Review Article

Essentials of Molecular Biology in Cancer Research

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Introduction

Genetic information is perpetuated in a sequence of nucleic acid and gene function expressed as a protein. This "central dogma" represents a paradigm of molecular biology, being a guiding principle for molecular biologists involved in all areas of basic biological, biomedical and genetic research.

Damage in the genome is the major mechanism underlying malignant transformation, and chromosomal aberrations are hallmarks of genomic instability and gene alteration in solid tumors.[1-2] DNA from human chromosomes has been used to demonstrate the presence of deletions, translocations, gains, or losses of whole chromosomes and a variety of other subtle defects associated to the diagnosis of cancer and the disease outcome.[3-5] The advance of chromosomal abnormalities studies has been shedding light on genetic diseases and more specifically on all forms of human malignancies.

In the same way, the measurement of gene expression by detecting absolute and relative mRNA expression levels has become a major approach to cancer molecular diagnostics.[6-9] Also, protein assessment has emerged as a complementary strategy to genomics for oncology: studies search for a marker of tumor aggression or the likelihood of recurrence,[10] the prognostic value of a specific protein.[11-12] In this sense, the field of molecular cytogenetics and genetics plays a pivotal role in cancer research today.

Fluorescent in situ hybridization (FISH)

Fluorescent *in situ* hybridization (FISH) is a cytogenetic technique used to detect specifically labeled DNA sequences of interest, including whole chromosomes. The first experiments showed that chromosome aberration could be detected in human cells by FISH using chromosome-specific DNA molecules acquired by flow cytometry.[13-15] Traditional FISH analysis has employed, at most, two colors of detection, a red-fluorescing fluorochrome and a green-fluorescing fluorochrome[16] using probes that can bind to a highly similar chromosome portion.

This methodology has been used in a variety of researches and clinical applications,[17] such as detection of chromosome gains and losses or aneusomies using complementary probes for the centromeres of human chromosomes and has proven to be a bright assay for detecting specific chromosomal translocations,[18]

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genomic aberrations[19] and gene amplification.[20] Although not sensitive enough to identify small chromosomal changes such as point of mutations or to detect specific chromosomal regions or chromosomes, this method is still widely used.

In order to overcome these restrictions, Multicolor-FISH (M-FISH)[21] and spectral karyotyping (SKY)[22] were developed. The advantage of such experiments lies in obtaining more information from a specific cell at a time, rather than carrying out separate experiments on multiple specimens prepared from the same sample. M-FISH is a fluorochrome-based system that uses five different fluorophores in combination. Optical fluorochrome-specific filters and computer software allows exhibiting the 24 human chromosomes in unique colors,[23] whereas SKY portrays chromosomes of dividing cells based on M-FISH assays[22] while conventional cytogenetics is supported only by a black-and white banding profile.

The experimental procedures are identical for both assays. Commercial probes available for all 24 human chromosomes are differentially labeled, hybridized to metaphase spreads and, if required, antibody detection. The difference is based on imaging, since M-FISH lies on fluorochrome's presence or absence in identifying the differentiation of chromosomes when visualized with specific filters, whereas SKY is supported by their spectral properties to identify the differentiation of chromosomes.[24] The analysis results are the same for both methodologies, and enlighten unknown translocations and insertions as well as the chromosomal components of marker chromosomes.

FISH has been used as a method for sub-classifying soft tissue sarcomas,[25] detection of BCR/ABL gene rearrangement to confirm the diagnosis of chronic myelogenous leukemia.[26-27] The most significant application of the FISH technique in cancer diagnosis has been detecting HER-2/neu gene amplification by selecting breast cancer patients to receive the humanized anti-HER-2/neu monoclonal antibody, transtuzumab (Herceptin ™).[20,28] M-FISH is applicable to identify numeric abnormalities, markers chromosomes, simple and complex translocations,[23] and genomic alteration.[29] SKY methodology has been applied to cell-line studies and it has redefined several previously misclassified chromosomal aberrations, delineating highly complex rearrangements and balanced translocations.30-31 Hence, knowledge of chromosome aberrations and breakpoints identified could be useful for further genetic and epigenetic studies of cancer.

Comparative Genomic Hybridization (CGH)

Comparative Genomic Hybridization (CGH) allows screening of the whole tumor genome for regional imbalances in DNA content based on two-color FISH experiment with differentially labeled test and reference genomes and it has the main advantage of not requiring tumor metaphase chromosomes.[32]

This technology has uncovered plentiful genetic defects in tumors previously undetected by conventional cytogenetics.[33-34] Clinical applications of CGH have been limited by the lack of resolution in the original procedure. More lately, this technology combined with arrays analysis has improved the accuracy, sensitivity, and utility of CGH for tumor-specific genetic alteration mapping.[18,30]

Array-based CGH (aCGH) has been replaced by spots of either cloned cDNA or bacterial or yeast artificial human chromosomes.[18,35] This methodology provides a rapid way to breakpoint localization and genomic analysis of subtle and complex rearrangements.[36] Recent studies of breast cancer cell lines and clinical samples have shown significant correlation between DNA abnormalities found by aCGH and RNA expression measured on the same specimens when they were outlined on genomic microarrays.[37]

The technique can be automated to increase throughput and expand the possibility of further discovery of specific genetic targets in cancer and also anticancer drugs.[38-39] aCGH has been applied in tumor classification i.e. gliomas[40] and its analyses have allowed highly accurate localization of specific genetic alterations that may be associated with tumor progression, response to therapy, or patient outcome.[41]

Southern Blot

Southern blot is a method routinely used in molecular biology to check the presence of a specific DNA sequence. This technique combines agarose gel electrophoresis for size separation of DNA with methods to transfer the size-separated DNA to a filter membrane for probe hybridization with the DNA sequence of interest.[42] The time-consuming, cumbersome nature of the assay and potential for the target DNA to be diluted by adjacent benign stromal, endothelial, and inflammatory cell DNA have limited the use of Southern blotting for

evaluating solid tumors.

The introduction of Southern blot technology to clinical practice in the 1980s represented one of the earliest clinical applications of the emerging field of molecular diagnostics. Southern blotting continues to be used in many laboratories for detecting clonal gene rearrangements in patients with malignant lymphoid neoplasms[43-46] and for detecting gene amplification, and it remains a major protocol test for the clinical management of children with newly diagnosed neuroblastomas.[47] In cancer studies, southern blot has been used with different organ systems and different goals, including the detection of cancer cells, diagnosis, distinction of benign and malignant disease, prediction of response to chemotherapy, risk assessment, and selection of patients for targeted therapy.[43,48-49]

Denaturing High-Performance Liquid Chromatography- DHPLC

Several technologies suitable for the experimental discovery of single nucleotides polymorphisms (SNPs) and disease causing mutations should be capable of fully automated high-throughput analysis that require no modified PCR primers, customized specific reagents arrays, detection labels, or any sample pre-treatment other than PCR. At present, various methods exist for genotyping SNPs, such as restriction endonuclease digestion, allele-specific hybridization, nick translation PCR, ligase chain reaction, allele-specific PCR, T_m-shift genotyping, and mini-sequencing. However, none provides sufficient economies of cost and labor to allow large-scale genotyping.[50]

A more recent technique referred as denaturing high-performance liquid chromatography (DHPLC) allows automated detection of single base substitutions, as well as small insertions and deletions. Under partial denaturing conditions, heteroduplexes formed upon mixing, denaturing, and re-annealing two or more chromosomes that differ in sequence are less retained than their corresponding homoduplexes on a unique DNA separation matrix. Although the method only shows the presence of a mismatch, location and chemical nature is established by sequencing.[50]

In recent years, a number of researches have emerged documenting the excellent sensibility and specificity of DHPLC in detecting mutations.[51-52] DHPLC is at least 10 times less expensive than sequencing, mainly because of savings in reagent, labor costs and also it is significantly cheaper than resequencing on DNA chips.

The numerous applications of DHPLC have accelerated the generation of genetic markers for linkage analysis and evolutionary studies as well as mutation identification within a gene implicated in human diseases. Many clinical studies have employed DHPLC to screen a variety of genes important to different diseases as *MLH1* and *MSH2* in hereditary non-polyposis colon cancer (HNPCC), *BRCA1* and *BRCA2* in breast/ovarian cancer.[50,53-54] However, direct sequencing is still considered the golden standard in mutation analysis.

Sequencing

Rapid DNA sequencing is one of the great challenges of genomic science today since much information can be obtained from DNA sequencing. Most DNA sequencing has been performed using the chain termination method developed by Frederick Sanger.[55] This technique uses sequence-specific termination of a DNA synthesis reaction using modified nucleotide substrates. In chain terminator sequencing, extension is initiated at a specific site on the template DNA by using a short oligonucleotide "primer" complementary to the template at that region. The oligonucleotide primer is extended using a DNA polymerase. Included with the primer and DNA polymerase are the four deoxynucleotide bases, along with a low concentration of a chain-terminating nucleotide (most commonly a dideoxynucleotide). Fragments are then size-separated by electrophoresis in a polyacrylamide. An alternative to the labeling of the primer is to label the terminators instead, commonly called 'dye terminator sequencing'. The major advantage of this approach is that a complete sequencing set can be performed in a single reaction, instead of the four needed with the labeled-primer approach. This is accomplished by labeling each of the dideoxynucleotide chain-terminators with a separate fluorescent dye, which fluoresces at a different wavelength. This method is easier and quicker than the dye primer approach, but may produce more uneven data peaks, due to a template-dependent difference in the incorporation of the large dye chain-terminators. However, this problem has been significantly reduced with the introduction of new enzymes and dyes that minimize incorporation variability.[56]

DNA sequences can be used to study phylogenetic relationships among different species. Sequencing also allows detection of new or unusual species and can be used to asses variations caused by transversions, transitions,

insertions or deletions.[57] Currently, four revolutionary proposals have been presented for DNA sequencing: direct linear analysis, nanopore sequencing, linear sequencing by hybridization (LSBH), and pyrosequencing,[58] but the last one has been more used in clinical cancer research.

Pyrosequencing

Pyrosequencing is based on the detection of released pyrophosphate (PPi) during DNA synthesis. In a cascade of enzymatic reactions, visible light proportional to the number of incorporated nucleotides is generated. The cascade starts with a nucleic acid polymerization reaction in which inorganic pyrophosphate (PPi) is released as a result of nucleotide incorporation by polymerase. The released PPi is subsequently converted to ATP, which is immediately sensed by luciferase, producing a proportional amount of light. Since the added nucleotide is known, the sequence of the template can be determined. Pyrosequencing has the potential advantages of accuracy, flexibility, parallel processing, and it can be easily automated. Furthermore, it discards the need for labeled primers, labeled nucleotides and gel-electrophoresis.

Pyrosequencing methodology is used for determining difficult secondary DNA structures, mutation detection, cDNA analysis, re-sequencing of disease-associated genes, microbial typing, and single-nucleotide polymorphism analysis.[59] Several articles were published addressing different applications of this technique for SNP and mutation analyses.[60-61] These include allele frequency in pooled samples, methylation analyses, molecular haplotyping, and SNP discovery.[62] Due to recent advances, pyrosequencing is not the best technology for large-scale analysis, where hundreds of markers in hundreds of individuals have to be studied. Pyrosequencing is a good technique for medium to low throughput SNP analysis in cancer.[62]

Microsatellite Instability - MSI

Two genomic instabilities define distinct neoplastic pathways, the chromosomal instability (CIN) that is associated with the suppressor pathway for aneuploid cancer, and microsatellite instability (MSI) that underlies the mutator pathway for (pseudo) diploid cancer. MSI phenotype is found in about 15% of sporadic colorectal cancer cases and it is associated to small insertions and deletions mainly in repetitive sequences (microsatellites, MS).[63] MSI accounts for the mutational activation and inactivation of cancer-related genes, those with positive and negative roles in cell growth or survival, respectively, driving carcinogenesis. Cancer in the MSI pathway accumulates hundreds of thousands of somatic mutations in simple repeated sequences or MS. The discovery of MSI, by the detection of these ubiquitous somatic mutations, provides conclusive evidence for the hypothesis of cancer as a mutator phenotype.[63]

Diagnostic characterization of MSI status has implications in clinical settings and oncology, because MSI is a useful screening marker for identifying better prognostic markers and efficacy of chemotherapy in Lynch syndrome patients.[63] The standard testing procedure recommended by the National Cancer Institute/ International Collaborative Group/HNPCC (NCI/ ICG-HNPCC) is the analysis of tumor and normal tissues using five MSs markers including two mononucleotide repeats (BAT25 and BAT26) and three dinucleotides repeats (D2S123, D5S346, and D17S250).[63-64]

RNA detection

Northern Blot

Northern blot is a technique used to study gene expression. The principle used is similar to Southern blot method, differing on the nucleic acid immobilized. This method detects specific sequences of RNA rather than DNA based on the construction of RNA label probe. Northern blot was the first mRNA detection method used to measure gene expression patterns in human cancer, being a reliable technique to detect gene expression.[65-67]

Currently Northern analysis is limited to a research role and is not widely used for clinical assessment of human samples. The technique is cumbersome, slow, and, like southern blotting, is at risk for the loss of sensitivity due to dilution of the target malignant cell mRNA levels by surrounding non neoplastic tissues.[68]

Microarray

In cDNA microarrays, DNA probes representing cDNA clones are arrayed onto a glass slide and hybridized with fluorescently labeled cDNA targets. The intensity signal of each individual probe should correlate with the abundance of mRNA complementary to that particular probe. The power of the technology is the ability to perform a genome-wide expression profile of thousands of genes in a given sample using a single hybridization reaction.[69-70] Microarrays technology has shown to be a great apparatus for screening studies, for hypothesis conception and for clarifying wide gene expression profile in health and disease.[71]

In spite of the massive quantity of data generated, the results from microarrays studies have the limitation of noise sensitivity and analysis method. This can lead to uncertain results and biologically false-positive genes and pathways for stratification.[72] Consequently, a new field of informatics has emerged, and the best data analysis tools applicable in the generated dataset are presently a theme of debate.

This technique has achieved extensive use for expression profiling of human clinical samples, understanding the molecular basis of cancer,[73-75] new subclasses in disease states,[76] discovering novel prognostic markers and also predict disease outcome[77-79] and response to chemotherapy.[80-81] In addition, microarrays have the ability to provide a picture of the genetic state of cells by identifying altered genes and pathways in cancer genesis, progression and metastasis.[82]

Quantitative Reverse Transcriptase Polymerase Chain Reaction (qRT_PCR)

The qRT_PCR methodology addresses the obvious requirement for quantitative data analysis in biological studies of gene expression in molecular medicine, biotechnology, microbiology and disease diagnostics.[83] It has become the method of choice for mRNA quantification from small amounts of tissue.[84] This technique combines reverse transcription (RT) dependent conversion of mRNA into cDNA, the amplification of cDNA using PCR, detection and quantification of amplification products in real time.[83] The transcription level of the interest target gene can be measured relatively to an endogenous reference "housekeeping" gene to correct variation in the quality of RNA preparations.[84] qRT_PCR uses fluorescent reporter dyes to combine amplification and detection steps of the PCR reaction into a single step.[85-86] The assay measures the increase in fluorescent signal, which is proportional to the amount of DNA processed during each PCR cycle.[83]

Benefits of this procedure over conventional methods for measuring RNA include its sensitivity, large dynamic range, and the potential for high throughout as well as accurate quantification enhancing the specificity.[85,87] With increasing demand for gene expression quantification, simple, rapid and reproducible methods are required for use in clinical and routine laboratory applications.[88] The quantification of specific mRNA transcripts gives a conception of the molecular processes that drive cell activity.[89]

There are several applications for qRT_PCR. For diagnosis qRT_PCR is applied to rapidly detect the presence of genes involved in human neoplasias and infectious diseases. In the research setting, it is the method of choice for gene expression quantification. Setup and analysis are simple and can be more easily extended to the clinical environment than traditional PCR techniques.

Micro RNA

MicroRNAs (miRNAs), a recently identified class of non-protein coding small RNAs, have been associated to genomic regions or fragile sites in cancer pathogenesis.[90] miRNAs regulate gene expression by translational repression, mRNA cleavage, and mRNA decay initiated by miRNA-guided rapid deadenylation. Therefore miRNA expression profiles may become useful biomarkers for cancer diagnostic, and miRNA therapy could be a powerful tool for cancer prevention and therapeutics.

Several researches have modified DNA microarray technology to form miRNA microarray.[91-96] Recently, Lu et al.[97] developed a novel strategy to detect abundant miRNA expression profiles in different cell types, including several human cancers. To overcome the concerns about probe specificity in miRNA microarray analysis, they performed hybridization in solution and quantified the polymer heads that are hybridized to

miRNA species using multicolor flowsorting. This method can be used to detect miRNA expression profiles in cancers, even in poorly differentiated tumors.[97-99]

Protein detection

Immunohistochemistry (IHQ)

The introduction of immunohistochemistry (IHQ) in routine diagnostic procedure has revolutionized surgical pathology. This method recognizes protein products expressed by different cell populations in conjunction with morphologic examination for nucleic acid alterations assessment. The technique consists in antigens localization in tissue sections using labeled antibodies as specific reagents through antigen-antibody interactions that are visualized by a marker.[100] Antibodies for immunohistochemical studies are produced by animal hosts in response to antigen stimulus. Primary antibodies recognize and bind to specific epitope and secondary antibodies serve as antigen within the constant region of the primary antibody. Normally, secondary antibodies are conjugated to a fluorescent dye, or to biotin, which, in turn, binds an avidin-enzyme complex, or directly to an enzyme that can produce a localized visible result when reacted with a chromogenic substrate allowing visualization of the anatomic and histological distribution of the interest antigen.[101]

The IHQ technique allows accurate characterization and diagnosis of solid tumors, hematolymphoid neoplasms and infectious disease.[101] An increasing number of antibodies directed against normal and abnormal cell proteins as well as infectious agents is available to diagnose and subclassify pathologic entities. With this method, the products of genes are assayed in tissue sections allowing characterization of cell population as benign or neoplastic, determine cell lineage, and, in some cases, determine the nature of molecular genetic alteration leading to a specific process.[102] One of its major utilities in the diagnosis of soft tissue tumors is to correctly identify a tumor as being of mesenchymal or non-mesenchymal origin. Once mesenchymal origin has been established, histologic subtyping according to specific cell lineage may be achieved with the use of lineage-specific markers. Despite of the rapid development of molecular genetic techniques, IHQ still remains the most important diagnostic tool in the diagnosis of soft tissue tumors aside from recognition of morphologic features and clinical correlation.[103]

With the development of tissue microarrays (TMA), it is now possible to simultaneously analyze multiple tissue samples for genes involved in signaling pathways that control cell proliferation, death and differentiation. This technique allows for up to 1,000 cylindrical cores of tissue to be embedded in a single paraffin block and the use of simultaneous immunohistochemical analysis of biomarkers of interest.[104] A classical goal in cancer biology has been to determine protein biomarker profiles of cancerous lesions at the preclinical stage.

Western blotting

Western analysis refers to the technique of detecting proteins immobilized on a filter with specific antibodies. Protein isolated from tissues or cells is subjected to electrophoresis on polyacrylamide gels, often in the presence of sodium dodecyl sulfate (SDS) detergent, to separate the molecules by molecular weight.[101-102] The protein is transferred to a filter using high-voltage electrophoresis. The filter is exposed to a primary antibody directed against the specific protein being investigated. Enzyme-linked or isotopically labeled antispecies secondary antibodies are used to detect the primary antibody. The protein bands recognized by the primary-secondary antibody complex are visualized by colorimetry or by autoradiographic methods.[105]

Western blot analysis is used to identify post-translational modifications such as phosphorylation or glycosylation, and screen changes in protein expression. These changes may also be seen with immunocytochemistry, but generally antibodies do not distinguish between protein variants *in situ* unless specifically designed for this.[102] Western blot is applied in research to explore future biomarkers for cancer and neurological diseases.[106] This methodology has been used in definitive test for Bovine spongiform encephalopathy (BSE, commonly referred to as 'mad cow disease') and to detect anti-HIV antibody in human serum samples.[107]

General Comments

In the light of several advances in cancer research, sensitive tests for identifying changes in a single cell

or cell clusters that may define tumor progression and early detection of patients with risk of cancer is a goal today. The molecular basis of disease process provides an opportunity for the clinical laboratory to develop and implement new approaches for diagnosis and prognostic assessment.

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