PROSTATE CANCER, ONCOSOMES, AND BLOOD BORNE MARKERS

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White Paper No 104
December, 2013

We examine the elements required in modeling cancer and we propose several variants. We focus primarily on intracellular models using a differential equation approach although questioning and modifying the reaction rate models in common use. We also develop a model for total cellular dynamics which we integrate with the intracellular model thus allowing for whole body predictive capabilities.

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1 INTRODUCTION

Almost daily one can see new proposed markers for Prostate cancer and its potential metastatic growth. There simply are often too many to have true prognostic value. The major concern that we have expressed over the past few years is the lack of causative structure behind many of these proposed markers. That is, the marker may be reflective of some more complex causative process and it is often that process, albeit unknown or not expressed, that we are seeking so that one may have the ability to develop targeted therapeutics against that target. For example we understand PSA as a marker reflective of an enlarging number of prostate cells but the driver may be any one of several things other than cancer.

In this note we examine two specific areas. The first is recent reporting on exosomes, called Oncosomes in the discussed work. This work covered in a recent and several extensive previous papers examine encapsulated blood borne markers for aggressive PCa. In contrast we examine a second recent result, also a blood borne marker of aggressive behavior, an mRNA of a quite large gene. The second is another blood borne marker, an mRNA from a quite large gene, NAALADL2 or N-acetylated alpha-linked acidic dipeptidase-like 2, located at 3q26.31, a large gene with hundreds of introns[1].

An excellent paper by Grasso et al has discussed the genetic landscape of androgen resistant PCa[2]. The authors summarize their work as follows:

Characterization of the prostate cancer transcriptome and genome has identified chromosomal rearrangements and copy number gains and losses, including ETS gene family fusions, PTEN loss and androgen receptor (AR) amplification, which drive prostate cancer development and progression to lethal, metastatic castration resistant prostate cancer (CRPC).

However, less is known about the role of mutations. Here we sequenced the exomes of 50 lethal, heavily pre-treated metastatic CRPCs obtained at rapid autopsy (including three different foci from the same patient) and 11 treatment-naive, high-grade localized prostate cancers.

We identified low overall mutation rates even in heavily treated CRPCs (2.00 per megabase) and confirmed the monoclonal origin of lethal CRPC. Integrating exome copy number analysis identified disruptions of CHD1 that define a subtype of ETS gene family fusion negative prostate cancer. Similarly, we demonstrate that ETS2, which is deleted in approximately one-third of CRPCs (commonly through TMPRSS2:ERG fusions), is also deregulated through mutation.

Furthermore, we identified recurrent mutations in multiple chromatin- and histone-modifying genes, including MLL2 (mutated in 8.6% of prostate cancers), and demonstrate interaction of the MLL complex with the AR, which is required for AR-mediated signalling. We also identified novel recurrent mutations in the AR collaborating factorFOXA1, which is mutated in 5 of 147

[1] http://www.ncbi.nlm.nih.gov/gene/254827

[2] http://www.nature.com/nature/journal/v487/n7406/pdf/nature11125.pdf

(3.4%) prostate cancers (both untreated localized prostate cancer and CRPC), and showed that mutatedFOXA1 represses androgen signalling and increases tumour growth. Proteins that physically interact with the AR, such as the ERG gene fusion product, FOXA1, MLL2, UTX (also known as KDM6A) and ASXL1 were found to be mutated in CRPC.

In summary, we describe the mutational landscape of a heavily treated metastatic cancer, identify novel mechanisms of AR signalling deregulated in prostate cancer, and prioritize candidates for future study.

The Table that they present is quite useful in this study. The measures are primarily mutational in nature.

We have previously reported on the development of exosomes, small encapsulated particle ejected from cells, often carrying within them marker proteins reflective of the status of an organ. Exosomes have been used for first, a word on terminology. As Simpson and Mathivanan state:

The first problem relates to the terminologies used in naming eMVs. In the past, isolated eMVs were named based on the sample source from which they were derived. For example, exosomes isolated from dendritic cells were named dexosomes, while cancer cell derived exosomes were referred to as texosomes/oncosomes and prostate cancer cell derived exosomes as prostasomes.

This sample material based vesicle naming customization has lead (sic) to different nomenclatures such as epididimosomes, argosomes, exosome-like vesicles, apoptotic blebs, microparticles, promininosomes, prostasomes, dexosomes, texosomes, dex, tex, exosomes, microparticles, nanoparticles, microvesicles, shedding microvesicles, ectosomes, archeosomes and oncosomes.

Note that a bleb is an outward protrusion from a cell. Recent work by Funkhouser et al has some interesting detailed analyses of this phenomenon.

2 ONCOSOMES

Let us begin by considering the Oncosome results. We use the term oncosome rather than the more encompassing exosome because that is what is done in the paper.

In that paper the authors make claims as regards to these "oncosomes" and they state the following[3]:

Prostate cancer cells release atypically large extracellular vesicles (EVs), termed large oncosomes, which may play a role in the tumor microenvironment by transporting bioactive molecules across tissue spaces and through the blood stream. In this study, we applied a novel method for selective isolation of large oncosomes applicable to human platelet-poor plasma, where the presence of caveolin-1-positive large oncosomes identified patients with metastatic disease.

This procedure was also used to validate results of a miRNA array performed on heterogeneous populations of EVs isolated from tumorigenic RWPE-2 prostate cells and from isogenic non-tumorigenic RWPE-1 cells. The results showed that distinct classes of miRNAs are expressed at higher levels in EVs derived from the tumorigenic cells in comparison to their non-tumorigenic counterpart.

Large oncosomes enhanced migration of cancer-associated fibroblasts (CAFs), an effect that was increased by miR-1227, a miRNA abundant in large oncosomes produced by RWPE-2 cells. Our findings suggest that large oncosomes in the circulation report metastatic disease in patients with prostate cancer, and that this class of EV harbors functional molecules that may play a role in conditioning the tumor microenvironment.

Thus the description could best use the general term exosome but we shall remain with and use oncosome throughout. Before continuing it is worth examining some prior research in this area as well. In the 2009 paper the authors state:

Oncosomes are recently discovered membranous microvesicles that have been implicated in rapid intercellular transfer of oncogenic information from glioblastoma to indolent glioma cells. Although this process resembles paracrine signaling, it involves intercellular transfer of a membrane-bound micro-organelle rather than a soluble protein such as a growth factor or cytokine.

In the present study, we show that PCa cells shed membrane-bound vesicles in response to signal transducers.

[3] Large oncosomes mediate intercellular transfer of functional microRNA, Matteo Morello, Valentina R Minciacchi, Paola de Candia, Julie Yang, Edwin Posadas, Hyung Kim, Duncan Griffiths, Neil Bhowmick, Leland WK Chung, Paolo Gandellini, Michael R Freeman, Francesca Demichelis, Dolores Di Vizio, Cell Cycle V 12 N 22 2013. also see https://www.landesbioscience.com/journals/cc/article/26539/

These structures are fairly large (0.5 to f5 Am), originate from nonapoptotic blebs in response to signaling cues, and have biological activity in their free-floating state. We also identify the formin homology protein, DRF3/Dia2, as a protein that seems to functionally inhibit oncosome formation. We also provide the first evidence that chromosomal loss at the DRF3 locus (DIAPH3) is associated with metastatic PCa.

Note that DIAPH3, diaphanous-related formin 3, is a protein that "*this gene encodes a member of the diaphanous subfamily of the formin family. Members of this family are involved in actin remodeling and regulate cell movement and adhesion. Mutations in this gene are associated with autosomal dominant auditory neuropathy. Multiple transcript variants encoding different isoforms have been found for this gene*"[4]. The authors continue with a discussion of DRF3[5] an actin facilitator gene:

Our data indicate that the actin nucleator DRF3 is capable of inhibiting oncosome formation, because DRF3 knockdown by RNAi increased blebbing in DU145 cells, particularly in the presence of EGF. DRF3is expressed by LNCaP, DU145, and PC-3 human prostate cell lines. Formin homology proteins mediate cytoskeletal dynamics and, as a group, have been implicated in a wide range of cellular functions, including motility and vesicular trafficking. The formin FHOD1, which exhibits 45% sequence homology to DRF3, was recently implicated in Src-dependent plasma membrane blebbing.

It should be recalled that blebs are cell protrusions due to cytoskeleton breakdown and blebbing is the process of creating such nascent vesicles. The authors continue:

Human DRF3 is not well-studied, although analyses of the mouse homologue Drf3, and the close mouse paralog, Drf1/mDia1, indicate that DRF3likely mediates actin filament nucleation and elongation and microtubule stability

Our experiments suggest that oncosome transfer between tumor cells, or between tumor and stroma, could play a role in propagation of aggressive behavior within the tumor microenvironment…

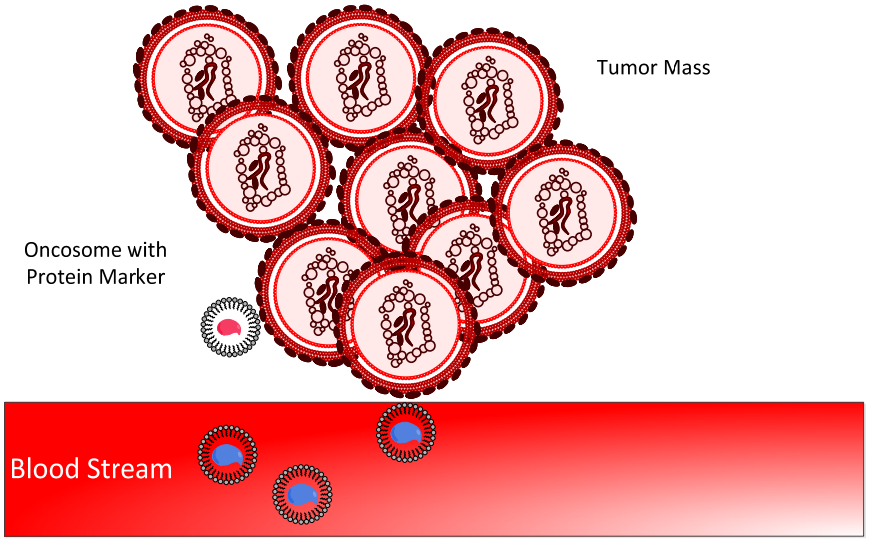
… oncosome exchange is markedly different from paracrine effects induced by soluble ligands. However, this process could result in amplification of paracrine pathways through intercellular sharing of membrane-associated signaling complexes. Although our study focuses on PCa, a similar microvesicular transfer mechanism may operate in other tumor systems.

From the 2012 paper we have the following diagram which has been modified and simplified. The description is one of a tumor mass eluding encapsulated exosomes in which are specific proteins. It could possibly be equally likely that the exosomes may encapsulate mRNA as well

[4] http://www.ncbi.nlm.nih.gov/gene/81624

[5] http://www.kegg.jp/kegg-bin/highlight_pathway?scale=1.0&map=map04810&keyword=DRF3

which is a putative marker. This description also shows that any localization of the source is in question.



The authors conclude in that paper:

Quantitation of circulating tumor cells (CTCs) is being evaluated to assess the risk of disease progression for prostate and other types of cancer. However, the clinical significance of CTCs remains to be established because of their extremely small number in peripheral blood compared with the number of blood cells.

We have demonstrated that large oncosome-like structures can be separated from plasma in a manner that does not require the capture of CTCs or other cells. The molecular characterization of large oncosomes may potentially offer a more sensitive and specific liquid biopsy than CTCs for patient selection, monitoring of treatment efficacy, and assessment of drug resistance.

There seems to be a significant interest in various forms of circulating tumor cells, exosomes or otherwise. In a previous note we observed work done on extracting exosomes from urine as a way to assess PCa[6]. This approach was putatively a localized approach.

The observations of the press are oftentimes of use in understanding how these results are interpreted. Several comments are below[7]:

[6] http://www.telmarc.com/Documents/White%20Papers/101%20Exosomes.pdf

[7] http://medicalxpress.com/news/2013-11-aggressive-prostate-cancer.html ; also one should compare this with the following: http://www.sciencecodex.com/novel_study_charts_aggressive_prostate_cancer-123155

Investigators in the Cedars-Sinai Samuel Oschin Comprehensive Cancer Institute have made extensive progress in understanding the molecular mechanisms of disease progression. These results may help scientists better understand the prognosis of patients diagnosed with advanced prostate cancer. …

"One of the long-standing difficulties in treating men with advanced prostate cancer has been predicting the response to given therapies or treatments," said Di Vizio, associate professor in the Department of Surgery, Department of Pathology and Laboratory Medicine and Department of Biomedical Sciences. "These latest research findings provide tangible insight into the molecular and structural phenomena that result in prostate cancer metastases. They have the potential to create a new source of biomarkers and an innovative standard of care. These findings may also help distinguish individualized treatment plans best suited for each patient."

The new source of biomarkers is large oncosomes, which are vesicles released from aggressive prostate cancer cells with highly migratory features. These large oncosomes carry tumor molecules and have been shown in previous studies to contribute to tumor progression. This study demonstrates, for the first time in human samples, that identification of circulating large oncosomes can be an indicator of patients with more aggressive, treatment-resistant disease. Also notable, researchers found that large oncosomes contain microRNA, a molecule that regulates several biological processes now proven to influence tumor progression.

At the Cedars-Sinai Urologic Oncology Program, researchers and physicians work in close collaboration to discover personalized options for patients with indolent prostate disease to those with aggressive cancer. This latest study will amplify the genetic and molecular understanding of aggressive prostate cancer.

"Many of the latest research developments and treatments for urologic cancers have been initiated from Cedars-Sinai investigators and physicians," said Robert Figlin, MD, FACP, associate director of the Samuel Oschin Comprehensive Cancer Institute, professor of Medicine and Biomedical Sciences and the Steven Spielberg Family Chair in Hematology Oncology. "These latest research observations may identify novel ways to measure a patient's prognosis and are just a stepping stone for the many discoveries moving down the pipeline in our cancer institute."

3 ANOTHER MARKER

We now briefly examine another recent market for metastatic behavior. Specifically we discuss the NAALADL2 gene. The work of Whitaker et al has received some recent interest because it alleges to allow, upon biopsy, to ascertain if the lesion was of an aggressive nature. The authors also argue that this measure can be ascertained vial circulating mRNA related to the gene as well. Whether this circulating mRNA is exosome encapsulated is open for discussion.

From the paper by Whitaker et al[8]:

N-acetyl-L-aspartyl-L-glutamate peptidase-like 2 (NAALADL2) is a member of the glutamate carboxypeptidase II family, best characterized by prostate-specific membrane antigen (PSMA/NAALAD1).

Using immunohistochemistry (IHC), we have shown overexpression of NAALADL2 in colon and prostate tumours when compared with benign tissue. In prostate cancer, NAALADL2 expression was associated with stage and Grade, as well as circulating mRNA levels of the NAALADL2 gene.

Overexpression of NAALADL2 was shown to predict poor survival following radical prostatectomy. In contrast to PSMA/NAALAD1, NAALADL2 was localized at the basal cell surface where it promotes adhesion to extracellular matrix proteins.

Using stable knockdown and overexpression cell lines, we have demonstrated NAALADL2-dependent changes in cell migration, invasion and colony-forming potential. Expression arrays of the knockdown and overexpression cell lines have identified nine genes that co-expressed with NAALADL2, which included membrane proteins and genes known to be androgen regulated, including the prostate cancer biomarkers AGR2 and SPON2. Androgen regulation was confirmed in a number of these genes, although NAALADL2 itself was not found to be androgen regulated.

NAALADL2 was also found to regulate levels of Ser133 phosphorylated C-AMP-binding protein (CREB), a master regulator of a number of cellular processes involved in cancer development and progression. In combination, these data suggest that changes in expression of NAALADL2 can impact upon a number of pro-oncogenic pathways and processes, making it a useful biomarker for both diagnosis and prognosis.

[8] http://www.nature.com/onc/journal/vaop/ncurrent/full/onc2013464a.html also see the discussion in http://www.newscientist.com/article/dn24628-prostate-cancer-tests-could-prevent-needless-surgery.html#.Uo9WfCetj0k also in PubMed they have http://www.ncbi.nlm.nih.gov/pubmedhealth/behindtheheadlines/news/2013-11-19-study-identifies-marker-for-high-risk-prostate-cancers/

Unfortunately the complete pathway models for NAALDL2 do not appear to be fully known. On the one hand the authors argue that it is androgen receptor independent, but that it plays a role in:

1. Cellular adhesion
2. Cell migration
3. Colony forming potential
4. Invasion

In the UK, this result has received considerable attention. For example, from the NHS report[9]:

"Prostate cancer patients could be screened to detect aggressive tumours after scientists identified a protein linked to severe forms of the disease," reports The Daily Telegraph. The news is based on the results of a complex laboratory study looking at a protein called NAALADL2. Scientists found that levels of NAALADL2 were high in prostate cancer when compared with healthy tissue, and levels were higher in more aggressive and more extensive prostate tumours. The level of the protein found in the tumours was also linked to whether men survived without recurrence of the cancer and overall survival after having radical prostatectomies (surgery to remove prostate cancer).

This is exciting news as one of the biggest problems in helping men with prostate cancer is estimating the likely outcome. Some prostate cancers cause no or few symptoms and do not have any impact on life expectancy – doctors may tell you that "many men die with prostate cancer, not of prostate cancer".

Other prostate cancers can be extremely aggressive. Around 10,000 men die of the disease in the UK per year. A test that could accurately identify high-risk cancers could potentially save lives and spare men with low-risk cancers unnecessary testing and treatment. So far this is early-stage research. The next hurdle is to see if the results of the lab research can be applied in the real world, and, most importantly, whether it can be used to help improve outcomes for men with prostate cancer. The researchers initially looked to see if the NAALADL2 protein was present in a range of normal and tumour tissues from different parts of the body.

The report, also in PubMed, then reports on the specific results regarding aggressive PCa. They state (as modified):

They then looked at whether the presence of NAALADL2 could differentiate between benign and cancerous tissue, and whether it could predict survival. Prostate tissue was taken from men who had radical prostatectomies (surgery to remove the prostate cancer) in Cambridge or Stockholm.

The researchers then investigated the localisation of NAALADL2 within the cell, what cells making NAALADL2 can do, and which other genes are switched on (expressed) in combination with NAALADL2. What were the basic results?

[9] http://www.nhs.uk/news/2013/November/Pages/Study-identifies-marker-for-high-risk-prostate-cancers.aspx

NAALADL2 was present at high levels in colon and prostate cancers. By measuring the amount of the protein, researchers were able to distinguish between benign and cancerous prostate tissue with a relatively good level of accuracy.

They found that in a group of samples from men in Cambridge:

1. *The level of sensitivity was 86% (sensitivity is the percentage of cancerous samples that were correctly given a positive result)*

2. *The level of specificity was also 86% (specificity is the percentage of benign samples correctly given a negative result)*

3. *Similar findings were seen in samples from a group of men from Stockholm.*

Although this is a good value for sensitivity and specificity it is only for a small sample and only for ascertaining benign vs malignant. Aggressiveness may very well be another issue. They also continue as follows:

Levels of NAALADL2 protein increased with the increasing aggressiveness of the prostate cancer, based on the microscopic appearance of the tissue (Gleason grade).

Levels of NAALADL2 protein also increased with cancer stage (the extent and spread of the tumour), particularly between T2 (cancer confined to the prostate gland) and T3 (cancer that has begun to grow and spread outside the prostate into the seminal vesicles, the glands that produce the fluid component of semen).

*Levels of NAALADL2 RNA (*mRNA of NAALADL2 in the blood stream) *in the blood were found to be higher in men with biopsy-confirmed prostate cancer, compared with men who had raised prostate specific antigen (another protein associated with prostate cancer) but a negative biopsy.*

One of the concerns one would have here is that it is known that biopsies are often non-conclusive and require multiple tries. This is especially true with the older methodologies of sextant cores. With high density cores, 24 and higher, and with repeat biopsies at say 9 months, one can reduce but not eliminate this risk. Secondly, there is the issue of how the mRNA is obtained from the blood. We have been discussing exosomes but one suspects that the UK approach is not that but actual mRNA extraction. Blood extraction can be problematic because one does not know from whence it came. It could be prostate originated thus a local but aggressive disease or it could already have metastasized, having established itself in the bone. This is one of the major difficulties of blood borne markers.

The researchers then looked at whether levels of NAALADL2 protein could predict survival. One hundred and four men had radical prostatectomies in Cambridge, and 38 had recurrence of the cancer over a median follow-up period of 86 months.

There was a trend that higher levels of NAALADL2 led to poorer outcomes, but this wasn't statistically significant. The researchers suggested that this might be because of the small

number of men: the smaller the sample size, the less "statistical power" the results have. They then looked at data from Stockholm: in this cohort, there were 252 men, and 101 of them had recurrence over a median follow-up of 61 months.

Of men with low levels of NAALADL2, 79.9% had no relapse at five years. Five-year recurrence-free survival was reduced to 72.5% for men with moderate levels of protein, and 65.3% for men with high levels of protein (hazard ratio 1.9).

The result was still significant after adjusting for a number of factors, including the Gleason grade and cancer stage.

Levels of NAALADL2 could also predict poor survival in low-risk patients (patients with low Gleason grades and cancer stage). Five-year survival was 93% in men with low levels of NAALADL2 and 45% in men with high levels of NAALADL2.

The researchers found that NAALADL2 protein on the basal (base) cell surface, where it promotes cell adhesion, migration (movement) and invasion (movement into tissue). They suggest that this could allow cells to escape the prostatic capsule and form tumours elsewhere. NAALADL2 was found to be expressed alongside androgen-related genes and prostate cancer biomarkers.

How did the researchers interpret the results? The researchers conclude that, "NAALADL2 protein is expressed in a number of cancers, and highly expressed in prostate cancer, where it predicts for relapse following radical prostatectomy". They go on to say that, "These data suggest that changes in expression of NAALADL2 can impact upon a number of pathways [involved in cancer development], making it a useful biomarker for both diagnosis and prognosis."

The size of the samples is exceedingly small and thus although this result is compelling and of interest it is clearly not of clinical significance. Thousands of patients would have to be examined and one of the most confounding elements would be the type of biopsies and the way in which they were performed.

Now the work in this area has also led to a paten filing and issuance. From the Patent[10]:

The invention features methods for detecting prostate cancer, especially hormone-refractory prostate cancer (HRPC) or castration-resistant prostate cancer (CRPC), by detecting over-expression of PKIB or NAALADL2 compared the normal organs. Also disclosed are methods of identifying compounds for treating and preventing prostate cancer including HRPC, based on the over-expression of PKIB or NAALADL2 in the prostate cancer, the cell proliferation function of PKIB or NAALADL2, the intracellular localization of PKIB or NAALADL2 or the interaction between PKIB and PKA-C.

[10] http://www.faqs.org/patents/app/20120022128

Also, provided are a method for treating prostate cancer by administering a double-stranded molecule against the PKIB or NAALADL2 gene. The invention also provides products, including the double-stranded molecules and vectors encoding them, as well as compositions comprising the molecules or vectors, useful in the provided methods. An isolated double-stranded molecule, which when introduced into a cell, inhibits in vivo expression of PKIB or NAALADL2 and cell proliferation, which double stranded molecule comprises a sense strand and an antisense strand complementary thereto, hybridized to each other to form the double-stranded molecule.

It is clear that the researchers have staked out their territory. But also have many others who have patented various other markers.

4 OBSERVATIONS

As one reads the current literature and discusses the issue of identifying what PCa is aggressive the general consensus is still that the problem has no simple solution. These two recent attempts identify new markers which may be useful. Blood borne markers, mRNAs or exosomes, have some improved predictive value in some limited trials. However there is not as of yet a true statistically significant set of such markers which we can rely upon.

The problem quite simply is as follows. The US Task Force has recently stated that there should be no PSA testing because most PCa are indolent. As we have argued again and again, the problem is that most is not all and that for that 5-10% who has this aggressive form that such a recommendation is most likely a death sentence.

The work in these two areas demonstrates two things. First, the aggressive form is quite prevalent and is indeed aggressive. Second that there are many putative markers, blood borne, which can provide reasonably determinations as to the aggressiveness.

Thus the USPTF recommendations are in our opinion without merit since the deny the severity and prevalence of the aggressive PCa status. The work herein is of significant merit and should be closely followed as one gets a better understanding of this disease.

There are however several issues which need clarification. They are:

1. Pathway Implications: As we have argued extensively before, understanding the pathway interactions is essential. For example, what does the NAALADL2 gene do and how specifically does it do what it does. This would lead us to understand whether this is an issue of that gene itself or of some promoter of that gene, and if so what promoter. The genetic network is essential to be a part of diagnosis, prognosis and therapeutics.

2. Stem Cell Issues: As with so many issues regarding PCa there is always the issue of a stem cell. Is the stem cell the one forcing the overproduction? The questions are quite substantial once we address this area.

3. Localization: Blood borne markers have significance but the question is from where these molecules originate. The concern we have expressed internally is that they may be early mets and not the localized tumor. To some degree that is the advantage of the urine based test, it provides a modicum of localization.

4. Statistical Significance: These tests are on small samples. One needs larger scale trials to determine the proper prognostic result. However with all of the putative markers available one could expect to see this being done in a parallel trial. The reason is because perhaps there are linkages which may come about in such a trial. We believe that single thread trials may fail to bring forth the power of multiple markers.

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