ANTICANCER RESEARCH *23:* 805-812 (2003)

Review

Proteomics as a Tool for Detection of Nuclear Matrix Proteins and New Biomarkers for Screening of Early Tumors Stage

P. OEHR

Department of Nuclear Medicine, University of Bonn, Germany

Abstract. *Nuclear matrix proteins are involved in control and co-ordination of gene expression. They have been isolated by extraction procedures, followed by gel electrophoresis and matrix- or surface-enhanced laser desorption and ionisation, with direct detection of retained proteins by time-of-flight mass spectrometry. Some nuclear matrix proteins from tumor tissues demonstrated tumor-specific expression which led to the development of highly tumor-specific nuclear matrix protein assays. In bladder cancer, NMP22 is twice as sensitive as cytology in detecting early stage cancers, and up to 90 % sensitive and 99% specific. NMP179 in squamous intraepithelial cervical lesions detects high-grade lesions with 96% sensitivity. Recently new technological approaches using ProteinChips, tracer-free biomolecular interaction, mass spectroscopy and nanotechnology have helped to successfully identify and apply specific biomarkers for cancer of the prostate, breast and colon and to develop new approaches for simultaneous screening of early cancer with several new biomarkers.*

There is an increasing interest in exploiting the power of proteomics as an interface between genomic data and biological function. The emphasis during the last decade was on genomics. The term genome refers to the entire set of genes of a species. Genes encode proteins. The proteome is the expressed protein complement of a genome and proteomics is functional genomics at the protein level. Proteomics can be divided into expression proteomics, the study of global changes in protein expression, and cell-map proteomics, the systematic study of protein-protein interactions through the isolation of protein complexes. Proteomes are not stationary and they are more complex than genomes.

Genome sequencing provides a wealth of information on predicted gene products (mostly proteins), but the majority of these have no known function. Two-dimensional gel electrophoresis and mass spectrometry have, coupled with searches in protein and EST databases, transformed the protein-identification process (1).

Proteomics is the large-scale study of proteins. There is considerable activity in development of proteomic technologies, which are now being incorporated into oncology in the postgenomic era. By comparison of normal and tumor tissue, genes that are differently expressed in tumors have been identified. As a consequence, genomics has expanded the field of biomarkers in molecular oncology, and proteomics is complementing genomics in the area of pathology, molecular diagnosis and anticancer drug development.

The simplest definition of a biomarker is a molecule that indicates an alteration in physiology from normal. A more practical definition of a biomarker would require clinical utility of this molecule. In this sense, the biomarker would specifically and sensitively reflect a disease state and could be used for diagnosis as well as for disease monitoring during and following therapy. The need for such biomarkers in all clinical fields is urgent, since the current arsenal of biomarkers is deficient and, in most cases, non-specific.

Recent comparative studies of global protein expression in normal tissue and in human tumors have led to the identification of biomarkers (here tumor markers) useful as diagnostic tools for cancer of various organs including the brain, esophagus, breast, liver, cervix, prostate and bladder. Demonstration of tissue- or cell type-specific expression of tumor-specific nuclear matrix proteins has led to development of nuclear matrix assays for diagnostics. The nuclear matrix A cell fraction that would today be termed "the nuclear matrix" was first described by the Russians Zbarskii and Debov in 1948 and rediscovered as a fundamental organizing principle of eukaryotic gene expression by Berezney and Coffey in 1974 (2, 3). The nuclear matrix is the non-chromatin protein and ribonucleoprotein network that is left in the nucleus following the removal of membrane, cytoskeletal and chromatin elements. Among the best characterized nuclear matrix proteins are the lamins A, B and C, which define the nuclear membrane. These proteins comprise part of what is known as the peripheral nuclear matrix. Two internal nuclear matrix proteins have been well characterized: nuclear mitotic spindle

Correspondence to: Prof. Dr. Peter Oehr, Am Büchel 53a, 53173 Bonn, Germany. e-mail: peter@oehr.info

Key Words: Proteomics, biomolecular interaction, cantilever, mass spectroscopy, SELDI, MALDI, nuclear matrix, diagnostic, proteinchip, screening, cancer.

apparatus protein (NûMA) and the DNA relaxing enzyme topoisomerase II. Both proteins are found on the matrix in interphase cells and relocate to the chromosomes during mitosis.

An extensive actual review on the nuclear matrix has been presented by Pederson (3), whereas the following review refers especially to nuclear matrix proteins and their importance for cancer detection. Several types of cancer have revealed discrete alterations in their respective nuclear matrices. One potential application of these nuclear matrix changes is the development of detection and monitoring tests that would reveal the presence of abnormal cells. These tests could be utilized at a number of points in the disease process including prior to gross physical symptoms, and thereby significantly reduce patient morbidity and mortality. A second potential application of the nuclear matrix is to utilize it as a tissue-specific protein targeting system to address narrowly directed therapeutic treatments and thereby avoid the systemic side-effects from broad-spectrum therapies like radiation.

Methods for detection and identification of low abundance specific proteins

The field of proteomics is expanding rapidly due to the completion of the human genome and the realization that genomic information is often insufficient to comprehend cellular mechanisms. In recent years, technologies in microfluidic and array technologies have appeared for proteomics. These novel approaches might help solve the technical challenges in proteomics. For detection of nuclear matrix proteins, samples are generally pretreated to remove lipids, immunoglobulin and human serum albumin. Afterwards, fractionations are made by NaCl gradients. The fractions can be analyzed by electrophoresis and/or applied to proteinchips. Protein profiles from proteinchips can be made by mass maps from mass spectroscopy. By comparison of protein profiles from tumor samples to tumor-free samples, tumor-specific proteins can be selected, sequenced and identified in databases.

Gel electrophoresis

Two Dimensional Polyacrylamide Gel Electrophoresis (2D-PAGE). Despite being first used more than 25 years ago, 2D-PAGE remains a basis of proteomics research. However, 2D-PAGE is labor intensive, time consuming and therefore not suitable for large scale screening or clinical testing, and difficult to standardize among laboratories. That is why it has not been useful for the routine analysis of large numbers of samples. It is also limited due to lack of sufficient analytical sensitivity, which was overcome by several new technologies. About ten years ago, different applications of mass spectrometry in addition to 2D-PAGE gave the necessary resolution to characterize the isolated proteins.

Capillary Zone Electrophoresis (CZE). Proteins in the subpicomole range are collected in 1 - 2μL volumes in microcentrifuge tubes and then mixed with matrix solution and can then be transferred to the sample probe of the mass spectrometer.

Mass spectrometry (MS)-associated methods

Matrix-assisted Laser Desorption Ionisation-time of Flight (MALDI-TOF): In MALDI-TOF analysis, proteins that have been digested to a complex mixture are affixed to a solid metallic matrix, desorbed with a pulsed laser beam to generate gas-phased ions that traverse a field- free flight tube, -and are then separated according to their mass-dependent velocities. By the use of different matrix mixtures, all peptides in a mixture can be detected. MALDI-TOF is a highly sensitive method for analyzing even large proteins intact and it is suitable for automation. Individual proteins and peptides can be identified through the use of informatics tools to search protein and peptide sequence databases (1). This method requires only a few masses to uniquely identify the protein. Typically this technique is used in conjunction with 2D-PAGE or capillary electrophoresis.

Surface-Enhanced Laser Despiption/Ionisation (SELDI): SELDI is an affinity-based MS method in which the proteins are selectively adsorbed to a chemically modified chemical surface. Impurities are removed by washing, an energy absorbing matrix is applied and the proteins are identified by laser desorption mass analysis (4). A commercial SELDI system (Ciphergen Biosystems, Inc., Freemont CA, USA) has been developed that is based on ready made arrays of addressable binding sites on multisample strips. These ProteinChip® (Ciphergen Biosystems) arrays are available with different chemically defined surfaces that permit protein binding based on hydrophilic, ionic, or hydrophobic interactions or on affinity for metal chelates, so that a range of protein classes may be identified in a relatively simple screening strategy. SELDI has been used to detect prostate cancer-associated biomarkers in complex protein mixtures such as cell lysates, serum and seminal plasma (5, 6).

Ion Mobility Mass Spectrometry (IMMS): IMMS is a high-throughput protein analysis system, which should allow researchers to develop new generations of mass spectrometers that rapidly characterize thousands of proteins from complex biological samples, such as blood, serum or cell extracts.

Biomolecular Interaction Analysis (BIA) Mass Spectroscopy: Impressive progress has been made with nano-electromechanical systems. An example is the recent realization of microfabricated silicon cantilevers functionalized with a selection of biomolecules in order to provide molecular recognition (7). They report the specific transduction, *via*

surface stress changes, of DNA hybridization and receptor-ligand binding into a direct nanomechanical response of microfabricated cantilevers. Cantilevers in an array were functionalized with a selection of biomolecules. The differential deflection of the cantilevers was found to provide a true molecular recognition signal despite large non-specific responses of individual cantilevers. Hybridization of complementary oligonucleotides shows that a single base mismatch between two 12-mer oligonucleotides is clearly detectable. Similar experiments on protein A-immunoglobulin interactions demonstrate the wide-ranging applicability of nanomechanical transduction to detect biomolecular recognition.

In an important distinction, BIA and SELDI are highly complementary techniques: BIA allows the determination of affinity binding while SELDI contributes to protein mass-to charge ratio and protein identification capabilities.

Synchrotrones: A detailed knowledge of the protein structure will help in understanding specific differences between normal and cancer-specific proteins for diagnosis and to develop drugs targeted to specific types of cancer. One tool for exploring proteomics is the 3-dimensional characterization of proteins by X-ray crystallography, using the pattern of a scattered X-ray beam. Synchrotrones generate most intensely focused X-ray beams focused on a very small spot, allowing the resolution of structures in the future faster and more easily than using traditional methods.

Protein sequence, data-banks, specific polyclonal and monoclonal antibodies: Proteins proven to be tumor-specific after comparison of protein profiles from extracted samples of normal, benign or tumor origin which were *e.g.*, separated by two dimensional gel electrophoresis and characterized by mass spectrometry, have been isolated and sequenced. Often these proteins are difficult to isolate and tend to be only partially characterized. In order to raise antibodies for immunoassays, the DNA-sequence information has often been characterized in databanks. For this special case, the Genovac company (a spin-off from the Institute of Immunobiology at the University Freiburg, Germany) developed a method called "genetic immunisation". This method raises antibodies against proteins based solely on their genetic sequence. Using an eukaryotic expression vector, researchers introduce the genetic information for the test protein into the animal. As a result, the immunized animal cells produce the protein. The animal recognizes the protein as foreign, which triggers an immune response. As a result, between eight and twelve weeks after researchers introduced the genetic information, the animal produces polyclonal antibodies against the test protein. Genovac removes the antibody producing cells from the animal, which fuse with murine (mouse) myeloma cells. This can result in immortalized hybridomas producing antibodies against the protein of interest. Cloning yields a stable cell line that produces just one type of antibody. A genetic immunization does not need the target protein, only the DNA sequence, and this approach could be an important step forward in proteomics.

Nuclear matrix proteins, assays and their clinical relevance

The nuclear matrix is involved in control and co-ordination of gene expression during differentiation. Loops of chromatin are persistently as well as transiently attached to this network. It has been proposed that each chromatin loop constitutes an independent regulatory unit containing several genes. DNA-replication, transcription, RNA processing and transport and the regulation of DNA superhelicity have been shown to be associated with this nuclear matrix. Nucleoplasm has elements of internal nuclear structure: actin-binding nuclear proteins, nonmuscle actin, nuclear lamins and nuclear pore complex-associated proteins are filaments extending 100-350 nm into the nucleus. Mitogenic stimulation and induction of differentiation alter the composition of nuclear matrix proteins, however there are certain matrix proteins shared by all cells:

1. Common sets of matrix proteins: 12 major proteins, including lamins, protein B23 and matrins (8).
2. Main subfractions: a) peripheral: proteins that remain insoluble after reduction; b) intemal: proteins released after reduction (9).
3. Major internal nuclear matrix proteins, common to different human cell types. The twenty-five most abundant proteins represent 75% of total nuclear matrix protein (10).

However modern research using physical approaches revealed key developments during the past years and no filaments or any sort of polymer-appearing structures are typically seen in the interchromatin space of unextracted nuclei. Nuclear RNA seems to move by diffusion in the interchromatin space and nucleoplasm seems to be a concentrated sea of individual protein molecules (3). That is why the biological reality of the nuclear matrix remains uncertain. Nevertheless, nuclear matrix proteins have already been shown to be a tool to detect cancer in a variety of tumor entities, and the development of tumor-associated nuclear matrix proteins has facilitated the clinical management for early diagnosing of some types of primary cancer. Demonstration of tissue or cell type-specific expression of tumor-specific nuclear matrix proteins has led to development of nuclear matrix assays for diagnostics.

NMP22 for detection of urinary bladder cancer: NMP22 is a nuclear protein which was analyzed and isolated by 2D-PAGE. It is responsible for chromatid and daughter cell separation in cellular replication (11). NMP22 has been approved by the US FDA (Food and Drug Administration) as an adjunct to cystoscopy and an aid in diagnosis, follow-up and screening of urinary bladder cancer and employs void urinary samples. This makes NMP22 the 2nd screening tool in genitourinary (GU) practice (after PSA) to obtain FDA

screening approval, which was recently also obtained for Japan and China. NMP22 is a non-invasive test for voided urinary sample and this feature puts it in a league of its own in GU screening altogether (12).

Two completely different test kit systems have been developed, a laboratory based EIA test, and an instrument-free point of care (POC) format test kit (Bladder Check®). The Matritech® NMP22 assay (Matritech Corp., Newton, Mass., USA) and the DPC Immulite (license by Matritech) are laboratory-based EIA tests for the quantitative detection of NMP22 in a stabilized voided urinary sample. Urine stabilization is achieved by collection into pretreated vial containers. The Matritech® NMP22-EIA assay is performed in approximately 3-4 hours. The automated DPC laboratory version enables a much faster performance within 30 to 60 minutes. The Matritech-POC test is an instrument-free assay employing 4 drops of voided urinary samples without stabilization and the assay is performed in about 30 minutes. The performance of the POC test format was compared to the Matritech® NMP22 EIA format in a precision study (13). Fresh urine samples were collected from 196 individuals at urology clinics. An aliquot of each sample was applied to a POC test. The development of a line at the "Test" mark was considered a positive test result. An additional aliquot was stabilized and tested by the NMP22 Test Kit to quantify the NMP22 concentration. When measured by the microplate format, 16 urine samples had an NMP22 concentration greater than 12.0 Units/ml; nine samples had a concentration within the POC threshold range of detection (between >8.0 Units/ml but <12.0 Units/ml); the remaining 171 urine samples had an NMP22 concentration less than or equal to 8.0 Units/ml. Fifteen of the 16 urine samples with an NMP22 concentration greater than 12.0 Units/ml gave a positive test result with the point-of-care test (94% concordance). One hundred and sixty-three of the 171 urine samples with an NMP22 concentration <8.0 Units/ml gave a negative test result with the point-of-care test (95% concordance). The overall concordance between the two assay formats was 95%.

Significantly, the expression of NMP22 has been reported to demonstrate a 25-fold increase in bladder tumors compared to normal bladder epithelia (14).

The Matritech® NMP22 assay is superior to voided urine cytology in the detection of transitional cell carcinoma (TCC) of the bladder. According to Lee (15), the sensitivity of the NMP22 test (75.7%) is significantly better than that of voided urine cytology (55.7%). There was no significant difference between the specificity of NMP22 and that of urine cytology. Sozel *et al.* (16) published that NMP22 results represented significant improvement over urinary cytology. The sensitivity of NMP22 for the detection of TCC in this group of patients was as much as twice that of cytology, especially in low-grade TCC.

In another investigation by Miyanaga *et al.* (17), the sensitivity of the NMP22 test for urothelial cancer was 90.9% whereas the sensitivity of voided urine cytology was only 54.5%. Zippe *et al.* (18) reported that NMP22 is a sensitive, cost-effective test in patients at risk for bladder cancer. The sensitivity and specificity of the NMP22 test were compared with urinary cytology, and the results of both tests were then compared to cystoscopic findings. The sensitivity of NMP22 was 100% with a specificity of 85% at a reference value of 10.0 units per ml, while cytology had a sensitivity of only 33% and specificity of 100%. Given a negative predictive value of 100% for NMP22, a cost savings of $28,302 to $111,072 (depending on the type of insurance carrier) would have been achieved if it was used alone as the indication for cystoscopy.

The sensitivity of the Matritech® NMP22 assay has been published by many international authors. Sensitivity and specificity depend on the patient group with respect to stage and grade, as well as cut-off level determined by the investigators. Table I gives a limited overview, demonstrating a range of sensitivity from 61 % to 90%, with a corresponding specificity between 70% and 99%. Detailed analysis by Sanchez-Carbayo (19) indicates that the sensitivity in detecting pTa and pT1 disease is around 71% with a specificity of 98.8%, and the sensitivity to detect pT2-4 tumors increases to 92.6% with a specificity of 93.8%. In this study, 2 out of 3 patients with carcinoma *in situ* were also detected. In a recent report by Sanchez-Carbayo (20), monitoring of the disease with urinary tumor markers in 106 bladder cancer patients could detect recurrence sooner than scheduled cystoscopies in 84% for NMP22 out of 31 patients who recurred during follow-up. The most relevant finding was that persistence of negative urinary markers during follow-up was largely indicative of disease-free status in 87% of these patients. Although false- positive results were present, they were mainly associated with sporadic urinary tract infections in 13% of the cases. Monitoring of bladder carcinoma patients with serial urinary tumor markers could therefore anticipate detection of recurrence. Persistent negative results might postpone and reduce the number of cystoscopies (20). Once the limitations leading to false-positive results are controlled by urinalysis and by starting sample collection when basal levels are reached in patients with intravesic therapy, urinary tumor markers might eventually individualize the intervals between cystoscopies in the surveillance of patients with bladder carcinoma (20).

Screening: Of the 608 patients 529 (87%) presented with *de novo* hematuria or chronic voiding symptoms without a diagnosis of bladder cancer, there were 79 (13.0%) patients being monitored with a known history of bladder cancer. In 135 patients with increased NMP22 values the 46 identified tumors were accompanied by 89 false-positive values yielding a specificity of 83.9% and a positive predictive value of 34. 1 %. These false-positive results were divided into 6 clinical categories. Exclusion of these categories improved the specificity and positive predictive value of NMP22 to 99.2% and 92.0%, respectively, yielding results similar to urinary cytology (99.8% and 94.1 %). Awareness and exclusion of the categories of false-positive results can increase the specificity

Table I. *Investigations for sensitivity and specificity of NMP22 in voided urinary samples.*

Source	Ref.	Sensitivity	Specificity %	Patients	Cut-off (U/ml)
Carpinito	(24)	88	70	353	6
Landman	(25)	81	77	47	6
Del Nero	(26)	83	77	30 (pTa)	6
Poulakis	(27)	85	94	739	8.25
Soloway	(28)	70	79	125	10
Zippe	(18)	100	85	330	10
Akaza	(29)	86	79	183	12
Miyanaga	(17)	90	76	309	12
Sanchez-Carbayo	(19)	78	95	267	13.7

and positive predictive value of NMP22, enhancing the clinical use of this urinary tumor marker (2 1).

A new bladder cancer-specific nuclear matrix protein (BLCA-4) was recently studied in urine samples of patients with TCC using immunoblot analysis. The BLCA-protein was detected in 75% (9 out of 12) of the tumor samples and in 100% of normal appearing tissue of the same bladders. It was not detected (0 out of 11) in bladders from organ donors. Using an ELISA test BLCA-4 can be detected in urine samples. All patients with bladder cancer could be separated from healthy individuals. The authors conclude that BLCA-4 is the first cancer-specific marker which is able to distinguish, absolutely, patients with TCC from those without the disease (22). However, studies with larger groups of cancer patients and controls are still necessary.

NMP 179 for detection of squamous intraepithelial cervical lesions: NMP179Human cervical cancer-associated nuclear matrix proteins were isolated on the base of high resolution two-dimensional gel electrophoresis. Keesee *et al.* (23, 30) compared nuclear matrix protein patterns in cervical carcinomas with those from normal cervical tissue. Tumors obtained from 20 patients undergoing hysterectomy for clinically localized cervical cancer were compared with normal cervical tissue. They identified five polypeptides (CvC-1: Mr = 69,408 Da, pl = 5. 78; CvC-2: Mr = 53,752 Da, pl = 5.54; CvC-3: Mr = 47,887 Da, pl = 5. 60; CvC-4: Mr =46,006 Da, pI = 5.07; and CvC-5: Mr = 44,864 Da, pl = 6.6 1) in the nuclear matrix from cervical carcinomas that were present in 20 out of 20 cervical tumors. partially present in lung or colon cancer, but 0 out of 10 normal tissues (Table II). These data extend similar findings of cancer-associated nuclear matrix proteins in other human cancers and suggest that nuclear matrix proteins may represent a new class of cancer markers. One of those tumor-associated nuclear matrix proteins from invasive squamous cell cervical carcinomas was further characterized by partial amino acid microsequencing, exhibited homology with a nuclear phosphoprotein, a cDNA clone was selected and recombinant protein was expressed, purified and used as an immunogen for antibody production.

One of the generated monoclonal antibodies, NMP 179, was evaluated in a preclinical feasibility study for the early detection of high-and low-grade cervical intraepithelial neoplasia. In a blind study involving two clinical sites, NMP 179 immunocytochernical staining data from 261 cervicovaginal Thin-Prep specimens were evaluated. The assay sensitivity and specificity were calculated based upon a positive threshold of > 10 immunostained cells per case, using cytological diagnosis as an end-point. Based upon the examination of squamous epithelial cells, NMP 179 detected 96.7% of cases with cytologically-diagnosed high-grade squamous intraepithelial lesions (HSIL) and 70.5% of low-grade squamous intraepithelial lesions. The antibody also reacted with 29.6% of normal (within normal limits or benign cellular changes) smears. The NMP 179 assay detected HSIL with very high accuracy (96.7%). The assay was 79.3% sensitive for the detection of low- and high-grade cervical intraepithelial neoplasia (grades 1-3), with a specificity of 70.4%. NMP179. This makes NMP 179 an effective marker for the early detection of preneoplastic squamous intraepithelial lesions of the cervix and it may be useful as an

Table II. *Matrix proteins distribution in cancer and normal cells.*

CvC* Tissues	N	1	2	3	4	5
Cervix cancer	20	20120	20/20	20/20	20/20	20/20
Lung cancer	7	-	-	5/7	317	5/7
Colon cancer	7	-				2/7
Normal tissue	10	-	-			

Table based on data from Keesee (23). *CvC 1 - 5: 5 polypeptides present in 20 human cervical cancer tissues.

adjunctive tool for better management of cervical intra-epithelial neoplasia (30).

New approaches for screening of biomarkers for cancer of the prostate, breast and colon: Cancer-specific serum protein markers for breast cancer were identified by a multistep screening strategy described by Watkins *et al.* (6). Sera from patients with proven cancer disease (prostate, breast and colon) were compared with sera from cancer-free controls. After removal of IgG and HSA, human serum was separated into 12 to 14 fractions by ion exchange chromatography. Each fraction was applied to five different SELDI chip surfaces in a process known as "retentate mapping". These 60 to 70 samples were analyzed with a Ciphergen Biosystem PBS I mass spectrometer to identify specific peaks corresponding to putative cancer-associated proteins that were only found in the cancer samples. Candidate mass peaks were identified by comparing spectra generated from each preparation of cancer sera to the corresponding preparations from healthy sera. A protein was defined as a cancer-associated protein if it was found in all investigated cancer samples but not in any of the cancer-free controls. In the next step, one or two cancer-associated proteins were selected for further analysis. The serum sample sets were expanded to include different stages of malignancy or different types of benign disease. The new serum samples were processed as described to determine whether the cancer-associated protein was present in each sample. Sequence information on several of the most promising markers has been obtained through large-scale separation and MS/N4S sequence analysis, using SELDI- MS to track each protein through the purification process.

Prostate cancer (50.8 kD protein marker): Watkins *et al.* (6) used sera from 36 patients, 20 healthy subjects and 4 different chip surfaces. Table III gives an example for mass peaks from 14 ion exchange salt fractions/serum samples from prostate cancer which showed seven candidate proteins (Table III). A 50.8 kD protein identified with the WCX2 ProteinChip was found in all 36 men previously identified with prostate cancer, but not in any of the healthy controls. The identification of the 50.8 kDa serum polypeptide is in progress. Eight of those men with cancer who were correctly identified with the 50.8 kD serum protein had been missed by the serum test for PSA, a blood marker routinely used to screen men for prostate cancer. The PSA test has an average sensitivity of about 70%, with a high rate of false-positives that include benign lesions. New developments demonstrate that the serum TOF-MS assay as a new prostate cancer marker was found to be 96 percent accurate.

Table III. *Selection of prostate cancer protein markers from NaCl ion exchange fractions from a Pak® Mono-Q column by comparison of fractions from sera of prostate cancer patients to fractions from sera of tumor-free persons.*

Mass (MZ) (kDA)	[NaCl] (mM)	Chip Surface	Prostate Ca. peak detected	Healthy (PSA < 2ng/mL)
21.31	125	H4	95	25
22.76	175	IMAC3	100	10
25.49	125	WCX-2	65	0
25.55	100	H4	85	25
50.76	125	WCX2	100	0
50.71	125	IMAC3	75	0
59.66	0	IMAC3	55	10

Table based on data from Hlavaty (33).

Breast cancer (28.3 kD protein marker): Nuclear matrix proteins show promise as informative biomarkers in following the pathogenesis of breast cancer. Altered profiles in nuclear

matrix proteins associated with DNA *in situ* during progression of breast cancer cells were reported by Spencer *et al.* (30). The results suggest that progression of breast cancer is accompanied by a reorganization of chromosomal domains, which may lead to alterations in gene expression (31).

In a similar approach (fractionation, SELDI-MS) as described for prostate cancer, Watkins *et al.* (6) selected and identified a 28.3 kD protein with a IMAC-Ni^2 ProteinChip in the 50-mM NaCl ion exchange fraction. The protein correctly detected 41 out of 41 (100%) metastatic, lateral stage and early stage cancers/and ruled out 27 out of 228 (96%) of the nonmalignant controls. This IS protein, designated NMP66, has been selected for further study and validation as a putative diagnostic marker for breast cancer.

Colon cancer (13.8 kD protein marker): In a similar approach (fractionation, SELDI-MS) as described for prostate cancer, Watkins *et al.* (6) selected (ProteinChip SAX2) and identified a 13.8 kD protein for colon cancer, using sera from 51 patients with benign, malignant, or premalignant colon disease, which were compared to 20 sera from healthy controls that had neither overt cancer nor polyps. The protein was found in all the sera from patients with colon cancer or precancerous polyps, but not in any sera from cancer-free controls or individuals with other benign intestinal disorders such as Crohn's disease or ulcerative colitis. Eight of the men with cancer who were correctly identified with the 50.8 kD serum protein had been missed by the serum test for prostate-specific antigen (PSA), a blood test routinely used for screening men for prostate cancer. The PSA-immunoassay test has an average sensitivity of about 70%, with a rate of false-positives including benign disease.

Screening, diagnosis and monitoring of cancer requires quantitative detection of multiple proteins. It is obvious that a combined screening of PSA, the 50.8 kD Protein and possibly other new biomarkers might lead to improved results, giving information on differentiation, precancerous stages, invasiveness or prognosis of a detected cancer disease. In order to do this successfully, there are two problems to be solved: SELDI-MS-TOF is time-limiting and commercial immunoassays using tracers are not optimal for high throughput mass screening. However, these limitations might be overcome. Development of the above described BIA-MS has shown that, when specific biomolecular binding occurs on one surface of a microcantilever beam, intermolecular nanomechanics bend the cantilever, which can be optically detected by a laser beam position-sensitive detector. In a recent publication (32), a tracer-free bioassay of (free or complexed) PSA using microcantilevers was developed. Using 200µm to 60 µm-long and 50µm to 65µm-thick cantilevers, it is possible to determine PSA concentrations in a range from 0.2ng/ml to 60µg/ml in a background of human serum albumin and human plasminogen at 1mg/ml, making this a clinically relevant high-throughput label-free microarray diagnostic technique, including simultaneous determination of several new biomarkers for cancer.

References

1 Blackstock WP and Weir MP: Proteomics: quantitative and physical mapping of cellular proteins: Trends Biotechnol *17(3):* 121-7, 1999.

2 Berezney R and Coffey DS: Identification of a nuclear protein matrix. Biochem Biophys Res Commun *60:* 1410-1417, 1974.

3 Pederson T: Half a century of "The Nuclear Matrix". Molecular Biology of the Cell *11*(3): 799-805, 2000.

4 Hutchens TW and Yip TT: New desorption strategies for the mass spectrometric analysis of macromolecules. Rapid Commun Mass Spectrom *7:* 576-80, 1983.

5 Verma M, Wright GL, Hanash SM, Gopal-Srivastava R and Srivastava S: Proteomic approaches within the NCI early detection research network for the discovery and identification of cancer biomarkers. Ann N Y Acad Sci *945:* 103-15, 2001.

6 Watkins B, Szaro R, Ball S, Knubovetz T, Briggmann J, Hlavaty JJ, Kusinitz F, Stieg A and Wu YJ: Detection of early-stage cancer by serum protein analysis. American Laboratory *33(12):* 32-36, 2001.

7 Fritz J, Baller MK, Lang HP, Rothuizen H, Vettiger P, Meyer E, Guntherodt H, Gerber C and Gimzewski JK: Translating biomolecular recognition into nanomechanics. Science *14;288(5464):* 316-8, 2000.

8 Nakayasu H and Berezney R: Nuclear matrins: identification of the major nuclear matrix proteins. Proc Natl Acad Sci USA *15;88(22):* 10312-6, 1991.

9 Stuunnan N, Meijne AM, van der Pol AJ, de Jong L, van Driel R and van Renswoude J: The nuclear matrix from cells of different origin. Evidence for a common set of matrix proteins. J Biol Chem *5;265(10):* 5460-5, 1990.

10 Mattern KA, van Goethem RE, de Jong L and van Driel R: Major internal nuclear matrix proteins are common to different human cell types. J Cell Biochem *65(1):* 42-52, 1997.

11 Compton DA and Cleveland DW: NuMA is required for the proper completion of mitosis. J Cell Biol *123:* 947-957, 1993.

12 Saad A, Hanbury DC, McNicholas TA, Boustead GB and Woodman AC: The early detection and diagnosis of bladder cancer: a critical review of the options. Eur Urol *39:* 619-633, 2001.

13 Kassabian V, Carpinito GA, Soloway M, Stein B, Roos R, O'Connell L, Oreper A, Bennett K, Healy B, Domurad M and Briggman J: Evaluation of the NMP22® bladdercheck™ for the rapid measurement of NMP22 antigen in urine. International Conference: Perspectives in Bladder Cancer, Monte Carlo, Monaco, November 16-17, 200 1, poster and abstract.

14 Keese SK, Briggman JV, Thill G and Wu YJ: Utilisation of nuclear matrix proteins for cancer diagnosis. Crit Rev Eukaryot Gene Expr *6:* 189-214, 1996.

15 Lee KH: Evaluation of the NMP22 test and comparison with voided urine cytology in the detection of bladder cancer. Yonsei Med J *42(1):* 14-8, 2001.

16 Sozen S, Biri H, Sinik Z, Kupeli B, Alkibay T and Bozkirli I: Comparison of the nuclear matrix protein 22 with voided urine cytology and BTA stat test in the diagnosis of transitional cell carcinoma of the bladder. Eur Urol *36(3):* 225-9, 1999.

17 Miyanaga N, Akaza H, Tsukamoto T, Ishikawa S, Noguchi R, Ohtani M, Kawabe K, Kubota Y, Fujita K, Obata K, Hirao Y, Kotake T, Ohmori H, Kurnazawa J and Koiso K: Urinary nuclear matrix protein 22 as a new marker for the screening of urothelial cancer in patients with microscopic hematuria. Int J Urol *6(4):* 173-7, 1999.

18 Zippe C, Pandrangi L and Agarwal A: NMP22 is a sensitive, cost-effective test in patients at risk for bladder cancer. J Urol *161(1):* 62-5, 1999; Comment in: J Urol. 1999 Aug; *162(2):* 500-1 and J Urol. 1999 Jan; *161(1):* 66.

19 Sanchez-Carbayo M, Herrero E, Megias J, Mira A and Soria F: Evaluation of nuclear matrix protein 22 as a tumour marker in the detection of transitional cell carcinoma of the bladder. BJU Int *84(6):* 706-13, 1999.

20 Sanchez-Carbayo M, Urrutia M, Gonzalez de Buitrago JM and Navajo JA: Utility of serial urinary tumor markers to individualize intervals between cystoscopies in the monitoring of patients with bladder carcinoma. Cancer *1;92(11):* 2820-8, 2001.

21 Ponsky LE, Sharma S, Pandrangi L, Kedia S, Nelson D, Agarwal A and Zippe CD: Screening and monitoring for bladder cancer: refining the use of NMP22. J Urol *166(1):* 75-8, 2001.

22 Nguyen TT, Vietmeier BN, Bastacky S, Konety BR and Getzenberg RH: Utilization of bladder cancer specific nuclear matrix protein, BLCA-4, for the detection of bladder cancer. J Urol *161 (suppl):* 149, 1999.

23 Keesee SK, Marchese J, Meneses A, Potz D, Garcia-Cuellar C, Szaro RP, Solorza G, Osornio-Vargas A, Mohar A, de la Garza JG and Wu YJ: Human cervical cancer-associated nuclear matrix proteins. Exp Cell Res *10;244(1):* 14-25, 1998.

24 Carpinito GA, Stadler WM, Briggman JV, Chodak GW, Church PA, Lamm DL, Lang, PH, Messing EM, Pasciak RM, Reservitz GB, Ross RN, Rukstalis DB, Sarosdy MF, Soloway MS, Thiel RP, Vogelzang N and Hayden CL: Urinary nuclear matrix protein as a marker for transitional cell carcinoma of the urinary tract. J Urol *156(4):* 1280-5, 1996.

25 Landman J, Chang Y, Kavaler E, Droller MJ and Liu BC: Sensitivity and specificity of NMP22, telomerase, and BTA in the detection of human bladder cancer. Urology *52(3):* 398402, 1998.

26 Del Nero A, Esposito N, Curro A, Biasoni D, Montanari E, Mangiarotti B, Trinchieri A, Zanetti G, Serrago MP and Pisani E: Evaluation of urinary level of NMP22 as a diagnostic marker for stage pTa-pTI bladder cancer: comparison with urinary cytology and BTA test. Eur Urol *35(2):* 93-7, 1999.

27 Poulakis V, Witzsch U, De Vries R, Altmannsberger HM, Manyak MJ and Becht E: A comparison of nuclear matrix protein-22 and bladder tumour antigen test with voided urinary cytology in detecting and following bladder cancer: the prognostic value of false positive results. BJU Int *88:* 692-701, 2001.

28 Soloway MS, Briggman V, Carpinito GA, Chodak GW, Church PA, Lamm DL, Lange P, Messing E, Pasciak RM, Reservitz GB, Rukstalis DB, Sarosdy MF, Stadler WM, Thiel RP and Hayden CL: Use of a new tumor marker, urinary NMP22, in the detection of occult or rapidly recurring transitional cell carcinoma of the urinary tract following surgical treatment. J Urol *156(2 Pt 1):* 363-7, 1996.

29 Akaza H, Miyanaga N, Tsukamoto T, Ishikawa S, Noguchi R, Ohtani M, Kawabe K, Kubota Y, Fujita K, Obata K, Hirao, Y, Kotake T, Ohmori H, Kumazawa J and Koiso K: Evaluation of urinary NMP22 (nuclear matrix protein 22) as a diagnostic marker for urothelial cancer-screening for urothelial cancer in patients with microscopic hematuria. Gan To Kagaku Ryoho *24(7):* 837-42, 1997.

30 Keesee SK, Meyer JL, Hutchinson ML, Cibas ES, Sheets EE, Marchese J, Oreper A, Potz D and Wu YJ: Preclinical feasibility study of NMP 179, a nuclear matrix protein marker for cervical dysplasia. Acta Cytol *43(6):* 1015-22, 1999.

31 Spencer VA, Samuel SK and Davie JR: Altered profiles in nuclear matrix proteins associated with DNA *in situ* during progression of breast cancer cells. Cancer Res *15;61(4):* 1362-6, 2001.

32 Wu G, Datar RH, Hansen KM, Thundat T, Cote RJ and Majumdar A: Bioassay of prostate-specific antigen (PSA) using microcantilevers. Nat Biotechnol *19(9):* 856-60, 2001.

33 Hlavaty JJ: Lecture and abstract. XXIX International Meeting ISOBM, and VIII International Symposium: Biology and Clinical Usefulness of Tumor Markers; Barcelona, Spain, September 2001.

Received February 7, 2003
Accepted February 26, 2003