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Perspective Article

Hylonome

Applications of high-throughput methods to cancer metastases

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Introduction

The phenotypic changes that occur in tissues as they transition from normal tissue through metastatic cancer can be, in part, represented in the gene expression of the tissue. (For the purposes of this discussion, gene expression refers to either mRNA or protein expression). Although gene expression may not represent all changes that occur in cancer cells (e.g., doesn't account for post-translational changes), it does provide a readout of differences in gene expression that occur from the normal or non-metastatic cells. Thus, identifying gene expression changes in cancer cells should help identify targets for therapeutic intervention.

In the past, the daunting task of identifying how gene expression is altered between normal tissues, primary cancers, and metastases has been addressed by a myriad of investigators evaluating one candidate gene at a time or at best several genes that have been identified through subtraction hybridization techniques or other comparative methods. While these techniques have provided critical information that has advanced the field's understanding of cancer biology, they present limited information in terms of understanding the global changes that occur in cancer.

To facilitate global evaluation within and among cancers, several high-throughput methodologies have been developed in recent years that include evaluation of a variety of aspects of cancer cells. Although these high-throughput methods provide a great deal of information, they also present challenges in terms of confirming their results and analysis of the massive amounts of information they provide. Several of these developed or developing methodologies are described below.

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Gene arrays

Gene microarrays are perhaps the most utilized and widely developed modern high-throughput methodology. Gene microarrays have been the subject of many excellent reviews[1-3], so the current discussion will only provide a brief overview. Gene arrays, initially developed as macroarrays on membranes, were quickly miniaturized onto glass slides. Currently, gene microarrays consist of thousands of cDNAs or oligonucleotides immobilized on a glass slide. Fluorescently-labeled mRNA, directly isolated from tissue or PCR amplified from target tissue, is hybridized to the immobilized DNA and differences in mRNA expression is determined on a fluorescent scanner.

Gene microarrays provide several challenges. For example, due to the thousands of genes being quantified, false positives may frequently occur. Sound statistical methodology is required to minimize these problems and conservative definitions of gene upregulation should be adhered to[4]. Once altered levels of gene expression are identified, confirmation of the expression should be performed. This is typically done by PCR analysis. Perhaps the greatest challenge is analysis of the data. Specifically, robust methodologies to compare gene expression between different samples are required.

Gene arrays have been used to characterize the transcriptome in primary and metastatic prostate cancer[5-9]. An expression profile different from benign prostatic hyperplasia and primary tumors can clearly be delineated as have individual genes associated with different stages of prostate cancer.

Protein arrays

(Based on Clontech Array System Manual)

As the initial runs of genome sequencing projects finish, efforts are now being made to elucidate the proteome, which represents the complete profile of protein expression within cells and tissues[10]. A major target for proteomics is to quanti-

fy changes in the expression levels of proteomes to fully delineate biological and pathological processes and disease states[11].

Initially accomplished using 2D-electrophoresis, identifying differences between the protein expression in tissues is a slow, laborious and difficult process[12]. Again, using a pseudo-candidate protein approach, antibody microarrays have been developed. The antibody microarray is composed of hundreds of distinct monoclonal antibodies printed at high density on a glass microscope slide. Using this detection platform an investigator can measure the abundances of hundreds of proteins with a single experiment. Antibody microarrays can be used to measure protein levels in virtually any biological sample, including cells, whole tissue, and body fluids[13]. They can also detect a wide variety of cytosolic and membrane-bound proteins, which, together, represent a broad range of biochemical pathways.

Production of antibody microarrays consists of two main steps. First, the surface of a glass microscope slide is chemically modified to present functional groups for covalent binding with antibodies. Second, antibodies are printed on the slide using a high-precision robot. Because the antibodies are immobilized on a glass slide, only small volumes of samples are required to complete the analysis and because one detects captured antigens by fluorescence, data collection is especially convenient; slides can be scanned with commercially available microarray scanners.

Technical challenges do exist. For example, because no two antibodies have exactly the same binding affinity, the lower detection limit of the Ab Microarray must be defined for each antibody. On average, one can detect as little as 20 pg/ml of a given target protein using commercially available antibody microarrays.

Protein chips

(Based on www.ciphergen.com)

The surface-enhanced laser desorption and ionisation (SELDI) ProteinChip (Ciphergen) enables protein capture, purification, analysis, and processing from complex biological mixtures. ProteinChip Arrays contain chemically (cationic, anionic, hydrophobic, hydrophilic, etc.) or biochemically (antibody, receptor, DNA, etc.) treated surfaces for specific interaction with proteins of interest. Selected washes create on-chip, high-resolution protein maps. The protein mass profile, or retentate map of the proteins bound to each of the ProteinChip Array surfaces is quantitatively detected in minutes by the ProteinChip Reader.

The protein chip reader uses time-of-flight (TOF) mass spectrometry to determine the precise molecular weight of multiple proteins from the native biological sample. Accurate mass determination of protein samples requires a few basic steps, including sample crystallization, sample ionization, flight through a vacuum tube, and detection of the ionized proteins. After washing off non-specifically bound proteins and other contaminants from the ProteinChip Array, a simple chemical Energy Absorbing Molecule (EAM) solution is applied and allowed to dry, during which time minute crystals form on the chip. These crystals contain the EAM and the protein(s) of interest. After inserting the ProteinChip Array into the ProteinChip Reader, a laser beam is focused upon the sample, which causes the proteins embedded in the EAM crystals to desorb and ionize. Released ions then experience an accelerating electrical field which causes them to ãflyä through a vacuum tube, towards the ion detector. Finally, the ionized proteins are detected and an accurate mass is determined based on the time of flight.

Unlike standard elution chromatography (where the sample is eluted from the stationary phase), SELDI retentate mapping analyzes the sample retained on the ProteinChip Array surface. Contaminants and buffers are washed away from the ProteinChip Array surface while retaining the protein of interest. Using a series of washes with increasing stringency, a purification scheme for a particular protein can be quickly developed. Retentate mapping can be combined with microchromatography spin columns to facilitate rapid purification of selected proteins from a complex sample mixture using only microliters of biological fluids.

To identify the protein, proteases such as V-8, trypsin and Lys-C can be used to produce a peptide map of a purified protein bound to the ProteinChip Array by on-chip protease digestion. The molecular weights of the resulting fragments can be compared to a peptide database for identification. However, this may be difficult to do considering the large number of proteins potentially present on the chip. Perhaps, instead of individual protein identification, the chips' value is identification of a pattern of protein expression. Although the proteins in the pattern will not be identified, the pattern may have diagnostic or prognostic significance in terms of identification of primary tumors that are prone to metastasize.

Although not on the performed on the commercially available protein chip, proteomic spectra were generated by SELDI from the serum from 50 unaffected women and 50 patients with ovarian cancer and were analysed by an iterative searching algorithm that identified a proteomic pattern that completely discriminated cancer from non-cancer[14]. The discovered pattern was then used to classify an independent set of 116 masked serum samples: 50 from women with ovarian cancer, and 66 from unaffected women or those with non-malignant disorders. The algorithm identified a cluster pattern that completely segregated cancer from non-cancer. The discriminatory pattern correctly identified all 50 ovarian cancer cases in the masked set, including all 18 stage I cases. Of the 66 cases of non-malignant disease, 63 were recognised as not cancer. This result yielded a sensitivity of 100% (95% CI 93–100), specificity of 95% (87–99), and positive predictive value of 94% (84–99). These findings justify a prospective population-based assessment of proteomic pattern technology as a screening tool for all stages of ovarian cancer in high-risk and general populations[14].

Protein: DNA arrays

(Based on Panomics TranSignal Assay)

Transcription factors (TF) interact with specific DNA-binding elements present in the promoters of certain genes to promoter transcriptional initiation. The expression or activity of TFs may be regulated in a cell-type, tissue-specific, or cell cycle-dependent manner. Regulation can also be mediated by interactions with other proteins. Understanding of how gene expression is regulated is the biochemical activity of different TFs.

The TranSignal Protein/DNA Array permits the nuclear expression of many TFs at a time, thus providing a picture of the transcriptional state of the assayed tissue. Each TranSignal Protein/DNA Array is spotted with 54 different consensus-binding sequences that correspond to a specific transcription factor.

The TranSignal procedure involves: (1) a set of biotin-labeled DNA binding oligonucleotides (TranSignal probe mix) that are preincubated with nuclear extract to allow the formation of DNA/protein complexes; (2) then the protein/DNA complexes are separated from the free probes; and (3) the probes in the complexes are then extracted and hybridized to the TranSignal Array. Detection of signals can be obtained using either X-ray film or chemiluminescent imaging system.

Tissue Microarrays

Primary and metastatic prostate cancer tissue from prostate cancer patients is a very valuable resource for research purposes; however, it is a very limited resource. A method to extend the utility of tissue samples should help advance investigators' ability to perform research. To this end, tissue microarrays (TMA) are a valuable tool for prostate cancer[15-17]. In brief, TMA are glass slides that have an array of small tissue samples. These tissues can be from one tumor, represented multiple times, or from different patients with similar or different stages of tumors. The presence of different tissues on one slide enables investigators to perform one assay on the slide with the result that the different tissues samples will be treated similarly (e.g. immunohistochemistry or in situ hybridization). This allows for accurate comparison between samples within the same slide[18].

An example of construction of TMA is described as follows: original tissue sample sources of morphologically representative regions of regular formalin-fixed paraffin-embedded tumor blocks, or from freshly frozen tumors fixed in cold ethanol and embedded in paraffin to preserve intact RNA and DNA are obtained. Core tissue biopsies (diameter, 0.6 mm; height, 3-4 mm) are taken from individual 'donor' paraffin-embedded tumor blocks and precisely arrayed into a new 'recipient' paraffin block (45 x 20 mm). As many as 1000 specimens can be arrayed in each recipient block with minimal damage to the original blocks. Sections cut from the array allow parallel detection of DNA (fluorescence in situ hybridization, FISH), RNA -nRNA ISH) or protein (immunohistochemistry, IHC) targets in the hundreds of specimens in the array. At least 200 consecutive sections 4-8 4m 1n thickness can be cut from each tumor array block. This allows consecutive analyses of a large number of molecular markers and construction of a database of correlated genotypic or phenotypic characteristics of uncultured human tumors.

The TMA facilitates cancer research by enabling rapid screening of tissue specimens, conservation of limited tissue resources, allowing for readily quantifiable histology results. An important aspect of the TMA is associating each tissue sample with its clinical history and pathologic diagnosis using a computer database.

Summary

Many high-throughput methods are being developed and validated. We are still at the beginning of an era of many of these methodologies. Use of these methods to analyze prostate cancer metastases may lead to identification of therapeutic targets or patterns of expression for diagnosis and prognosis. However, caveats include the need for confirmation of results and rigorous statistical analysis.

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