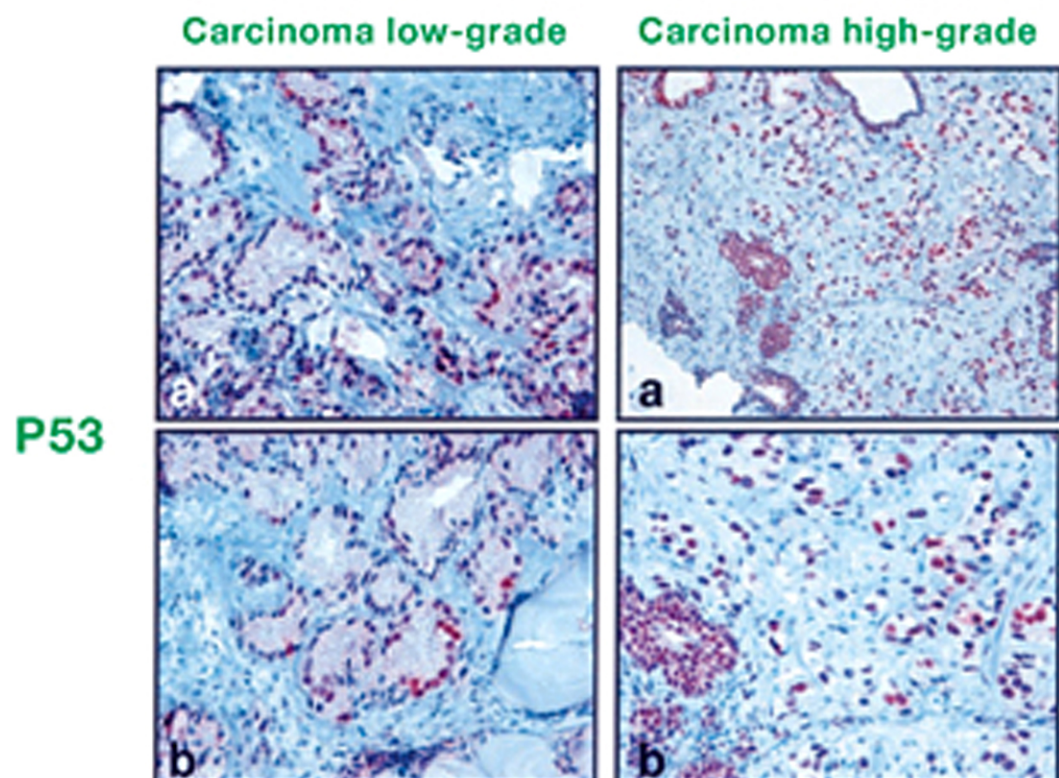


**H A N D B O O K O F**

# IMMUNOHISTOCHEMISTRY AND *IN SITU* HYBRIDIZATION OF HUMAN CARCINOMAS

MOLECULAR PATHOLOGY, COLORECTAL CARCINOMA,  
AND PROSTATE CARCINOMA



*Edited by* **M.A. HAYAT**

**Handbook of Immunohistochemistry  
and *in situ* Hybridization of  
Human Carcinomas, Volume 2**

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Edited by M. A. Hayat

***Volume 1***

**Molecular Genetics; Lung and Breast Carcinomas**

***Volume 2***

**Molecular Pathology, Colorectal Carcinoma, and Prostate Carcinoma**

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**Molecular Pathology, Colorectal  
Carcinoma, and Prostate Carcinoma**

Edited by

**M.A. Hayat**


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*To*

Molecular Geneticists/Clinical Pathologists

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# Foreword

According to mortality data from the National Center for Health Statistics, approximately 1,334,100 new cases of cancer will have been diagnosed, and 556,500 people will have died from cancer in the United States by the end of 2003. Though the number of cancer-related deaths has been on the decline since 1992, the incidence has increased over the same period. This increase is largely due to the implementation of improved screening techniques that have in turn been made possible by advances in immunochemical diagnostic testing. As immunochemical techniques such as *in situ* hybridization (ISH) and immunohistochemistry (IHC) continue to be refined, their use in improving patient care through research and improved methods of diagnosis is becoming ever more valuable.

*In situ* hybridization is a well-established approach for identifying the organization and physical position of a specific nucleic acid within the cellular environment, by means of hybridizing a complementary nucleotide probe to the sequence of interest. The use of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) as probes to assay biologic material has been in use for approximately 30 years. However, recently, advances in ISH have seen a replacement of radioactive detection by more adaptable colorimetric and fluorescent (FISH) methods for the interrogation of nuclei, metaphase chromosomes, DNA fibers, patient tissue, and, most recently, deriving information from patient samples using DNA microarrays. Technologic advances, including array comparative genomic hybridization, spectral karyotyping, and multicolor banding, have provided a refinement in the study of genome organization and chromosomal rearrangements. In addition, ISH using RNA has allowed for a determination of the expression pattern and the abundance of specific transcripts on a cell-to-cell basis. Advances in DNA and RNA ISH have migrated from the research setting and are becoming routine tests in the clinical setting permitting examination of the steps involved in tumorigenesis, which would not have been possible by the use of classical cytogenetic analysis.

Since the introduction of monoclonal antibodies, immunohistochemistry has developed into a vital tool,

which is now extensively used in many research laboratories and for clinical diagnosis. Immunohistochemistry is a collective term for a variety of methods, which can be used to identify cellular or tissue components by means of antigen-antibody interactions. Immunostaining techniques date back to the pioneering work by Albert Coons in the early 1940s, using fluorescein-labeled antibodies. Since then, developments in the techniques have permitted visualization of antigen-antibody interactions by conjugation of the antibody to additional fluorophores, enzyme, or radioactive elements. As there are a wide variety of tissue types, antigen availabilities, antigen-antibody affinities, antibody types, and detection methods, it is essential to select antibodies almost on a case-to-case basis. The consideration of these factors has led to the identification of several key antibodies that have great utility in the study and diagnosis of tumors.

The scientific advances in the field of immunochemistry have necessitated rapid developments in microscopy, image capture, and analytical software in order to objectively quantify results. These cutting-edge experimental systems have already produced many significant differences between cancers that might not have been distinguished by conventional means.

The focus of these volumes is the use of ISH and IHC to study the molecular events occurring at the DNA, RNA, and protein levels during development and progression of human carcinomas. Continued investment of time and expertise by researchers worldwide has contributed significantly to a greater understanding of the disease processes. As the technical requirements for many immunochemical techniques is quite demanding and as the methodology itself poses many pitfalls, the step-by-step methods provided in these volumes will serve as an excellent guide for both clinical and basic researchers studying human malignancies.

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# Preface to Volume 2

The primary objectives of this volume remain the same as those of volume 1—that is, discussion of immunohistochemical and *in situ* hybridization (ISH), including fluorescence *in situ* hybridization (FISH) and chromogenic *in situ* hybridization (CISH) procedures as they are used in the field of pathology, especially cancer diagnosis. The practical importance of the antigen retrieval protocols in immunohistochemistry was realized in 1991, and since then they have been used routinely in pathology laboratories. Many chapters in this volume contain the details of these protocols. However, detection of certain antigens even in formalin-fixed tissues can be accomplished without using antigen retrieval methods.

Immunohistochemistry, ISH, FISH, and CISH of two major carcinomas (colorectal and prostate) are presented. The biomarkers of two other major carcinomas (lung and breast) were explained in Volume 1, and others will be discussed in the forthcoming Volume 3. The procedures are explained in maximum details in a step-by-step fashion so that the readers can use them without additional references. Materials required to carry out the procedures are also included. These procedures are also useful in clinical laboratories.

Another objective of this volume is the discussion of the role of molecular pathology (molecular genetics, molecular medicine, molecular morphology) to understand and achieve correct diagnosis and therapy in neoplastic diseases. Molecular pathology/genetics has the advantage of assessing genes directly. Knowledge of the genetic basis of disease will, in turn, allow more specific targeting of the cause, rather than the symptoms only, of the disease. The time is overdue to apply our knowledge of molecular genetics in conjunction with immunohistochemistry and histology to diagnostic, therapeutic, and prognostic decisions.

Genetic information will improve the prognosis used to monitor both the efficacy of treatment and the disease recurrence. Molecular markers, largely from tumors but also from germline, have great potential for diagnosis, for directing treatment, and as indicators of

the outcome. In other words, these markers are of considerable importance to clinical practitioners. For this and other reasons the role of gene mutations in cancer is emphasized because the characteristics of the tumor depend on the mutations that lead to their emergence. For example, down-regulation of tumor suppressor genes BRCA1/2 and their proteins is a well-known test for breast cancer susceptibility, resulting in poor prognosis. Indeed, methods of molecular testing of tumors are finally well established and are discussed in this and other volumes of this series of handbooks. Widespread molecular testing is the future for clinical practice.

Unfortunately, clinical practice has lagged behind the current knowledge of research in molecular genetics. Both technicians and pathologists need to be aware of the importance of molecular pathology testing. Somatic mutations are rarely performed, although some histopathology and cytogenetics laboratories have done limited testing such as chromosomal rearrangements in lymphoma. Molecular testing should be regarded as a means of complementing, rather than replacing, established methods such as immunohistochemistry and FISH.

There are several reasons for the limited use of molecular genetics in clinical practice. One reason is the high cost of establishing facilities for molecular techniques; another is our comparatively meager understanding of the nature of many diseases, including cancer. Although equipment for molecular testing is available, some investment is needed. Another reason is the dearth of clinician–scientist training programs, resulting in limited clinician–scientists. Also, an inequity in pay exists between those working in clinical practice versus research faculty. Accordingly, the differential in pay may be a disincentive for choosing a full-time career in medical research. The length of time (8 years as an average) to receive the M.D./Ph.D. is probably also a barrier in the development of new clinician–scientists. Many clinician–scientist trainees are married, or are in stable relationships, and personal time for family life and children is increasingly important.

Narrowing the gap in income between clinical practitioners and full-time medical researchers would provide a positive incentive for this profession.

Pathologists are well advised to adapt to modern therapeutic shifts (i.e., morphologic interpretation needs to be combined with molecular diagnostic modalities). The latter protocols can provide a second level of testing that is particularly useful for the analysis of neoplasms for which histologic and immunophenotypic data are inconclusive. Therapies already are beginning to progress more and more toward specific molecular targets. Examples are deoxyribonucleic acid (DNA) microarrays, differential display of gene expression, serial analysis of gene expression, comparative genomic hybridization, rolling circle amplification, reverse transcription polymerase chain reaction, FISH, Southern Blot hybridization, and specific cloned probes; most of these methods were discussed in Volume 1 and are also discussed in this volume. Flow cytometry technology is also presented. We already are down a path that has the potential to alter oncology clinical practice. My hope, through this series of volumes, is to expedite the translation of molecular genetics into clinical practice.

I am indebted to the authors of the chapters for their promptness and appreciate their dedication and hard work in sharing their expertise with the readers. In most cases the protocols presented were either introduced or refined by the authors and routinely used in their clinical pathology laboratories. The methods presented here offer much more detailed information than is available in scientific journals. Because of its relatively recent emergence from the research

laboratory, many molecular pathology protocols are still found in scientific journals only and have not appeared in a book. Each chapter provides unique individual practical knowledge based on the expertise of the author. As with all clinical laboratory testing, the results obtained should be interpreted in conjunction with other established and proven laboratory data and clinical findings.

This volume has been developed through the efforts of 97 authors, representing 15 countries. The high quality of each manuscript made my work as the editor an easy one. The authors were gracious and prompt. This volume is intended for use in research and clinical laboratories by medical technicians and pathologists, especially in the field of oncology. This volume will also be of interest and help to medical students.

I appreciate the cooperation extended to me by Hilary Rowe, a valued, competent publishing editor. As the sponsoring editor, her understanding of the importance of this project in the field of human carcinomas helped me to embark on this uniquely difficult and complex endeavor and bring it to fruition. I am grateful to Dr. Frank Esposito and Dr. Dawood Farahi for their recognition of my teaching and scholarly contributions, and for their help. I acknowledge the hard, efficient work of Denise DeLancey, the production editor. I greatly appreciate receiving indispensable, expert help from Eliza McGovern in the preparation of the manuscript.

M.A. Hayat  
February 2004

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# Prologue

We possess scientific and industrial knowledge in new biotechniques, including human genetic technologies. However, ethical and social implications of these advances must be addressed. Such concerns are especially relevant in some of the applications of genetic engineering, such as pharmacogenetics, gene therapy, predictive diagnostics (including prenatal genetic diagnosis, therapeutic cloning, and cloning of humans and other animals), human tissue banking, transplanting, and patenting of inventions that involve elements of human origin including stem cells. Bioethics should be

a legitimate part of governmental control or supervision of these technologies. Scientific and industrial progress in this field is contingent on the extent to which it is acceptable to the cultural values of the public. In addition, in medical research on human subjects considerations related to the well-being of the human subject should take precedence over the interests of science and industry. Any form of discrimination against a person based on genetic heritage is prohibited.

M.A. Hayat



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# Selected Definitions

**Ablation:** Ablation consists of removal of a body part or the destruction of its function.

**Adenocarcinoma:** Adenocarcinoma is a malignant neoplasm of epithelial cells in glandular or glandlike pattern.

**Adenoma:** Adenoma is a benign epithelial neoplasm in which the tumor cells form glands or glandlike structures. It does not infiltrate or invade adjacent tissues.

**Adjuvent:** Adjuvent is a substance that nonspecifically enhances or potentiates an immune response to an antigen; something that enhances the effectiveness of a medical treatment.

**Affinity:** Affinity is a measure of the bonding strength (association constant) between a receptor (one binding site on an antibody) and a ligand (antigenic determinant).

**Allele:** Allele is one of two or more alternative forms of a single gene locus. Different alleles of a gene have a unique nucleotide sequence, and their activities are all concerned with the same biochemical and developmental process, although their individual phenotypes may differ. An allele is one of several alternate forms of a gene at a single locus that controls a particular characteristic.

**Alternative Splicing:** Genes with new functions often evolve by gene duplication. Alternative splicing is another means of evolutionary innovation in eukaryotes, which allows a single gene to encode functionally diverse proteins (Kondrashov and Koonin, 2001). In other words, the alternative splicing refers to splicing the same pre-mRNA in two or more ways to yield two or more different protein products. Alternative splicing can produce variant proteins and expression patterns as different as the products of different genes. Alternative splicing either substitutes one protein sequence segment for another (substitution alternative splicing) or involves insertion or deletion of a part of the protein sequence (length difference alternative splicing). Thus, alternative splicing is a major source of functional

diversity in animal proteins. Very large types and number of proteins are required to perform immensely diverse functions in a eukaryote.

Lack of correlation between the high complexity of an organism and the number of genes can be partially explained if a gene often codes for more than one protein. Individual genes with mutually alternate, alternative exons are capable of producing many more protein isoforms than there are genes in the entire genome. A substantial amount of exon duplication events lead to alternative splicing, which is a common phenomenon. Indeed, alternative splicing is widespread in multicellular eukaryotes, with as many as one (or more) in every three human genes producing multiple isoforms (Mironov *et al.*, 1999). In other words, alternative splicing is a ubiquitous mechanism for the generation of multiple protein isoforms from single genes, resulting in the increased diversity in the proteomic world.

**Amplification:** Amplification refers to the production of additional copies of a chromosomal sequence, found as intrachromosomal or extrachromosomal DNA. Amplification is selective replication of a gene to produce more than the normal single copy in a haploid genome.

**Anaplasia:** Anaplasia results in the regression of cells and tissues to undifferentiated state (dedifferentiation) in most malignant neoplasms.

**Aneuploidy:** Aneuploidy is the abnormal condition in which one or more whole chromosomes of a normal set of chromosomes either are missing or are present in more than the usual number of copies. Aneuploidy refers to not having the normal diploid number of chromosomes.

**Annealing of DNA:** Annealing of DNA is the process of bringing back together the two separate strands of denatured DNA to re-form a double helix.

**Antibody:** Antibody (immunoglobulin) is a protein produced by B lymphocytes that recognizes a particular foreign antigenic determinant and facilitates clearance

of that antigen; antigens can also be carbohydrates, even DNA.

**Antigen:** An antigen is a foreign substance that binds specifically to antibody or T-cell receptors and elicits an immune response.

**Antigenic Determinant:** Antigenic determinant is the site on an antigenic molecule that is recognized and bound by antibody.

**Apoptosis:** Apoptosis is the capacity of a cell to undergo programmed cell death. In response to a stimulus, a pathway is triggered that leads to destruction of the cell by a characteristic set of reactions. Failure to apoptose allows tumorigenic cells to survive and thus contribute to cancer.

**Avidity:** Avidity is referred to the functional binding strength between two molecules such as an antibody and an antigen. Avidity differs from affinity because it reflects the valency of the antigen-antibody interaction.

**Carcinoma:** Carcinoma is of various types of malignant neoplasm arising from epithelial cells, mainly glandular (adenocarcinoma) or squamous cell. Carcinoma is the most common cancer and displays uncontrolled cellular proliferation, anaplasia, and invasion of other tissues, spreading to distant sites by metastasis. The origin of carcinoma in both sexes is skin, and in prostate in men and in breast in women. The most frequent carcinoma in both sexes is bronchogenic carcinoma.

**cDNA (complementary deoxyribonucleic acid):** mRNA molecules are isolated from cells, and DNA copies of these RNAs are made and inserted into a cloning vector. The analysis of that cloned cDNA molecule can then provide information about the gene that encoded the mRNA. The end result is a cDNA library.

**Chromosomal Aberration:** Chromosomal aberration is a change in the structure or number of chromosomes. The variation from the wild-type condition is either chromosome number or chromosome structure. Four major types of aberrations are deletions, duplications, inversions, and translocations. Variations in the chromosome number of a cell give rise to aneuploidy, monoploidy, or polyploidy.

**Clinical Guidelines:** Clinical guidelines are statements aimed to assist clinicians in making decisions regarding treatment for specific conditions. They are systematically developed, evidence-based, and clinically workable statements that aim to provide consistent and high-quality care for patients. From the perspective of litigation, the key question has been whether guidelines can be admitted as evidence of the standard of expected practice or whether this would be regarded as hearsay. Guidelines may be admissible as

evidence in the United States if qualified as authoritative material or a learned treatise, although judges may objectively scrutinize the motivation and rationale behind guidelines before accepting their evidential value (Samanta *et al.*, 2003). The reason for this scrutiny is the inability of guidelines to address all the uncertainties inherent in clinical practice. However, clinical guidelines should form a vital part of clinical governance.

**Clones:** A clone is a group of cells that are genetically identical to the original individual cell.

**Codon:** A codon is a three-base sequence in mRNA that causes the insertion of a specific amino acid into polypeptide, or causes termination of translation.

**Concatemers:** Concatemers are DNAs of multiple genome length.

**Constitutive Gene:** A gene whose products are essential to normal cell functioning, regardless of the life-supporting environmental conditions. These genes are always active in growing cells.

**Constitutive Mutation:** Constitutive mutation is a mutation that causes a gene to be expressed at all times, regardless of normal controls.

**Cytokines:** Cytokines are a group of secreted low molecular weight proteins that regulate the intensity and duration of an immune response by stimulating or inhibiting the proliferation of various immune cells or their secretion of antibodies or other cytokines.

**Deletion:** Deletion is a mutation involving a loss of one or more base pairs; a chromosomal segment or gene is missing.

**Dendritic Cell:** Dendritic cell is a type of antigen-presenting cell that has long membrane processes (resembling dendrites of nerve cells) and is found in the lymph nodes, spleen, thymus, skin, and other tissues.

**Determinant:** Determinant is the portion of an antigen molecule that is recognized by a complementary section of an antibody or T-cell receptor.

**Diagnosis:** Diagnosis means the differentiation of malignant from benign disease or of a particular malignant disease from others. A tumor marker that helps in diagnosis may be helpful in identifying the most effective treatment plan.

**DNA Methylation:** Genetic mutations or deletions often inactivate tumor suppressor genes. Another mechanism for silencing genes involves DNA methylation. In other words, in addition to genetic alterations, epigenetics controls gene expression, which does not involve changes of genomic sequences. DNA methylation is an enzymatic reaction that brings a methyl group to the 5th carbon position of cytosine located 5' to guanosine in a CpG dinucleotide within the gene promoter region. This results in the prevention of transcription. Usually, multiple genes are silenced by DNA methylation in a tumor.