and DD3 expression was analysed by real time PCR (qRT-PCR) using DD3 specific oligonucleotides. LNCaP cells transfected with siDD3 demonstrated a marked decrease in cell proliferation and viability, as compared to siScr transfected cells.

Further, LNCaP cells in which DD3 was knocked-down presented a significant increase in proportion of cells in SubG0/G1 phase of cell cycle and presenting pyknotic nuclei, indicative of cells undergoing apoptosis. In order to investigate the putative mechanisms underlying the decrease of LNCaP cell survival as a result of DD3 knockdown, we then evaluated the involvement of DD3 on androgen receptor (AR) pro-survival signaling. DD3 expression was significantly uregulated as a result of LNCaP treatment with dihydrotestosterone (DHT), the active androgen metabolite. This effect was reverted by the addition of the AR antagonist, flutamide.

Consistent to an AR activation by DHT treatment, LNCaP cells presented a significant upregulation of AR target genes. Notably, siDD3/LNCaP transfected cells significantly inhibited the expression of tested AR responsive genes. Besides, DD3 knockdown was able to counteract DHT stimulatory effects over AR target gene expression. Despite negatively modulating the transcription of AR target genes, DD3 knockdown did not alter Akt and ERK phosphorylation, suggesting that DD3 is mainly controlling the expression of signaling pathways downstream to AR activation.

In summary, our findings indicate that DD3 is a ncRNA whose expression is AR regulated and is involved on the control of PCa cell survival and proliferation, in part by modulating the AR signaling pathway and its target genes.

These findings correspond to the first description of DD3 roles on PCa cells and could provide new insights into understanding prostate carcinogenesis, besides opening new prospects to use DD3 not only as a biomarker for PCa, but also as an specific target for therapeutic approaches aiming to inhibit PCa growth by negatively modulating AR pro-survival signal and their target genes.

In this slightly earlier paper the authors focus on the PCA3 as a target and examine its pathway significance.

Other researchers have examined PCA3 as well as other markers. It is well known that the TMPRSS2:ERG fusion is often seen in PCA. As Salagierski and Schalken conclude:

In recent years advances in genetics and biotechnology have stimulated the development of noninvasive tests to detect prostate cancer. Serum and urine molecular biomarkers have been identified, of which PCA3 has already been introduced clinically. The identification of prostate cancer specific genomic aberrations, ie TMPRSS2:ERG gene fusion, might improve diagnosis and affect prostate cancer treatment. Although several recently developed markers are promising, often showing increased specificity for prostate cancer detection compared to that of prostate specific antigen, their clinical application is limited. The only 2 true prostate cancer specific biomarkers identified to date remain PCA3 and TMPRSS2:ERG gene fusion.

3 OBSERVATIONS

As with so many of these other putative markers we have here four mRNAs that seem diverse yet somehow are reflective of a diagnostic malignancy test. Clearly PCA3 is already a marker with some merit. ELF3 is also arguably as part of the ETS family in the same neighborhood as PCA3. The H2B mRNA fragment may or may not be reflective of a process. Finally SPP1 seems to be an outlier. Clearly causative linkages should be drawn here. But this is an interesting find.

The NCCN has set recommendations for a variety of detection markers. The CMS has approved use of 4K. We have examined 4K in some clinical settings and it seems to corroborate integrated MRI guided TRUS saturation core biopsies. Thus they may be of significant use going forward.

It is always useful to examine newer possibilities. The AUC alleged here is good but 4K seems to exceed that one. Also it will require substantial clinical evaluation to be able to make reliable statements that are diagnostic.

Finally as with so many of these putative markers it would be useful to better understand the "causative" linkages in some detail.

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