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Biomarker Discovery in Nasopharyngeal Carcinoma Using Proteinchip Profiling

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ABSTRACT

After the accomplishment of the Human Genome Project, there has been intense interest in applying differential proteomic analysis to discover biomarkers for cancer diagnosis, treatment, and prognosis. Nasopharyngeal carcinoma is one of the most common cancers in Hong Kong and certain regions of China, yet not many oncoproteomic studies have been conducted for this malignancy. Previous studies have used surface-enhanced laser desorption/ionisation time-of-flight mass spectrometry to identify serum amyloid A, inter-α-trypsin inhibitor heavy chain H4 precursor, and platelet factor 4 precursor as potential cancer biomarkers for nasopharyngeal carcinoma. The biomarkers discovered in these mass spectrometry-based proteomic studies have expanded the current understanding of the molecular pathogenesis of nasopharyngeal carcinoma, which may provide the basis for the identification of potential targets for novel therapeutic agents. The results of these studies are reviewed in this report. The advantages and disadvantages of proteinchip profiling technology are also evaluated. This paper concludes by expounding the current challenges and future perspectives of using proteinchip profiling for the discovery of cancer biomarkers.

Key Words: *Biological markers; Mass spectrometry; Nasopharyngeal neoplasms; Protein array analysis; Proteomics*

INTRODUCTION

Cancer is a result of complex changes that occur in normal cells as they transform to become malignant. These changes are not a consequence of a single protein, but involve multiple proteins that function in pathways and networks. Understanding the molecular basis of the pathways regulating the cancer cell promises to revolutionise clinical practice. Molecular medicine is moving beyond genomics to proteomics. Proteomics is the study of various types of proteins and their interactions in a cell. Serum proteomic analysis provides information about the proteome's dynamic and rapid changes, which result from exogenous exposure and/or endogenous factors. Applying high-throughput proteomic analysis of sera can facilitate the identification of a large number of proteins expressed in cell samples. Oncoproteomic studies have generated numerous datasets of potential diagnostic, prognostic, and therapeutic significance for human cancers. Utilisation of this information for the diagnosis and selection of suitable drugs or targets will be useful.[1]

Nasopharyngeal carcinoma (NPC) is common in Hong Kong and certain regions of China. The cause of increased risk for NPC in these endemic regions is not entirely clear. As few proteomic studies have been conducted in NPC, the authors embarked on studies applying the proteomic approach to identify cancer biomarkers for NPC.

PROTEOMIC TECHNOLOGIES

Traditional methods for quantitation of proteins have relied on techniques such as 1-dimensional and 2-dimensional gel electrophoresis, immunoblotting, enzyme-linked immunosorbent assay (ELISA), and radioimmunoassay. Most of these established methods for protein characterisation are not amenable to high-throughput application.[2] Advances in mass spectrometry (MS)-based proteomics have shifted the paradigm of translational cancer research. Proteomic profiling with proteinchip technology has been used successfully to

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discover biomarkers with potential clinical usefulness in various cancers.[3,4]

Proteinchip Profiling

Surface-enhanced laser desorption/ionisation-time-of-flight (SELDI-TOF)-MS is a high-throughput approach to biomarker discovery that combines 2 powerful technologies, retentate chromatography and MS. The core of the SELDI-TOF-MS platform is the proteinchip arrays, which have varying chromatographic properties such as anion exchange, cation exchange, metal affinity, and reverse phase. SELDI-TOF-MS is capable of detecting proteins in the range of 2 to 200 kDa and those that are relatively acidic or basic. Various complicated biological materials can be uniformly captured, concentrated, and purified on the small chemical surface of the chip. A complex mixture of proteins from blood or body fluids can be reduced to sets of proteins with common properties by binding the sample to chips with differing surface chemistries in parallel and in series. After washing to remove unbound proteins, salts, and contaminants, the bound proteins are ionised by laser and read by TOF-MS. The resulting spectra provide a multidimensional binding profile on the basis of different types of interactions.[5]

Bioinformatics and Statistical Analysis

For SELDI-TOF-MS, the data come in the form of arrays of intensity values and their corresponding mass-to-charge ratio. The processing and analysis of these data arrays can be broadly divided into 3 stages. In stage 1, the data arrays are processed individually, including noise filtering, baseline subtraction, and peak detection. In stage 2, the data arrays are analysed collectively and the analysis consists of peak clustering and normalisation. Stage 3 operates on peak clusters and consists of statistical univariate and multivariate analyses of the clusters. Univariate analyses uncover single potential biomarkers that can classify the sample groups. In some cases, univariate analyses alone are sufficient, but biological systems are usually complex and cancer can be caused by multiple factors. If univariate analyses have identified multiple individual biomarkers that can be linked to a cancer pathway, multivariate analyses are needed for proper classification.

APPLICATION

Biomarkers for cancer may be used for risk stratification, early detection, treatment selection, prognostication, and monitoring for recurrence. Major discovery efforts brought about by advances in proteomic technologies have resulted in the identification of new targets for drug therapy. With these potentials in mind, the authors have explored the value of proteinchip profiling for NPC to elucidate potential novel therapeutic strategies. Recently, the authors have identified serum biomarkers to help with diagnosis, relapse monitoring, and prediction of treatment response for patients with NPC. These biomarkers include serum amyloid A (SAA), inter-α-trypsin inhibitor heavy chain H4 precursor (ITIH4), and platelet factor 4 precursor (PF-4). To the authors' knowledge, this represents one of the first attempts to use serum proteinchip profiling for patients with NPC. In the following sections, the discovery of cancer biomarkers for NPC using proteinchip profiling will be described.

Targeting Relapse for Patients with Nasopharyngeal Carcinoma

To determine whether proteomic profiles differ between patients with NPC at relapse and those in remission, a pilot study was performed using 636 serum samples longitudinally collected from 42 patients with NPC (31 at relapse and 11 in remission).[6] A high-throughput prefractionation strategy was used to generate anion-exchange fractions, and to examine their protein profiles on proteinchip arrays. Comparisons of NPC at relapse and in remission have not been evaluated previously using SELDI-TOF-MS. In this initial screening, a biomarker was found to be differentially expressed according to the recurrence status. This polypeptide was subsequently identified to be SAA by tryptic digestion and MS/MS fragmentation. ELISA was then used to validate the finding of the initial screening. Monitoring the patients longitudinally for SAA level, both by proteinchip and immunoassay, showed a dramatic increase in SAA correlating with relapse, and a dramatic fall in SAA correlating with response to salvage chemotherapy (CT). Acute-phase SAA is a well-known marker of inflammation; molecular interactions of SAA are possibly involved in carcinogenesis.[7] The discovery of serum biomarker SAA has revealed its potential for detecting NPC relapse. The study by Cho et al is the first proteomic study using SELDI-TOF-MS to identify SAA in the serum of patients with cancer.[6] The various biomarkers discovered are listed in Table 1.

Following the novel discovery of SAA's role in NPC, other researchers have recently demonstrated the detection of SAA using proteomic analysis for the diagnosis of various cancers. Results show that SAA might be a candidate biomarker for detecting and monitoring the

Table 1. Biomarkers for nasopharyngeal carcinoma discovered by proteinchip profiling.

Biomarker	Primary use	Sensitivity	Specificity	Remarks	Study
Serum amyloid A	Relapse monitoring	71%	100%	Relapse vs remission	Cho et al[6]
ITIH4	Diagnosis	100%	94%	Cancer vs control	Cho et al[14]
Platelet factor 4 precursor	Treatment monitoring	NA	NA	84% down-regulated after treatment	Cho et al[14]
MW 2950 Da and 6701 Da	Chemoresponse prediction	80%	87%	Non-response vs response	Cho et al[14]
MW 13510 Da	Chemoresponse prediction	60%	100%	Non-response vs response	Cho et al[14]
MW 14855 Da	Chemoresponse prediction	60%	96%	Non-response vs response	Cho et al[14]

Abbreviations: ITIH4 = inter-α-trypsin inhibitor heavy chain H4 precursor; MW = molecular weight; NA = not applicable.

progressive growth of pancreatic cancer,[8] and SAA has been shown to be strongly correlated with prognosis in patients with neuroblastoma.[9] Immunohistochemical staining also revealed that SAA protein expression was co-localised with its mRNA expression in colon carcinoma.[10] SAA has been demonstrated to be not only useful for predicting survival of patients with gastric cancer, but also a valuable tool for postoperative follow-up of disease progression.[11] The SAA peak cluster has also been validated as a robust renal cell carcinoma biomarker candidate.[12] Profiling analysis further demonstrated that SAA could be a useful biomarker for monitoring the progression of lung cancer.[13]

High-level Expression of Inter-α-trypsin Inhibitor Heavy Chain H4 Precursor in Nasopharyngeal Carcinoma

A subsequent study to identify potential biomarkers for the diagnosis and treatment response of NPC was conducted using proteinchip profiling in 209 serum samples from 66 relapsed patients before and after salvage CT, 11 patients in remission, and 35 healthy individuals.[14] In the comparison of patients with NPC with the healthy controls, a biomarker was found to be significantly increased at all stages of disease. This polypeptide was subsequently identified to be ITIH4 by tandem MS and proteinchip immunoassay. The ITIH4 serum concentration was found to be increased during the acute-phase processes and was up-regulated by interleukin-6 in hepatocarcinoma HepG2 cells.[15] The longitudinal profile of ITIH4 in patients with NPC varied with clinical disease activity, but its level was almost always detectable. This result was different from that of plasma Epstein-Barr virus DNA, which invariably drops to an undetectable level during remission after treatment.[16] The discovery of ITIH4 may serve as a supplementary biomarker for the diagnosis of NPC.[14] The marker details are described in Table 1.

Treatment-associated Biomarkers

In the analysis of paired pre- and post-CT serum samples of the aforementioned study,[14] another biomarker was found to be significantly decreased in the post-CT serum samples. Using the same identification approach as for ITIH4, this polypeptide was identified to be PF-4.[14] PF-4 is an ELR(-) CXC-chemokine present in platelet α-granules, which is released during platelet aggregation and inhibits heparin-mediated reactions. PF-4 is also released from activated T lymphocytes and mast cells.[17] PF-4 has been shown to influence numerous other biological properties including inhibiting endothelial cell proliferation, migration, and angiogenesis.[18-20] PF-4 inhibits T-cell function by down-modulating cell proliferation and cytokine release, and supports the survival of normal haematopoietic precursors and protects them from the toxicity of chemotherapeutic agents.[21,22] PF-4 is predicted to play a role in wound repair and inflammation, which is also highly expressed in inflammatory disease and early tumour growth.[23,24] In agreement with these findings, PF-4 concentration was found to be increased in other cancers during active disease or relapse, but returned to normal levels during remission after CT.[25] This suggests a potential role of PF-4 as a surrogate marker for treatment assessment during CT, which might serve to triage patients with NPC for appropriate treatment. These preliminary findings warrant confirmation in a larger cohort of patients in further studies.

Biomarkers for Chemoresponse Prediction

Chemoresponse is an important clinical parameter for patients with cancer receiving salvage CT for relapse. To develop a proteomic profile that will enable clinicians to more accurately predict the chemoresponse of patients with NPC, pre-CT sera from those who have complete or partial response and those who have no response or progressive disease were profiled by SELDI-TOF-MS. Four potential biomarkers (at 2950 Da, 6701 Da, 13510 Da, and 14855 Da) were found to be correlated with chemoresponse.[14] The protein peaks at 2950 Da and 6701 Da were combined in a classification tree analysis to serve as a multiplexed biomarker panel with a classification accuracy of 83%. Subsequent identification of these potential biomarkers may confirm

the important role of proteinchip profiling in identifying predictors of CT response in NPC, and may eventually provide clinicians with insights into the mechanisms of drug resistance. The various biomarkers are listed in Table 1.

DISCUSSION

The value of biomarker research in the field of diagnostics and predictive medicine has long been acknowledged. Diagnostic tests have evolved from single-molecule monitoring to multiplexed assays for entire panels of biomarkers, resulting in comprehensive data that clinicians can use to efficiently and effectively treat patients. Differential proteomic analysis has been extensively applied to study cancer using proteinchip profiling of samples derived from humans and animals. There is intense interest in applying proteomics to foster a better understanding of oncogenesis and to discover new biomarkers for diagnosis, because proteins are ultimately responsible for the malignant phenotype. The aforementioned studies have successfully used SELDI-TOF-MS analysis on the sera of patients with NPC to elucidate a picture of the NPC proteome.

Advantages and Disadvantages of Proteinchip Technology

SELDI-TOF-MS offers many advantages, such as low sample volume requirement, high speed of discovery, quantitative results, ease of use, automated sample handling, high resolution, and high-throughput. The capability of proteinchip profiling to simultaneously and comprehensively examine changes in large numbers of proteins in the context of disease or other changes in physiological conditions holds great promise for unlocking diagnostic and therapeutic solutions for difficult clinical problems. Proteinchip technology is one of the most potentially valuable molecular diagnostic tools for clinical care, and may be used for monitoring cancer progression and evaluating the therapeutic and adverse effects of drugs.[26-28] One of the key features of SELDI-TOF-MS is its ability to provide a rapid protein expression profile from a variety of biological and clinical samples, such as blood, urine, cerebrospinal fluid, synovial fluid, saliva, gastric juice, bronchial eluate, cell lysis solution, and various other secretions. Recently, a new application of direct tissue proteomic analysis has been developed to expand the use of clinical proteomics as a complement to anatomopathological diagnosis.[29]

Nevertheless, even with all its potential, studies of proteinchip technology must be carefully designed to differentiate true differences in protein expression from differences originating from variation in sample collection and experimental conditions, and normal biological variability. Perhaps the biggest challenge facing the widespread use of proteinchip technology in the clinical laboratory setting is the standardisation of each step involved in array production and bioinformatic analysis, and ensuring that the samples are processed and handled rapidly and optimally. The reproducibility of MS proteomic profiling has become an intensely controversial topic that needs to be addressed before MS proteomic profiling can become a tool for routine clinical use. To solve these problems, some centres adopt multicentre and/or multistage studies to ensure and validate the consistency of research results, at great cost.[30] Another challenge facing SELDI-TOF-MS is protein identification. However, in recent years, SELDI has interfaced with matrix-assisted laser desorption/ionisation-TOF-MS. Equipment such as Q-STAR can optimise protein/polypeptide identification, contributing great benefits to the future development of proteinchip technology.

Challenges of Cancer Biomarker Discovery by the Proteomic Approach

There are several obstacles to identifying cancer biomarkers by the proteomic approach. Firstly, protein concentrations are dynamic, sometimes changing markedly with stress, disease, or treatment. Examination of the proteome of a cell or organism is like taking a snapshot of its activity at a single point in time. This may underestimate or miss the significance of processes taking place over time. Secondly, proteins can be modified by cleavage or addition of new functional groups such as phosphorylation or glycosylation, changes that may affect detection. Thirdly, the functional validation of proteins and signalling pathways using clinical samples remains a critical step in MS-based proteomic studies. Fourthly, the study of low-abundant proteins in highly complex biological samples is also a major obstacle in biomarker discovery. Blood possibly constitutes the most complex of all biological samples with a concentration dynamic range from low pg/mL to high mg/mL. Protein concentrations vary enormously, perhaps up to 10 orders of magnitude in serum, making the characterisation of the proteins within the lower 1% of protein abundance an analytically challenging task using current technologies. Unfortunately, 99% of this protein concentration is made up of just a few high-abundant proteins, such as albumin and immunoglobulin. Therefore, most observed proteins are typically of high abundance. Many potentially valuable biomarkers are expressed at

low levels and are difficult to detect. Finding new and better methods for detecting and identifying these low-abundant proteins represents a new challenge for routine diagnostics. In view of this, the newly developed equaliser beads technology has been adapted to study the deep proteome of the sera from patients with cancer in a rapid and high-throughput fashion. The increased capacity allows the enrichment of low-abundant proteins from larger sample volumes; proteins that would previously have been swamped by high-abundant proteins become visible in the spectra.[31-34] Finally, the integration of proteomics with genomic and metabolomic data, as well as their functional interpretation in conjunction with clinical results and epidemiology, constitute another major challenge. The road ahead for overcoming these challenges will be fraught with difficulties, but there is reason to be optimistic that the proteomic approach will be of great benefit for patients with cancer.

Perspectives of Cancer Biomarker Discovery by the Proteomic Approach

Monitoring the protein expression pattern in blood or tumour cells by proteomic technologies offers opportunities to discover potential new biomarkers for the early detection and diagnosis of cancer. Different proteomic tools have been used for differential analysis of various biological samples to better understand the molecular basis of cancer pathogenesis and the characterisation of cancer-associated proteins.[35] The combinations of proteinchip technology, retentate affinity chromatography, and statistical algorithms for pattern recognition have engendered enormous interest in proteomic profiling as a comprehensive set of tools for early detection of cancer and development of drugs in the discipline of molecular oncology (Figure 1).

The field of proteomics is expanding rapidly to provide greater volume and quality of protein information for a better understanding of the multifaceted nature of biological systems. Recent advances in high-throughput screening have led to the development of biochip-based assays for detecting changes in protein expression. The antibody-based protein microarray is a new technology that assesses polypeptide differences directly by binding fluorescently labelled protein mixtures from cell extracts onto glass slides spotted with different monoclonal antibodies specific for various human proteins. These novel techniques provide opportunities for cancer diagnostics, biomarker discovery, patient stratification, recurrence prediction, and drug target discovery.[36,37] Monitoring the proteome in a real-time/time-lapse manner will greatly enhance knowledge of how proteins behave. It is not hard to forecast that future proteomic technology will focus on real-time proteomics.

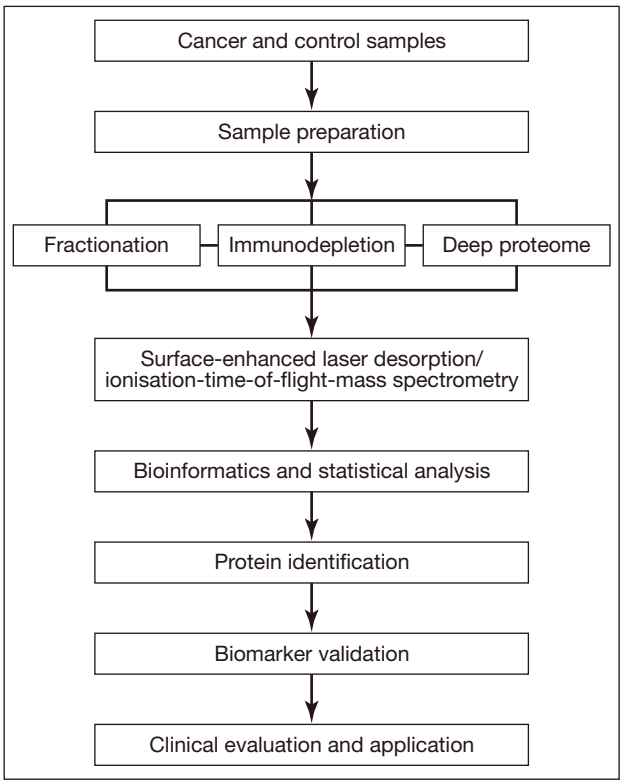

Figure 1. Integrative framework for the application of proteinchip profiling in the discovery of cancer biomarker.

Many oncologists believe that targeted therapies are the oncotherapy of the future. It is envisioned that the next generation of anticancer treatments might be tailored according to the molecular alterations identified in tumour cells of individual patients. As solid tumour continues to be viewed as a chronic condition and systemic disease, methods for long-term treatment, with fewer adverse effects, continue to be investigated. On the basis of proteomic portraits of the cancer, an individualised selection of therapeutic combinations that best target the protein network for a specific patient can be selected, resulting in a paradigm shift in the treatment and management for patients with cancer. Protein expression profiling is increasingly being used to discover, validate, and characterise biomarkers that can potentially facilitate the combination of therapeutics with diagnostics and will thus play an important role in the development of personalised medicine. This has led to the emergence of a novel discipline, pharmacoproteomics, which investigates the relationship between protein expression in tumour samples and the response to anticancer agents. This will be instrumental

in developing optimal chemotherapeutic regimens for patients with cancer.

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