M.A. Hayat
Editor

Tumors of the Central Nervous System

Volume 5
Astrocytomas, Hemangioblastomas, and Gangliogliomas

Springer

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Astrocytomas, Hemangioblastomas, and Gangliogliomas

Edited by

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"Although touched by technology, surgical pathology always has been, and remains, an art. Surgical pathologists, like all artists, depict in their artwork (surgical pathology reports) their interactions with nature: emotions, observations, and knowledge are all integrated. The resulting artwork is a poor record of complex phenomena."

Richard J. Reed MD

Preface

It is recognized that scientific journals and books not only provide current information but also facilitate exchange of information, resulting in rapid progress in the medical field. In this endeavor, the main role of scientific books is to present current information in more detail after careful additional evaluation of the investigational results, especially those of new or relatively new therapeutic methods and their potential toxic side-effects.

Although subjects of diagnosis, drug development, therapy and its assessment, and prognosis of tumors of the central nervous system, cancer recurrence, and resistance to chemotherapy are scattered in a vast number of journals and books, there is need of combining these subjects in single volumes. An attempt will be made to accomplish this goal in the projected ten-volume series of handbooks.

In the era of cost-effectiveness, my opinion may be minority perspective, but it needs to be recognized that the potential for false-positive or false-negative interpretation on the basis of a single laboratory test in clinical pathology does exist. Interobservor or intraobservor variability in the interpretation of results in pathology is not uncommon. Interpretative differences often are related to the relative importance of the criteria being used.

Generally, no test always performs perfectly. Although there is no perfect remedy to this problem, standardized classifications with written definitions and guidelines will help. Standardization of methods to achieve objectivity is imperative in this effort. The validity of a test should be based on the careful, objective interpretation of the tomographic images, photo-micrographs, and other tests. The interpretation of the results should be explicit rather than implicit. To achieve accurate diagnosis and correct prognosis, the use of molecular criteria and targeted medicine is important. Equally important are the translation of molecular genetics into clinical practice and evidence-based therapy. Translation of medicine from the laboratory to clinical application needs to be carefully expedited. Indeed, molecular medicine has arrived.

This is the fifth volume in the series, Tumors of the Central Nervous System. As in the case of the four previously published volumes, this volume mainly contains information on the diagnosis, therapy, and prognosis of brain tumors.Various aspects of three types of brain tumors (Astrocytomas, Hemangioblastoma and Ganglioglioma) are discussed. Insights into the understanding of molecular pathways involved in tumor biology are explained, which lead to the development of effective drugs. Information on pathways facilitates targeted therapies in cancer. Tumor models are also presented, which utilize expression data, pathway sensitivity, and genetic abnormalities, representing targets in cancer.

Advantages and limitations of chemotherapy (e.g., Cisplatin/carboplatin combination) for patients with pilomyxoid astrocytoma are discussed. Identification and characterization of biomarkers, including those for metastatic brain tumors, are presented. Genomic analyses for identifying clinically relevant subtypes are included. A number of imaging modalities, including time-resolved laser fluorescence spectroscopy and magnetic resonance- guided laser interstitial thermal therapy are detailed to diagnose and treat brain tumors.

Introduction to new technologies and their applications to tumor diagnosis, treatment, and therapy assessment are explained. For example, nanotechnology-based therapy for malignant tumors of the CNS is explained. Molecular profiling of brain tumors to select therapy in clinical trials of brain tumors is included. Several surgical treatments, including resection, and radiosurgery, are discussed. The remaining two volumes in this series will provide additional recent information on this and other aspects of other types of CNS malignancies.

By bringing together a large number of experts (oncologists, neurosurgeons, physicians, research scientists, and pathologists) in various aspects of this medical field, it is my hope that substantial progress will be made against this terrible disease. It would be difficult for a single author to discuss effectively the complexity of diagnosis, therapy, and prognosis of any type of tumor in one volume. Another advantage of involving more than one author is to present different points of view on a specific controversial aspect of the CNS cancer. I hope these goals will be fulfilled in this and other volumes of this series. This volume was written by 85 contributors representing 14 countries. I am grateful to them for their promptness in accepting my suggestions. Their practical experience highlights their writings, which should build and further the endeavors of the reader in this important area of disease. I respect and appreciate the hard work and exceptional insight into the nature of cancer provided by these contributors. The contents of the volume are divided into seven subheadings: Introduction, Diagnosis and Biomarkers, Therapy, Tumor to tumor cancer, Imaging methods, Prognosis, and Quality of life for the convenience of the reader.

It is my hope that the current volume will join the preceding volumes of the series for assisting in the more complete understanding of globally relevant cancer syndromes. There exist a tremendous, urgent demand by the public and the scientific community to address to cancer, diagnosis, treatment, cure, and hopefully prevention. In the light of existing cancer calamity, government funding must give priority to eradicating this deadly malignancy over military superiority.

I am thankful to Dr. Dawood Farahi and Dr. Kristie Reilly for recognizing the importance of medical research and publishing through an institution of higher education.

Union, New Jersey M.A. Hayat
April 2011

Contents

Contents of Volume 1

Contents of Volume 2

Contents of Volume 3

Contents of Volume 4

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Part I
Astrocytomas: Diagnosis and Biomarkers

Chapter 1

Methylation in Malignant Astrocytomas

María del Mar Inda, Juan A. Rey, Xing Fan, and Javier S. Castresana

Abstract The term epigenetics is used to describe the study of stable and heritable alterations in gene expression potential that arise during development and cell proliferation. Two epigenetic mechanisms have been thoroughly investigated in the past few years: DNA methylation and histone modifications. The failure of the maintenance of these heritable epigenetic marks can lead to inappropriate activation or inactivation of signaling pathways and result in disease, such as cancer. Promoters of tumor suppressor genes have been assessed for hypermethylation with a variety of techniques, both at specific loci or genome wide. Methylation of the *MGMT* gene, which favors treatment results with temozolomide, is a clear example of the influence of methylation in a specific gene in astrocytomas. At the clinical level, the emphasis is now on combining inhibitors of DNA methyl transferases and of histone deacetylases.

Keywords DNA methylation · CpG islands · DNMT · O^6-Methylguanine · MGMT methylation

Understanding the Word Epigenetics

Even though they are genetically identical, cells from a multicellular organism present differential gene expression and are structurally and functionally heterogeneous. These differences occur during development and are retained throughout mitosis. They do not involve mutations of the DNA itself and are referred to as epigenetic alterations. Originally, the term epigenetics, which literally means outside conventional genetics, was defined as the casual interactions between genes and their products, which bring the phenotype into being (Waddington, 1942). Nowadays, the term epigenetics is used to describe the study of stable and heritable alterations in gene expression potential that arise during development and cell proliferation (Jaenisch and Bird, 2003). Two epigenetic mechanisms have been thoroughly investigated in the past few years: DNA methylation and histone modifications.

Epigenetic mechanisms are essential for development and differentiation, but they can also arise in adults, either by random change or under the influence of the environment, allowing the organism to respond to the environment by modulating gene expression. The failure of the maintenance of these heritable epigenetic marks can lead to inappropriate activation or inactivation of signaling pathways and result in disease, such as cancer.

DNA Methylation

Methylation might be responsible for the stable maintenance of a particular gene expression pattern through mitotic cell division. Ample support to this hypothesis has been provided and now, DNA methylation is recognized as an important mechanism for establishing a silent chromatin state by collaborating with proteins that modify nucleosomes. These epigenetic modifications can be copied after DNA synthesis, resulting in heritable changes in chromatin structure. Genes

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can be transcribed from methylation-free promoters even though adjacent transcribed and non-transcribed regions are extensively methylated.

In mammals, DNA methylation is predominantly found in cytosines of the dinucleotide sequence CpG and consists in the addition of a methyl group to the 5′-position of cytosines, altering the appearance of the major grove of DNA to which DNA binding proteins bind. CpG dinucleotides are not evenly distributed in the genome but rather are concentrated in short CpG-rich DNA stretches called CpG islands, defined as regions of DNA greater than 200 bp, with a C + G content >50%, and an observed/expected presence of CpG >60%.

In non-embryonic cells, methylation is found in approximately 80% of CpG dinucleotides. An exception for this global methylation of the genome are the CpG islands. The majority of CpG islands are associated with genes unmethylated in the germline and often located within promoter regions of genes. Approximately 60% of the human gene promoters contain CpG islands at the 5′ end. How CpG islands in non-embryonic cells remain unmethylated is still unknown, but it is known that in cancer cells, methylation of CpG islands contributes to gene silencing of tumor suppressor genes. Methylation of certain CpG island promoters during development, resulting in long-term transcriptional silencing, has been observed.

Relevance of DNA Methylation in Normal Cells

The relevance of DNA methylation in mammal development has been demonstrated by targeted mutagenesis of the different DNA methyltransferases (DNMT) genes in mice (Bestor, 2000). Genes involved in the establishment, maintenance or interpretation of genomic methylation pattern are essential for normal development. The first *Dnmt* to be discovered was *Dnmt1* and it seems to act as a maintenance methyltransferase. *Dnmt1* knock-out mice resulted in global demethylation and embryonic lethality (Li et al., 1992). In contrast, *Dnmt3a* and *Dnmt3b* are highly expressed in mouse embryo and are responsible for global de novo methylation after implantation. No obvious phenotype has been observed in mice after deletion of *Dnmt2*, but this gene is highly expressed during oogenesis and lacks biochemically detectable methyltransferase activity, but it seems to be responsible for the small amount of non-CpG methylation observed in the fly embryo (Lyko et al., 2000).

CpG islands can normally be methylated in four cases: imprinted genes, X-chromosome inactivation in women, germline-specific genes, and tissue specific genes. X-chromosome inactivation in women is a well-characterized developmental phenomenon associated with DNA methylation in CpG islands assuring monoallelic gene expression (Jaenisch and Bird, 2003). The X-inactivation process and the genomic imprinting share some epigenetic mechanisms. The choice of the inactive X-chromosome and the initiation of the inactivation depends on *Xist* RNA, a noncoding transcript that originates at the X inactivation center (Xic) and coats the inactive X chromosome. *Dnmt1* activity is needed for the maintenance of imprinting as well as for the X inactivation. Some studies suggest that *Dnmt3L*, which has no detectable methyltransferase activity, is required to establish maternal imprinting through the cooperation with de novo methyltransferase *Dnmt3a.* Some tissue-specific gene silencing through CpG island methylation has been reported in a variety of somatic tissues to silence these tissue-specific genes in tissues that should not express them, a well characterized example are the methionine adenosyltransferases 1A and 2A in rodents. A similar case are the germline-specific genes to restrict the expression of these genes to the male or the female germline and that later in the adult tissues will not be expressed, such as MAGE and LAGE gene families.

Another interesting function for the normal DNA methylation is its role in repressing parasitic sequences. The methylation of the parasitic promoters inactivates them; over time, and thanks to the promutagenicity of the methylated cytosine, cytosines can be substituted by thymidine and destroy many transposons.

Epigenetics, Environment, Diet and Aging

Epigenetic states are reversible and can be modified by environmental factors, diet and ageing and may contribute to the development of abnormal phenotypes. In addition, normal response to certain environmental

stimuli may be mediated by epigenetic mechanisms. In mammals, hypo- and hypermethylation have been associated with ageing; however, the functional significance remains to be determined. It is known that age is a major risk factor for cancer development, probably through the methylation of CpG islands and silencing of tumor suppressor genes. Some examples of genes hypermethylated in ageing individuals are estrogen receptor, *IGF2* and *MYOD* (Jaenisch and Bird, 2003). In addition, some dietary supplements, such as folate or vitamins, can affect the activity of enzymes supplying methyl groups for the methylation processes and influence the rate of disease manifestation. A methyl-deficient diet has been shown to induce liver cancer associated with both hypomethylation and the enhanced expression of oncogenes such as *c-ras*, *c-myc* or *c-fos* (Dizik et al., 1991).

DNA Methylation in Human Disease and Cancer

The failure of the maintenance of the DNA methylation or disruption of its machinery can be the cause of disease or cancer. Mutations in the *DNMT3B* gene, the human homolog of *Dnmt3b*, cause the ICF syndrome (immunodeficiency, centromeric region instability, and facial abnormalities), a human heritable genetic disease with deficient methylation of the pericentromeric repetitive DNA and at CpG islands of the X chromosome. Another X-linked neurological disorder, the Rett's syndrome, is due to a failure in DNA methylation-related system; more specifically, it is due to mutations in the methyl binding protein MeCP2, responsible for recruiting histone deacetylases (HDACS) and other chromatin factors to methylated DNA. These two diseases suggest that DNA methylation is not only needed to complete embryonic development, but it is also required for development after birth.

DNA methylation plays a critical role in the development and differentiation of mammalian cells, and its deregulation has been involved in oncogenesis. The alteration of the DNA methylation pattern results in global dysregulation of gene expression profiles, leading to the development and progression of cancer. Since these alterations are hereditable, cells with epigenetic alterations conferring a growth advantage are rapidly selected and result in uncontrolled tumor growth. Cancer can be considered to be a genetic disease at the same level as an epigenetic disease, and DNA methylation can be an excellent candidate to explain how certain environmental factors or ageing can increase the risk of cancer. In fact, DNA methylation plays an essential role in all three mechanisms by which cancer cells eliminate tumor suppressor gene function: point mutation, silencing by promoter hypermethylation and deletion by LOH due to genomic instability (Gronbaek et al., 2007).

CpG sites have been considered to be mutation hotspots in the human germline and recently, it has become apparent that they are also hotspots for inactivating mutations of tumor suppressor genes such as p53 which is mutated in CpG sites in 25% of the cases. That CpG dinucleotides constitute hotspots for point mutations is due to the fact that methylated cytosines can be spontaneously deaminated to thymine and result in a C–T transition. If C–T transitions are not repaired and occur in the coding region of genes, they may activate an oncogene or suppress a tumor suppressor gene (Gronbaek et al., 2007). More than 30% of the point mutations in the germline related to disease occur at CpG dinucleotides. For example, in colorectal cancer, 44% of the mutations are C–T transitions. In addition, methylated cytosines also favor the formation of adducts on the neighboring G in the presence of some carcinogens, such as the benzo(a)pyrene present in tobacco smoke, resulting in a G–T transversion (Gronbaek et al., 2007).

Commonly, cancer cells are characterized by global genomic hypomethylation and hypermethylation of CpG islands that are generally unmethylated in normal cells. DNA hypomethylation plays a critical role in tumorigenesis and may lead to the upregulation and activation of oncogenes, such as *R-Ras* and *MAPSIN* in gastric cancer, or *MAGE* in melanoma. The mechanisms by which DNA methylation can contribute to tumorigenesis can be summarized in three: reactivation of retrotransposons, increasing chromosomal instability, and loss of imprinting. DNA hypomethylation can allow the transcription and/or translocation of retrotransposons, increasing the genomic instability, or lead to the upregulation of oncogenic microRNAs. Loss of methylation has been observed in Alu repeats and in *LINES* in cancer cells, and some imprinted genes, such as *H19* or *IGF-2*, present loss of methylation in pediatric tumors. Hypomethylation may allow

the formation of chromosomal breaks, translocations and/or allelic loss by illegitimate mitotic recombination, and the demethylation in pericentromeric regions of chromosomes plays a role in aneuploidy.

In contrast to DNA hypomethylation which can lead to the activation of proto-oncogenes or increase genomic instability, hypermethylation of CpG islands that are unmethylated in normal cells leads to inactivation of tumor suppressor genes by silencing their expression, and several reports have shown a correlation between expression and loss of DNA methylation. How these genes are targeted for hypermethylation still remains unclear, and in some tumors, silencing by promoter hypermethylation occurs at a very high frequency. Many genes of key pathways in cancer are affected by promoter hypermethylation; however, methylation of the downstream gene sequences usually has no effect on gene expression (Jones, 1999). Examples of tumor suppressor genes silenced by hypermethylation were found in cancer and include: *MGMT*, *Rb*, $p16^{Ink4a}$, *BRCA1*, $p14^{ARF}$, *APC*, retinoic acid receptor-$\beta 2$, *RASFF1*, etc (Gronbaek et al., 2007). Recently, experimental data has provided support to the idea that genes can be transcriptionally activated by removing DNA methylation (Baylin et al., 1998, 2001; Lorente et al., 2009), providing an attractive target for cancer therapeutics.

Methods to Detect Methylation

Aberrant methylation is the most common alteration found in cancer cells, while silencing of tumor suppressor genes by CpG island promoter hypermethylation is the change of DNA methylation most studied in neoplasms. The detection of methylation in clinical samples (Table 1.1) may be useful in the early detection of cancer screening; therefore, it has become the focus of research in many clinical and translational laboratories. The reason for this is partially due to the early occurrence of alterations in the methylation pattern (hypo or hypermethylation) in carcinogenesis. Furthermore, since they are DNA markers, they are more stable than RNA or proteins, and studies can be performed in formalin-fixed and paraffin-embedded tissues (Fan et al., 2002). It has been demonstrated that DNA methylation can be detected in blood, sputa, ductal lavage fluids, urine, saliva, mammary aspiration fluid, stool, and biopsy specimens by using highly sensitive PCR-based methods after bisulfite modification. In addition to being a tumor-specific change, different tumor types have different DNA methylation profiles that are helpful in diagnosing difficult cases (Shames et al., 2007). In glioblastoma multiforme, the detection of methylation in the promoter of the MGMT gene (O^6-methylguanine-DNA methyltransferase) predicts a favorable outcome in patients treated with alkylating agents (Hegi et al., 2005).

The initial studies of DNA methylation relied on the use of methylation-sensitive restriction enzymes that were able to distinguish between unmethylated and methylated recognition sites and Southern blot hybridization. This approach has many drawbacks: the limitation of the sites that can be analyzed, the problem of incomplete restriction cutting, the necessity of using high-molecular weight and elevated amounts of DNA to perform the Southern blot analysis, and the fact that the method is labor-intensive. In addition, only CpGs located within sequences recognized by methylation-sensitive enzymes can be analyzed.

The majority of the methods used to detect DNA methylation are based on the chemical modification of DNA with sodium bisulfite followed by PCR with primers specific for methylated sequences. These methods, especially the ones that use primers designed specifically to amplify the methylated sequence, provide a very sensitive and specific analytical tool for detecting methylation at single loci. The treatment of DNA with sodium bisulfite deaminates cytosines to uracil, and because deamination of 5-methylcytosine is much slower, it is generally assumed that only unmethylated cytosines are transformed. There are three processes in the DNA modification by the bisulphite reaction: the reversible cytosine sulphonation, the irreversible hydrolytic deamination of the sulphonated cytosine, and the removal of the bisulfite adduct to give uracil by alkali treatment (Clark et al., 1994). It has been determined that the conversion rate under ideal conditions of unmethylated cytosines is about 99% (Taylor et al., 2007). Several groups have worked on optimizing the bisulfite treatment (Cottrell et al., 2004; Fan et al., 2002; Grunau et al., 2001). Once DNA is treated and modified with sodium bisulfite, different techniques can be used so as to make it possible for every laboratory and hospital to assess DNA methylation. Bisulfite sequencing provides a quantitative way to determine the methylation state of a genomic

Table 1.1 Comparison among some of the different techniques to detect methylation

Method	Specimen treatment	Application	Sensitivity	Quantitative	Advantages	Disadvantages
Bisulfite sequencing	Bisulfite conversion	Specific locus	Low	Yes	Methylation status of individual CpG sites can be analyzed	Expensive and time-consuming
Southern blot	Methylation-sensitive enzyme	Genome-wide	Low	No	Easy to perform	Limited sites available, needs high amounts of high quality DNA and is labor intensive
RLGS	Methylation-specific restriction enzyme	Genome-wide	Low	Yes	Reproducible	Needs high quality DNA
ChIP-on-chip	Immnunoprecipitation + array	Genome-wide	Low	Yes	Novel marker discovery	No correlation with expression
MSP	Bisulfite conversion	Specific locus	High	No	Cost-effective and needs small amounts of DNA	False positives and does not allow discrimination between unmethylated and partially methylated
Q-MSP or Methylight	Bisulfite conversion	Specific locus	High	Yes	Easy and high throughput	Does not allow discrimination between unmethylated and partially methylated
Heavymethyl	Bisulfite conversion	Specific locus	High	Yes	Low false positives and high throughput	Many oligonucleotides are used
MALDI-TOF MS	Bisulfite conversion	Genome-wide/specific locus	Medium	Yes	Quantitative data on individual CpG sites can be obtained	Expensive equipment required

region at a single-nucleotide resolution and is the gold standard of the methods based on the bisulfite DNA treatment. Unfortunately, this method is too expensive and time consuming to be used in a clinical setting. In this chapter we will discuss the methods most often used for detecting methylation at a single locus or multiple loci, as well as genome-wide (Table 1.1).

The most widely used assay for sensitively detecting methylation is called methylation-specific PCR (MSP) (Herman et al., 1996). Before PCR amplification, genomic DNA is modified by sodium bisulfite treatment in order to convert all unmethylated cytosines to uracil which, after amplification, will be transformed into thymidine. Two sets of primers are designed for