Biomarkers, September–October 2006; 11(5): 406–416

informa
healthcare

Application of proteomic strategies to the identification of urinary biomarkers for prostate cancer: A review

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Abstract
In the post-genomic era, genes and proteins are now studied on a more comprehensive scale. Studying disease processes at only the genetic or transcriptomic level will give an incomplete amount of information. A proteomic approach potentially allows for a more global overview of how disease processes affect the proteins present in cells, tissues and organisms. The challenge arises in determining which proteins are affected in specific diseases and establishing which of these changes are unique to a particular disease. Existing and emerging proteomic technologies allow for high throughput analysis of proteins in a variety of sample types. Prostate cancer is a significant male health problem in the Western world. It is widely accepted that more specific prognostic and diagnostic markers of prostate cancer are urgently required. The present paper suggests that urine may be an attractive biofluid in which to pursue the identification of novel biomarkers of prostate cancer. This review introduces some proteomic techniques including mass spectrometry and the newer, quantitative proteomic strategies. It focuses on the potential application of these platforms to novel urinary biomarker identification in prostate malignancy. It also includes a synopsis of the current literature on urinary proteomics.

Keywords: *Proteomics, urine, prostate cancer, biomarker.*

(Received 16 January 2006; accepted 11 May 2006)

Introduction

The study of the proteomes of tissues and body fluids has presented a new and exciting way in which to pursue the discovery of disease biomarkers. A proteome can be defined as the group of proteins that are encoded by the genome and expressed in the same biological environment. Traditionally, biological approaches to the investigation of gene function investigated one or a few genes or proteins of interest at a time. In the post-genomic era genes and proteins are now studied on a more comprehensive scale. Furthermore, it is now well-recognized that a single gene may encode multiple messenger RNAs, which in turn are translated into multiple proteins that are potentially subjected to a bewildering array of post-translational modifications.

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ISSN 1354-750X print/ISSN 1366-5804 online
DOI: 10.1080/13547500600799821

Finally, it is increasingly recognized that, excluding monogenic disorders, diseases are multifactorial in nature, and that multiple genes and proteins are likely to be involved in their initiation and progression. Together this implies that studying disease processes at only the genetic or transcriptomic level will give an incomplete amount of information. A proteomic approach potentially allows for a more global overview of how disease processes affect the proteins present in cells, tissues and organisms. It is estimated human cells can contain 2000–6000 'primary' proteins (Duncan & McConkey 1982), which is further increased by post-translational modification (Mann & Jensen 2003). Arguably, only a relatively small number of proteins will be associated with the onset and progression of a disease, possibly by changing in concentration, localization and structure. The challenge arises in determining which proteins are affected in specific diseases and establishing which of these changes are unique to a particular disease. Existing and emerging proteomic technologies allow for high-throughput analysis of proteins in a variety of sample types. In particular, they have the ability to enable the identification of protein biomarkers in readily accessible biological fluids to provide prognostic and diagnostic markers of disease.

Prostate cancer is a significant male health problem in the Western world. For example, it is the second commonest cause of cancer related mortality in men in both the UK and Ireland (The National Cancer Registry Ireland 2001, The United Kingdom National Statistics 2003). At present, the disease is diagnosed primarily through the use of digital rectal examination and measurement of serum levels of prostate-specific antigen. However, the poor specificity of serum prostate-specific antigen (PSA), the only current biomarker for the disease, presents significant problems for both patient treatment and management. It is widely accepted that more specific prognostic and diagnostic markers of prostate cancer are urgently required. Here the present authors suggest that urine may be an attractive biofluid in which to pursue the identification of novel biomarkers of prostate cancer and for reasons that will be introduced below, it may allow for the identification of earlier features of phenotypic change. This review, therefore, is focused on the application of diverse proteomic strategies for biomarker discovery in urine in prostate cancer. Urine represents a challenging biofluid for proteomic analysis and requires careful attention to sample acquisition, storage and preparation before proteomic analysis; these important issues will be discussed.

There has been (and perhaps still is) a conception that discovery-based expression proteomics studies are 'open-ended' fishing expeditions and as such are unlikely to lead to the identification of valuable diagnostic markers, therapeutic targets or insight into alterations in the expression of proteins including those that are involved in signalling pathways that would provide fundamental insight into the pathogenesis of diseases — including prostatic disease. In part, these criticisms/objections are based on the inherent complexity of 'the proteome' and that all existing proteomics strategies including gel and liquid chromatography-based approaches are still evolving, cannot access the entire proteome and may lack requisite sensitivity (Pennington & Dunn 2003a,b). This noted there is no doubt that with appropriate well-controlled sample acquisition, sample preparation (which can in part address issues of sensitivity) and the application of the latest methods for protein separation (for example differential gel electrophoresis (DIGE)) and identification (such as matrix-assisted laser desorption/ionization-tandem time-of-flight (MALDI-TOF-TOF) and electrospray ionization-tandem mass spectrometry (ESI-MS/MS)) such proteomic studies can and have led to the identification of potentially clinically relevant proteins. Indeed, studies

from the present authors' own groups have identified auto-antibody markers that predict heart transplant rejection (Jurcevic et al. 2001), proteins that are prognostic for survival times for pancreatic cancer (Shekouh et al. 2003) and a protein that interacts with topoisomerase inhibitors, which act in a synergistic manner to restrict tumour growth in vitro and *in vivo* (Barker et al. 2005). These and other important examples from a large number of studies indicate that whilst not 'perfect', careful application of proteomic approaches can lead to the identification of functionally (and potentially clinically) relevant proteins. Additionally, recent developments in quantitative proteomic strategies indicate that mass spectrometry-based approaches have the potential to move from discovery to routine assay more rapidly than traditional antibody dependent assays (Jenkins et al. 2005).

Overview of current proteomic techniques and strategies

Gel-based separation

A variety of techniques are in use for proteomic analysis. They can broadly be divided into gel-based and gel-free techniques. Gel electrophoresis is a long-established method for the investigation of the proteome. Two techniques are in current use: one-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and two-dimensional (2-D) PAGE. SDS-PAGE allows for the one-dimensional separation of proteins according to molecular weight and is commonly used before Western blotting or mass spectrometry (MS) (Hirsch et al. 2004). It has limited separation power especially for low molecular weight proteins, but is inexpensive, easy to perform and can be used with higher salt concentrations than 2-D PAGE. Two-dimensional electrophoresis (2-DE) has been in use for over 30 years having been developed by O'Farrell (1975). In the first dimension, proteins are separated by isoelectric focusing (IEF) using an immobilized pH gradient (IPG) polyacrylamide gel. In the second dimension they are separated based on molecular weight by sodium dodecyl electrophoresis (Gorg et al. 2004). Alternative gels such as agarose may be used. The advantages of 2-DE are its good separation power and immediate visualization and quantitation of protein content. It is also a mature technique with which a variety of biofluids can be used. As previously mentioned, this technique does not work well with high salt concentrations. It requires a lot of manual input and also underrepresents low (e.g. membrane proteins) and high molecular weight proteins. There may also be issues with reproducibility between gels. Many disease-associated proteins are low-abundance proteins that can be present in femtomole amounts or less and the greatest technical issue with 2-DE is its limited sensitivity for low-abundance proteins in a complex biological milieu. The proteins separated by electrophoresis are then stained to make them visible.

Commonly used stains include Coomassie blue and silver stains. These are sensitive and user-friendly (Hirsch et al. 2004). Other stains include fluorescence detection systems and DIGE. Fluorescent systems have good reproducibility, but are very expensive. The newer technique of DIGE allows for up to three samples to be run simultaneously on a gel, which minimizes reproducibility problems and allows direct comparison of samples. However, special equipment is required for visualization. Spots of interest can be cut from the gels (either manually or with a robot), the proteins are digested with trypsin and can then be analysed to identify them.

Traditionally this was done by a Western blot or by matching against a master 2-D PAGE pattern — these techniques have now been superseded by mass spectrometry.

Non-gel separation

An alternative to gel electrophoresis is chromatography, especially liquid chromatography (LC). Techniques such as multidimensional protein identification technology (MudPIT), the so-called 'shotgun' proteomics, allows analysis of complex protein mixtures (Kuruma et al. 2005). It incorporates high-performance liquid chromatography (HPLC) coupled to online mass spectroscopic detection. The main disadvantage of this is the lack of quantitative information that can be obtained (unless isotope coding is incorporated into the strategy). With biomarker discovery it may be that a particular protein is increased/decreased and not just present/absent — hence the need for quantitative information. It is, however, a fast and sensitive technique with good reproducibility.

Mass spectrometry

No matter what the separation technique employed, a mass spectrometer is used to identify the protein of interest. These require ionization of the analytes followed by measurement of the molecular weight and charge (m/z) of the protein/peptide. These data alone are not used to identify the protein because post-translational modifications can lead to variations in the molecular weight. Additional techniques such as peptide mass fingerprinting and peptide sequencing are then used either alone or in combination. The data are then correlated with that in databases using search algorithms to identify the protein/peptide.

There are a number of biological ionization techniques used in mass spectrometry. The matrix-assisted laser desorption ionization (MALDI) technique was introduced in the late 1980s (Karas & Hillenkamp 1988) (Figure 1). The sample of interest is crystallized with the matrix on a metal slide and placed in front of the ion source. A laser causes excitation of the matrix causing it, along with the analyte ions, to be released into

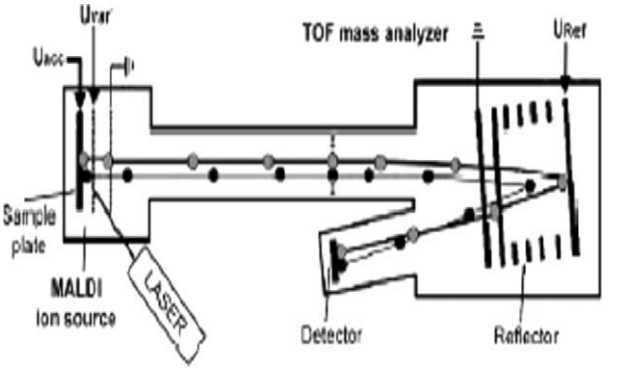

Figure 1. Schematic representation of matrix-assisted laser desorption/ionization-time-of-flight-mass spectrometry (MALDI-TOF-MS) that demonstrates laser excitation of sample, which is co-crystallized with matrix on the metal sample plate. The gaseous analyte ions accelerate towards the mass detector by use of an electric field. Reprinted, with permission, from the *Annual Review of Biochemistry* 70 (2001) © Annual Reviews, http://www.annualreviews.org

the gas phase. These are then accelerated towards a mass detection unit. More recently, MALDI instruments have been combined with time-of-flight (TOF) mass analysers. MALDI-TOF-TOF (Medzihradszky et al. 2000) instruments are capable of identifying the sequence of peptides. The sensitivity of MALDI-TOF is such that it can detect peptides in the femtomole range (Clauser et al. 1999). An alternative to MALDI is electrospray ionization (ESI) (Fenn et al. 1988). With this technique the proteins to be analysed are ionized by pumping through a capillary at high voltage. This can be connected with an online liquid chromatography separation strategy. The main disadvantage is blocking of the capillary resulting in more maintenance (Figure 2).

The newer 'protein profiling strategies' include surface-enhanced laser desorption ionization (SELDI) and imaging mass spectrometry. With the SELDI technique, proteins are captured on a protein chip array and detected by TOF-MS. It is a modification of the MALDI technique where the chip plays a role in the extraction of protein from the sample. A variety of chips can be used depending on the protein of interest, e.g. chips can be modified to capture anionic/cationic or hydrophobic/hydrophilic proteins. SELDI has been marketed as a biomarker discovery platform and applauded for its ability to deal with high throughput analysis of samples. In addition, minimal samples preparation is required before application to the chip. The biggest draw back is that the actual protein/peptide of interest can rarely be identified from the spectrum (Merchant & Weinberger 2000) and there have been issues with reproducibility. A lot of urinary proteomic work to date has been done using SELDI (Rogers et al. 2003, Schaub et al. 2004a,b). Whilst biomarker discovery programmes seek to identify novel molecular markers, which will have clinical significance, it must also be remembered that the proteins and peptides identified can also contribute to the understanding of the disease/cancer pathobiology and may lead to the identification of novel, therapeutic targets — this information may not be available if the SELDI approach is used. However, if an identified biomarker has been validated, then the SELDI approach could then be used for a high-throughput, rapid-screening programme. Imaging mass spectrometry uses MALDI-MS techniques for analysing tissue samples directly (Charaund & Caprioloi 2002). The sample of interest is cut

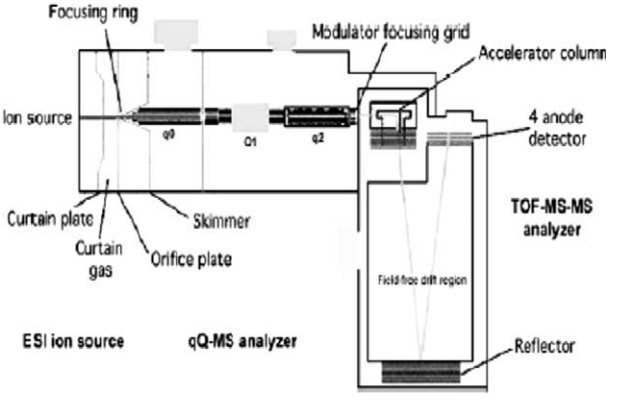

Figure 2. Electrospray mass spectrometer with quadruple mass analyser and time-of-flight (TOF) unit. Proteins are ionized by pumping through a capillary at high voltage and then sprayed into the mass spectrometer. Ions enter the vacuum system where they are focused, separated and dissociated. The ions then pass into the TOF to the detector where they are recorded. Reprinted, with permission, from the *Annual Reviews of Biochemistry* 70 (2001) © Annual Reviews, http://www.annualreviews.org

into a thin slice and coated with matrix. This technique allows for spatial information about the distribution of proteins and peptides in the sample.

Quantitative proteomics

Newer techniques in the field of quantitative proteomics include isotope tagging with isotope-coated affinity tags (ICAT) and iTRAQ reagents. These can be used to overcome the limitations that have been experienced in terms of protein quantitation when MS methods are coupled with LC separation. ICAT react with cysteine residues in the protein. The original reagents used labelled or unlabelled deuterium atoms but newer cleavable reagents use C^{13} rather than deuterium (Applied Biosystems 2005a). This technique allows two different samples to be labelled with ICAT reagents of different isotopic weights and following a protein separation strategy, MS can be used to quantify the differences in protein between the two samples. The main problem with this is that not all proteins will have a cysteine residue. The newer iTRAQ reagents are amine specific labelling reagents, which can quantitate up to four different samples simultaneously (Applied Biosystems 2005b). They allow for expanded coverage of the proteome by labelling all peptides including those with post-translational modifications.

Urine

Proteomics is a rapidly expanding area with new, more sensitive techniques appearing regularly. It allows the characterization of protein mixtures in complex biological samples and can help determine the function and interaction of proteins. Differential profiling of biofluids will hopefully lead to the identification of new, more sensitive biomarkers of disease. Urine as a biofluid presents technical challenges in terms of sample preparation. It is known that protein concentration is normally low in urine from humans with no evidence of renal disease (33 ± 10 mg per 24 h) (Norden et al. 2004), which means that sample handling needs to be minimal to prevent protein loss and degradation. Precipitation steps are necessary to concentrate the protein from this dilute sample — protein can possibly be lost at this stage. Coupled to this are the high salt concentrations found in urine. This presents a difficulty when using 2-DE as high salt concentration results in streaking on the second dimension gel — this is less of an issue with techniques such as SELDI where relatively crude samples can be used. High-abundance proteins such as uromodulin, albumin (Oh et al. 2004) and immunoglobulins may need to be removed to prevent 'masking' of low abundance proteins, which may be biomarkers. In removing the high-abundance proteins you run the risk of losing small proteins in the fractionation procedure. Finally, the inter-individual variability in any biofluid presents a challenge to the identification of a consistent, reproducible biomarker (Conrads et al. 2003). Despite these technical considerations the proteomic analysis of urine using some of the techniques alluded to provide a platform for discovery of possible biomarkers of prostate cancer.

Prostate cancer

Prostate cancer is the most common solid organ malignancy in males in Europe and the USA and the second most common cancer-related mortality rate (The National

Cancer Registry Ireland 2001, The United Kingdom National Statistics 2003). The incidence rate has increased due to increasing life expectancy and the widespread use of serum PSA screening. This marker exhibits poor specificity for the disease leading to difficulty with management of these patients. It is unable to differentiate between patients with clinically relevant and indolent disease. This means everyone is assumed to have a significant burden of disease and hence is treated as such with resulting morbidity (Bradley et al. 2004). Radical therapy in the form of surgery and radiotherapy are offered to men deemed to have local, organ-confined disease: 35% of men fail surgery within 10 years (Freedland et al. 2005). This means that either local recurrence has occurred or that systemic metastases were present but undetectable at the time of initial treatment. The heterogeneity of prostate cancer is such that a panel of biomarkers is necessary to provide information regarding the presence, aggressiveness and stage of the disease.

It is possible that elevation of serum biomarkers may represent a more advanced form of malignancy and that by mining the urinary proteome, earlier markers of more localized disease could be detected. The earlier a cancer in evolution is diagnosed the better the long-term prognosis for the patient. The enormous number of potential biomarkers that could be present in urine (either by filtration from the serum via the kidney or by direct secretion from the epithelial cells of the prostate in to the acinar lumen) (Figure 3) means that wide-scale, high throughput biomarker analysis is required and proteomic strategies are possibly the best approach currently available.

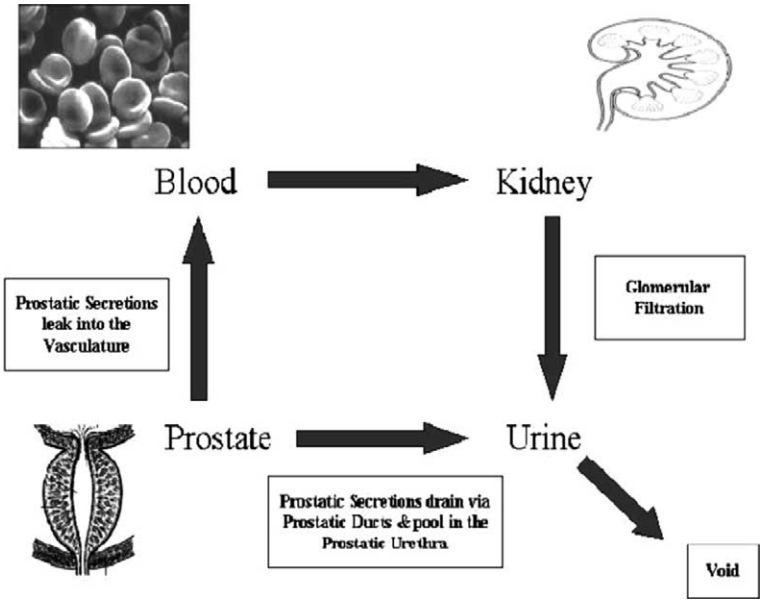

Figure 3. Pathways by which prostatic secretions gain access to urine showing direct release of products into the prostatic urethra and the release of prostatic products into the vasculature from where they may be filtered through the glomerular apparatus into the urinary tract.

Urinary proteomics

The literature

To date most of the work on urinary proteomics has been done on establishing a 'map' of the normal human urinary proteome (Thongboonkerd et al. 2002, Wittke et al. 2003, Oh et al. 2004, Schaub et al. 2004a). Many papers have been published on aspects of nephrology such as Fanconi syndrome (Norden et al. 2000, Cutillas et al. 2004), diabetic real disease (Mischak et al. 2004) and transplant rejection (Schaub et al. 2004b, 2005). However, very little has been done on urological malignancies. Some papers have been published looking at proteomic analysis of prostatic fluid and ejaculate (Fung et al. 2004), but very little on 2-D urinary proteomics, with the exception of Rehman et al. (2004). Difficulties have been noted with the processing of urine for gel-based proteomic techniques in terms of removing the salts and albumin. Both eluting columns (Wittke et al. 2003, Mischak et al. 2004) and/or dialysis (Oh et al. 2004) have been used to purify the urine samples. Between 150 and 157 protein spots have been identified on gels by different investigators (Oh et al. 2004, Freedland et al. 2005). Capillary electrophoresis-mass spectrometry has identified 1400 peptides in normal urine (Pieper et al. 2004). The development of these 'normal' urinary proteome maps provides an important starting point to allow for the detection of 'abnormal' proteomes as with prostate cancer (Figure 4: 'normal' urinary proteome map). The first paper using DIGE on urine samples has just been published (Sharma et al. 2005). It looked at the use of 2-D DIGE for analysing differences in the urinary proteomes from patients with advanced diabetic nephropathy. These were compared with gender-matched urine samples from control patients (non-diabetics). Using 2-D DIGE, they identified 99 spots that exhibited statistically significant differences in protein expression between the diabetic and normal urine samples (63 spots increasing and 36 decreasing in the diabetic samples). They then used SELDI-TOF-MS to identify one spot of interest — AAT. The ability to run up to three samples simultaneously means that DIGE is a powerful technique for comparing differences in the proteome between 'diseased' and 'normal' states.

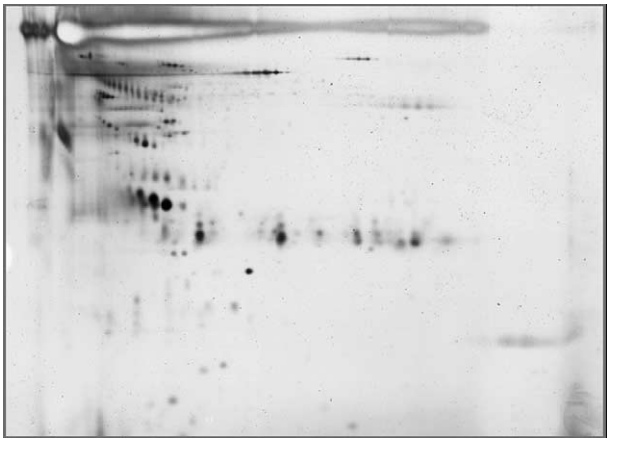

Figure 4. Two-dimensional gel image of OWL silver stained undepleted urine (100 μg protein loaded); 24 cm pH 3-10NL strip.

Prostate cancer urinary proteomics

One paper published to date by Rehman et al. (2004) was a pilot study looking at the proteomic analysis of urine post-prostatic massage. They looked at the voided urine post-transrectal ultrasound and biopsy in six men who had prostate cancer and compared it against age-matched controls with benign prostatic hypertrophy (BPH). The urine was concentrated using a Biomax 5 ultrafree centrifugal unit and proteins separated with 2-D PAGE. Protein identification was performed by MALDI-TOF-MS on Coomassie blue-stained spots. They found a higher median number of spots on the BPH gels compared with the prostate cancer gels, but this was not statistically significant. Twenty protein spots were common to all 12 gels but only six were present in significant amounts to be analysed by MS. Transferrin was seen in two of six patients with prostate cancer and none of the BPH patients. Calgranulin B/MRP-14 was identified in four of six urine samples from the prostate cancer cohort, but in none of the BPH group. Immunohistochemistry was performed on radical prostatectomy and cystoprostatectomy specimens (26 in total) and found Calgranulin B/MRP-14 in two well-differentiated and one moderately differentiated tumour. None was identifiable in poorly differentiated cancer. They also noted staining to be positive in 43% of benign epithelium adjacent to histologically identifiable cancer with absent staining of the tumour itself. Tissue microarrays of kidney and upper urinary tract epithelium (19 cases) showed an absent expression of Calgranulin B/MRP-14. This paper demonstrates that gel-based proteomic techniques can be used for the identification of new markers in the urine of prostate cancer patients. Unfortunately, the numbers in this study were quite small and the urine samples could not be directly correlated with the immunohistochemistry as the prostate samples were from different patients.

A previous study by Fernandez et al. (1986) showed elevated levels of transferrin in the urine of patients with prostate cancer. Both these markers could possibly be used as part of a panel of urinary biomarkers. Grover & Resnick (1997) performed a study looking at the high-resolution 2-DE analysis of urinary proteins of patients with prostate cancer. They isolated proteins that they called A (36 kDa) and B (23 kDa) which were absent from the urine of men with BPH and prostate cancer. They also noted a protein F (18–28 kDa), which was abundant in the urine of BPH sufferers but absent from prostate cancer and normal controls. There was a predominance of low molecular weight proteins in the BPH proteome. The proteins were unable to be identified as they did not have access to mass spectrometry at the time. It is clear that proteomic approaches are being used to identify novel markers for a variety of conditions including prostate cancer.

Conclusion

Traditional approaches to biomarker discovery in a 'one-marker-at-a-time' manner using antibody assays are unrealistic in view of the thousands of potential markers that exist. For this reason, proteomic strategies represent an ideal, high-throughput approach to the identification of novel molecular markers. Whilst there are limitations to all techniques, the array of proteomic technologies currently available represents the best platform for biomarker discovery.

Prostate cancer is a significant health issue with one in six men being diagnosed within their lifetime. The lack of robust, specific markers presents a challenge in the management of these patients and for this reason there is an urgent need for new molecular markers of this disease. It is not currently possible to distinguish patients with clinically

relevant disease from those with indolent tumours, hence it must be assumed that all patients have a significant burden of disease and must be treated as such. The analysis of urine, whilst challenging, may allow for the discovery of early phenotypic changes of prostate cancer. Thus, by mining the urinary proteome with some of the described approaches, it could be possible to identify early, localized, curable prostate cancer, which would significantly impact on patient treatment strategies and management.

Acknowledgements

Grants were obtained from the Royal College of Surgeons in Ireland/University College Dublin Research Fellowship in Surgery Incorporating the Gussie–Mehigan Scholarship 2005, for which the authors are grateful.

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