J. Microbiol. Biotechnol. (2001), 11(6), 907–914

JOURNAL OF
MICROBIOLOGY
AND
BIOTECHNOLOGY
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Proteomics and Microarrays in Cancer Research

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Received: June 12, 2001
Accepted: October 3, 2001

Abstract A whole genome analysis for monitoring specific changes in gene expression, using microarrays or proteome profiling of the same, are the two tools that have already revolutionized current approaches for studying disease. These methods are particularly important in cancer research as there are many overexpressed genes, and their products remain uncharacterized. This article presents a general overview of these technologies and their applications for studying cancer.

Key words: Cancer, proteomics, LCM, protein biochip, cDNA microarray

For the last few years, we have seen a phenomenal growth in analytical methods for protein characterization. This emerging field, referred to as Proteomics, is the study of protein expression and function on a large scale. Proteomics is potentially helpful to find answers to many, if not all, the questions posed in the post-genomic era. The term proteome, first coined in the year 1995 [78], describes the analysis of a set of proteins on the global level. The branching out of proteomics as a separate field stemmed from the success of genome projects. The sequence data made available by these projects provided a much-needed platform for focusing efforts towards understanding the regulation of gene expression. The analysis of protein expression profiles is important since the levels of mRNA do not always represent the amount of functional protein. Also, gene sequences do not provide any information on post-translational modifications, which are essential for the protein function and activity, plus the study of the genome alone is insufficient to understand the dynamics of cellular processes [38]. As the list of available complete genome sequences continues to grow at a very rapid pace, the task for those working in the area of proteomics becomes more challenging, despite the support of bioinformatics.

Proteomics is basically a combination of many sophisticated technologies for separating, identifying, and characterizing a number of proteins at one time. These technologies include two-dimensional (2-DE) electrophoresis, mass spectroscopy (MS), genome-wide yeast hybrid systems, protein chip array technologies, laser capture micro-dissection (LCM), or any other tool that can be used to study a set of proteins at one time [26].

Cancer is one of the most common causes of death. The main feature of cancer cells when compared to normal ones is DNA damage, with several mutations leading to altered or impaired cellular responses. The protein expression pattern of cancerous cells varies significantly when compared to normal ones. Hence, the use of proteomics and microarrays to study various types of cancer is increasing. This article attempts to give a brief review of the applications of these tools in cancer research along with the recent progress that has been made as a consequence of these tools.

Proteomics Tools

Two-dimensional electrophoresis. Two-dimensional electrophoresis remains the most powerful tool for separating and characterizing thousands of proteins at one time. Although 2-DE was originally performed as far back as 1956 [70], its true potential was first realized when O'Farrell [55] and Klose [41] demonstrated that the combination of isoelectric focusing and the molecular weights of proteins could be effectively used to separate proteins. Since then, this technique has formed the basis for further and rapid improvements and has been continuously refined over the past few years.

Isoelectric focusing is achieved in the first dimension, while the separation based on the molecular weight by SDS-PAGE is achieved in the second dimension. The visualization of proteins after 2-DE is usually done by staining with Coomassie Brilliant blue or silver staining for higher sensitivity [38]. Despite its widespread application,

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the systematic use of 2-DE has not been possible until recently due to the lack of sensitive methods for the characterization of the proteins resolved by 2-DE. However, the idea of combining MS with 2-DE has since made it a powerful tool. The resolved proteins are first digested, usually by trypsin, and then the masses of the peptides are measured and compared to the predicted masses of peptides from gene sequence databases for proteins [3]. Many such databases have been made available during the last few years [21, 20, 36, 46, 81, 83]. More details, including a list of Internet sites for MS-based protein identification tools, are available [61].

Alternate methods. In addition to 2-DE, alternate separation techniques have also been developed. Proteins from *E.coli* lysate were successfully isolated using 2D-HPLC [55]. In this method, the proteins are first separated by SDS-PAGE, then in the second dimension, a reverse phase HPLC is used that produces 2D chromatograms showing the resolved proteins on an X-Y axis.

In another method, a combination of Liquid Chromatography (LC)-MS/MS is employed for the direct analysis of un-separated complex peptide mixtures generated by the digestion of the sample. All the eluting peptides can thus be automatically selected and analyzed with MS and then compared to a database [33, 63]. High performance liquid chromatography coupled with an IEF system has also been developed, wherein the proteins are first separated by HPLC and then transferred to an integrated IEF system for further analysis [72].

There have been some efforts directed towards a multi-dimensional analysis [45]. In a method called Direct Analysis of Protein Complexes (DAPC), the protein separation is based on the charge and hydrophobicity of the individual proteins. There is also a simpler single dimension HPLC method developed for the analysis of an adenovirus type 5 proteome [44].

Even though the techniques mentioned above hold a promising future, at present they all have serious disadvantages, the most important of which is the quantification of the proteins. While it is possible to determine the relative abundance of proteins in a 2-DE coupled with MS, an LC-MS/MS suffers from a complete loss of quantification [47]. Another major problem is the isolation of essentially the same protein in several places on a gel due to the separation of post-translationally and proteolytically modified proteins from their parent [4, 11]. Another drawback is the hydrophobicity and copy number of a protein [80, 10]. It has been observed that highly hydrophobic proteins are not adequately separated while a high copy number protein can mask several other proteins present at low levels.

Accordingly, to overcome these limitations and allow for the quantitative estimation of a proteome, stable isotope labeling methods have been developed [34 ,60, 53]. All these methods differ in their incorporation of heavy isotopes and their methodology. More details can be found in a recent review article [33].

Laser-Capture Micro-Dissection (LCM)

Laser-capture micro-dissection, first conceptualized by Emmert-Buck *et al.* [23] at NIH, uses a laser to overcome many of the drawbacks found in manual micro-dissection methodologies. The method is very simple and does not involve manual handling, thereby enabling precise one-step transfers [24].

Basically, LCM involves the following stages. A transparent film (usually made of ethylene vinyl acetate) is placed over a tissue section and the cells of interest are selectively adhered to the film by visualizing the tissue through a microscope and using a focused pulse from an infrared laser. The film is then used for further processing. Thus, LCM facilitates the procurement of pure cell populations from tissue with a precision of 3 to 5 μm under direct microscopic visualization [62]. This method can even target and transfer single cells.

Efforts have also been made to develop an integrated methodology where there is no need for a separate LCM transfer step. This method involves the micro-electrophoresis of the molecules of a tissue section into an overlying gel matrix, thereby extracting and separating molecules at specific sites using a "patch clamp" approach. In an attempt to further refine this method, parallel micro-channel plate electrophoresis is being investigated as a technique for the rapid mapping of the distribution of multiple molecules within tissue. NIH has already established LCM as commercial laboratory equipment with the collaboration of Arctures Engineering Inc. [6]. Currently available LCM methodologies enable dissections approaching 100% purity [6, 25, 67]. It may not be long before an integrated LCM becomes common housekeeping equipment for clinical and research laboratories.

Protein Biochips

In parallel with 2-DE technology, chip-based proteome profiling has also gained momentum in recent years [79]. The commercially available Proteinchip® Technology (Ciphergen Biosystems. Inc.), in combination with mass spectroscopy, is now widely employed. In Proteinchip® arrays, the isolation of a protein population is based on chromatographic principles, such as anion/cation exchange, reverse phase, or even metal affinity. Thus, a complex mixture of proteins from cells/tissue specimens or body fluids is reduced to a smaller subset of proteins with similar properties. After protein binding, the chips are washed to remove any unbound proteins, then the bound ones are characterized by MS. The data obtained is then referred back to databases for characterizing individual proteins.

A protein biochip provides a stronger platform for proteomic profiling due to its low sample requirements

and high-throughput format. However, at this point, the technology suffers from certain limitations; for example, the identity of the proteins at specific positions on the chip is required, the proteins must be soluble, and the identification of some proteins may be a demanding task as it may require the development of an individual detection method [79]. Recent developments in protein biochip technology have already been reviewed [30].

Application of Proteomics Tools in Cancer Studies

Currently, there is a great deal of interest in the application of proteomics, LCM, and protein biochips for studying cancer, basically to identify new tumor markers. Two-dimensional electrophoresis has already been employed to resolve labeled proteins from human normal and transformed lung fibroblasts [8], and in mice and hamsters [7]. A brief account of the proteome tool as used in cancer research is given below.

Leukemia. Expression profiling was performed by 2-DE using human B-cell chronic lymphocytic leukemia (B-CLL) from 24 patient samples [76]. It was shown that the B-CLL patient populations with short survival times exhibited changed levels of redox enzymes, HSP-27, and protein disulfide isomerase, which may have been responsible for drug resistance. In another study, 2-DE and a multivariate-coupled principal component analysis were used to quantify changes in the protein composition of a CEM T-lymphoblastic leukemia cell line after treatment with bohemine, a potent cyclin-dependent kinase inhibitor [39]. The down-regulation of proteins, such as α-enolase, triphosphate isomerase, eukaryotic initiation factor 5A, and the α and β subunits of Rho GDP-dissociation inhibitor-1, was found in the bohemine-treated cells, thereby correlating them to the probable action mechanism of bohemine. To make the identification of the up/down regulated proteins in diseased cell lines easier, Hanash and Teichroew [35] created a large database comprising about 10,000 2-DE patterns of various cell types under various conditions.

Other studies have focused on the identification of hitherto unknown markers for tumor detection, including oncoprotein 18 (op18), in a clinical study of 177 childhood acute leukamias by using 2-DE [50]. Phosphorylated op18 has been suggested to correlate with a high WBC count in the S-phase, thereby implying its possible role in proliferation. Accordingly, the authors propose the inhibition of either op18 expression or its phosphorylation to effectively control leukemia cell proliferation.

High performance liquid chromatography coupled to electro spray ionization mass spectroscopy (EI-MS) has also been employed to map the proteins of human leukemia cells [16], resulting in a much-improved coverage of protein sequences compared with other MS methods.

Liver cancer. Hepatocellular carcinoma is one of the more common forms of cancer. In a model study using rat liver, 2-DE was successfully applied to hepatocarcinogenesis induced artificially by different chemical carcinogens to characterize the tumor-associated proteins [86, 87]. One of the important proteins characterized was an isoform of aldose reductase. This enzyme, which is expressed in the liver during embryogenesis yet absent in an adult rat-liver, was found to be re-expressed and remained functionally active during liver carcinogenesis.

Two-DE was employed to determine the intracellular factors influencing the sensitivity of rat hepatoma cell lines to tumor necrosis factor-α (TNF-α) in TNF-α resistant and sensitive rat hepatoma cell lines [43]. A phosphatidylethanol-amine-binding protein, detected only in the TNF-α resistant cell lines, was implicated as responsible for the resistance of these cells to TNF-α induced cell death.

A 2-DE map of the human hepatocellular carcinoma cell line HCC-M was created after resolving the proteins, then a further characterization of 400 individual spots by silver staining, MS, and database search resulted in the identification of many proteins [68]. In addition to house keeping proteins, such as alcohol dehydrogenase, α-enolase, aspargine synthetase, isocitrate dehydrogenase, and glucose-6-phosphate-1 dehydrognase, other proteins considered related to the cancer process were also identified. These included 14-3-3 protein, annexin, prohibitin, and thioredoxin peroxidase. Recently, the same research group identified a novel protein, HCC-1, based on the analysis of the hepatocellular carcinoma (HCC)-M cell proteome. The level of this protein was found to be closely associated with the carcinogenicity of the cell line [19].

Yu *et al.* [85] identified 99 protein spots that showed significant quantitative and qualitative variations in the human hepatoma cell line BEL-7407 and normal human liver cell line L-02. Twelve protein spots were identified using the SEQUEST database. While the levels of ionosine-5'-monophosphate dehydrogenase 2, HSP-27, calreticulin, and calmodulin were elevated in the hepatomas, the levels of the tubulin β-1 chain, a natural killer cell enhancing factor B, were found to be down-regulated.

Proteases are known to facilitate cancer progression at various stages. Fifteen sets of carcinoma biopsies representing a primary tumor, adjacent normal colon, and liver metastases were selected to identify the most suitable protease for drug targeting by functional proteomic screening [48]. The major proteases detected were matrix metalloproteases (MMP1, MMP2, and MMP9), Cathepsin B, Cathepsin D, many cell serine proteases, Tryptase, and Chymase. The matrix metalloproteases were expressed at higher levels in the primary tumor than in the adjacent normal tissue. This survey of all major classes of proteases helped to identify those proteases that may be essential for tumor progression, as such, low molecular weight inhibitors against these proteases could become potential chemotherapic agents for treatment.

Urological cancers.

a. Bladder cancer

Celis and co-workers created a comprehensive database for bladder carcinomas, including profiles of both transitional and squamous cell carcinomas [10].

In a recent study, 150 fresh bladder tumors were subjected to 2-DE/MS, to obtain 6 profiles corresponding to squamous cell carcinomas (SCC) [57]. A few tumor-associated markers, including keratins, differentiation associated proteins, psoriasin, a psoriasis associated fatty acid binding protein (PA-FABP), and galectin-1, were identified. All 6 SCCs externalized psoriasin into the urine, indicating that this protein alone or in combination with other polypeptides may serve as a marker for the early detection of such lesions. Accordingly, to assay psoriasin, an ELISA method was developed [58] and its clinical use over a period of time will reveal the usefulness of psoriasin as a diagnostic marker.

The proteome profiling of fresh bladder transitional cell carcinoma revealed many proteins with altered levels of expression [1]. Of these, 5 proteins (tryptophenyl-tRNA synthetase, IFN-γ3, Mn-SOD, and 2 unknown proteins) were up-regulated significantly (75%), while aldose reductase was down-regulated. This study also provided the first results of the effect of IFN-γ in the protein expression profiles of transitional cell carcinomas (TCC).

In an exploratory work, protein-chip technology (surface enhanced laser desorption/ionization time of flight mass spectroscopy) was employed to analyze the protein profiling of urine samples of 94 patients with TCC and healthy individuals [75]. Five potential novel markers were identified, thus it was suggested that use of a combinatorial approach may lead to the development of a highly sensitive urinary diagnostic test.

b. Renal cancer

First generation 2-DE databases for human kidney proteins have been created using a combination of many techniques for protein characterization, for example, amino acid analysis, N-terminal sequencing, and immuno-detection following 2-DE [66]. From among the 2,789 separated polypeptides, 43 have been identified and a comparison between normal and tumor kidney tissues has shown that 4 polypeptides are only present in normal cells. Of the 4 polypeptides, 2 proteins have been identified as cytochrome C reductase and a NADH-ubiquinone oxidoreductase complex. Unknown deletions, or changes in gene transcription or translation have been proposed as the causes for the down-regulation of these proteins in tumor cells. The same research group also identified glutathione peroxidase and Mn-SOD as putative markers for possible application in renal tumor diagnosis [65].

c. Prostate cancer

A subset of the cell population, as procured by LCM and analyzed by 2-DE, confirms that these methods can efficiently detect alterations in protein expression patterns. As such, differentially expressed proteins, such as prostate specific antigen (PSA), can be used as new targets for therapeutic intervention, serum markers, or image markers [56].

Protein expression profiles of androgen stimulated prostate cancer cells have been generated by 2-DE [51], and a mass spectroscopic analysis has then identified the metastasis suppressor gene (NDKA.nm23), thereby explaining the marked reduction in the metastatic potential when stimulated by androgen.

Breast cancer. Systematic studies by Franzen and co-worker have yielded protein profiles of human breast cancer cells, which reveal elevated levels of several proteins, including cytokeratins, PCNA, HSP-60, HSP-90, and calreticulin [27, 28, 29]. As a result, the possible use of cytokeratin as a tumor marker has been suggested. Protein expression patterns have been studied in human breast luminal and myoepithelial cells and 185 proteins have been characterized, yet not many matches have been found in the databases, indicating the novelty of the proteins [59].

Changes in the protein synthesis pattern induced by the fibroblast growth factor (FGF-2), a potent regulator in breast cancer cell proliferation, have been investigated in the human breast cancer cell line MCF-7 [74]. The up-regulation of 4 proteins, namely, HSP-60, HSP-90, PCNA, and a transcriptionally controlled tumor protein (TCTP) was observed. It was also shown that HSP-90 would appear to be crucial for FGF-2 mediated stimulation in MCF-7 cells.

Microarrays

The advent of microarray technology has revolutionized the experimental approach in molecular biology. This technique allows unprecedented, parallel investigation of thousands of genes for several purposes like expression monitoring [13] and polymorphism analysis [77]. There have been some review articles already published that cover many aspects of microarray technology, including a general overview [22, 31, 52], automation and technique developments [49], biomedical applications [84], cancer diagnosis [2], recent developments [5], and sequence databases [32]. However, recently, there have been warnings about serious limitations to the microarrays supplied by companies, mainly due to wrong sequences obtained from public database domains, and as a result, probes have been unable to detect their target mRNAs [42].

Basic microarray technology involves a minimum of 5 different components [18, 37], viz., solid support with a special surface for binding the probe, a device for producing microarrays by spotting cDNA probes onto the chip, a system for DNA and its probe hybridization, a scanner to record the chips, and computer software for data analysis.

The methodology involves fixing the DNA onto a solid support, like a coated glass surface. This procedure of fixing the DNA is repeated in an automated fashion with a large number of genes. Each gene is fixed in a precise spatial location that allows for the subsequent identification of an individual spot. The probes are usually labeled with either radioactive or fluorescent markers for detection and quantification.

For comparison purposes, the data obtained from microarrays requires standardization. This is usually achieved based on the expression levels of actin and GADPH genes as controls. Yet one of the current limitations is the absence of universal standards for data comparison and analysis from different laboratories. As a result, frequently, it is not necessary to use Northern hybridization, RT-PCR, or immunostaining to substantiate the data [9].

Basically, cDNA microarrays have been used to study differentially expressed genes in cancer. A brief account of this is outlined below:

To understand the molecular mechanisms underlying prostate cancer, a microarray was employed to search for those genes whose expression had been altered in this disease, and the differentially expressed genes were then further evaluated by RT-PCR [17]. It was observed that one gene was overexpressed and 12 others were underexpressed in prostate cancer. Of these, five, namely, glutathione S-transferase M1 (GSTM1), monocyte chemotactic protein-1 (MCP-1), tumor necrosis factor-alpha receptor-1 (TNFR-1), transforming growth factor β3 (TGF-β3), and inhibitor of DNA binding-1 (ID-1), showed a statistically significant reduction in their mRNA levels.

To identify the set of genes involved in the development of colorectal carcinogenesis, expression profiles of colorectal cancer cells from eight tumors with corresponding noncancerous colonic epithelia were compared using a DNA microarray consisting of 9,216 human genes [40]. The differentially expressed genes included those associated with signal transduction, metabolizing enzymes, the production of reactive oxygen species, cell cycle, transcription, mitosis, and apoptosis. A closer examination of 10 genes (five up-regulated and five down-regulated) by RT-PCR substantiated the reliability of the analysis. Accordingly, the list of overexpressed genes identified in these experiments provides a large body of potentially valuable information on colorectal carcinogenesis and represents a source of novel targets for cancer therapy.

A microarray with 14,000 cDNA clusters was employed to study the expression profiles in clinical hepatocellular carcinoma samples and distal nontumorous liver tissue from the same patients. It was found that 72 genes (including 30 novel genes) were down-regulated and 84 genes (including 48 novel genes) were up-regulated in more than 50% of the cancer samples that were identified. The alterations in the gene expression levels were confirmed by a Northern blot and RT-PCR in all of 4 randomly selected genes. Most of the genes were found to be regulated by liver-enriched transcription factors and the 12 up-regulated genes were found to be associated with translation [82].

In another study using microarrays, it was observed that colorectal carcinoma tissues are characterized by the up-regulation of molecules related with angiogenesis. These results suggest that angiogenesis-related molecules are suitable candidates for target-based therapies for colorectal cancer patients [73].

Similar studies using cDNA microarrays have been carried out on bladder cancer [71], prostate cancer [12], liver cancer [69], and leukemia [15]. A cDNA microarray was also employed to study the effect of soya flavone genistein on the cell growth inhibition of bladder tumor cells [14] and a number of studies have reported that the use of microarrays for cancer studies is rapidly growing.

CONCLUSION

Although there are a still number of serious shortcomings in current technologies, as the proteomic and microarray tools continue to improve, the possibility of obtaining a more comprehensive picture on cancer at the molecular level is becoming a reality.

Acknowledgments

This work was supported by KRF-2001-015-OP0553, and SRC Fund to IRC, UOU from KOSEF and the Korean Ministry of Sciences and Technology.

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