Early Prostate Cancer Antigen-2: A Controversial Prostate Cancer Biomarker?

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It is well known that prostate-specific antigen (PSA)⁴ has both advantages and disadvantages as a marker of prostate cancer. Advantages include its ability to effectively detect early-stage prostate cancer and to monitor disease progression. A disadvantage of PSA is that prostate cancer cells and normal prostate cells both produce PSA; thus, it is frequently increased in nonmalignant conditions such as prostatitis and benign prostatic hyperplasia. The low diagnostic specificity of PSA leads to many false positives and a large number of biopsies in patients who are suspected to have prostate cancer. These well-recognized limitations of PSA suggest that new, and improved, prostate cancer biomarkers could play a useful role in reducing the number of unnecessary biopsies.

Over the last 10 years, many candidate prostate cancer biomarkers have been proposed (1). None of them has as yet reached the clinic, owing to their inferiority when compared with PSA. Getzenberg et al. published two provocative reports, one in the journal Urology (2) and a more recent one in the journal The Prostate (3), claiming that a newly discovered prostate cancer biomarker, early prostate cancer antigen-2 (EPCA-2), may be more effective than PSA in detecting prostate cancer, and more accurate in differentiating between localized and extracapsular disease. There are two important differences between the two papers. In the second paper, the authors claim that by using a different antibody epitope on the same molecule, the discrimination between organ-confined and nonorgan-confined prostate cancer, which was a major finding in the first paper, is now nonexistent. Also, despite measuring the same protein (but targeting two different epitopes), the claimed optimal cutpoint in the

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first paper was 30 ng/mL (μ g/L), while in the second the cutpoint was 0.5 ng/mL, nearly 2 orders of magnitude lower. Nevertheless, the authors concluded that the new data confirmed their previous findings, providing some validation of the earlier studies.

As soon as the first paper on EPCA-2 was published (2), by analyzing what we know about ELISA assay design and performance I concluded that the assay would not be either a sensitive or a specific measure of any analyte present in serum at the low ng/mL concentrations (4). Here is the explanation; in the first step of their assay, the authors coat a microtiter plate well with 50 μ L of serum. For simplifying the points below, let us assume that serum only contains two analytes, albumin (the most abundant, and representing all other serum proteins), at a concentration of 80 g/L (80 μ g/ μ L) and EPCA-2 at a concentration of 1 ng/mL (1 $pg/\mu L$). Note that the albumin concentration is approximately 80 000 000-fold higher than the EPCA-2 concentration. It is also known that a microtiter well can bind irreversibly approximately 40 ng of protein in total (in 100 μ L solution) and that the plastic (polystyrene) has no preference for binding any particular protein. Consequently, addition of 50 µL serum is equivalent to approximately 4 000 000 ng of albumin and 0.05 ng of EPCA-2. Assuming competitive binding, the plastic well will retain only 40 ng of albumin (1:100 000 of input molecules) and only 0.5 fg (1:100 000 of input molecules) of EPCA-2. With a molecular mass of approximately 40 000 daltons (3), this translates to 0.01 amol of EPCA-2 (6000 molecules). Studies from our lab and others have indicated that an ELISA with highly sensitive detection (such as time-resolved fluorescence) can only measure down to approximately 1 amol of analyte (approximately 600 000 molecules) (5). Thus, these calculations show that the utilized assay could not measure EPCA-2 in serum at any concentration below 100 ng/mL under the best analytical scenario. My estimate is that, with the assay design and peptide antibodies used by the authors, the detection limit could be no better than 1000 to 10 000 ng/mL.

I have now demonstrated these claims experimentally, by performing a model ELISA as per the author's protocol. The intent of this experiment is to show the effect of a high-abundance protein on ELISA sensitivity (as performed by the authors), when trying to quantify a low abundance biomarker (PSA in this example).

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⁴ Nonstandard abbreviations: PSA, prostate-specific antigen; EPCA-2, early prostate cancer antigen 2; S/N, signal-to-noise; EDRN, Early Detection Research Network.

PSA calibrators (pure, recombinant PSA) were prepared in PBS alone [50 mmol/L phosphate buffer (pH 7.4), containing 200 mmol/L of NaCl] or in PBS containing 60 g/L of BSA at concentrations of 0, 0.001, 0.01, 0.1, 1, 10, 100, 1000 and 10 000 ng/mL. After coating the plate with the calibrators of both series, in triplicate, and incubating for 3 h at room temperature, the plate was washed and the immobilized PSA was detected by adding a biotinylated monoclonal anti-PSA antibody and finishing the assay essentially as described elsewhere (6). The results were as follows. With PBS as diluent, the assay could detect PSA at 0.001 ng/mL (with a signal-to-noise (S/N) ratio of 1.5). Then, signals progressively increased, as expected, until saturation at approximately 100-1000 ng/mL of PSA. When PBS plus 60 g/L of BSA was used as diluent, signals were essentially the same as the background signal, until a PSA concentration of 10 ng/mL; an S/N of 1.5 was obtained at 100 ng/mL of PSA. Thus, a 100 000fold decrease in sensitivity was observed when 60 g/L of BSA was used in this assay. These data are in close agreement to my predictions outlined above. I conclude that the authors' assay design could not support their claims of measuring EPCA-2 in serum at low ng/mL concentrations.

One would then wonder, despite the calculations and experimental data mentioned above, why the authors apparently obtained discrimination between noncancer and cancer patients. There are many possibilities for an assay to produce seemingly distinguishing results between two groups of patients. For example, the samples from the patient groups may differ in some components that could cause a bias in signal (increase or decrease). This discrimination may not be subsequently reproducible with other sets of samples or independent validations. Examples of biasing factors include total protein concentration, lipid or salt composition, viscosity, medications, and time of sample collection (e.g. fasting vs postprandial). My own suspicion is that the data presented resulted from random variations in signals related to sample composition, collection, storage, or another unknown bias.

The literature is full of reports of high-profile papers that have reported excellent diagnostic discrimination between groups, but subsequent independent validation was a failure. In most cases, various biases were critical factors, as outlined by Ransohoff (7). Some examples include:

1. Nuclear magnetic resonance profiling of urine for cancer detection, which failed repeated validation efforts (8).

2. The serum proteomic profiling method proposed for diagnosis of ovarian and other cancers (9) and criticized until an independent validation published in

Clinical Chemistry, sponsored by the Early Detection Research Network (EDRN), confirmed the inability of the method to diagnose prostate cancer (10).

3. The discovery of lysophosphatidic acid as a diagnostically sensitive and specific test for ovarian cancer, leading to the formation of a company, Atairgin, which invested tens of millions of dollars to unsuccessfully validate the test in multicenter clinical trials and eventually closed its doors.

4. A 4-analyte panel with reportedly high diagnostic sensitivity and specificity for ovarian cancer detection *(11)* that was recently independently evaluated by EDRN and found to be no better than CA125 alone *(12)*.

How do we then deal with this situation of initial spectacular reports with wide publicity and raised hopes but subsequent validation failures? As I indicated earlier (4), organizations such as EDRN could independently validate biomarkers and publish the findings. Other helpful measures would be for journals publishing the original reports to give space to critics and encourage discussions, and for media to also publicize failures, not just promising initial data. Discredited papers should be retracted promptly by the authors and mechanisms to reexamine promising reports within, let's say, 5 years from publication, should be developed (e.g., see BioMed Critical Commentary at www.bm-cc.org, which is a forum for posting opinions on published papers).

Journal editors bear responsibility for establishing fair practices for their authors, readers and the public. In a recent letter in *CAP Today (13)*, I discussed my negative prior experience in trying to publish a letter in *Urology* commenting on the shortcomings of the assay used by Getzenberg (2). This letter was accepted for publication, but has never been published and the reasons for the delay not resolved.

Onconome, the company that collaborated and funded Dr. Getzenberg's research on this marker, has filed a lawsuit against both Dr. Getzenberg and his institutions, alleging breach of contract and scientific fraud. The story has been covered in Science (14) and Nature Medicine (15) as well as numerous popular news outlets (http://chronicle.com/article/Company-Says-Research-It/48319). Science can advance faster if developments are not only published, but also critically discussed in appropriate forums such as the journals themselves, as well as at conferences and other venues. Editors and publishers bear responsibility for promoting such discussions in any way possible. This is precisely the reason that many journals publish letters. While the final judge of any new development or discovery is time, it is useful to accelerate the process with discussions and debates so that valuable time and

money are invested appropriately and promptly, or redeployed to other projects.

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