REVIEW

Role of Advanced Diagnostic Aids in Oral Pathology

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ABSTRACT:

The field of pathology has developed at an exponential rate in the past few decades. Molecular techniques have contributed greatly in understanding the pathogenesis of genetic disorders and in diagnosis of several undifferentiated malignant neoplasms. Microbiology is also one of the disciplines which has been dramatically affected by molecular techniques. Many molecular techniques are being used in clinical practice. Similarly, the advent of these newer methods has thrown light on various fields of dentistry. Newer methods like in situ hybridization have led to a deeper insight into understanding basic concepts like odontogenesis. Cytogenetics and other methods have changed the very face of diagnostic oral pathology as well as forensic odontology. The present article highlights few of the various techniques which when used optimally by the pathologist have improved the quality of life of numerous patients.

Key words: Molecular techniques, Cytogenetics, Diagnostic pathology, forensic odontology

INTRODUCTION

Surgical pathology was a largely unknown enterprise a century ago. The early interface between surgery and pathology has been traced eloquently by *Underwood*. The first microscopic illustrations of human tumors were found in a text by *Sir Edward Home* in 1830, followed shortly by another by *Johannes Muller* in 1838.[1] The term "biopsy" was introduced into medical terminology in 1879 by *Ernest Besnier*. While studying the milestones in development of surgical pathology it is possible to make out three stages in more than 100 years history of the method development: an occasional use of histologic procedure involving living organs and tissues accessible for observation and study (approximately until the late 19th century); restricted application of biopsy (until the mid-20th century); present stage at which the method is widely adopted and its use in general and total (with respect to human organism) not only in oncology but practically in all clinical specialties.[2] Progress in surgical pathology was slow as the existing technology back then for handling and sectioning of human tissues was not highly developed. Embedding specimens in wax did not come into general use till the 1800s and histotechnology was still rudimentary.[3] Frozen sections were being introduced in American medical centres and were considered as reliable by the turn of twentieth century.[4]

The origins of histochemistry can be traced back directly to the French botanist *Francois-Vincent Raspail,* who according to *Pearse* in 1825 was the first to fully appreciate the power of combining a chemical reaction with the microscopic observation of tissues and cells.[4] Despite the auspicious beginning, progress was slow and soon became even slower as aniline dyes took over the field of histology. There was resurgence of interest in the 1930s because of the publication of *Lison's Histochimie Animale.* The period from 1940s to 1970s saw the development of immunohistochemistry, in-situ hybridization and molecular biological techniques.[1] The present article briefly describes few of the various methods as well as their applications in the field of oral pathology.

METHODS IN MOLECULAR PATHOLOGY

The diagnosis and characterization of genetic diseases and of malignancy are increasingly dependent on analysis of specific genetic sequences that determine the pathologic processes in such disorders. This type of molecular genetic analysis is technology intensive; as with other, more established diagnostic methods, proper design and interpretation of these new diagnostic approaches requires understanding of the concepts behind the techniques and of the biological processes and molecular structures that are being examined.[5]

All nucleated cells contain DNA and RNA. Common sources of DNA obtained for clinical use include blood, bone marrow and tissue samples such as biopsies and tissues removed during surgical resections. Buccal scrapings may also be used. It is possible to extract DNA from formalin fixed, paraffin embedded tissues. This is quite useful for analysis of stored samples in archival material. Before the nucleic acids can be extracted, the paraffin must be removed and the tissues treated with protienase to breakdown the protein cross links formed during formalin fixation. The quality of these preparations is variable and depends on the age of the tissue and the length of time it was exposed to formalin. If the DNA obtained from fixed tissues is found to be degraded, it is possible to amplify this DNA by Polymerase Chain Reactions (PCR). RNA isolations from from tissues fixed in formalin, although they have been reported are less successful.[6]

EXTRACTION TECHNIQUES

Extraction of DNA and RNA involves several steps including lysis of cells, removal of proteins and other cellular components and purification of nucleic acids. To avoid degradation of DNA and RNA use of EDTA has been advocated. EDTA chelates Mg^{2+}, an essential cofactor for many DNAses. Protein removal can be accomplished either by extraction with organic solvents such as phenol and chloroform or by selective precipitation of proteins and high concentrations of salt.[6]

MEASUREMENT OF NUCLEIC ACID CONCENTRATION

The bases in nucleic acids absorb light in ultraviolet portion of the spectrum with an absorption maximum at approximately 260nm. This property is used to quantify nucleic acids in solution by spectrophotometry. Other general methods for nucleic acid quantification exist. Several fluorescent dyes are available that bind DNA with a resulting increase in their fluorescence efficiency.The presence of degraded DNA or RNA can be identified by electrophoretic separation followed by Ethidium bromide staining of an aliquot of purified sample.[6]

PROBES AND PROBING

A probe refers to a stretch of nucleotides (from a few base pairs to a few thousand base pairs) that is used to detect a specific region of DNA or RNA as a function of it's complementarity to the target sequence. Those derived from native DNA are referred to as genomic probes and essentially mirror a region of DNA and include both extronic and intronic regions. A probe derived from RNA is a cDNA probe and recognizes only exons. While DNA and cDNA probes are derived from cellular material, a third form of probe is synthesized in the laboratory. Synthetic oligonucleotide probes can be designed to distinguish between normal and abnormal genetic sequences referred to as allele specific oligonucleotide probes.[3]

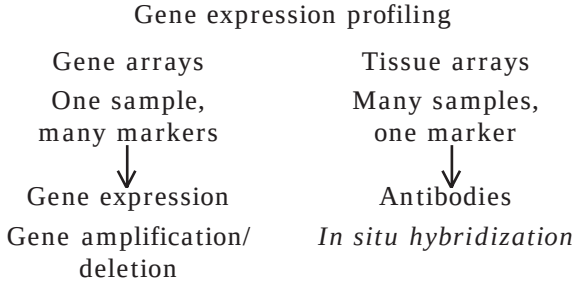
MICROARRAYS

In 1998, Olli Kallioniemi, Guido Sauter and colleagues published their seminal paper describing high density tissue microarrays (TMAs) a surprisingly robust method for creating a single paraffin block containing hundreds of well ordered tissue samples each roughly the diameter of a propelling pencil lead.[6] Early on, interest in TMA technology benefited from the (then) recent development of nucleic acid arrays and the anticipated potential of multiplexed biomarker analysis. The key benefit underlying TMA technology is the ability to assay hundreds of patient tissues arrayed on a single microscope slide.[7]

Gene microarray technology rests on the ability to deposit many (tens of thousands) different DNA sequences on a small surface, usually glass slide (often referred to as a chip). The different DNA fragments are arranged in rows and columns such that the identity of each fragment is known through its location on the array.[8]

Tissue microarrays (TMAs) are constructed by transferring cores of paraffin embedded tissue to pre-cored holes in a recipient paraffin block.[6] Over 500 cores can be placed in a single block by this technique. Sections cut from TMA blocks can then be used for immunohistochemistry or in situ hybridization.[9]

APPLICATIONS



Because they do not require fresh frozen clinical samples, TMAs allow for a rapid validation and extension of the gene expression studies on many more samples than are generally available for gene microarray studies.[8]

Gene expression profiling as a diagnostic adjunct can improve the sub classification of tumors; in the case of lymphomas in particular. Gene expression profiling could be applied to cases that are diagnostically challenging using current techniques e.g. small blue cells tumors, soft tissue sarcomas and carcinomas of unknown primary site. Gene expression profiling can be used to discover new markers that might predict a response to targeted therapy.[8]

APPLICATIONS OF MICROARRAYS IN FIELD OF ORAL PATHOLOGY

DNA microarrays are emerging as a technology that allows for the identification of novel cancer-related genes (Golub *et al.*, 1999) and the fine molecular classification of human cancer (Francioso *et al.*, 2002). Carinci *et al.* carried out a study to define the genetic expression profile of odontogenic tumors. This technology for the first time was applied to odontogenic tumors and compared three cases of ameloblastoma with three cases of malignant tumors (*i.e.*, one ameloblastic carcinoma, one clear cell odontogenic tumor, and one granular cell odontogenic tumor) by Carinci et al. The results of their study showed that notable genes were over-expressed in all six odontogenic tumors and selected by Gene Otology (GO) analysis were those that encode for proteins involved in intercellular adhesion and for receptors used by cells to bind to the extracellular matrix, such as integrins (alpha 3, 5, 6, 11; beta 2 and integrin associated PTK2), genes involved in the signaling pathways from the integrin receptor, and others encoding for enzymes of collagen biosynthesis.[9]

Extracellular matrix was similarly affected, with proteoglycans and type V collagen, a key determinant in the assembly of tissue-specific matrices. Connexins CX26, CX32, and CX43, that form gap junctions and provide long range cell signaling within epithelia, appear down-regulated as the expression profiles of three malignant odontogenic tumors with those of the three benign ameloblastomas. Up-regulated genes in malignant biopsies include coronin, a ubiquitous actin-binding protein essential for organizing the normal actin cytoskeleton that plays a significant role in cell division (Fukui *et al.*, 1999), and MYD88, a protein containing death domains with a role in negative growth control, cell cycle arrest, and apoptosis (Liebermann and Hoffman, 2002). On the other hand, repressed genes in malignant tumors correspond to: STK19, a Ser/Thr nuclear protein kinase; ABT1, a transcription co-activator; and CTBP2, a transcriptional repressor which acts as a tumor suppressor and plays an important role in oncogenesis (Chinnadurai, 2002). Down-regulated genes also include RFP, a DNA-binding protein.[9]

IN-SITU-HYBRIDIZATION

Chromosome painting, competitive hybridization using entire chromosome specific libraries for chromosomes as probes and human genomic DNA as competitor, was one of the first applications of FISH. It provided intense and specific fluorescent staining of human chromosomes in metaphase spreads and interphase nuclei, allowing the distinctive identification of chromosomes involved in complex arrangements. The advent of the Human Genomic Project has made available a repertoire of single locus probes that have provided a significant boost to gene mapping strategies and led to the identification of the breakpoints of consistent translocations.[10,11]

APPLICATIONS IN ORAL PATHOLOGY

Nucleic acid probe technology provides information for diagnosis, determining prognosis, selecting therapeutic modalities, and monitoring disease progression. Examples abound for the application of this technology in microbiology, immunology, forensic science, genetics, and oncology.[6]

To examine the nature of chromosome instability in the field of head and neck tumors, archival surgical specimens were sought that exhibited a contiguous histologic progression from normal adjacent epithelium through hyperplasia to dysplasia to invasive cancer. In-situ hybridization using probes for highly repeated sequences located in the centromere regions of specific chromosomes was then used on tissue sections of these specimens to detect chromosome polysomy, or the presence of cells with three or more chromosome copies.[12]

The technique of in situ hybridization using labeled mRNA probes allows the domains of expression of specific genes to be visualized in developing tooth germs. Cultured tooth germs can now be transferred into the kidney capsules of adult male mice, allowing tooth development to proceed to full crown formation and localized alveolar bone differentiation. Further adaptations to the culture techniques have provided great insight into the signaling mechanisms that occur during odontogenesis.[13]

CYTOGENETICS

Clinical cytogenetics was born in 1959 with the description of trisomy 21 in Down's syndrome, monosomy X in turners syndrome, and XXY in Klinefelter's syndrome. The field came of age in 1970 with the introduction of chromosome banding techniques. Cytogenetics is maturing further today because of the rapid development of fluorescence labeled DNA probes that can mark whole chromosomes, centromere regions, or specific genes of interest.[14]

The most recent advances in cytogenetics has been the advent of molecular cytogenetics, in which the techniques of molecular biology are applied to cytogenetic preparations. These procedures include in situ hybridization for localizing DNA probes to specific chromosomes and bands. Cytogenetics has now become an integral part of the gene mapping attempts and has wide applications to medical relationships between such chromosome abnormalities as infertility, miscarriage, birth defects, mental retardation and cancer.[15]

APPLICATIONS OF CYTOGENETICS IN ORAL PATHOLOGY

The Ewing family of tumors is characterized by a recurrent t(11;22) (q24;q12) chromosomal translocation, detectable in approximately 85% of the cases. Secondary chromosomal aberrations, notably gains of chromosome arm 1q and chromosomes 8 and 12 occur in more than half of the cases. Molecular cloning of the t(11;22) breakpoints revealed an in frame fusion between the 5' end of the EWS gene from chromosome band 22q12 with the 3' portion of the 11q24 FL1 gene, a member of the ETS family of transcription factors.[15]

SCCs account for 90% to 95% of all malignancies in the upper aerodigestive tract. Some 200 cases with clonal abnormalities have been reported after short-term culturing. The majority of these have had fairly complex karyotypes, typically showing a large number of numerical and unbalanced structural rearrangements. The most common imbalances detected at banding analysis are loss of 2q34-qter, 3p, 4p, 4q28-qter, 8p, 9p13-pter, 10p, chromosomes 13 and 14, 15p, 17q23-qter, 18q21-qter, 21p, 22p, and the Y chromosome, and gain of 3q, chromosome 7, 8q, and 11q13. The still relatively few cases that have been analyzed by CGH have shown a similar distribution of gains and losses, albeit at slightly different frequencies.[15]

The non-squamous cell carcinomas of the head and neck region that arise predominantly from major and minor salivary glands, which include adenocarcinoma, acinic-cell carcinoma, mucoepidermoid carcinoma, adenoid cystic carcinoma, and carcinoma ex pleomorphic adenoma, are less well characterized, with less than 25 informative cases per tumor type. The karyotypes of these malignancies usually are less complex than those typically encountered in SCC. Adenocarcinomas and acinic cell carcinomas often show deletions of 6q and trisomies for chromosomes 7 or 8, in mucoepidermoid carcinomas a recurrent t(11;19)(q14-21;p12) has been described, adenoid cystic carcinomas are characterized by a recurrent t(6;9) (q21-24;p22-24) or del(6q), and carcinomas arising from pleomorphic adenomas usually have structural rearrangements resembling those in pleomorphic adenomas together with other structural or numerical abnormalities, such as polyploidization, indicating that the malignant

transformation often is paralleled by cytogenetically visible clonal evolution.[16]

Mostly all, osteosarcomas contain clonal chromosomal aberrations. The aberrations are complex, comprising an abundance of numerical and structural alterations.[14] Chromosomal regions 1p11-13, 1q11-12, 1q21-22, 11p14-15, 14p11-13, 15p11-13, 17p and 19q13 are most frequently affected by structural changes, and the most common imbalances are +1, -6q, -9, -10, -13, and -17.[17]

METHODS USED IN FORENSIC DENTISTRY

Forensic identification is based on finding differences polymorphisms between different individuals. These differences can take many forms, such as differences in facial appearance, differences in hair color, differences in height, etc. – some variations are unique and some are not. Dental identification takes advantage of the polymorphic nature of the hardest structures in the body precisely those structures which are most likely to remain available for identification purposes. DNA has a greater likelihood of survival in teeth.

The DNA typing method that was first described, and most commonly employed by crime labs initially, is known as restriction fragment length polymorphism (RFLP) analysis. The six steps in RFLP testing include:

1. Extraction of DNA from a biologic source
2. Cutting the DNA into relatively small fragments at specific sites with "restriction enzymes"
3. Separating the fragments by size using agarose gel electrophoresis;
4. Transferring and immobilizing the separated DNA fragments onto a nylon membrane
5. Denaturation of the DNA into single strands and hybridization to radioisotopically-labeled probes (small fragments of single-stranded DNA)
6. Autoradiography, in which an X-ray film is placed over the membrane for several days, resulting in exposure of the film at the point of the probe

PCR Methods

The polymerase chain reaction (PCR) is a method of copying or "amplifying" a particular segment of DNA. A few strands or even a single strand of DNA can be used to reproduce millions of copies of target DNA fragments. Most significantly for remains identification, it is often successful even though the tissue specimen is degraded because only a few copies of relatively short segments need remain intact.[18]

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