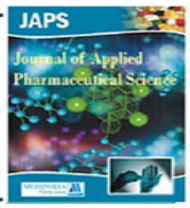
Journal of Applied Pharmaceutical Science 02 (03); 2012: 182-191

Journal of Applied Pharmaceutical Science

Available online at www.japsonline.com



ISSN: 2231-3354
Received on: 08-01-2012
Revised on: 16-01-2012
Accepted on: 03-02-2012

BIOMARKERS IN ONCOLOGY

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ABSTRACT

A biomarker or biological marker is in general a substance used as an indicator of biological state. Biomarkers are characteristic biological properties that can be detected and measured in parts of the body like the blood or tissue. They may indicate either normal or diseased processes in the body. Biomarkers can be specific cells, molecules, or genes, gene products, enzymes, or hormones. As an important biological indicator of cancer status and progression for the physiological state of the cell at a specific time, biomarkers represent powerful tools for monitoring the course of cancer and gauging efficacy and safety of novel therapeutic agents. The recent progress of proteomics has opened new avenues for cancer-related biomarker discovery. Advances in proteomics are contributing to the understanding of pathophysiology of neoplasia, cancer diagnosis, and anticancer drug discovery. One of the major contributions proteomics has made to the medical and pharmaceutical communities is the identification of potential drug targets.

Keywords: Biomarkers, carcinoma, peptides, proteomics, genomics, analytical analysis.

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INTRODUCTION

The National Academy of Sciences defines a biomarker or biological marker as a xenobiotically induced alteration in cellular or biochemical components or processes, structures, or functions that is measurable in a biological system or sample. A biomarker or biological marker is in general a substance used as an indicator of biological state (Singer *et al.*, 2007). In medicine a biomarker is a term often used to refer to a protein measured in blood whose concentration reflects the severity or presence of some diseased state (Crawford *et al.*, 2006). A biomarker can be understood as a molecule that is present (or absent) from a particular cellular type. This facilitates the characterization of a cell type, their identification, and eventually their isolation (Jacobs *et al.*, 2005). Biomarkers are detectable and measurable by a variety of methods including physical examination, laboratory assays and medical imaging (Anderson and Anderson, 2002). Biomarkers are characteristic biological properties that can be detected and measured in parts of the body like the blood or tissue. They may indicate either normal or diseased processes in the body. Biomarkers can be specific cells, molecules, or genes, gene products, enzymes, or hormones (He, 2006). Biomarker is used to indicate or measure a biological process (for instance, levels of a specific protein in blood or spinal fluid, genetic mutations, or brain abnormalities observed in a PET scan or other imaging test) (Alterovitz *et al.*, 2008).

The use of biological markers in the evaluation of disease risk has increased markedly in the last decade. More than 11 million people are diagnosed with cancer every year. It is estimated that there will be 16 million new cases every year by 2020. From a total of 58 million deaths worldwide in 2005, cancer accounts for 7.6 million (or 13%) of the global mortality. Deaths from cancer in the world are projected to continue rising, with an estimated 9 million people dying from cancer in 2015 and 11.4 million dying in 2030. As an important biological indicator of cancer status and progression for the physiological state of the cell at a specific time, biomarkers represent powerful tools for monitoring the course of cancer and gauging efficacy and safety of novel therapeutic agents. They can have tremendous therapeutic impact in clinical oncology, especially if the biomarker is detected before clinical symptoms or enable real-time monitoring of drug response. There is a critical need for expedited development of biomarkers and their use to improve diagnosis and treatment for cancer. Malignant transformation involves alterations in protein expression with subsequent clonal proliferation of the altered cells. These alterations can be monitored at the protein level, both qualitatively and quantitatively. Protein signatures in cancer provide valuable information that may be an aid to more effective diagnosis, prognosis, and response to therapy. The recent interest in biomarker discovery is because new molecular biologic techniques promise to find relevant markers rapidly, without detailed insight into mechanisms of disease. Genomics and proteomics are some technologies that are used in this process. There is considerable interest in biomarker discovery from the pharmaceutical industry. Blood test or other biomarkers could serve as intermediate markers of disease in clinical trials, and also be possible drug targets. Identification of clinically significant protein biomarkers of phenotype and biological function is an expanding area of research that will extend diagnostic capabilities. Recently, biomarkers for various diseases have emerged, including prostate specific antigen (PSA) for prostate cancer and C-reactive protein (CRP) for heart disease. Using biomarkers from easily assessable bio- fluids (e.g. blood, urine) is beneficial in evaluating the state of harder-to-reach tissues and organs. Bio- fluids are much more readily accessible, unlike more invasive or unfeasible techniques such as tissue biopsies. Bio- fluids contain proteins from tissues and serve as effective communication/hormonal. The tissue acts as a transmitter of information and the biofluid (sampled by physician) as receiver. The recent progress of proteomics has opened new avenues for cancer-related biomarker discovery. Advances in proteomics are contributing to the understanding of pathophysiology of neoplasia, cancer diagnosis, and anticancer drug discovery. With the advent of new and improved proteomic technologies such as the development of quantitative proteomic methods, high-resolution, high-speed, high-throughput, high-sensitivity mass spectrometry (MS) and protein chip as well as advanced bioinformatics for data handling and interpretation, it is possible to discover biomarkers that are able to reliably and accurately predict outcomes during cancer treatment and management. Besides, the newer technologies provide higher analytical capabilities, employing 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 can be obtained.

Oncoproteomics offers cutting-edge capabilities to accelerate the translation of basic discoveries into daily clinical practice. Continued refinement of techniques and methods to determine the abundance and status of proteins holds great promise for the future study of cancer and the development of cancer therapies (Cho WC, 2004 and Cho WC, 2006).

BIOMARKERS IN CARCINOMA

Current tumor markers

Early diagnosis of cancer is difficult because of the lack of specific symptoms in early disease and the limited understanding of etiology and oncogenesis. For example, blood tumor markers for breast cancer such as cancer antigen (CA) 15-3 are useless for early detection because of low sensitivity. Therefore measurement of carcinoembryonic antigen (CEA) and HER-2 in abnormal nipple discharge has been approved for diagnosis of breast cancer in some countries (Kurebayashi, 2004). More than 98% of cervical cancer is related to human papillomavirus (HPV) infection. The identification and functional verification of host proteins associated with HPV E6 and E7 oncoproteins may provide useful information for the understanding of cervical carcinogenesis and the development of cervical cancer-specific markers (Yim and Park, 2006).

For hepatocellular carcinoma (HCC), the common method of screening high risk patients by alpha-fetoprotein (AFP) and ultrasonography has been shown to result in earlier detection. Of the other tumor markers, the newer high sensitive desgamma-carboxy-prothrombin has been found to be useful. In addition, the AFP fractions L3, P4/5, and the +II band are highly specific for HCC. Among routinely assayed tumor markers in the laboratory, CA-125 is more sensitive for HCC than AFP but far less specific (Lopez JB, 2005). Currently available screening tests for ovarian cancer include CA-125, transvaginal ultrasound, or a combination of both. CA-125 has provided a useful serum tumor marker for monitoring response to chemotherapy. A rapid fall in CA-125 during chemotherapy predicts a favorable prognosis and can be used to redistribute patients on multiarmed randomized clinical trials. Prostate-specific antigen (PSA) is the most important tumor marker in all solid tumors, indispensable in the management of prostate cancer (Vukotic *et al.*, 2005).

Diagnostic biomarkers

Diagnostic oncoproteomics is the application of proteomic techniques for the diagnosis of malignancies. The early detection of cancer has a potential to dramatically reduce mortality. The thermostable fractions of serum samples from patients with ovarian, uterus, and breast cancers, as well as samples from benign ovarian tumor were analyzed using two-dimensional gel electrophoresis(2-DE) combined with matrix-assisted laser desorption/ ionization time-of-flight (MALDI-TOF)/TOF MS. Of

them, alpha-1-acid glycoprotein and clusterin were expressly down-regulated in breast cancer, whereas transthyretin was decreased specifically in ovarian cancer (Goufman *et al.*, 2006).

Bladder cancer

Celis and co-workers have utilized 2-DE and MS analysis to identify differential protein expression between bladder cancer and healthy tissue including squamous cell carcinomas versus normal urothelium, which has defined some of the steps involved in the squamous differentiation of the bladder transitional epithelium (Celis and Gromov, 2003).

Breast cancer

Isotope-coded affinity tag tandem MS allows for qualitative and quantitative analysis of paired protein samples. Alpha2-HS-glycoprotein was under-expressed in nipple aspirate fluid from tumor-bearing breasts, while lipophilin B, beta-globin, hemopexin, and vitamin D-binding protein precursor were over-expressed (Pawlik *et al.*, 2006).

Colorectal cancer

Six proteins (ANXA3, BMP4, LCN2, SPARC, MMP7, and MMP11) were found to be over-expressed in colorectal tumoral tissues by using immunoblotting and tissue microarray analysis. Two of them (LCN2 and MMP11) were clearly over-expressed in late Dukes stages (Madoz Gurpide *et al.*, 2006). To identify proteins with colorectal cancer (CRC) - specific regulation, comparative 2-DE of individual matched normal and neoplastic colorectal tissue specimens was performed. Endocrine cell-expressed protein secretagogin exhibited a marked down-regulation in CRC tissues. This finding may represent the basis for the clinical application of secretagogin as a biomarker for a distinct subgroup of CRCs (Xing *et al.*, 2006).

Esophageal cancer

Using agarose 2-DE and agarose 2-D difference gel electrophoresis (DIGE), a number of proteins with altered expression between primary esophageal cancer and adjacent non-cancer tissues have been identified. Among them, periplakin was significantly down-regulated in esophageal cancer, which was confirmed by immunoblotting and immunohistochemistry. These results suggested that periplakin could be a useful marker for the detection of early esophageal cancer and the evaluation of tumor progression (Nomura, 2006 and Nishimori *et al.*, 2006).

Gastrointestinal stromal tumor

To investigate the molecular characteristics of gastrointestinal stromal tumor (GIST) according to mutation type, protein expression profiles in GIST were analyzed using 2-DE and MALDI-TOF MS. Among the 15 proteins differently expressed according to the mutation status, overexpression of 5 proteins (annexin V, high mobility group protein 1, C13 or f2, glutamate dehydrogenase 1, and fibrinogen beta chain) and decreased expression of RoXaN correlated with a higher tumor grade. These findings suggested that differential protein expression could be used as diagnostic biomarkers (Kang *et al.*, 2006).

Glioma

Application of direct tissue MALDI-TOF MS to human brain tumors identified protein patterns that distinguished primary gliomas from normal brain tissue and one grade of gliomas from another, with high sensitivity and specificity (Schwartz *et al.*, 2005).

Hepatocellular carcinoma

Comparative proteomic analysis was used to search for characteristic alterations in the sera of HCC patients who had undergone curative radiofrequency ablation treatment subjected to 2-DE, and the proteins were identified by MS based on MALDI-TOF/TOF analysis and public database searches. The statistical analysis suggested that 4 proteins decreased after treatment, including pro-apolipoprotein, alpha2-HS glycoprotein, apolipoprotein A-IV precursor, and PRO1708/PRO2044 (the carboxy terminal fragment of albumin). Seven proteins were increased after treatment, including leucine-rich alpha2-glycoprotein and alpha1-antitrypsin. These data provided candidate biomarkers for the development of diagnostic and therapeutic tools (Kawakami *et al.*, 2006). On the other hand, 2-D DIGE combined with nano flow liquid chromatography (LC) tandem MS was employed to investigate differentially expressed proteins in HCC (Melle *et al.*, 2007).

Leukemia

DotScan microarray (a cluster of differentiation antibody microarray) has been developed to enable an extensive immunophenotype obtained for a suspension of leukocytes in a single analysis. The antibody microarray is printed as microscopic (10 nL) dots on a nitrocellulose film on microscope slide. Cells are captured by the immobilized antibodies and a dot pattern is recorded with an optical array reader giving the immunophenotype of leukemia. Procedures are developed to enable diagnosis of myeloid leukemia by comparison of the dot pattern obtained from an unknown blood sample with a library of consensus patterns for common leukemia (Christopherson *et al.*, 2006).

Lung cancer

Based on the assumption that proteins can emanate from tumor to serum, Maciel *et al* investigated whether serum proteins could discriminate lung adenocarcinoma patients from healthy donors. Results of 2-DE/MALDITOF showed 5 up-regulated proteins (immunoglobulin lambda chain, transthyretin monomer, haptoglobin-alfa 2, and 2 isoforms of serum amyloid protein) and 1 downregulated protein (fragment of apolipoprotein A-I) in lung adenocarcinoma patients (Maciel *et al.*, 2005).

Lymphoma

Fan *et al* making use of two-way hierarchical clustering analysis of the protein expression profiles differentiated reactive follicular hyperplasia, follicular lymphoma, and Burkitt lymphoma,

with 5 major clusters of differentially expressed protein peaks for molecular classification of B cell lymphoma subtypes. They identified histone H4 as a potential differentially expressed protein marker that seemed to distinguish grade 1 from grade 3 follicular lymphoma (Fan *et al*., 2005).

Nasopharyngeal carcinoma

Unfractionated whole sera of newly diagnosed Malaysian Chinese patients with advanced nasopharyngeal carcinoma were subjected to 2-DE and image analysis, ceruloplasmin showed higher expression. The enhanced expression of ceruloplasmin in the patients' sera was confirmed by competitive enzyme-linked immunosorbent assay (ELISA) (Doustjalali *et al.*, 2006).

Ovarian cancer

Identified by MALDI-TOF MS and validated by Western blotting, haptoglobin precursor significantly up-regulated while transferrin precursor significantly down-regulated in grade 3 ovarian cancer patients. Changes in serum expression of haptoglobin correlated with the change of CA-125 levels before and after chemotherapy (Ahmed *et al.*, 2005). Of great significance, the technique worked well on patients with early stage disease, offering the prospect of earlier diagnosis which would greatly enhance the chance of successful treatment outcome. This has led to the development of a commercial test, termed OvaCheck, for diagnosis of ovarian cancer.

Pancreatic cancer

The survival rate of pancreatic cancer patients is the lowest among those with common solid tumors, and early detection is one of the most feasible means of improving outcomes. Proteomic analysis combining 2-DE and MS successfully identified 154 potential serum markers for pancreatic cancer. Of these, fibrinogen γ a protein associated with the hypercoagulable state of pancreatic cancer, discriminated cancer from normal sera. Fibrinogen γ was subsequently confirmed to be over-expressed in pancreatic cancer sera by enzymatic analysis and tissue by immunohistochemistry relative to normal pancreas, thus it is a potential tumor marker in pancreatic cancer (Bloomston *et al.*, 2006). Besides, a PowerBlot analysis with more than 900 well-characterized antibodies was performed with tissue specimens from patients with chronic pancreatitis, pancreatic adenocarcinoma, and normal pancreas. A large number of proteins are differentially expressed in the chronic pancreatitis and pancreatic adenocarcinoma compared with the normal pancreas. Among them, expression analysis of UHRF1, ATP7A, and aldehyde oxidase 1 in combination could potentially provide a useful additional diagnostic tool for fine-needle aspirated or cytological specimens obtained during endoscopic investigations (Crnogorac Jurcevic *et al.*, 2005).

Prostate cancer

A promising prostate cancer biomarker identified by 2-DE and MS is annexin I. Studies have already confirmed that annexin I is under-expressed in a majority of early stage prostate cancer. Other non-gel-based proteomic technologies that may have improved sensitivity as compared to 2- DE have recently been developed; one of the examples is the ProteomeLab PF 2-D (Beckman Coulter Inc, Fullerton, CA, USA). The goal of most proteomic studies is to identify biomarkers that can be measured by ELISA or immunohistochemistry. Improvements in proteomic technology are changing this paradigm because there are now efforts to develop proteomic technologies directly into clinical diagnostic tests, an example of these technologies is SELDI-TOF MS. Using this technology combined with pattern recognition based bioinformatics tool, discriminatory spectrum proteomic profiles were generated which could help discriminating men with prostate cancer from those with benign prostate (Ornstein *et al.*, 2006).

Urothelial carcinoma

Using capillary electrophoresis-coupled MS to obtain polypeptide patterns from urine samples of patients with urothelial carcinoma and healthy volunteers, a prominent polypeptide from the diagnostic pattern for urothelial carcinoma was identified as fibrinopeptide A (a known biomarker of ovarian cancer and gastric cancer) (Theodorescu *et al*.,2006).

BIOMARKERS FOR TARGETS IN CANCER THERAPY

Results from genomic and proteomic studies are eagerly awaited for selecting patients, avoiding the use in non-targeted situation and reducing the cost of treatments. One of the major contributions proteomics has made to the medical and pharmaceutical communities is the identification of potential drug targets. Many cancers are characterized by alternations in certain signalling pathways and identification of the aberrant pathway in a particular patient allows for targeted therapy to that specific pathway.

For example, epithelial ovarian cancer is often characterized by activation of EGFR signaling pathway, and targeted therapies including monoclonal antibodies, such as cetuximab and small molecule inhibitors such as gefitinib are either in clinical use or under clinical trial for different stages of cancer. Proteinchip has been employed to measure enzyme activity of secreted and membrane proteomes of cancer cell lines, and are now being used to measure kinase activity via specific detection of phosphoproteins (Jessani *et al.*, 2002). It has been shown that actively proliferating cancer cells are more susceptible to the action of proteasome inhibitors than non-cancerous cells. Constitutively active NF-κB pathway is common in several solid tumors and proteasome inhibitors block this activation and make cancer cells more susceptible to radiation therapy and chemotherapeutic agents.

Breast cancer

The monoclonal antibody inhibitor of HER-2, trastuzumab (Herceptin), has been used successfully as monotherapy and in combination with chemotherapy in women with HER-2 over-expressing metastatic breast cancer.

Colorectal cancer

Bevacizumab receives European Union approval for the first-line treatment of metastatic CRC in combination with irinotecan- or 5-FU-based chemotherapy. Bevacizumab prevents interaction of VEGF with VEGFR1 (FLT-1) and VEGFR2 (KDR) on the surface of endothelial cells to inhibit angiogenesis. Bevacizumab is used for the first-line and second-line treatment of metastatic CRC. Besides, cetuximab became the first EGFR-targeting monoclonal antibody approved for use in metastatic CRC in 2004.

Hepatocellular carcinoma

By reverse transcriptase-polymerase chain reaction, a 1,741 bp cDNA encoding a protein that is differentially expressed in HCC have been isolated. This novel protein was identified by proteomic analysis and was designated as Hcc-2, which is up-regulated in poor-differentiated HCC but unchanged in well-differentiated HCC. This work demonstrated that an integrated proteomic and genomic approach could be a very powerful means of discovering potential diagnostic and therapeutic protein targets for cancer therapy.

Prostate cancer

The identification of antigens expressed by prostate tissue and/or prostate cancer that are recognized by T cells or antibodies creates opportunities to develop novel immunotherapeutic approaches including tumor vaccines. Proteins expressed in prostate cancer including PSA, prostatic acid phosphatase, and prostate membrane antigen has been used as immunologic targets for immunotherapy (Fong and Small, 2006).

RESPONSE OR EFFECT BIOMARKERS

Response or effect biomarkers are indicative of biochemical changes within an organism as a result of xenobiotic exposure. The ideal biomarkers should be early detected and be able to show adverse effects before they are irreversible. Those are the most studied biomarkers and they include modifications in some parameters of blood composition, alterations of specific enzyme activities, DNA-adducts appearance, localised mRNA and protein increases, and appearance of specific antibodies (autoantibodies) against a xenobiotic or a particular cellular fraction.

Respiratory system

Several studies have suggested that low-molecular weight proteins (LMWP) specific for the lung might serve as peripheral biomarkers of lung toxicity. A lung biomarker, measurable in serum, bronchoalveolar fluid (BAL) and sputum has recently been identified. This biomarker is a microprotein initially isolated from urine in 1974 (Urine Protein 1) of patients with renal tubular dysfunction and subsequently identified as the major secretory product of the lung Clara cells, which are non-ciliated cells localised predominantly in terminal bronchioles. This protein called Clara cell protein (CC16) is a homodimer of 15.8 kDa. Clara cells are particularly sensitive to toxic lung injury and they contain indeed most of the lung cytochrome P-450 activity, which confers them a high xenobiotic metabolising activity. Several lines of evidence indicate that CC16 is a natural immunoregulator protecting the respiratory tract from unwanted inflammatory reactions. CC16 has been shown to inhibit the activity of cytosolic phospholipase A2, a key enzyme in inflammatory processes. Phospholipase A2 is the rate-limiting enzyme in the production of arachidonic acid, the substrate for the synthesis of prostaglandin and leukotriene mediators of inflammation. By inhibiting phospholipase A2, CC16 could also prevent the degradation of lung surfactant phospholipids. A significant reduction of CC16 in serum is an indicator of Clara cells number and integrity. After adjustment for age, a linear dose-response relationship was apparent between smoking history and serum CC16, latter decreasing on average by about 15% for each 10 pack-year smoking history. Serum CC16 was also found to be decreased in several occupational groups chronically exposed to silica, dust and welding fumes, and lung diseases (cancer, asthma and patients with chronic obstructive pulmonary disease) (Bernard *et al.,* 1932-1937).

Blood system

The most studied biomarkers of effect are those related to the alterations of heme synthesis**.** ALAD is an enzyme involved in the heme biosynthetic pathway and the assay is highly specific for lead exposure and effect. The inhibition of ALAD has been shown to be a reliable indicator of effect to lead in studies on humans and animals (especially several species of fish and birds –eagles, starlings, ducks and geese). Heme biosynthesis is normally closely regulated, and levels of porphyrins are ordinarily very low. Some organochlorines (OCs) cause the formation of excess amounts of hepatic highly carboxylated porphyrins. The two OCs that are most involved in inducing porphyria in mammals and birds are hexacholorobenzene (HCB) and the PCBs. Hemoglobin adducts are formed from exposition of several compounds (ethylene oxide, acrylamide, 3-amino-1, 4-dimethyl- 5OH-pyrido-indole, 4-aminobiphenyl, 2, 6- dimethylaniline, etc). Acrylamide is an important neurotoxic agent causing a peripheral neuropathy to experimental animals as well as to humans and it has been shown to be a potential carcinogen. The conversion rate of acrylamide to glycidamide (reactive metabolite epoxide responsible for the neurotoxicity) is significantly correlated with the hemoglobin adducts of acrylamide. These adducts are useful as biomarkers of acrylamide-induced peripheral neuropathy. Because of the relatively long life span of the red blood cells (four months in humans), hemoglobin adducts have advantageously been used for integrating concentrations in the blood of genotoxic substances.

Nervous system

The most significant and useful example of specific biomarker of neurotoxicity is the inhibition of acetyl choline esterase (AChE) caused by organophosphorus compounds or carbamate pesticides. The enzyme activity is present in several

tissues though their inhibition is generally determined from blood samples (whole blood or plasma) and brain. This biomarker has been used in human toxicology and is widely studied in ecotoxicology (birds, mammals and aquatic species). For example, inhibition of AChE in brain can be taken as proof of mortality in birds, whereas in other animals, such as fish, there is a bigger variability founding lethal inhibition in a range of 40-80%. The decrease in AChE activity in brain may remain for several weeks after the toxic exposure, which is adequately correlated with the effect, in contrast to that occurring in blood with a lower life span. Nevertheless, measuring the blood AchE activity has the advantage of easy sampling since there is no need of animal sacrifice.Several active bioamines are liberated from the nerve ending by exocytosis, a process which is triggered by influx of Ca^{2+} and are inactivated by reuptake and methylation mediated by catechol-O-methyltransferase (COMT). Because of its intracellular localisation, monoamine oxidase (MAO) plays a strategic role in inactivating catecholamines that are free within the nerve terminal and not protected by storage vesicles. Isoenzymes of MAO have been characterised with differential substrate specificities; MAO-A preferentially deaminates norepinephrine and serotonin, whereas MAO-B acts on a broad spectrum of phenylethylamines. MAO-B is a microsomal enzyme and the aminoacidic sequences of the enzymes from human cerebral cortex and consequently platelets were shown to be identical and platelet MAO-B activity appears to reliably reflect enzyme activity. MAO-B activity is used clinically as a marker of the pharmacological effects of MAO inhibitors, such as in the treatment of Parkinson's disease. MAO-B activity in platelets has been used as a biomarker of effects of styrene and perchloroethylene occupational exposures, which is known to cause dopamine depletion. Changes in MAO-B could represent an adaptive response to dopamine depletion and alternatively, styrene or its metabolite(s) might exert a direct inhibitory effect on the enzyme (Manzo *et al.*, 1996).

Urinary biomarkers

Long-term exposure to certain nephrotoxic compounds (heavy metals –lead, mercury, cadmium and chromium-, halogenated hydrocarbons –chloroform- organic solvents –toluene-therapeutic agents –aminoglucosides, amphotericine B, acetaminophen, etc.) may cause progressive degenerative changes in the kidney. In practice, one usually recommends the determination in urine of at least two plasma-derived proteins, a high molecular weight protein (HMWP) such us albumin for the early detection of glomerular-barrier defect and a low molecular weight protein (LMWP) such as retinol-binding protein for the early screening of proximal damage. Injury to the kidney can be detected by measuring the urinary activity of kidney derived enzymes. As index of nephro toxicity it has been proposed the lysosomalenzyme β-N-acetyl-D-glucosaminidase (NAG). Advantages of this enzyme include its stability in urine and its high activity in the kidney. The diagnostic value of NAG can be further improved by measuring the B isoenzyme (lesional form released with fragments of cell membranes). Destruction of renal tissue can also be detected by measuring in urine kidney components which, when they are quantified by immunochemical methods, are referred to as renal antigens. These have been proposed as urinary markers of nephrotoxicity and include: carbonic anhydrase, alanine aminopeptidase and adenosinedeaminase-binding protein for the proximal tubule, fibronectin for the glomerulus and Tamm-Horsfall glycoprotein for the thick ascending limb of the loop of Henle. Within the field of Ecotoxicology, the resistance to infection in ducks exposed to organochloride pesticides has been studied measuring the cellular activities involved in the immune response; particularly the *in vitro* phagocytic capacity from kidney isolated macrophages in an number of species has been evaluated (Hotz et al.,1995).

Biomarkers of DNA damage

Some examples of toxics capable to form human DNA adducts includes polycyclic aromatic hydrocarbons, aromatic amines, heterocyclic amines, micotoxins and alquilant chemotherapeutic agents. The biologic monitoring to detection of human and animal DNA adducts include 32P post-labelling and recently immunoassays using adduct-specific antibodies. They can be detected in blood (lymphocytes), urine or tissue homogenates from biopsy (gastric mucosa, liver, etc.) although the study of DNA-adducts is not feasible in the routine analysis.

Biomarkers of gene expression

The development of many tumours related with xenobiotics is associated with the aberrant expression of genes that encode proteins involved in cellular growth. This aberrant expression can involve a quantitative difference (over expression of the protein) and a qualitative difference (expression of a mutant) been shown to correlate with the incidence of a carcinogenic process and is a promising biomarker for elucidating the molecular epidemiology of cancer.

DISCOVERY OF NOVEL PROTEIN BIOMARKERS IN ENDOMETRIAL CANCER USING FORMALIN-FIXED PARAFFIN-EMBEDDED TISSUE AND MASS SPECTROMETRY

Proteomic Analysis Of Laser Microdissected Ffpe Tissue Using Mass Spectrometry

Figure 1: Heatmap based on the Spectral count analysis of the 33 endometrial cancer samples and 12 normal endometrial samples. The number of peptides identified for each of the 12 proteins in each sample in this study shown. The colour code reflects the spectral count abundance of these proteins in each sample. Endometrial cancer is the most common cancer of the female reproductive system. If the cancer is detected early, a large percentage of patients survive at least five years. A study was undertaken to identify candidate protein biomarkers for early detection of endometrial cancer using formalin fixed paraffin embedded tissue samples. A collection of well documented formalin-fixed endometrial tissues was assembled, and microdissected utilizing the Director® laser microdissection technology to collect both early stage cancerous epithelium and normal epithelium.

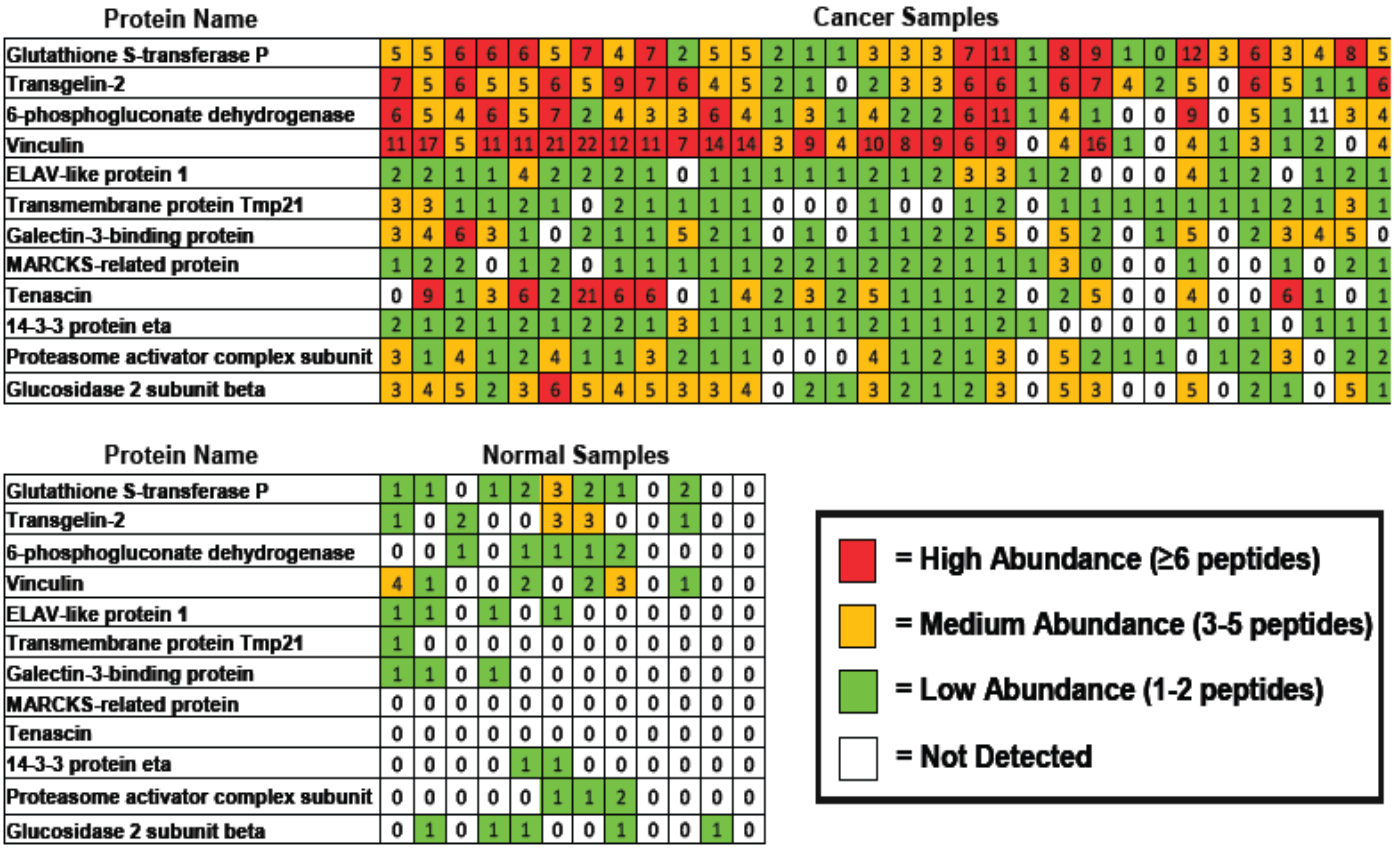

Fig. 1: Heatmap based on the spectral count analysis of the 33 endometrial cancer samples and 12 normal endometrial samples. The number of peptides identified for each of the 12 proteins in each sample in this study shown. The colour code reflects the spectral count abundance of these proteins in each sample.

Table. 1: Spectral count analysis of the 12 most differentially expressed proteins; defined by total number of unique peptides identified per protein across all cancer/normal samples, average number of peptides per protein identified per cancer/normal sample, ratio between avg. peptides per cancer sample Vs avg. peptides per normal sample, and total percentage of cancer/normal samples where each protein expressed.

Protein name	Accession	Peptides in Cancer	Peptides in Normals	Average peptides per Cancer Sample	Average Peptide per Normal Sample	Ratio	% Cancers Expressing Protein	% Normal Expressing Protein
Glutathione S-transferase P	P09211	158	13	4.7	1.1	4.4	95%	67%
Transgelin-2	P37802	137	10	4.2	0.8	5.0	88%	42%
6-Phosphogluconate dehydrogenase	P52209	124	08	3.8	0.5	7.5	81%	42%
Vinculin	P18208	250	13	7.6	1.1	7.0	81%	50%
ELAV-like protein 1(Hu-antigenR)	Q15717	47	04	1.4	0.3	4.3	78%	33%
Transmembrane proteinTmp21	P49755	38	01	1.1	0.1	13.1	71%	8%
Galectin-3-binding protein	Q08380	72	03	2.2	0.3	8.7	71%	25%
MARCKS-like protein1	P49008	35	00	1.1	0.0	-	67%	0
Tenascin	P24821	95	00	2.9	0.0	-	67%	0
14-3-3 protein eta (protein AS1)	Q04917	35	02	1.1	0.2	6.4	67%	17%
PA2B-alpha	Q08323	54	04	1.6	0.3	4.9	67%	25%
Glucosidase -2-subunit beta	P14314	83	05	2.5	0.4	6.0	67%	42%

Methods

Liquid Tissue® lysates were prepared from microdissected epithelial cells obtained from both early stage endometrial cancer and normal endometrium. Global mass spectrometry profiling of all lysates followed by spectral count quantitation indicate differential expression of proteins that include known cancer biomarkers; low-abundance proteins such as transcription factors and signal pathway proteins; and housekeeping proteins. These differentially expressed proteins also include candidate protein biomarkers of early stage endometrial cancer which can be utilized as improved diagnostic, prognostic, and therapeutic targets (Figure 1). The proteomics platform utilized is diagrammed in (Figure 3). Histologically-defined cases of early stage endometrial cancer and normal endometrial epithelium were profiled for protein expression (David *et al.,).*

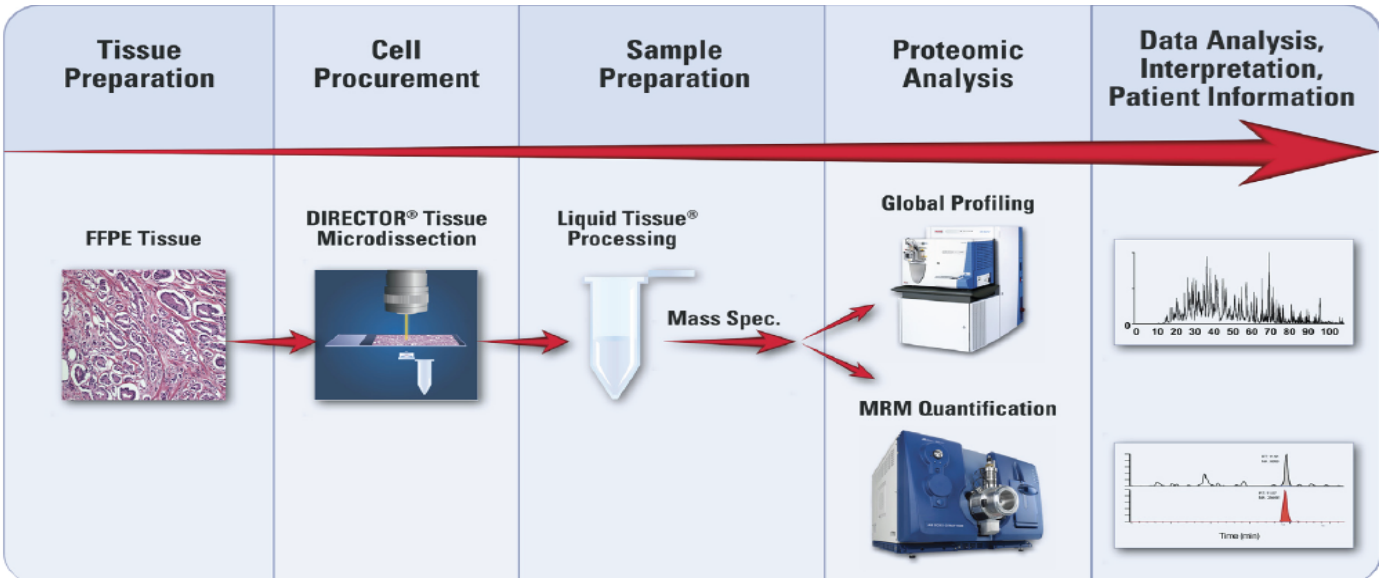

Fig. 3: Tissue Microproteomics Technology Platform.

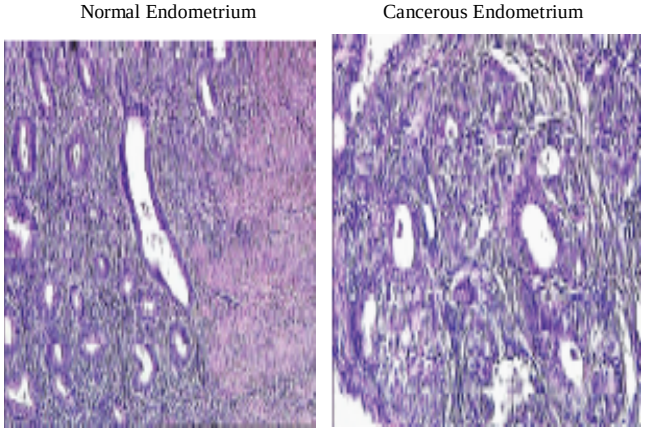

Fig. 2: Histology of normal endometrial epithelium and early stage endometrial cancer.

SOLUTIONS OF BIOMARKERS

Personalized medicine is the wave of the future, and ApoCell is at the forefront of discovering and analyzing biomarkers that will make this new treatment revolution possible. ApoCell possesses the technologies which allow biomarker analysis to be performed on tissues, blood, other biological fluids, and on enriched rare cells.

Integrated Platforms

The key to ApoCell's leadership in biomarker research is our ability to use cutting edge techniques for the quantitative analysis of rare circulating cells. ApoCell's technologies allow for the study of cells at the level of single rare cells (CTCs, CEC, stem cells) to provide with qualitative and quantitative morphologic observations, gene expression (protein RNA) and mutations data. Platform technologies are integrated to characterize rare cells in a progressive, step-wise manner for protein expression, apoptosis, molecular profiling, and finally genetic mutations.

Rare cell enrichment

ApoCell utilizes state-of-the-art technologies for analysis of rare cell populations. Enriching for a specific rare population from whole blood for downstream molecular or protein analysis yields valuable information on pharmacodynamics and subset phenotype, and even may allow for patient stratification.

Circulating tumor cells

ApoCell is an authorized service provider for the Veridex CellSearch® CTC IVD test. The sensitivity, specificity and reproducibility of CellSearch® allows for rapid CTC observation of changes of counts as early as the first cycle of treatment to help evaluate patient progression earlier. Apocell has taken enumeration of CTCs a step further by significantly improving recovery rate, thus allowing for downstream biomarker and molecular analysis of CTCs. ApoCell incorporates its enhanced CTC recovery process in ongoing clinical studies assessing biomarkers in CTCs to determine parameters such as stratification of patients, drug efficacy, and clinical correlation to tumor phenotype.

Circulating endothelial cells

Circulating endothelial cells (CECs) are increasingly studied as surrogate biomarkers in many cases of cancers undergoing anti-angiogenic therapy. Isolation of the CEC population and stratification of the maturity level is achieved by immunomagnetic enrichment based on a broadly-expressed surface marker, such as CD31 or CD146, coupled to more specific analysis of CD105, the vascular endothelial growth factor receptors (VEGFRs), or others. Induction of apoptosis within the CEC population has also shown to be a very valuable measurement, whereby cell death caused by anti-angiogenesis treatment can be gauged. ApoCell performs phenotyping analysis of the enriched population to meet the needs of the client and provides consultation to define the options best suited for the disease state or targeted therapy.

Stem Cells

Stem cell research is a rapidly expanding area of investigation. It is well know that hematopoietic stem cells (HSCs) can be defined, isolated, and utilized for gaining insight the mechanisms of stem cell growth and differentiation, as well as stem cell use for tissue engineering applications.The hematopoietic stem cells can be enriched from peripheral blood by immunomagnetic enrichment based on CD34 antigen, further stained for other markers, and followed by Multiplex Flow Cytometry and/or LSC quantification. ApoCell offers a variety of different antigen combination analysis that includes combinations of CD34 with other markers such as CD45 and CD15.

FUNTIONAL BIOMARKERS

Cell death related markers

- Ideal for novel drug candidates with mechanism of action to kill tumor cells directly or indirectly by interfering DNA-repair process.
- Signals of cell death detected on or within CTCs could provide proof of concept of novel therapeutics in patients in Phase I or Phase II clinical studies.
- Potential uses in clinic: proof-of-concept, dosing selection.
- Potential markers could be examined in CTCs:1)Apoptosis: Caspases 2, 3, 7, 8 and 9, neo-epitopoe of CK18 (M30), annexin –V, Tunel 2)DNA-repair: p53, phosphorylated p-53, p21, pRb, 3)Nuclear DNA damage marker: γ-H2AX

Phospho-Protein markers

- Ideal for novel drug candidates with mechanism of action in blocking growth factor/receptor interaction or directly blockade of downstream signaling events.
- Modulation of intracellular phosphor-protein expression in CTCs or CECs isolated from treated patients could signal drug activity in Phase I or Phase II studies.
- Potential uses in clinic: proof-of-concept study, dosing selection study, markers to assist patient stratification strategies in advance clinical study.
- Potential markers can be examined in CTCs and in CECs including, but not limited to:

pERK/ ERK,
PAKT/AKT,
PFGFR1/FGFR1,
cMET,
PVEGFR/VEGFR,
TIE2

Pathway-resistance markers

- Ideal for novel drug candidates that target a specific pathway-resistance patient population.
- Potential uses in clinic: patient selection/stratification.
- Potential markers can be examined in CTCs:

BRAF mutation
EGFR mutation and amplification
KRAS mutation
PI3KCA mutation
PTEN loss
TMPRESS-ERG fusion

Angiogenesis markers

- Ideal for novel drug candidates with mechanism of action to interfere/block angiogensis.
- Potential uses in clinic: proof-of-concept study, dosing selection study.
- Potential markers can be examined:
- CEC/CEP count
  pVEGFR/ VEGFR

Genomic/Genetic Analysis

- ApoCell's CTC molecular profiling services detect the genetic and molecular changes unique to each patient. Our scientists simultaneously characterize Circulating Tumor Cells (CTCs) or Circulating Endothelial Cells (CECs) for single nucleotide polymorphisms (SNPs), gene translocations, rearrangements, copy number alterations, and changes in mRNA or microRNA expression.

Gene Expression Profiling

- RNA isolation from Circulating Tumor Cells (CTCs), Circulating Endothelial Cells (CECs), or Peripheral Blood Mononuclear Cells (PBMCs).
- RNA quality control using Agilent BioAnalyzer®.
- Single cell RNA isolation, end-point RT-PCR, quantitative RT-PCR.
- Gene expression assays using TaqMan® quantitative RT-PCR.
- Gene expression assays using SYBR-Green quantitative RT-PCR.
- Gene expression profiling using TaqMan® Custom and Gene Signature Array Microfluidic Cards and Plates.
- Illumine Human HT-12 Expression BeadChip Arrays.

CONCLUSION

Biomarkers are the new technique for diagnosis, detection, treatment, identification and discovery of drug. It is a very specific and rapid method for diagnosis and detection of carcinoma. The identification of reliable biomarkers to track cancer, which should provide a better classification of tumors, allow for personalized therapy and predict pharmacological side effects or resistance, remains an exciting challenge for the scientific and medical community. Analysis of genes and proteins expressed by tumors, using novel concepts and methods, should accelerate our quest to attain this goal and bring to light a better and more comprehensive view of the molecular heterogeneity of cancers. The recent models integrating genomic, proteomic and clinical data appear to be the most relevant in providing information for future personalized prediction of disease outcomes.

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