PROTEOMIC TECHNIQUES IN COLORECTAL CANCER RESEARCH

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ABSTRACT

The increasing information about proteomes makes proteomics worth partner for other disciplines, including oncology. Genomic and proteomic approaches are applied in studying carcinogenesis, as proteomics allows more reliable analysis.Tasks of priority in translational research of colorectal cancer are identification and characterization of new molecular markers, study of oncogenic pathways, and discovery of novel targeted therapies. This review focuses on new available proteomic techniques in investigation of colorectal cancer.

Keywords: Proteomics, colorectal cancer

Introduction

Colon cancer ranks still as a serious public health concern which ocures almost equally in men and women (19). Transformation of normal cells into malignant cells is a multi-step process in colorectal carcinogenesis and involves loss of function of tumor suppressor genes, as well as activation of oncogenes (17). Cancer dues to an accumulation of genetic but also epigenetic events, leading to uncontrolled growth of cells, invasion of the tumor cells, and metastasing. Researchers use tissues of the patient, body fluids, cell lines and animal models for further studying of the molecular and cellular features of cancer (21). Unfortunately, the basic research for colorectal cancer is not linked to the clinical practice, probably because of the genetic and molecular complexity of this tumor, the lack of the ideal *in vivo* model for colorectal cancer, and the difficulties found in reproducing animal results into clinical trials in patients (21). Therefore, the main goals of the molecular biology are to identify tumor markers and chemotherapy targets usefull for patient management.

Studies of DNA and RNA for analysis of colorectal cancer are of limited value (30), since one gene can encode a considerable protein population, due to alternative splicing of mRNA and proteins, and protein posttranslational modifications. Genomic and proteomic approaches are applied in studying carcinogenesis, as proteomics allows more reliable analysis. Proteomics, when combined with genomics, may reveal the molecular basis of carcinogenesis and the development of more effective anti-cancer therapies (5). In the ''post-genomic era,'' proteomics identifies tumor markers, pathways controlling growth, differentiation, and death of cells. Proteomics contributes to determin protein expression profiles within a cell, a tissue or an organism. Proteome is extremely dynamic, comprising all proteins encoded from the whole genome and reflecting the impact of its surrounding environment (21). Proteomic technologies examine simultaneously and comprehensively differences in protein expression in disease and treatment. Currently, specific protein profiles could be used for early diagnosis of patients, study of oncogenic pathways, patient-tailored therapies and prediction of drug response (15). This review focuses on new available proteomic techniques in investigation of colorectal cancer.

Proteomic techniques

Sample preparation is a very important step in the proteomics since it can affect the reproducibility. Mechanical methods (37), calcium depletion and other nonenzymatic methods (31), laser capture microdissection (LCM) (6) have been used for capturing cancer cells. The proportion of tumor cells in a tissue sample influences the results when studying tumor homogenates. Pure populations of cancer cells from frozen, paraffin-embedded, stained or unstained tissues can be obtained using LCM, based on visualizing a tissue section via light microscopy and procurement of cells by activating an infrared laser beam, adhering the tissue to a plastic cap. Protein of good quality can then be extracted using conventional methods (6).

Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) is used for comparisson of protein expression patterns from tissues in normal and disease state. The first dimension separates proteins by pH (isoelectric focusing) and the second dimension by molecular mass (SDS PAGE). This technique is used in disease proteomics to discover diagnostic and prognostic biomarkers for cancer patients. Although 2-D PAGE is laborious and does not resolve highly basic or proteins, smaller than 10 kDa, it is ideal for studying cancer biomarkers (5, 21).

A technical modification of 2-D PAGE is the two-dimensional difference gel electrophoresis (2D-DIGE). This approach directly labels lysine groups in proteins with cyanine (Cy) dyes prior to isoelectric focusing and allows quantitative comparisons between diseased/treated and control samples, resp. Cy5 and Cy3. The individual protein data are normalized against the Cy2 dye-labeled sample, Cy5:Cy2 and Cy3:Cy2. This method reduces spot pattern variability and the gel number in an experiment, and allows simple and accurate spot matching (5).

Surface-enhanced laser desorption ionization time-of-flight mass spectrometry (SELDI-TOF MS) is a commonly used non-gel based method for profiling clinical biological fluids and for identifying unique biomarkers or complex patterns with diagnostic value in various cancers. The technique combines protein separation directly with presentation to the mass spectrometer. It is a very attractive technique because of several advantages - easy of use, high throughput, and relatively reasonable cost, allthough still controversial in its reproducibility and ability to detect actual tumor specific proteins (5, 15, 21). It has been developed bioinformatic algorithms for analysis of SELDI-TOF-MS data such as single classification trees, neural nets, genetic algorithms, and random forest algorithms, which share a common goal: to construct a classifier and discover peak intensities most likely to be responsible for segregating classes of samples. They could be applied in the diagnosis of cancer and other diseases (20).

The high-resolution hybrid quadrupole TOF analyzer (qTOF) is he the first major advances which could fit with SELDI for searching for serum proteomic pattern. The diagnostic models generated from mass spectra acquired using the high-resolution q-TOF MS were statistically superior. Different combinations of bioinformatic heuristic parameters (similarity space for cluster classification, learning rate in training of the genetic algorithm) were used to generate different diagnostic models (5, 21).

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), is a technique to analyze peptides and proteins in relatively complex samples, even tissue specimens. A small quantity of specimen containing peptides or protein is dried on a target plate together with a lightabsorbing matrix molecule. Its advancements are an electronic mirror (reflectron) to reflect ions substantially increases resolution, sample vaporization followed by delayed extraction, and microchannel plate detector, acting as an electron multiplier for ions. Shorter times are requared for small molecules, and longer times for large molecules.

Capillary-scale HPLC-MS/MS (LC-MS) is a method of choice for large scale proteomic analysis. Peptides of interest are induced by collision fragmentation followed by database matching for sequence identification. LC-MS systems consists of different instruments to separate peptide mixtures based on physicochemical properties (on the basis of m/z ratios) and to register the relative abundance of ions at discrete m/z (5, 21).

Isotope-coded affinity tags (ICAT) are applied for quantitative proteome analysis. This approach is based on stable isotope affinity tagging. The sulphydryl-directed alkylating agent comprises a cysteine reactive group and it is an ICAT reagent of iodoacetate attached to biotin through a coupling arm containing heavy or light isotopes. After the enzymatic digestion all the cysteine residues tagged with biotin are selectively separated by avidin column for further separation using MS (42). The iTRAQ technique is based on the ICAT technique. The iTRAQ technique uses four isobaric reagents allowing the multiplexing of four different samples in a single LC-MS-MS experiment. One of the major advantages of this technique is the increased confidence of identification and quantitation because of its ability to label multiple peptides per protein, (21, 42).

Microarrays, an emerging high throughput class of nanotechnology, are suitable for simultaneous analysing of many different proteins in a single sample (antibody array) or particular protein in many samples (reverse phase array). The advantage of the microarrays for cancer research is based on the low sample volumes in protein profiling, which means consumption of small amounts of clinical samples and expensive antibodies. After detection by direct fluorescent labeling or by labeled secondary antibodies, the assays have good reproducibility, high sensitivity, and quantitative accuracy over large concentration ranges (5, 21, 23).

Proteomics of colorectal cancer

These advances in the translational research in oncology aims identification and characterization of molecular markers, employable in diagnosis, early detection, prognosis, and pharmacology as well as studing oncogenic pathways (2, 21, 22, 36). The disease-related markers should assess drug responsiveness and optimal anticancer drug combinations, for targeted therapy design, identify targets for novel therapies (Herceptin), select the patient population and specific therapy, optimize drug dosing and maximize patient response (14, 21). Tumor initiation and progression markers could be applied as therapeutic antibodies (11, 26) or anti-tumor vaccines (25). In the near future the standard therapies as surgery, radiation therapy and chemotherapy will continue to play an important role in the treatment of patients with colorectal cancer but under armamentaria in cancer therapy will be aided by targeted drugs (21).

An example for application of proteomic techniques is a recent study which aimed to detect tumor-specific changes in the proteome of human colorectal cancer (32). Paired samples, tumor and normal tissue from unrelated patients with newly diagnosed sporadic colorectal cancer were analysed. Tumor tissue and neighboring normal intestinal tissue from each patient were separated by 2-D PAGE, followed by silver staining and mass spectrometric analysis. The 2-DE gels were quantitatively analysed using a Delta2D software tool (DECODON GmbH, Greifswald, Germany). Different abundance of 55 spots in all pathological tissues was found. 39 out of 55 trypsin-digested samples were unambiguously identified by qTOF LC MS/MS as being 32 different regulated proteins and a half of them new potential biomarkers. Although numerous proteomic studies by several groups (3, 4, 19, 33, 38, 43) have addressed the identification of novel targets in colorectal cancer, availability of a reliable tumor marker still remains a major challenge, whose expression may reveal insight into critical events in disease progression. It is required a systematic analysis of a large number of proteins in an easy, reproducible, time-efficient and cost-effective way (21).

Genomics and proteomics in colorectal cancer research revealed new potential oncogenic mediators and checkpoints, worth further investigating. These potential new disease related markers are commonly involved in many molecular pathways and their intimate upstream/downstream regulators (21). The molecular pathways in oncology are most performed on well controlled *in vitro* systems, however recently in more reliable and physiologically relevant *in vivo* experiments. For the colorectal cancer progression and the therapeutic resistance is important to find molecules inhibitting the gene expression. Sequence-specific gene suppression is based on the use of antisense oligonucleotides, ribozymes and deoxiribozymes, specifically cleave the target RNA or inhibit translation by steric hindrance (8, 9, 16, 34). The gene-silencing molecules are recently discovered for studying gene regulation and gene expression control are 20-25 nucleotide RNA molecules - microRNAs (miRNAs) and small interfering RNA (siRNAs). siRNAs can be used as a new powerful technology for down-regulation of target mRNAs in mechanistic research or even therapeutic development in colorectal cancer (10, 12, 28, 35).

After the discovery of potential targets and the successful inhibition of their expression *in vitro* the safety, efficacy and feasibility of their inhibitors need to be evaluated in *in vivo* models reproducing the human disease (21), which supposes developing a reliable, reproducible and human colorectal cancer-mimicking animal models (1, 7). The design and development of animal models for colorectal cancer (rodents) are based on the knowledge from the human inherited syndromes (24, 29). The preffered model is mouse because of their abundant genetic/genomic information, and easy mutagenesis using transgenic and gene knockout technology (21, 41). The first mouse model with mutation in the tumor suppressor gene *APC* (adenomatous polyposis coli) resulted in synthesis of a truncated protein and development of more than one hundred intestinal adenomas and a lifespan reduction (39). In another study it has been shown that different mutations in the *APC* gene, confer distinct tumor susceptibility phenotypes which resembles the heterogeneity observed in human familial adenomatous polyposis (FAP) (18). There are other models resembling hereditary non-polyposis colorectal cancer (HNPCC) with mutations of several mismatch repair genes, for example *MSH2* deficient mice. *MSH2* deficient mice that survive more than 6 months develop gastrointestinal adenomas, carcinomas and skin tumors (13). Another developed model is use of *SMAD4* heterozygous mice with *APC* mutations in which it was shown enhanced progression and a more malignant phenotype (40). The combination of mutations in *APC* and in *KRAS* seems to be synergistic in enhancing Wnt signaling (27), part of the most important pathway, destroyed in colorectal cancer.

Conclusions

The increasing information about proteomes makes proteomics worth partner for other disciplines such as biochemistry, cell biology, molecular genetics, medicine, pharmacology, statistics, and chemistry, pushing them forward. It is needed basic researchers and clinical investigators to work together because of the epidemiological importance and economic impact of the colorectal cancer. Developing of new proteomic approaches and equipment are required for a new conception of cancer diagnosis and treatment.

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