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Point/Counterpoint

Point Proteomic Patterns in Biological Fluids: Do They Represent the Future of Cancer Diagnostics?

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Writing on the future of cancer diagnostics, this author has predicted that multiparametric biomarker analysis, in combination with artificial neural networks and pattern recognition, will likely represent one of the most promising methodologies for diagnosing and monitoring cancer *(1, 2)*. Over the last few years, we have witnessed publication of many reports dealing with proteomic patterns in biological fluids, and especially serum, by using the so-called "SELDI-TOF" technique (surface-enhanced laser desorption/ionization time-of-flight mass spectrometry), in combination with artificial intelligence *(3–7)*. The reported sensitivities and specificities of this method for ovarian, prostate, and breast cancer diagnosis are clearly impressive, and they are superior to the sensitivities and specificities obtained with current serologic cancer biomarkers *(8–12)*. In particular, these techniques appear to detect early as well as advanced disease with similar efficiency, making them candidate tools for cancer screening, an application that is not currently recommended, by utilizing the classical cancer biomarkers, e.g., CA125, carcinoembryonic antigen (CEA), and α-fetoprotein (AFP) *(1)*.

In addition to scientific journals, these reports have also been presented in international news media and have attracted public attention. Despite of some important shortcomings of these methodologies, criticism has been minimal *(13, 14)*. It seems that the impressive bottom line (very high diagnostic sensitivity and specificity) overshadows potential problems. The recent publication of three reports, from two different research groups, on the use of this technology in the diagnosis of prostate cancer allows for comparison of the data and the methodology and for the presentation of some important questions that have not been adequately addressed. In the following paragraphs, I will focus on some critical questions and provide discussion that could form the basis for further investigations. I will concentrate only on prostate cancer, but the same questions are likely valid for ovarian and other cancers.

Technologic Comparison of Three SELDI-TOF Reports on Prostate Cancer

Adam et al. *(10)* report 83% sensitivity at 97% specificity for prostate cancer detection, whereas Petricoin et al. *(9)* report 95% sensitivity at 78–83% specificity. Qu et al. *(12)* reported 97–100% sensitivity at 97–100% specificity. I consider these data roughly comparable, impressive, and clearly superior to the specificity obtained by prostate-specific antigen (PSA) testing (~ 25%) at the same sensitivities *(15)*. However, it is surprising that the two groups in three studies obtained these results using different methodologies and distinguishing peaks (Table 1). Adam et al. *(10)* and Qu et al. *(12)* (the same research group) used an IMAC-Cu metal-binding chip for serum adsorption after evaluating other types of chips, including hydrophobic, ionic, cationic, and metal binding. Petricoin et al. *(9)* found that a hydrophobic C-16 chip was superior. Furthermore, Adam et al. *(10)* used nine peaks at *m/z* ratios of 4475, 5074, 5382, 7024, 7820, 8141, 9149, 9507, and 9656, whereas Petricoin et al. *(9)* selected different peaks at *m/z* ratios of 2092, 2367, 2582, 3080, 4819, 5439, and 18220. Qu et al. *(12)* identified 12 major peaks at *m/z* ratios of 9656, 9720, 6542, 6797, 6949, 7024, 8067, 8356, 3963, 4080, 7885, and 6991 for differentiating noncancer from cancer and 9 peaks at *m/z* ratios of 7820, 4580, 7844, 4071, 7054, 5298, 3486, 6099, and 8943 for differentiating healthy individuals from patients with benign prostatic hyperplasia. It should be surprising that none of the peaks identified by Petricoin et al. *(9)* were identified by either Adam

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Table 1. Comparison of three reports for prostate cancer diagnosis based on SELDI-TOF technology.

	Adam et al. *(10)*	Petricoin et al. *(9)*	Qu et al. *(12)*
Diagnostic sensitivity and specificity	83%; 97%	95%; 78–83%	97–100%; 97–100%
SELDI-TOF chip type	IMAC-Cu	Hydrophobic C-16	IMAC-Cu
Distinguishing peaks, *m/z*[a]	4475, 5074, 5382, **7024**, 7820, 8141, 9149, 9507, **9656**	2092, 2367, 2582, 3080, 4819, 5439, 18220	Noncancer vs cancer: 3963, 4080, 6542, 6797, 6949, 6991, **7024**, 7885, 8067, 8356, **9656**, 9720 Healthy individuals vs BPH:[b] 3486, 4071, 4580, 5298, 6099, 7054, 7820, 7844, 8943
Bioinformatic analysis	Decision tree algorithm	Proprietary; based on genetic algorithms and cluster analysis	Boosted decision tree algorithm

[a] *m/z* ratios were rounded to whole numbers for simplicity. *m/z* ratios in bold font represent those identified by Adam et al. *(10)* and Qu et al. *(12)* for differentiating cancer from noncancer patients. The underlined *m/z* ratio represents a peak identified by Adam et al. *(10)* for differentiating cancer from noncancer patients and by Qu et al. *(12)* for differentiating healthy individuals from patients with benign prostatic hyperplasia.

[b] BPH, benign prostatic hyperplasia.

et al. *(10)* or Qu et al. *(12)*). Even more surprising is the fact that although Adam et al. *(10)* and Qu et al. *(12)* used the same chip for serum extraction and the same instrument for peak identification, their distinguishing peaks are very different. Notably, only two peaks are the same, at *m/z* ratios of 7024 and 9656 (Table 1). Another peak, at a *m/z* ratio of 7820, was identified by Adam et al. *(10)* as distinguishing for cancer vs noncancer patients and by Qu et al. *(12)* as distinguishing between healthy individuals and patients with benign prostatic hyperplasia, but not between noncancer and cancer patients. One (albeit very unlikely) explanation to this finding is that there must be thousands of potential distinguishing peaks in serum and that the chance of two groups finding the same peaks would be very low. Another, more likely explanation is that the methods for extracting these potential molecules from serum are very sensitive to the experimental details or to serum storage conditions, even if the same extraction devices are used.

PSA as an Internal Control

To our current knowledge, the best distinguishing serum protein for patients with prostate cancer vs healthy individuals is PSA. The molecular mass of the free antigen, 27 755 Da as determined by mass spectrometry *(16)*, is clearly below the upper mass limit that was used in at least two of these studies (40 000 Da) *(10, 12)*. Consequently, PSA should be a distinguishing target, among other proteins and peptides, with this technology. Petricoin et al. *(9)* used serum PSA to differentiate between healthy individuals with no evidence of prostate cancer (PSA <1 μg/L) and patients with biopsy-confirmed prostate cancer (PSA >4 μg/L) in their training set. Adam et al. *(10)* used a similar approach. It is clear from these data that the PSA concentration in serum of this series of patients with cancer was at least 5 times higher [and in the data by Qu et al. *(12)* up to 200 times higher] compared with the control group, but PSA was not identified as a distinguishing molecule with this technology. It will be important to examine why PSA was not detected (please see below).

The Sensitivity of Mass Spectrometry

Despite the application of the SELDI-TOF technology to clinical samples, the actual sensitivity (detection limit) of the method in this context is not well established. Two critical experiments could be done in determining this parameter. In the first experiment, diluted free PSA or other peptide standard solutions in a nonproteinaceous matrix would be applied to the various types of SELDI-TOF chips to determine whether PSA and other peptides can be captured by these matrices and what would be the sensitivity of detection on a SELDI-TOF instrument. For example, treatment of PSA standards with the same methodology as used for serum samples, followed by application to the chip and mass spectrometry, would reveal whether this analyte can be measured at concentrations of mg/L, μg/L, or ng/L.

In the second, subsequent experiment, certain concentration of PSA or other peptides would be measured in the presence of large amounts of unrelated proteins. For example, it would be informative to prepare PSA or other peptide standards in female serum (which is practically devoid of PSA) or horse serum and then perform the same analysis on various chips. One of the limitations of sample processing before SELDI-TOF mass spectrometry is that the matrices used for sample treatment [hydrophobic, ionic, cationic, and metal binding, as reported by Adam et al. *(10)*] are not specific for any type of protein. It would appear very unlikely for lower abundance molecules to immobilize on such chips. It is also quite probable that the efficiency of recovering "informative" molecules on the

chip would be dependent on the abundance of "noninformative" competing molecules. For example, in serum, the PSA concentration in healthy males averages 1 μg/L, whereas the total protein concentration is in the order of 80 g/L (80 000 000 μg/L). Thus, when proteins are exposed to the chip, each PSA molecule (or other molecules of similar abundance) will encounter competition for binding to the same matrix by 80 000 000 irrelevant (noninformative) molecules. It seems highly unlikely that with this nonspecific interaction, any molecules of relatively low abundance in serum (e.g., in the low μg/L concentration) will ever be detected by this method. These points were not adequately addressed by Adam et al. *(10)*, Qu et al. *(12)*, or Petricoin et al. *(9)*. On the basis of the procedure of Adam et al. *(10)*, it seems that the amount of serum applied to this chip, after dilutions and pretreatments, will be no more than 2 μL, a small amount compared with the 20–100 μL of serum applied in typical immunoassays. This will further affect the potential final analytical sensitivity of the method.

In another report, Wright et al. *(17)* claimed that four classic prostatic biomarkers, including free and complexed PSA, could be detected by mass spectrometry in various biological fluids and tissue extracts, including seminal plasma, prostatic extracts, and serum. However, the authors admitted that they had no way to be certain that the masses assigned to free or complexed PSA indeed originated from these molecules or from other molecules with an identical molecular mass. Furthermore, they admitted that the presence of various other molecules, including salts, in the mixture could cause a mass shift, thus complicating the interpretation further. In the same report, in their efforts to show a quantitative relationship between peak area and PSA concentration, they constructed linear calibration curves, but at PSA concentrations between 1000 and 50 000 μg/L, concentrations rarely seen, even in sera from patients with highly metastatic prostate cancer *(17)*.

In the absence of more experimental data, and based on the analysis described above, I could predict that the concentrations of the SELDI-TOF-monitored peptides/proteins in the serum of patients with or without prostate cancer must be much higher (e.g., mg/L or g/L) than the concentration of typical cancer biomarkers (e.g., PSA) in the serum of healthy individuals and prostate cancer patients (μg/L range). This conclusion is important to the points raised below.

Is It Possible That the Distinguishing Peptides Originate from Prostatic Tissue?

Petricoin et al. *(9)* postulate that the serum proteomic patterns identified by this methodology originate by blood perfusion of prostatic tissue and transfer of characteristic molecules from the diseased organ to the circulation *(9)*. They hypothesize that these molecules may be chemokines, cytokines, metabolites, or enzymatic cleavage products. Regarding prostate cancer, it would be instructive to use PSA as a model system of such a concept. PSA is produced by the columnar cells of prostatic epithelium (the cells from which prostate cancer usually originates) and then diffuses into the general circulation. It would be useful to compare the relative concentrations of PSA in prostatic tissue, in seminal plasma (most of the produced PSA is secreted into this fluid), and in serum. It has been calculated that approximately only 1 molecule per 10^2–10^3 molecules of PSA produced by the prostate successfully enters the general circulation, the rest being secreted into the seminal plasma *(18)*. PSA in seminal plasma is present at g/L concentrations, whereas in serum, the concentration is 10^6-fold less *(18)*. For PSA to enter the general circulation, it must first pass many barriers, as described elsewhere *(18)*. If the proteins/peptides detected by proteomic patterns are present in serum at much higher concentrations than PSA (as postulated in the previous paragraphs), it could be further hypothesized that it will be very unlikely or impossible that they are produced by prostatic tissue (unless the prostate makes them in amounts of many grams per day). In fact, this could be a testable hypothesis because SELDI-TOF analysis of seminal plasma or prostatic tissue extracts would be able to identify at least some of these distinguishing molecules. Such experiments, I believe, are worth pursuing in the future.

Concluding Remarks

The analysis described above suggests that the molecules monitored in serum by SELDI-TOF proteomic patterns are likely to be present at concentrations manyfold higher than the classic cancer biomarkers (e.g., mg/L or higher vs μg/L, respectively). It is also likely that these distinguishing molecules do not originate from prostate. I suspect that these distinguishing molecules are epiphenomena of cancer and that they are produced by other organs in response either to the presence of cancer or to a generalized condition of the cancer patient (e.g., malnutrition, infection, cachexia, or acute-phase reaction). It remains to be seen whether these molecules could indeed collectively constitute specific biomarkers for cancer, in view of the fact that cancer epiphenomena are not specific for this disease.

What Needs to Be Done?

In my opinion, much effort should now be devoted to identifying the nature of the distinguishing molecules and their concentration ranges and to understand their pathobiologic changes in serum and the relation of these changes to either cancer or to cancer epiphenomena. Despite the publication of much data for over 2 years, the

Table 2. Some open questions related to diagnostic SELDI-TOF technology.

- Identities and serum concentrations of distinguishing molecules are not known. Mass spectrometry is a largely qualitative technique. The relationship between peak height and molecule abundance is not linear and could be very complex.
- Distinguishing peaks identified by different investigators (and by the same investigators in different studies) for the same disease are different.
- Data are not easily reproducible between laboratories, making validation difficult.
- Optimal sample preparation for the same disease differs among investigators. Sample handling and preparation may be a critical issue.
- Validated serum cancer markers (e.g., PSA and CA125) that could serve as internal controls are not identified by this technology.
- Nonspecific absorption matrices favor extraction of high-abundance proteins/peptides at the expense of low-abundance proteins/peptides. The rates of recovery for informative molecules vs uninformative molecules are not known. The analytical sensitivity of mass spectrometry in the context of these experiments is not known.
- The technique likely measures peptides or other molecules present in high abundance in serum (e.g., mg/L to g/L range). Such molecules are unlikely to originate from cancer tissue. More likely, they represent cancer epiphenomena that may not be specific to cancer.
- The relationship between distinguishing molecules and cancer biology is not known.

identity of the distinguishing molecules remains elusive. As the groups cited above suggested previously, and I would agree, the identity of these molecules is not absolutely necessary for their use as biomarkers, but without this knowledge, the method will remain empirical and probably difficult to validate, reproduce, standardize, and quality control. Clearly, clinical use at the moment is not warranted. Furthermore, it will be critical to determine the analytical sensitivity of this method, especially when it is used to identify traces of proteins/peptides in the presence of massive amounts of unrelated serum proteins. Sample storage effects should be addressed systematically. In addition, it would be desirable to incorporate PSA, a classic and well-established prostatic biomarker, as an internal control in these studies, and other established biomarkers for other cancers. This methodology should currently be viewed as qualitative and empirical until the missing information is provided. Some open questions related to this technology are further summarized in Table 2.

The contribution of biological mass spectrometry to science is already enormous and has been recognized by the 2002 Nobel Prize in Chemistry. The power of artificial neural networks and other pattern recognition algorithms is also unquestionable *(19)*. In my opinion, these powerful analytical and bioinformatic tools should be combined with molecules of known identity and abundance to devise novel and robust strategies for cancer detection and monitoring. It just seems that the third element of this strategy is still missing.

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