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Short Course 7

Molecular pathology

Chairperson: G. Bevilacqua, *Italy* **Co-chairpersons** J Costa *USA* and FL Fern~ndez, *Spain.*

DNA analysis: Biological and morphological methods

G. Bevilacqua

Dept. of Oncology Division of Pathology University of Pisa, Italy.

Current biomedical sciences have been revolutionized by a series of new investigative techniques that allow the definition of molecular mechanisms involved in complex processes such as cell growth and division, metabolism, differentiation and development. These techniques represent powerful tools for pathologists, who can now approach, at the molecular level, many of the problems and controversies that have vexed them for decades. The study of the genetic material, DNA, is a crucial step in the understanding of numerous pathological processes.

High molecular weight genomic DNA can easily be obtained from fresh or frozen tissue (1). Methods are also available to recover genomic DNA from fixed and embedded materials (2, 3). The quality of DNA recovered from paraffin-embedded tissues depends on various parameters, including type of fixative, length of fixation and the reagents used for the embedding process.

Gross, but sometimes useful, information about a genetic DNA sequence can be obtained by Southern blotting (4). High molecular weight genomic DNA is subjected to enzyme digestion and electrophoresis on agarose gel, blotted onto nylon membranes and hybridized with isotopically or nonisotopically-labeled probes. Applications of Southern blot analysis have been reported in different fields of diagnostic molecular pathology. The method has been successfully applied in studies of chromosomal rearrangements, particularly for the analysis of lymphoid genes. Another important application of Southern blot analysis in pathology is the study of polymorphic alleles to assess linkage of a particular allele to a disease phenotype or to evaluate the genome of tumor cells for loss of heterozygosity at a particular locus, which may imply deletion of a tumor-suppressor gene. Moreover, Southern blot can be used to identify other genomic alterations, such as gene amplifications or viral insertions. Unfortunately, Southern blot analysis is not very sensitive: the method can detect a DNA alteration if it is present in no less than 5% of the DNA molecules in the sample.

The advent of the polymerase chain reaction (PCR) and related technologies has greatly increased the sensitivity of molecular analyses (5). The PCR product can be directly examined by agarose gel electrophoresis after being stained with ethidium bromide, subjected to Southern blot, cloned, sequenced or used as a probe in hybridization studies.

PCR can be useful for detecting a small number of DNA molecules of a target sequence *(e.g.,* novel DNA targets such as viral genomes) or for producing a large quantity of a DNA sequence for subsequent analysis. For instance, the PCR product can be subjected to the single-strand conformation polymorphism analysis, or to the denaturant gradient gel electrophoresis assay in order to detect subtle mutations or polymorphisms (6, 7). In addition, the diagnosis of clonality in lymphoid malignancies, and the identification of loss of heterozygocity or microsatellite alterations in tumor samples is much easier and quicker by PCR technology (8).

DNA analysis can be performed *in situ;* this approach allows the combination of biological and morphological information. *In situ* analysis offers a potential advantage in sensitivity over filter hybridization in cases where the sequence of interest is present in only a small number of cells within a large mixed population. Such sequences may be undetectable in tissue extracts due to dilution by other sequences from the surrounding tissue. *In situ* hybridization has become a powerful and versatile tool for the detection of nucleic acid sequences and is capable of giving a high degree of spatial information in locating a specific DNA target within individual cells or chromosomes. More recently, techniques such as *in situ* PCR or fluorescence and *in situ* hybridization (FISH), which can greatly increase the potential of *in situ* technology, have been developed.

In situ PCR is a sophisticated technique that should combine the sensitivity of PCR reaction to intracellular localization of genomic sequences with the same specificity as *in situ* hybridization (9). The visualization can be realized by direct *in situ* PCR, the product obtained being directly identifiable by incorporation of labeled nucleotides or primers or preferentially by indirect *in situ* PCR. In this case, the amplification is followed by *in situ* hybridization with labeled probes. This last procedure seems to be more specific. Unfortunately, several variables can affect *in situ* PCR results, including type of fixative and time of fixation, protease digestion, the hot start maneuver, stringency and the composition of the amplifying solution and oligoprobe cocktail. Appropriate and meaningful controls are needed to allow the accurate interpretation of results and the possible problems and pitfalls of the *in situ* PCR methods, including artifacts related to diffusion of PCR products and nonspecific incorporation of labeled nucleotides into fragmented DNA undergoing repair. Due to the possibility of false results, this technique should be performed in laboratories specialized in the evaluation of viral diseases, as well as in hematological and other malignancies that have unique genetic markers.

FISH is a variation of the traditional hybridization process, whereby the probes utilized are fluorescently labeled and produce bright clear signals upon detection (10). Unique sequences, chromosomal subregions and entire genomes can be specifically highlighted in metaphase chromosomes or interphase cells. Spectacular advances in the use of FISH have been made over the last few years, making the technique a useful tool in clinical research. Chromosomal translocations, deletions, amplification of specific genes and changes in chromosome number can all be detected in the nondividing interphase nucleus using probes ranging from whole chromosome paints" to individual, gene-specific probes.

The methods reported above are the most frequently used techniques for DNA analysis when approaching clinical problems. Other DNA methods have been developed which could be usefully applied in diagnostic molecular pathology. So many technical advances have been made in the last decade that now the question lies not so much in deciding how to apply a method to solve a problem, but more in deciding which is the right question to answer.

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RNA analysis: Biological and morphological methods

G. Stanta

Institute of Pathology University of Trieste and International Center for Genetic Engineering and Biotechnology Trieste, Italy.

In the last 10 years, most of the work in molecular pathology has been performed at the DNA level. The next challenging step is to understand how gene products govern pathological cellular pathways. For this reason, we recently developed new qualitative and quantitative RNA methodologies to study pathological alterations at the messenger RNA (mRNA) level.

The handling of RNA presents many more problems than does that of DNA. This is because ribonuclease (RNase) enzymes, which rapidly degrade RNA, are ubiquitous. Moreover, temperature and alkaline environment can rapidly destroy it. Consequently, RNA methods require more precautions than do DNA methods. This is one of the reasons why molecular pathology laboratories are less familiar with this kind of method. Gloves, RNase-free reagents and ware, specifically treated water, low temperature and accurate handling of samples and reagents are prerequisites.

The first problem with RNA is an accurate method of extraction; the RNA must be protected against RNase throughout the entire procedure. Even in these conditions, it can easily be degraded. Even more precautions are needed to extract total RNA from fixed and paraffin-embedded tissues. In this case the RNA is already partially degraded and only fragments of 200 bases or less are present (1). The use of paraffin-embedded tissues is advantageous since the huge pathological archives of pathology departments could be utilized. In this kind of material the specific lesions can be microdissected after histopathological examination. For this reason, we developed a reliable method of RNA extraction from paraffin-embedded tissues (2). Good commercial extraction kits, specifically for paraffin tissues, are also available (3).

The qualitative classical methods for RNA analysis, such as *in situ* hybridization or Northern blot, are not highly sensitive; high expression at cellular level for *in situ* hybridization and a large amount of extracted RNA for Northern blot are needed. This problem could be overcome with more sophisticated techniques such as the RNase protection assay (RPA) or reverse transcription-polymerase chain reaction (RT-PCR) and more recently by *in situ* PCR. This last method is still not completely reproducible for RNA but the possibility of combining high sensibility and morphology is so exciting that many laboratories are keen to use it. RPA is a reliable and sensitive method but is more complicated and time-consuming than RT-PCR and is less sensitive.

Nowadays, the most important challenge is the development of a quantitative analysis of gene expression. In this way, the dynamics of cellular alterations at the molecular level can be deciphered. The role of quantitative changes in gene expression in cancer and other diseases can give more information on the pathogenesis and evolution of diseases. Many methods have been proposed for the quantitative analysis of RNA from cells or fresh tissues, such us competitive POR analysis. We developed quantitative methods for RNA analysis in paraffin-embedded tissues, using RT-PCR in specific conditions (4, 5). The most relevant point is linearity between the log of target RNA concentration and the log of the amplification products; only in this case is a quantitative analysis possible. For this reason, specific conditions of target concentration and number of amplification cycles must be found (4).

To quantify the results, we used gamma-counter or phosphor imaging analysis (4) and capillary electrophoresis quantification (5). More recently, we have become interested in real-time PCR, where the quantitative analysis conditions are defined directly during the amplification procedure.

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