METHODS IN MOLECULAR MEDICINE™

Molecular Diagnosis of Cancer

Methods and Protocols

SECOND EDITION

Edited by

Joseph E. Roulston
John M. S. Bartlett

HUMANA PRESS

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Second Edition

Edited by

Joseph E. Roulston

Division of Reproductive and Developmental Sciences, The University of Edinburgh, The Royal Infirmary, Edinburgh, Scotland, UK

and

John M. S. Bartlett

Division of Cancer and Molecular Pathology, University Department of Surgery, Glasgow Royal Infirmary, Glasgow, Scotland, UK

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Preface

We are currently experiencing a fundamental shift in the way in which we approach the characterization of cancer. Never before has the make up of cancer tissues and individual cells been so exhaustively researched and characterized. We are now capable of producing molecular "fingerprints" that characterize the expression of all known and unknown genes within tumors and their surrounding tissues. More than 30,000 different genes may be measured in each patient's tumor in a single experiment. Simultaneously, novel therapies that exploit the molecular roadmap have been developed and are now being offered to patients. These novel agents, such as Glivec, Herceptin, Iressa, and others, specifically target individual genes within tumors and can produce dramatic responses in some patients. These drugs are only the forerunners of a coming tidal wave of novel therapeutics that individually target specific molecules within cancer cells—more than 300 such agents are currently in phase I or II clinical trials. This is an exciting time for cancer specialists and patients alike.

However, if we have learned anything from the past 50 or more years of research into cancer, it is that Lord Beaverbrook, in founding the British national health service in the 1950s, was frighteningly prescient when he defined the primary goal of health care to be "Diagnosis, Diagnosis, Diagnosis." Now, more than ever, it is essential that appropriate diagnostic methods and approaches are applied to the selection of patients for treatment. Each of the novel agents above, and those in development, requires, almost by definition, the development of an appropriate molecular test to characterize the patients who are most likely to benefit. For example, Herceptin, which is producing dramatic effects in the treatment of advanced breast cancers, targets the HER2 oncogene. In patients who display this genetic abnormality, response rates are between 25 and 35%, in unscreened breast cancers the predicted response rate would be 3–5%. We are faced, therefore, with the likelihood of an exponential rise in requests for molecular characterization of tumors to identify gene mutations, losses, amplifications, rearrangements, and so on. Experience has shown that many diagnosticians are currently untrained in the specific technical areas critical to this relatively novel field of "Molecular Diagnostics." *Molecular Diagnosis of Cancer* aims to provide not only an academic, but also a fundamentally technical insight into this novel area of diagnostic medicine. We are particularly grateful to those who have taken time to contribute to this volume, their efforts have created a comprehensive

overview of current molecular diagnostic approaches and have, by providing detailed technical protocols, produced a laboratory handbook to facilitate the introduction of these techniques.

Although this volume does not seek to cover every possible aspect of molecular research, it does focus on specific molecular techniques that will provide an invaluable aid to those seeking to implement novel technologies into their diagnostic practice. The detailed step-by-step protocols and explanatory notes will, we hope, enable many more laboratories to enter this new and exciting arena.

In addition to those who have contributed to *Molecular Diagnosis of Cancer* we would like to thank those whose assistance and patience have greatly facilitated the production of this book. First, thanks are owed to Patricia Livani, whose hard work and organizational skills kept us on track for a timely publication of this volume. Second, our wives Dorothy and Jacqui and our children who put up with long hours in the evenings when we were closeted with our computers.

John M. S. Bartlett
Joseph Roulston

Contributors

ELAINE K. ALLAN • *Hemato-Oncology Section, Division of Cancer Science and Molecular Pathology, University of Glasgow, Glasgow, Scotland, UK*
DIANA M. BARNES • *Cancer Research UK Breast Pathology Laboratory, Guy's Hospital, London, UK*
JOHN M. S. BARTLETT • *Division of Cancer and Molecular Pathology, University Department of Surgery, Glasgow Royal Infirmary, Glasgow, Scotland, UK*
JEAN BENHATTAR • *Institute of Pathology, CHUV, Lausanne, Switzerland*
KAREN E. BIJWAARD • *Department of Cellular Pathology and Genetics, Armed Forces Institute of Pathology, Rockville, MD*
ROBERT BROWN • *Department of Medical Oncology, Beatson Laboratories, Glasgow, Scotland, UK*
LUKAS BUBENDORF • *Institute for Pathology, University of Basel, Basel, Switzerland*
PATRICE CAILLAT • *CEA Grenoble, LETI, Department of Microtechnologies, CRCC Val d'Aurelle, Montpellier, France*
K.C. ALLEN CHAN • *Department of Chemical Pathology, The Chinese University of Hong Kong, Prince of Wales Hospital, New Territories, Hong Kong*
MARK W. DRUMMOND • *Hemato-Oncology section, Division of Cancer Science and Molecular Pathology, University of Glasgow, Glasgow, Scotland, UK*
JOANNE EDWARDS • *Division of Cancer & Molecular Pathology, University Department of Surgery, Glasgow Royal Infirmary, Glasgow, Scotland, UK*
AMANDA FORSYTH • *Division of Cancer & Molecular Pathology, University Department of Surgery, Glasgow Royal Infirmary, Glasgow, Scotland, UK*
SANDRA GEBHARD • *Institute of Pathology, CHUV, Lausanne, Switzerland*
GILLIAN GIFFORD • *Department of Medical Oncology, Beatson Laboratories, Glasgow, Scotland, UK*
JAMES J. GOING • *University Department of Pathology, Glasgow Royal Infirmary, Glasgow, Scotland, UK*
BRUNO GRILLI • *Institute for Pathology, University of Basel, Basel, Switzerland*
PETER A. HALL • *Department of Pathology and Cancer Research Center, Queen's University Belfast, The Royal Hospitals, Northern Ireland, UK*
JENNI HAKKARAINEN • *Department of Pharmacology and Toxicology, University of Oulu, Oulu, Finland*

STACEY F. HOARE • *Cancer Research UK Department of Medical Oncology, University of Glasgow, Cancer Research UK Beatson Laboratories, Glasgow, Scotland, UK*
TESSA L. HOLYOAKE • *Hemato-Oncology Section, Division of Cancer Science and Molecular Pathology, University of Glasgow, Glasgow, Scotland, UK*
JORMA ISOLA • *Institute of Medical Technology, University of Tampere, Tampere, Finland*
MARIE JAROSOVA • *Department of Hemato-Oncology, University Hospital, Olomouc, Czech Republic*
YUTAKA KAWAKAMI • *Division of Cellular Signaling, Institute for Advanced Medical Research, Keio University School of Medicine, Tokyo, Japan*
PETER H. KAY • *Department of Pathology, The University of Western Australia, Nedlands, Australia*
W. NICOL KEITH • *Cancer Research UK Department of Medical Oncology, University of Glasgow, Cancer Research UK Beatson Laboratories, Glasgow, Scotland, UK*
WALTER KING • *Vysis/Abbott, Downers Grove, IL*
JACK H. LICHY • *Department of Cellular Pathology and Genetics, Armed Forces Institute of Pathology, Rockville, MD*
THIERRY LIVACHE • *CEA Grenoble, DRFM, CRCC Val d'Aurelle, Montpellier, France*
YUK-MING DENNIS LO • *Department of Chemical Pathology, The Chinese University of Hong Kong, Prince of Wales Hospital, New Territories, Hong Kong*
EVELYNE LOPEZ-CRAPEZ • *Centre de Recherche en Cancérologie, CRCC Val d'Aurelle, Montpellier, France*
MAIKO MATSUSHITA • *Division of Cellular Signaling, Institute for Advanced Medical Research, Keio University School of Medicine, Tokyo, Japan*
MARTINA MIRLACHER • *Institute of Pathology, University Hospital, Basel, Switzerland*
AILEEN J. MONAGHAN • *Cancer Research UK Department of Medical Oncology, University of Glasgow, Cancer Research UK Beatson Laboratories, Glascow, Scotland, UK*
ANDREW PEARCE • *South-East Cytogenetics Service, Lothian Universities Hospital NHS Trust, Edinburgh, Scotland, UK*
EKATERINA PESTOVA • *Vysis/Abbott, Downers Grove, IL*
MICHAEL F. PRESS • *Department of Pathology, Norris Comprehensive Cancer Center, University of Southern California, Los Angeles, CA*
ANTHONY RHODES • *Faculty of Applied Sciences, University of the West of England, Bristol, UK*

JOSEPH E. ROULSTON • *Clinical Biochemistry Section, Division of Reproductive and Developmental Sciences, The University of Edinburgh, The Royal Infirmary Edinburgh, Scotland, UK*
GUIDO SAUTER • *Institute of Pathology, University Hospital, Basel, Switzerland*
MICHAEL SCOTT • *Department of Pathology and Cancer Research Center, Queen's University Belfast, The Royal Hospitals, Northern Ireland, UK*
RONALD SIMON • *Institute of Pathology, University Hospital, Basel, Switzerland*
DOMINIC V. SPAGNOLO • *Division of Tissue Pathology, The Western Australian Centre for Pathology and Medical Research, Nedlands, Western Australia*
HELEN SPEIRS • *Molecular Endocrinology Unit, Western General Hospital, Edinburgh, Scotland, UK*
MINNA TANNER • *Institute of Medical Technology, Tampere, Finland*
JEREMY M. E. TAYLOR • *Division of Tissue Pathology, The Western Australian Centre for Pathology and Medical Research, Nedlands, Western Australia*
ELIZABETH R. UNGER • *Centers for Disease Control and Prevention, Atlanta, GA*
KIRSI H. VÄHÄKANGAS • *Unit of Toxicology, Department of Pharmacology and Toxicology, University of Kuopio, Kuopio, Finland*
SUZANNE D. VERNON • *Centers for Disease Control and Prevention, Atlanta, GA*
JUDITH A. WELSH • *Laboratory of Human Carcinogenesis, National Cancer Institute, Bethesda, MD*
LINDA M. WASSERMAN • *Division of Medical Genetics, Department of Medicine, University of California, San Diego, La Jolla, CA*
WEN-HSIANG WEN • *Department of Pathology, Norris Comprehensive Cancer Center, University of Southern California, Los Angeles, CA*
KIM WILBER • *Vysis/Abbott, Downers Grove, IL*
RIE YAMAZAKI • *Division of Cellular Signaling, Institute for Advanced Medical Research, Keio University School of Medicine, Tokyo, Japan*
DANIELA ZSOLDOS • *Apibio, Zone ASTEC, Grenoble, France*

Molecular Diagnosis of Cancer

Contents

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Prognostic and Predictive Factors

Michael Scott and Peter A. Hall

1. Introduction

Despite manifold advances in cancer care during recent times, the outlook for many patients with epithelial and mesenchymal malignancies remains poor. Hence, as cancer diagnosis and management moves into the 21st century, cancer has become the paradigm disease of the molecular era, with a burgeoning body of research into aspects of cell biology amenable to earlier molecular diagnosis and efficacious treatment.

An intrinsic component of effective management is the art (or science) of prognostication: the ability to forecast clinical outcome for the benefit of patients and their families. Prognostic factors can, therefore, be defined as objective properties that indicate the likely course or outcome of a disease process.

Given that prognostic factors can, in many instances, determine the course of treatment (ranging from curative to palliative, according to the clinical context), one of the many developing roles of the histopathologist within the multidisciplinary team environment is the assimilation of a spectrum of data arising from traditional morphology in conjunction with relevant immunohistochemical markers and appropriate molecular studies to provide the oncologist with both a tissue diagnosis and a prognostic context into which an individual patient may be placed with confidence and accuracy. Furthermore, the pathologist can now dare to venture beyond the provision of prognosis by providing some degree of prediction of response to various therapeutic modalities.

2. Prognostic Factors in Practice

The concept of prognosis remains at the forefront of oncological theory, but despite the publication of a veritable plethora of articles investigating possible

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markers of prognosis, only a few have entered common clinical parlance for reasons that will be described in due course. This situation is likely to improve when recent advances in microarray technologies and bioinformatics are fully assimilated into clinical practice.

A key function of a prognostic factor is to provide an estimate of outcome for an individual patient. Conventional prognostic factors in oncology have been well validated over recent years, none more so than stage and histological grade, established indices that provide a convenient means of separating patient subgroups on the basis of differing probabilities of survival as embodied in the familiar concept of the 5-yr survival rate. Amplification of oncogenes such as n-*myc* in neuroblastoma provides extra information with regard to outcome in addition to the conventional parameters of stage and grade ***(1)***. Many such genetic alterations have been described in various tumors, but such detail is outside the scope of this review. A significant limitation of grade and stage is the failure of these criteria to detect patient subgroups likely to relapse or to benefit from adjuvant therapies.

Of similar importance to outcome is the planning of clinical treatment, a sterling example being the widespread adoption of the Nottingham Prognostic Index in breast cancer management ***(2,3)***.

Prognostic factors play an important role in clinical trials by providing criteria with which to define stratified randomized treatment groups, thereby ensuring analytical comparability. A further use is the detection of patients who may benefit from new therapies or adjuvant treatment, simple examples being chemotherapy for lymph node metastasis in colorectal carcinoma and tamoxifen for estrogen-receptor-positive breast carcinomas; new treatments have also been directed against tumors with loss of p53 function ***(4)***. It is evident that a great drive exists within oncology with the ultimate aim of improving outcome by harnessing the knowledge generated by prognostic and predictive factors based on new molecular targets.

To illustrate the level of interest in cancer prognosis, PubMed listed 127,168 articles on "cancer prognosis" on July 8, 2002; only 1681 of these referred to prediction. A multitude of articles exists with regard to prognosis, but as Hall and Going have concluded, "the plethora of prognostic studies leaves one disappointed by how few parameters have been accepted into clinical practice" ***(5)***.

2.1. Tumor Biology: Hidden Complexities

Consideration of reasons for the lack of well-characterized and widely accepted prognostic and predictive factors must begin with the inherent biological complexity of malignancy (compounded by inadequate methodological rigor in many studies). It seems evident that any attempt to predict the

behavior of a neoplasm by merely assaying a single molecular entity such as p53 mutations is doomed to fail given the complexity of interactions among a legion of molecular pathways. It is no surprise, therefore, that tumor stage and grade retain their place as the most reliable predictors of outcome by virtue of providing a crude combinatorial bioassay of many molecular events.

Chaos theory, if applied to neoplasia, would suggest that small molecular variations can lead to vastly different patterns of behavior. Such molecular and behavioral heterogeneity is well accepted and provides great impetus for research, particularly when one considers the morphological similarity of many tumors. Urothelial carcinomas can be divided into two broad groups by virtue of histology and behavior, namely superficial (noninvasive) and muscle invasive, the latter having a much worse prognosis. Molecular alterations in bladder cancer clearly associated with adverse outcome include inactivating mutations of p53 and retinoblastoma protein (Rb); hence, the identification of urothelial tumors with the capacity to progress is a current priority and presents a fertile field for prognostic markers ***(6)***.

This molecular heterogeneity of morphologically uniform diagnostic categories is also exemplified in borderline ovarian tumors, a subset of tumors ripe for reclassification by molecular criteria, given the widely differing outcomes seen in this peculiar and poorly understood category where some patients have an excellent prognosis when compared with others who share 5-yr survival rates akin to those with overtly malignant tumors ***(7)***.

The apparent morphological uniformity of such diagnostic categories serves as a motivating force in the search for molecular markers; the developmental concept of the "phenocopy" illustrates this problem, a phenocopy being a mutation having a particular phenotype identical to that caused by a different mutation. Hence, tumors of a particular histological type may be phenocopies but may, therefore, behave very differently given their underlying molecular heterogeneity.

Although a great desire to uncover molecular correlates of tumor behavior undoubtedly exists, our knowledge of genetic events in cancer has made relatively little impact in clinical practice; in breast cancer, none of the described genetic changes have, until recently, defined a subset of patients requiring different therapy ***(8)***. HER-2 amplification is a significant discovery with potential for real clinical impact ***(9)***.

A track record of relatively slow progress points to the need for stronger clinico-pathological frameworks in prognostic studies. There can be no substitute for simple and testable hypotheses, realistic and achievable goals within the context of appropriately designed studies.

It is evident that new molecular techniques will generate vast quantities of data that require interpretation if clinical benefit is to eventuate; there is no

place for unfocused data trawling in the absence of a clear hypothesis. The success of the Human Genome Project depends on new bioinformatic technologies to utilize these data in a meaningful way, particularly with regard to gene expression patterns *(10)*.

2.2. Microarray Technology

DNA microarray technology is an exciting development with almost limitless potential within the context of cancer prognosis studies by means of gene expression profiling in tumors *(11)* or population-based polymorphism analysis *(12)*. The rationale of visualizing these "transcriptome snapshots" is that patterns of gene expression may point toward new genetic targets for therapeutic manipulation or provide an indication of drug resistance.

In essence, DNA microarrays allow simultaneous expression analysis of thousands of genes by hybridization of labeled cDNA (reverse-transcribed from mRNA) to specific cDNA or oligonucleotide substrates. Analysis of hybridized target cDNAs provides data on relative levels of gene expression and the presence of polymorphisms or mutations. Hence, gene profiles can be created for different tumors by analyzing the entire transcriptome of a neoplasm *(13)*. An effective application of microarray technology has been the genetic profiling of diffuse large B-cell lymphomas with the subsequent identification of two tumor groups with distinct genetic "fingerprints," each group having significantly different prognoses *(14)*. Global gene expression profiling has more recently been applied to Barrett's esophagus and esophageal adenocarcinoma with intriguing results by hierarchical cluster analysis *(15)*. Such advances are both encouraging and exciting, with much potential for providing specific treatments for tumor subtypes.

With regard to methodological considerations, DNA microarrays represent a particularly powerful resource given that they provide unbiased detection of genetic variations; such objective means of analysis is a valuable commodity. The focus of prospective researchers can, therefore, be turned upon the selection of clinically robust patient groups for study; without well-characterized study material, microarray technology is in danger of being exploited with the generation of meaningless data. Moreover, experimental data used to suggest a hypothesis must be validated in a second dataset (i.e., data independent of the data that suggests the original hypothesis). Many prognostic studies are severely flawed by such attempts to both suggest and prove a hypothesis by utilizing a single dataset.

Microarray technology is at present expensive and of limited availability. The majority of DNA microarrays have previously utilized RNA from fresh tissue, which is then reverse-transcribed and labeled prior to hybridization; significant advances have been made in the extraction of RNA from formalin-

fixed paraffin-embedded tissue with the hope of utilizing archival mRNA for microarray analysis. This would allow the vast potential of archival tissue in pathology departments to be realized in prognostic studies (subject to ethical approval) ***(16)***. Although much progress has been made by the Human Genome Project, most of the identified genes are only partially characterized; many genes with potential for prognostication in cancer probably remain unrecognized. As we have seen, current research efforts are directed toward subclassification of malignancies based on gene expression profiles. Breast cancer is an area of great interest at present in an attempt to refine treatment protocols by molecular characterization ***(17)***.

Tissue arrays are a related development with similar potential for revolutionizing cancer prognosis and predictive studies. Given that cDNA microarrays are expensive and time-consuming to manufacture and that the majority rely on frozen tissue, pathologists have traditionally measured protein expression in formalin-fixed paraffin sections using immunohistochemistry for the evaluation of diagnostic and prognostic markers. Archival tissue also has the advantage of long-term follow-up data in many cases, coupled with the availability of large numbers of cases in surgical pathology departmental archives.

Although conventional immunohistochemistry is widely used in prognostic studies, such approaches can be time-consuming and impractical for high-throughput studies ***(18)***. This has precipitated the development of tissue arrays comprised of small cores from large numbers of paraffin-embedded tumor samples arranged in an ordered array in a new paraffin block ***(19)***. A single section can therefore contain hundreds of individual tumor samples, thereby permitting simultaneous immunohistochemical analysis to be performed on a large sample size; this carries obvious benefits with regard to standardization of test conditions. A primary benefit of this new tool is the rapid characterization of expression patterns of protein targets for new antibodies, allowing comparison with existing markers in the same sample set. Tissue arrays have the potential to take immunohistochemical studies into a new dimension in terms of sample size, scope, and speed of throughput.

Benefits thus far of the microarray revolution include the distinction of a subset of breast carcinomas with a basal epithelial cell phenotype characteristic of poor clinical outcome ***(17)*** and an estrogen-receptor-positive breast cancer subset surprisingly associated with a very poor outcome ***(20)***.

Although this serves to illustrate the power of microarray technology in uncovering markers of molecular heterogeneity with possible prognostic significance, the reader should bear in mind the need to validate gene expression profiling using prospective studies of satisfactory quality. Furthermore, retrospective validation by tissue arrays is a step of major importance in the search