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Application of Mass Spectrometry to the Discovery of Biomarkers for Detection of Prostate Cancer

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Abstract There has been an impressive emergence of mass spectrometry based technologies applied toward the study of proteins. Equally notable is the rapid adaptation of these technologies to biomedical approaches in the realm of clinical proteomics. Concerted efforts toward the elucidation of the proteomes of organ sites or specific disease state are proliferating and from these efforts come the promise of better diagnostics/prognostics and therapeutic intervention. Prostate cancer has been a focus of many such studies with the promise of improved care to patients via biomarkers derived from these proteomic approaches. The newer technologies provide higher analytical capabilities, employ automated liquid handling systems, fractionation techniques and bioinformatics tools for greater sensitivity and resolving power, more robust and higher throughput sample processing, and greater confidence in analytical results. In this prospects, we summarize the proteomic technologies applied to date in prostate cancer, along with their respective advantages and disadvantages. The development of newer proteomic strategies for use in future applications is also discussed. J. Cell. Biochem. 98: 496–503, 2006.

Key words: proteomics; prostate cancer; profiling; proteins; peptides; biomarkers

Prostate cancer is the most common non-skin cancer in the US. In 2005, more than 200,000 men have been diagnosed with prostate cancer, and over 30,000 men will die from it, making it the second-most leading cause of cancer-related deaths among men in the US. If diagnosed early, prostate cancer can be effectively treated by surgery or radiation. However, every year, 70,000 men require additional treatment due to recurrence of the disease. Prostate cancer is a complex heterogeneous disease that acts differently in different men. The slow rate of prostate cancer growth, coupled with the widely varied presentation, has made it difficult, if not impossible, to determine conclusively which treatment is best for which man.

Early prostate cancer usually has no symptoms and is most commonly detected through prostate cancer screening tests such as the prostate specific antigen (PSA) blood test and digital rectal exam. An elevated PSA level in the bloodstream does not necessarily indicate prostate cancer, since PSA levels can be altered by infection or other prostate conditions such as benign prostatic hyperplasia (BPH). Although the standard PSA test remains the most widely used screening assay for prostate cancer, approximately 25% of men with prostate cancer have a PSA level below 4.0 ng/ml and only 25% of men with a PSA level of 4–10 ng/ml have prostate cancer. Indeed significant numbers of men with an elevated PSA do not have prostate cancer. Thus there is a need for more accurate and non-invasive techniques to detect, diagnose, and stratify the disease based on molecular markers present in the body fluids.

WHY PROTEOMICS?

Most of the physiological changes in cancer are mediated by molecular alterations at the protein level many of which would not be expected to be revealed at the DNA/RNA level.

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Disease specific changes arising from the tumor cell or microenvironment can be utilized to provide biomarkers that can guide treatment decisions at the molecular level. These biomarker proteins can be uncovered by comprehensive protein analysis of cells, tissues, and body fluids (like blood, seminal plasma, and prostatic fluid) as well as artificially generated animal models and cell lines. Blood has been a particularly attractive target proteome source because cellular biomarkers routinely "leak" into the body fluids. In addition, blood is easy to handle, and acquisition is non-invasive and this proteome likely harbors a true picture of the physiological state of the patient.

Potential proteomic biomarkers of prostate cancer can not only benefit in earliest detection of disease but can also be used for determining cancer risk, stratifying disease stage and grade, monitoring response to therapy, and in general assisting in therapeutic decision making. Through careful sample selection, proper study design, automation in sample handling and processing, proteomic platforms are fast becoming very powerful tools in prostate cancer research.

APPROACHES TO CLINICAL PROTEOMICS

Proteomic studies dating from the 1970s utilized the technique of two-dimensional gel electrophoresis to display a large number of proteins from a given cell-line or organism [O'Farrell, 1975]. The technique works as a powerful tool for comparative analyses of protein expression levels between samples. However it soon became clear that this approach was limited in application with respect to the needs of clinical proteomics. The resolving power is limited by mass and pI, the technique is not high-throughput, the platform is limited in reproducing similar 2-D patterns and needs larger amounts of samples for processing. In response to these limitations researchers have incorporated fluorescent dyes in a process termed 2-D differential in-gel electrophoresis or DIGE [Unlu et al., 1997], to differentially label proteins from multiple sources and analyze the patterns of each on the same 2-D gel. The technique allows for increased throughput and easier comparative expression analysis between samples. Although, these recent advances in staining techniques using fluorescent dyes, along with the use of pre-fractionation approaches [Van den Bergh et al., 2003] and narrower pH ranges in the first dimension along with large format gels [Gorg et al., 2002] are improving the sensitivity and effectiveness (reviewed by [Lilley and Friedman, 2004]), nevertheless a need remains for high-throughput applications capable of simultaneously assessing the proteome of population-representative sample sets.

Combinatorial approaches include a combination of pre-fractionation and gel electrophoresis with mass spectrometry techniques. The approach has been utilized for proteomic analysis of human prostate cancer [Nelson et al., 2000; Ahram et al., 2002; Meehan et al., 2002]. Using a combination of laser capture microdissection, 2-D PAGE followed by LC MS/MS analysis of the tryptic digests of the protein spots, Ahram et al. [2002] identified 40 tumor specific protein expression changes. With the combination of 2-D PAGE, MALDI-TOF MS, peptide mass fingerprinting (PMF) and N-terminal protein sequencing, Meehan et al. [2002] identified 20 protein alterations in prostate tissues and validated them by Western blotting and immuno-histochemistry (IHC). Similarly, the combination of cDNA microarrays, 2-DE and MS has been employed to generate global gene/protein expression profiles of androgen-stimulated prostate cell lines by Nelson et al. [2000].

Even more recently the numbers of technical approaches available to proteomic analysis are proliferating at a staggering pace; to the extent that evaluating the merit of individual approaches has become a top priority as evidenced by several recent requests for applications issued by the National Cancer Institutes. Among the many technical hurdles to successful proteome mining, the two most prominent are likely the daunting numbers of different protein entities and the existing range of protein concentration. Estimates of potential protein types can reach into the millions when considering post-translational modification events and the relative concentration range can span 12 orders of magnitude. Thus, researchers must contend with achieving utility in both resolution and sensitivity of a given technique. An additional level of complexity exists when one considers the heterogeneity of individuals, which is a significant confounding factor in the study design of successful biomarker efforts. Solutions to these issues have come in the form of so called "top down" proteomics in which whole

proteins are separated and case versus control differential established prior to mass spectrometry analysis. Alternatively, complex protein mixtures can be enzymatically digested prior to separation and differential expression determination using mass spectrometry in "bottom up" approaches. This latter approach was enabled via improved tandem mass spectrometry advances. In all of these conceptual approaches (see Fig. 1), "front-end" sample fractionation and separation strategies are required to reduce the complexity of native clinical samples (or cell lysates) and the technical improvements in this area have grown as well.

SCREENING ALTERNATIVES TO MS-BASED PROTEOMICS

There are several very exciting approaches to high-throughput screening proteomics approaches that will not be discussed in detail here. These include antibody arrays (reviewed by [Haab, 2005]) which have had and will continue to have significant applications in cancer research. The antibody-arrays have been utilized for protein profiling, biomarker identification, protein characterization and in some cases the detection of protein post-translational modifications. Some notable success stories of interest to

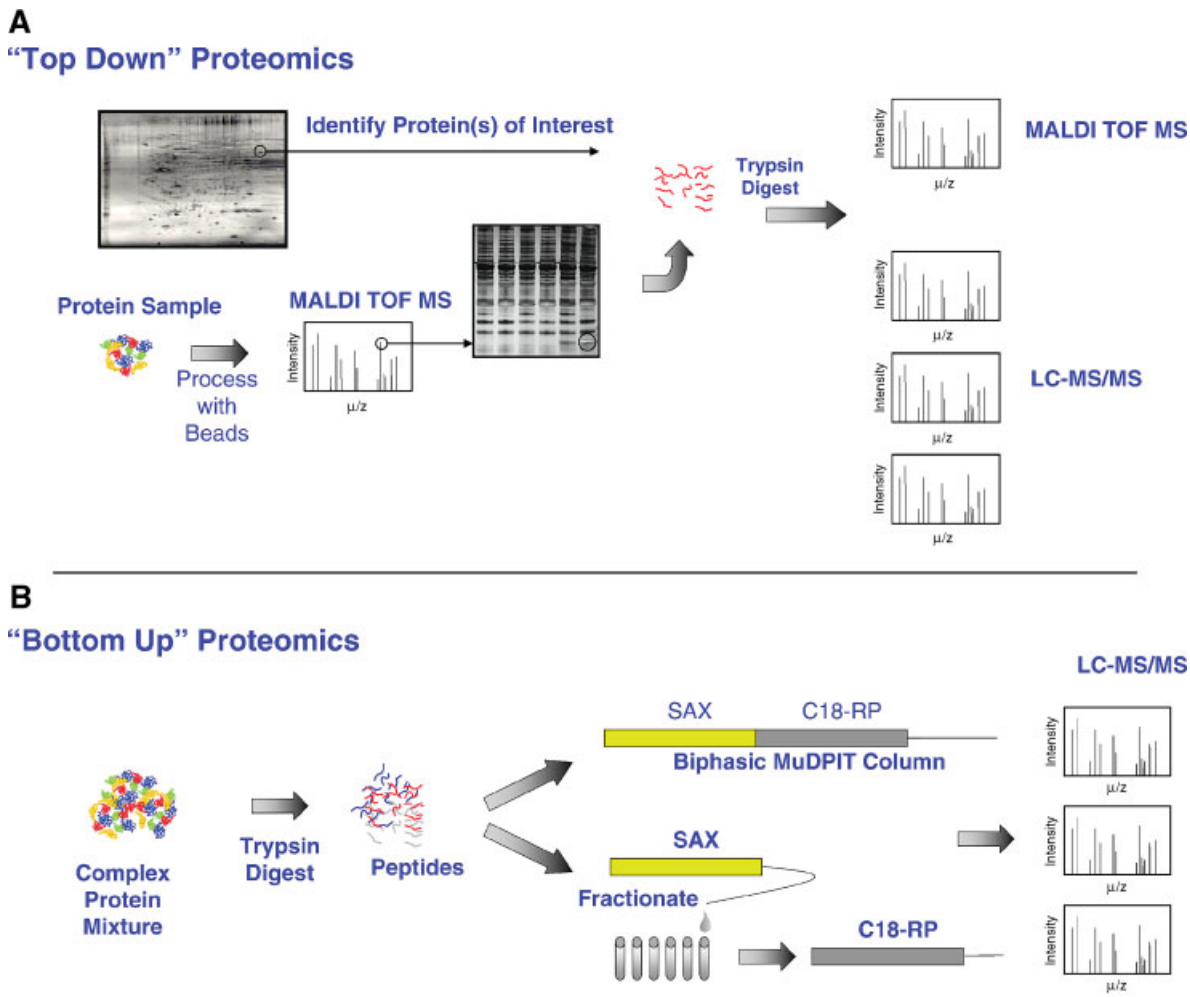

Fig. 1. Two major approaches to clinical proteomics. **A**: In the Top-down proteomics approach, whole proteins are pre-fractionated via various gel and non-gel based techniques. There are obvious scientific advantages for determining protein complexes and post-translational modifications when employing these approaches. Intact proteins of interest are then subjected to subsequent MS-based analyses using either single or tandem mass spectrometry. Throughput is usually indirectly proportional to the amount of information gathered. **B**: The bottom-up proteomics approach utilizes primarily non-gel based fractionation of peptides generated from complex protein mixtures. The rationale is that the digested peptides will reflect the native proteins and that the peptides behave more uniformly in both fractionation and detection. Digest-generated peptides are then subjected to mass spectrometry analysis. The approach can involve simple peptide mass profiling as well as quantitative tandem mass spectrometry to yield protein identification and relative protein concentration.

this audience would be in the analysis of proteins in tumors [Knezevic et al., 2001], the specific analysis of the prostate cancer humoral antibody response in patients to antigens [Webb et al., 1981; Wang et al., 2005a] as well as serum protein expression profiling [Miller et al., 2003]. The development of tissue microarray (TMA) technology [Kononen et al., 1998] has initiated large-scale studies using tumor tissues. The technique has also been widely extended to prostate cancer studies (reviewed by [Kuefer et al., 2004; Watanabe et al., 2005]) specifically for protein expression profiling, biomarker validation [Rubin et al., 2002; Zellweger et al., 2003], and study of tumor biology [Sun et al., 2003; Boddy et al., 2005; Chuan et al., 2005]. TMA technology has considerable value in translating the information gained from initial discovery into clinical applications. Protein arrays have been used to detect antibodies in samples against a set of cancer antigens [Robinson et al., 2002]. This approach may also involve the arraying of uniquely designed antigens and has been successfully applied in prostate cancer studies using patient samples or cancer models [Lagarkova et al., 2003; Nishizuka et al., 2003; Zhang et al., 2003]. Thus, it is essential that the value of these approaches not be lost in the focus of this review on the emerging mass spectrometry based techniques.

MS-BASED APPROACHES TO CLINICAL PROTEOMICS

Top-down: The highly complex and wide dynamic range of proteins/peptides in body fluids needs high-resolution systems for biomarker mining. Moreover, in complex body fluids like serum, the biomarkers could be spread over a wide range of concentrations. One of the ways to ease the "mining" of biomarkers in complex proteomes is via the separation of whole proteins prior to MS-based analyses. Two-dimensional liquid chromatography-based technologies (2D-LC) are the most widely used techniques for the this type of approach [Yan et al., 2003; Kolch et al., 2005; Qin et al., 2005]. Pre-fractionation of samples in liquid phase prior to biomarker mining not only reduces the proteome complexity of body fluids like serum but also allows for automation of sample processing before the analyses of the fractions. Capillary electrophoresis coupled to mass spectrometry has also been utilized in several studies for a high resolution fast separation of complex fluids like urine [Chalmers et al., 2005; Fliser et al., 2005].

Protein expression profiling using either MALDI-TOF or SELDI-TOF approaches has seen a wide application to many disease sites including prostate cancer [Peter et al., 2001; Adam et al., 2002; Cazares et al., 2002; Petricoin et al., 2002; Qu et al., 2002; Banez et al., 2003; Kidd et al., 2003; Lehrer et al., 2003]. The SELDI-TOF approach utilizes a chip-based affinity capture procedure to reduce sample complexity and then "profiles" the bound intact proteins by mass. The technique is sensitive, needs minimal amount of protein, and is relatively high throughput (reviewed by [Wright, 2002; Conrads et al., 2004; Solassol et al., 2005]). Our laboratory and others have been employing a combination of chromatographic paramagnetic beads and MALDI TOF/TOF MS to present a powerful and sensitive analysis of pre-fractionated samples (reviewed by [Pusch and Kostrzewa, 2005]). The paramagnetic beads allow for reasonable high throughput processing and reproducible fractionation of proteins/peptides followed by MALDI-TOF MS analysis (Fig. 2). Since the introduction of this technology to the field, the technique has been widely used for single or multidimensional separation of proteins/peptides on the beads. The fractions are then spotted on target plates for MALDI-TOF analysis [Villanueva et al., 2004]. Although not yet fully realized, this approach via sophisticated TOF/TOF capabilities offers direct protein identification with little or no additional work-up. We have been particularly interested in the utility of this instrumentation in improving so called immuno-MS (Fig. 2) which is an approach that we first reported on in early 2000 using SELDI-TOF. The incorporation of immuno-MS provides for early validation of biomarkers discovered on the same platform and offers distinct advantage over ELISA in that isoforms, modifications and cleavage products can be evaluated with the same antibody.

Bottom up: Various non-gel based liquid chromatography techniques focusing on peptides are gaining attention, as they allow multidimensional, automated separation of peptides representing very low abundance of proteins. The capabilities of these techniques to perform proteome analysis from minimal samples has

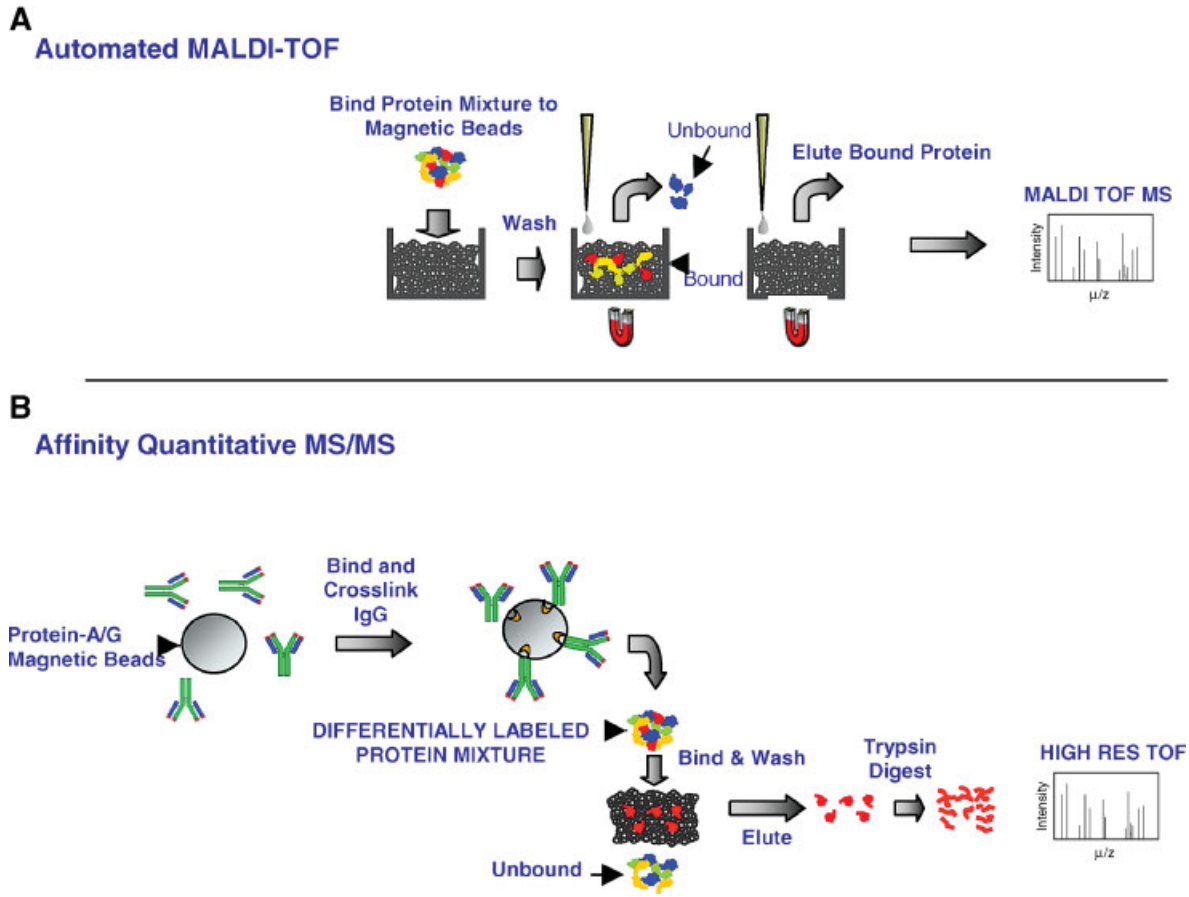

Fig. 2. Introduction of automated high-resolution MALDI-TOF based approaches (**A**) Using a combination of affinity selection on paramagnetic beads and downstream mass spectrometry, a high-resolution and high-throughput MALDI-TOF based approach has been developed. The technique utilizes the affinity-capture of proteins/peptides from complex fluids using a variety of capture molecules. The bound/unbound protein fractions can then be spotted on a target plate and analyzed by MALDI-TOF MS. The procedure profoundly reduces the sample complexity and then ''profiles'' the bound intact proteins by mass. **B**: The automated paramagnetic bead system can also be used in combination with differential protein labeling for a quantitative MS analysis. Using mass-shift and mass-defect tagging of protein mixtures from different sources, the high resolution MALDI-TOF/TOF approach allows for direct protein quantitation and identification.

generated new prospects for biomarker discovery especially using selected cell populations from tissue specimens [Wang et al., 2005b]. A high-resolution chromatographic separation of digest-generated peptides prior to mass spectrometry analysis without the involvement of gel electrophoresis has potential application to clinical proteomics. Such micro fluidic systems have already been integrated with increasingly sophisticated mass spectrometry for bottom-up differential identification [Brivio et al., 2002; Li et al., 2002; Zhu et al., 2003; Metodiev et al., 2004]. Peptide quantification using a combination of multidimensional liquid-chromatography, protein labeling and digestion has also been reported recently [Gygi et al., 1999; Griffin et al., 2003; DeSouza et al., 2005] adding direct quantitation and thus making it a much more powerful tool. The technique of differential peptide display (DPD) has been recently utilized to analyze the peptidome of the HUPO human serum and plasma specimens [Tammen et al., 2005]. The samples were fractionated on RP-HPLC and each fraction is applied to MALDI-TOF MS to generate an in silico 2-D display of peptide masses. A combinatorial approach of protein fractionation using HPLC, tryptic digestion and RPLC-MS/MS has also been recently utilized to characterize the mouse serum proteome [Hood et al., 2005]. Using these techniques the group identified 12,300 unique peptides originating from 4567 unique mouse serum proteins.

A number of groups are now trying to identify and analyze proteins from less complex mixtures such as seminal fluids [Utleg et al., 2003;

Fung et al., 2004], laser captured cells from cancer tissues [Paweletz et al., 2001; Cazares et al., 2002; Diaz et al., 2004], albumin-associated proteins from blood sera [Lowenthal et al., 2005], glycosylated proteins [Manning et al., 2004; Yang and Hancock, 2005; Yang et al., 2005], or sub cellular fractions from cancer cells [Gretzer et al., 2004]. The up-front reduction in sample complexity helps to reduce the numbers of proteins being interrogated thus effectively increasing the coverage of the disease proteome.

Clearly there is a demand for enabling the adaptation of cutting-edge mass spectroscopy approaches to clinical proteomics. These solutions will likely focus on improving sample acquisition and handling, reducing sample complexity, increasing sample throughput, and improving sensitivity/resolution in ion detection. It is also clear that this need, delineated by the pioneering work of a handful of clinical proteomics laboratories, has been noted by the mainstream mass spectrometry community. However, technology alone cannot drive future success in the application of proteomics to prostate cancer. Clearly, a concerted multi-disciplinary effort is needed. Central to this collaboration is the biochemist with a greater understanding of protein behavior and the tools to tease proteins from the proteome. In fact the advances in the application of mass spectrometry to proteins should signal a renaissance in classic biochemistry, an expertise that had largely given way to molecular biology. Studies involving mouse models, cell lines and direct human samples need to be coordinated toward the same clinical goals. For example, uncovering the proteome changes associated with exposure of LNCaP cells to androgen [Meehan and Sadar, 2004], would compliment nicely with similar studies in androgen resistant mouse models and proteomic analysis of prostatic fluids from patients with androgen-resistance transition. In the end, study design and solid biochemistry will push the success envelope of new technologies in clinical proteomics.

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