

Invited Review

Toward a molecular classification of the gliomas: histopathology, molecular genetics, and gene expression profiling

L.S. Caskey, G.N. Fuller, J.M. Bruner, W.K.A. Yung, R.E. Sawaya, E.C. Holland and W. Zhang

Departments of Pathology, Neuro-Oncology, and Neurosurgery, The University of Texas M.D. Anderson Cancer Center, Houston, Texas, USA

Summary. As many as 100,000 new cases of brain tumor are diagnosed each year in the United States. About half of these are primary gliomas and the remaining half are metastatic tumors and non-glial primary tumors. Currently, gliomas are classified based on phenotypic characteristics. Recent progress in the elucidation of genetic alterations found in gliomas have raised the exciting possibility of using genetic and molecular analyses to resolve some of the problematic issues currently associated with the histological approach to glioma classification. Recently, immunohistochemical studies using novel proliferation markers have significantly advanced the assessment of tumor growth potential and the grading criteria of some tumor subtypes. Preliminary studies using cDNA array technologies suggest that the profiling of gene expression patterns may provide a novel and meaningful approach to glioma classification and subclassification. Furthermore, cDNA array technologies may also be used to identify candidate genes involved in glioma tumor development, invasion, and progression. This review summarizes current glioma classification schemes that are based on histopathological characteristics and discusses the potential for using cDNA array technology in the molecular classification of gliomas.

Key words: Classification, Gliomas, cDNA microarray

Introduction

Gliomas are the most common malignant primary brain tumors. They are derived from neuroepithelial cells and are extremely diverse with respect to location, morphology, differentiation, and response to therapy. They can be divided into two major lineages: astrocytic

tumors and oligodendrogliomas. Recently, the Central Brain Tumor Registry of the United States obtained 5 years of incidence data (1990-1994). Among the primary brain tumors, a significant percentage of gliomas (>40%) were reported. An estimated 13,300 deaths in 1998 were attributed to primary malignant brain tumors (CBTRUS, 1998).

Diagnosis of tumors is conventionally achieved by assigning a presumed cell of origin and grade based largely on morphological criteria, such as the size, shape and pleomorphism of the tumor cells, the presence or absence of mitotic figures, vascular proliferation and necrosis, and pattern recognition of various architectural features that are traditionally associated with a given tumor type. However, accurate diagnosis is often problematic because the histologic features of most gliomas are heterogeneous and agreement on consensus criteria for the diagnosis and, particularly, the grading, of several glioma subtypes has been difficult to achieve. Lack of consensus on objective grading and classification criteria breeds subjectivity in diagnosis. A more objective classification system has long been needed to augment and complement the traditional histopathological approach.

Recent developments in immunohistochemical techniques have allowed a more accurate assessment of proliferation indices, and early attempts to incorporate these into glioma grading systems have been made (Fuller and Burger, 1996a). Historically, a number of markers have been employed, including: (1) Ki-67, a nuclear nonhistone protein that is expressed by actively dividing cells, (2) proliferating cell nuclear antigen (PCNA/cyclin), an auxiliary protein of DNA polymerase δ , and (3) bromodeoxyuridine (BrdU), a thymidine analogue that is incorporated into DNA during the S phase of the cell cycle (Vandenberg, 1992; Fuller, 1996a; Cavalla and Schiffer, 1997). The currently preferred and most widely used marker is that detected by the monoclonal antibody MIB-1, which recognizes an epitope on the Ki-67 antigen. The principal advantage of this marker over the earlier Ki-67 monoclonal antibody

Offprint requests to: Wei Zhang, Ph.D., Cancer Genomics Core Laboratory, Department of Pathology, The University of Texas M.D. Anderson Cancer Center, Box 85, 1515 Holcombe Blvd., Houston, TX 77030. Fax: (713) 792-5549. e-mail: wzhang@notes.mdacc.tmc.edu.

is that MIB-1 is reactive in formalin-fixed, paraffin-embedded tissue samples, whereas the Ki-67 antibody requires frozen tissue for optimal reactivity. Several different groups have proposed incorporating MIB-1 labeling indices into grading systems for the gliomas (Kros et al., 1996; Coons et al., 1997; Hsu et al., 1997; McKeever et al., 1998).

In a different arena, advances are rapidly being made in the understanding of the genetic alterations in brain tumors. For example, loss of heterozygosity (LOH) for large regions at 10p, 10q23, and 10q25, or loss of an entire copy of chromosome 10 are the most frequent genetic alterations found in glioblastomas (von Deimling et al., 1993; Steck et al., 1995; Albarosa et al., 1996). One of the potential tumor suppressor genes located on chromosome 10, *PTEN/MMAC1*, has been recently cloned and characterized (Li et al., 1997; Steck et al., 1997).

Similarly, evaluation of oligodendrogliomas has revealed an incidence of LOH for chromosomes 1p and 19q of 80-90% (Reifenberger et al., 1994). Genetic alterations may affect treatment strategies. In the case of oligodendroglioma, tumors with the characteristic 1p and 19q deletions respond better to combination chemotherapy (procarbazine, cytoxan, vincristine, or PCV) compared to oligodendrogliomas that lack this molecular signature (Cairncross et al., 1998; Paleologos and Cairncross, 1999). An understanding of gene activities is thus rapidly becoming an indispensable component of tumor diagnosis, classification, and treatment. Because cancer cells often have heterogeneous molecular interactions and the total transcript number in a given cell is 50,000-100,000, it is not feasible to fully characterize cancers one gene at a time. However, recent developments in genechip technology that permit parallel analysis of thousands of genes simultaneously will make it possible to study genome-wide gene expression in individual tumor samples. Thus, it may soon be possible to develop a cancer classification system that is based on gene expression profiles. The anticipated synergistic combination of molecular classification, clinical information and traditional histopathological assessment may significantly improve diagnosis, therapeutic intervention, and ultimately clinical outcome.

This review will describe the current classification of gliomas that is based on morphological criteria, the molecular genetics of gliomas, and the current methods of comparing gene expression profile changes in different histological specimens. Because numerous reviews have been written describing glioma histopathology (Burger and Scheithauer, 1994; Fuller and Burger, 1996a; Bigner et al., 1998) and molecular genetics (Furnari et al., 1995; von Deimling et al., 1997; Rasheed et al., 1999), this review will focus primarily on recent studies discussing the application and potential of gene expression profiling in the study and classification of gliomas.

Histopathology

Low grade astrocytoma

Low grade astrocytomas typically present in the third or fourth decade and most commonly originate in the white matter of the cerebral hemispheres. A characteristic feature is an indistinct, diffusely infiltrating margin with the surrounding brain parenchyma, which is the principal impediment to complete surgical resection. Microscopically, low grade astrocytomas vary from very well-differentiated tumors, in which the histologic features need to be distinguished from reactive gliosis, to more cellular and pleomorphic lesions, which must be distinguished from astrocytomas of higher grade. The nuclei of low grade astrocytomas are typically uniformly round-to-oval with a delicate chromatin pattern. Mitotic figures are very rare or absent; in most contemporary grading schemes, the presence of mitoses elevates the tumor to an anaplastic category (Fuller, 1996b). Neuropil vacuolation and microcysts may also be present (Fuller and Burger, 1996b).

Anaplastic astrocytoma

Similar to low grade astrocytoma, anaplastic astrocytoma (AA) primarily arises in the cerebral hemispheres, but may also occur in the brain stem and spinal cord. AAs are intermediate with respect to age of highest incidence (40-50 years), duration of preoperative symptoms, and degree of macroscopic abnormality compared to low grade astrocytomas and glioblastomas (Burger et al., 1985). It is often not possible to grossly distinguish between a low grade astrocytoma and an anaplastic astrocytoma, and grading is dependent on histological examination. However, the higher cellularity of the anaplastic astrocytoma sometimes produces a sharper circumscription from surrounding brain structures compared to low grade astrocytoma. Microscopically, AAs are more cellular and pleomorphic than low grade astrocytomas, and mitotic figures are present (Scherer, 1940). Some grading systems permit the presence of microvascular proliferation in AAs, whereas in other systems this feature, together with pleomorphism and the presence of mitotic figures, would require a diagnosis of glioblastoma (Fuller, 1996b). Depending on the particular grading scheme employed, the criteria that distinguish between the different grades of astrocytoma are often imprecise and highly subjective. Moreover, the type of surgical procedure used to obtain tissue also impacts the diagnosis: because astrocytomas are graded based on the worst area identified, small tissue samples, such as those obtained by stereotactic biopsy, frequently are not representative of the lesion and result in undergrading the tumor secondary to sampling bias or "error" (Glantz et al., 1991; Fuller and Burger, 1996a).

Glioblastoma multiforme

The most malignant astrocytic tumor is the glioblastoma multiforme (GBM). Unfortunately, GBM is also the most common of all gliomas, accounting for approximately half of all astrocytic tumors. GBMs most often present in the cerebral hemispheres of adults in the fifth decade or higher. They also arise in the brain stem of children and more rarely in the spinal cord or cerebellum of children and adults. Glioblastomas are composed of poorly differentiated astrocytes and may develop through anaplastic progression from lower grade astrocytomas over a period of years (secondary glioblastoma) or they may present after only a short clinical history with no evidence of a preceding lower grade tumor (primary, or *de novo*, glioblastoma). On neuroimaging studies, glioblastomas typically present as irregularly shaped lesions with a peripheral, ring-like zone of contrast enhancement surrounding a central area of necrosis (Burger et al., 1983). The contrast-enhancing ring structure corresponds to the hypercellular, highly vascularized peripheral zone of the lesion. Radiologic-pathologic correlation studies have shown that infiltrating glioma cells can be identified within and occasionally beyond a 2-3 cm margin surrounding this enhancing ring (Burger et al., 1983). Although the infiltrative nature of glioblastomas is readily apparent microscopically, in contrast to well-differentiated astrocytomas, many GBMs often appear deceptively well-defined on gross examination (Fuller and Burger, 1996a).

Microscopically, as the descriptor "multiforme" aptly implies, GBMs exhibit a very heterogeneous array of cell types, which vary greatly with respect to cell size, nuclear pleomorphism, and cytoplasmic volume and configuration (Burger et al., 1983). A number of GBM morphologic subtypes have been described, including giant cell glioblastoma, small cell glioblastoma, and epithelioid glioblastoma. Two morphologic features characterize most glioblastomas. The first is vascular or "endothelial" proliferation, in which proliferative vascular elements (endothelial cells, pericytes, smooth muscle cells and fibroblasts) form tortuously coiled masses (Haddad et al., 1992; Wesseling et al., 1993). The second feature is tumor necrosis. Neoplastic cells often surround necrotic foci in dense aggregates referred to as *pseudopalisades* (Burger and Vollmer, 1980; Burger and Scheithauer, 1994).

Oligodendrogliomas

Oligodendrogliomas are less prevalent than astrocytomas, representing approximately 4-5% of primary brain tumors (Mork et al., 1985). These tumors often exhibit a slowly progressive preoperative history (10 to 20 years). Oligodendrogliomas are most commonly found in the subcortical white matter with frequent extension into the overlying cerebral cortex (Bigner et al., 1999). Oligodendrogliomas may rarely

arise in the cerebellum, brain stem or spinal cord. Macroscopically, the lesions are diffusely infiltrating tumors and can closely resemble well-differentiated astrocytoma. Calcification is a common feature seen within the tumor and in adjacent cortex. Histologically, oligodendrogliomas display uniformly round nuclei surrounded by a clear halo (so-called "fried egg" appearance). The halos are artifacts of delayed fixation produced by water that has been osmotically drawn into the cytoplasm of ischemic tumor cells following resection (Fuller and Burger, 1997). The halo artifact is a reliable morphologic finding characteristic of oligodendrogliomas, but is not seen in tissues that are fixed rapidly, including those used for intraoperative rapid frozen section diagnosis (Fuller and Burger, 1996a). Since there are as yet no immunohistochemical markers specific for oligodendrogliomas, the diagnosis currently rests on standard light microscopic assessment of hematoxylin-eosin (H&E) stained tissue sections. Grading of oligodendrogliomas is very subjective and a number of different systems have been proposed (Smith et al., 1983; Dumas-Duport et al., 1988; Shaw et al., 1989). In general, high-grade lesions are characterized by microvascular proliferation, foci of tumor necrosis, and "brisk" mitotic activity; however, precise, objective criteria for tumor stratification have not been universally agreed upon. Moreover, disagreement exists over whether oligodendrogliomas with anaplastic features that include necrosis with pseudopalisading should be classified as high grade oligodendrogliomas or as glioblastomas. Nevertheless, clinical studies have demonstrated that there is a significant prognostic difference between high and low grade tumors: the median survival time for patients with low-grade tumors is approximately 9.8 years, compared to 3.9 years for patients with high-grade tumors (Shaw et al., 1992).

Mixed gliomas

Tumors composed of two different glial cell types are referred to as mixed gliomas. By far the most common type of mixed glioma is the oligoastrocytoma, which by definition is composed of a mixture of neoplastic astrocytes and neoplastic oligodendrocytes. Two morphologic variants have been described: 1) the "compact" form, in which the two representative tumor cell types are present as discrete, separately identifiable areas within the neoplasm, and 2) the "diffuse" form, in which the two tumor cell types are intimately intermingled with each other. In addition, there is increasing recognition of the existence of a third, "*tertium quid*", category of mixed gliomas in which every tumor cell exhibits ambiguous morphologic characteristics that are intermediate between those of classical oligodendroglioma and astrocytoma, such that it is very difficult for even experienced oncologic neuropathologists to make an unequivocal diagnosis of astrocytoma, oligodendroglioma, or mixed glioma. Currently, the diagnosis of mixed glioma is based on the

highly subjective recognition and relative weighting of the various morphological features historically associated with each neoplastic cell type.

Low grade oligoastrocytomas are moderately cellular neoplasms with low mitotic activity. Microcalcifications and microcystic change may be present. Necrosis and vascular endothelial proliferation are absent. Anaplastic oligoastrocytomas show "increased" cellularity, nuclear atypia, and pleomorphism, and "brisk" mitotic activity. Microvascular proliferation and necrosis are also frequently present. Identification of an oligodendroglial component in anaplastic gliomas has recently acquired increased significance as data showing a correlation with response to chemotherapy has been adduced (Cairncross et al., 1998). Molecular analysis is beginning to resolve some of the uncertainties inherent in the subjective histological evaluation of the gliomas and will be discussed in the next section.

Molecular genetics

There are two major classes of genes critical for the development of all types of cancer: tumor suppressor genes, which encode products that function to inhibit cell proliferation and tumor development, and oncogenes, which encode proteins that stimulate proliferation and mediate biological activities integral to invasion, neoangiogenesis, immune escape, and other characteristics of malignancy. Many of the tumor suppressor genes and oncogenes directly participate in or regulate signal transduction pathways linking extracellular stimuli to cell cycle progression and/or cell death, which are two critical areas where abnormalities occur in cancer cells. Although our understanding of the sequence and types of genetic alterations in the each of the brain tumor phenotypes is far from complete, several specific genetic changes that have been identified will be briefly discussed. For interested readers, several recent detailed reviews are available (Furnari et al., 1995; von Deimling et al., 1997; Rasheed et al., 1999).

Astrocytomas

p53 is a tumor suppressor gene located on chromosome 17 that is frequently implicated in the pathogenesis of astrocytomas (Bogler et al., 1995). The *p53* protein is a sequence-specific transcription factor that acts to induce or repress specific genes involved in multiple cellular functions, including progression through the cell cycle, DNA repair after damage, genomic instability, and the tendency for the cell to undergo apoptosis following treatment (Haffner and Oren, 1995). *p53* mutations have been reported in approximately 40% of astrocytic tumors of all grades and are most commonly found in gliomas of adults. Although *p53* mutations are rare in primary glioblastomas (<10%), they have a high incidence in secondary glioblastomas (>65%) (Watanabe et al., 1996, 1997a,b). While the prognostic implications of *p53*

mutations have not yet been defined clearly, they remain strong candidates for clinical significance in several areas, including response to irradiation, in which *p53* has recently been shown to be of importance (Haas-Kogan et al., 1999).

The *MDM2* gene (mouse double minute 2) contains a *p53* DNA binding site. Recently it has been demonstrated that, under a variety of conditions, *MDM2* transcription is induced by wild type *p53* (Barak et al., 1994; Zauberman et al., 1995). The *MDM2* protein forms a complex with *p53*, thereby abolishing its transcriptional activity; thus, in normal cells, this autoregulatory feedback loop regulates both the activity of *p53* and the expression of the *MDM2* gene (Zauberman et al., 1995). Amplification of *MDM2* is present in <10% of primary glioblastomas that lack a *p53* mutation (Refeinberger et al., 1994). However, overexpression of *MDM2* was observed in more than 50% of primary glioblastomas; in contrast, less than 10% of secondary glioblastomas showed overexpression of *MDM2* (Biernat et al., 1997; Korkolopoulou et al., 1997; Newcomb et al., 1998). Interestingly, the majority of glioblastomas contain short forms of alternatively spliced *MDM2* transcripts that lack a region containing the *p53* binding domain (Matsumoto et al., 1998).

Another mutation associated with primary glioblastomas is the lack of an intact *INK4a-ARF* tumor suppressor locus (He et al., 1995; Ichimura et al., 1996). The cells are thus unable to make the two *INK4a-ARF* gene products, *p16^{INK4a}* and *p19^{ARF}*, that arrest the cell cycle by different pathways in G1 and in both G1 and G2, respectively (Quelle et al., 1995; Serrano et al., 1996). *p16* binds to and inhibits the function of the cyclin dependent kinases CDK4 and CDK6, thereby reducing their capacity to phosphorylate the Rb protein and ultimately resulting in the loss of cell cycle control. Gliomas that progress from lower to higher grade lesions are usually wild type for *INK4a-ARF* but have an amplified *cdk4* locus or have lost the Rb gene, and frequently also display either loss of *p53* function or amplification of *MDM2*. Therefore, in both types of tumors the same two cell cycle arrest pathways are disrupted by different mutations. An *in vivo* glial-specific gene transfer system has been developed to examine the effects of individual mutations and combinations of mutations in mice (Holland and Varmus, 1998). This system, which utilizes retroviral vectors and a transgenic mouse line, has recently been used to investigate the role of *EGFR* mutation in gliomagenesis (Holland et al., 1998). The transfer of the mutant *EGFR* gene induces glioma-like lesions in mice deficient for *INK4a-ARF* but not in mice that are wild type at the *INK4a-ARF* locus. In addition, *EGFR*-induced gliomagenesis does not occur in conjunction with *p53* deficiency, unless the mice are infected with a vector carrying CDK4.

Loss of heterozygosity at the 10p, 10q23, or 10q25-26 loci, or the loss of an entire copy of chromosome 10, are the most frequent genetic alterations in glioblastomas

(von Deimling et al., 1993; Steck et al., 1995; Albarosa et al., 1996). *MMAC1/PTEN*, a tumor suppressor gene located on chromosome 10, encodes a protein that has phosphatase activity and causes growth suppression (Li et al., 1997; Steck et al., 1997). The majority of *MMAC1/PTEN* alterations are seen in primary glioblastomas (32%) and only rarely in secondary glioblastomas (4%) (Tohma et al., 1998). Thus, this gene may be important for progression to more malignant, high-grade tumors. In addition, *p53* mutations and *MMAC1/PTEN* mutations appear to be mutually exclusive (Rasheed et al., 1997; Tohma et al., 1998).

In contrast to tumor suppressor gene mutations, which result in the loss of function of proteins that normally inhibit cell proliferation, the activation of oncogenes results in the enhanced function of proteins that facilitate cell proliferation. Amplification is a common mechanism by which oncogenes are activated and is a manifestation of genetic instability. In astrocytomas, the gene most frequently found to be amplified is *EGFR* (Liberman et al., 1985; Ekstrand et al., 1992; Wong et al., 1992), which encodes a growth factor important for astrocytes. Amplification of a mutated *EGFR* allele is seen rarely in low-grade astrocytomas, but is found more frequently in about one-third of glioblastomas, especially in tumors occurring in elderly patients (Hunter et al., 1995). In addition, glioblastomas with *EGFR* amplification also have a simultaneous loss on chromosome 10 (von Deimling et al., 1992).

Oligodendrogliomas

Cytogenetic studies have found that genetic alterations associated with oligodendrogliomas are distinctive from those found in astrocytic tumors. Oligodendrogliomas characteristically exhibit a loss of chromosomal regions on 1p and 19q13 (Ritland et al., 1995). Other chromosomal regions lost from oligodendrogliomas include 1p36, 9p, and 22, and increased numbers of chromosome 7 have also been reported. Some studies have reported the incidence of 19q loss in oligodendrogliomas as high as 81% (Reifenberger et al., 1994), while LOH for 1p has been reported in up to 94% of tumors (Bello et al., 1995).

Mixed oligoastrocytomas

Currently, molecular genetic analysis fails to support the concept that the oligoastrocytoma is a tumor in which distinct populations of neoplastic astrocytes and oligodendroglia exist. Maintz et al. (1997) evaluated 38 grade 2 and 3 oligoastrocytomas and found that loss of 1p/19q and *p53* gene mutations seldom occurred in the same neoplasm. Furthermore, histologic evaluation determined that the lesions with 1p/19q loss often had predominantly oligodendroglial morphology. In addition, Kraus et al. (1995) analyzed histologically-separable oligodendroglial and astrocytic regions of three mixed

oligoastrocytomas and found that both histologies shared the 1p/19q loss in all three cases. These studies suggest that even when astrocytic and oligodendroglial elements are identified based on phenotypic features within the same lesion, most tumors will uniformly display the molecular profile of either an astrocytoma or an oligodendroglioma. The importance of correctly classifying these tumors is emphasized by the fact that oligodendrogliomas that exhibit chromosome 1p and 19q abnormalities have been reported to respond better to combination PCV chemotherapy compared to oligodendrogliomas that lack this molecular signature (Cairncross et al., 1998; Paleologos and Cairncross, 1999). Glass et al. (1992) have reported that oligoastrocytomas frequently respond to PCV. Thus, molecular genetic analysis may not only improve diagnostic and prognostic accuracy; it may also help determine specific treatment strategies.

Gene expression profiling

Although extensive research has led to the identification of some specific genetic changes that are characteristically associated with the gliomas, we are still at a very early stage with respect to a comprehensive understanding of the myriad genetic alterations involved in glioma phenotype determination. Many inferences have been drawn regarding the molecular basis of glioma genesis and biology based on the analysis of only one or a few genes. Considering that there are probably more than 100,000 expressed genes in a given glioma cell population, our single-gene based knowledge is at best fragmented and biased. Clearly, a systematic approach is needed to provide a global perspective and to put all of the disparate pieces together. In recent years, a variety of techniques have been developed that allow high-throughput analysis of differential gene expression. These include comparative expressed sequence tag (EST) sequencing, serial analysis of gene expression (SAGE), mRNA hybridization to cDNA or oligonucleotide arrays, and tissue microarrays. In this section, we will discuss the technical aspects of these technologies, followed by a discussion of their applications to the study of gene expression in cancer.

Technical aspects of gene expression technologies

One technique commonly used to analyze gene expression levels is expressed sequence tag (EST) sequencing from representative cDNA libraries. EST sequencing is accomplished by sequencing the 5' or 3' ends of cDNAs from libraries representing various tissue and cell types, which are then used to define sequence tags that uniquely identify a particular gene (Adams et al., 1991). Normalized or non-normalized cDNA libraries may be used for EST sequencing. A normalized library is one in which each transcript is present in more or less equal numbers and has the twin advantages of minimizing the redundant sequencing of highly

expressed genes and maximizing the potential for identification of rare transcripts (Bonaldi et al., 1996). The advantage of using non-normalized, non-amplified libraries, however, is that the relative abundance of the transcript in the original cell or tissue is accurately reflected in the frequency of the clones in the library (Ji et al., 1997).

Another method known as serial analysis of gene expression (SAGE) is essentially an accelerated version of EST sequencing. In this method, a unique sequence tag is generated for each transcript in the cell or tissue of interest. The sequence tags are concatenated, cloned and sequenced, thereby creating transcript profiles for each SAGE library. The frequency of a particular transcript within the starting cell population is reflected by the number of times the associated sequence tag is encountered within the sequence population (Velculescu et al., 1995). Because sequencing reactions yield information for 20 or more genes, it is possible to generate data points for thousands of transcripts. Thus, it is possible to compare SAGE tag data from a variety of samples.

Gene expression analysis utilizing cDNA array systems can be divided into three steps: sample preparation, array hybridization, and data interpretation. Although large numbers of tissue samples are available in many clinical departments, often the samples are suboptimal with respect to RNA integrity, fixation, or critical patient information. Thus, an efficient system must be in place to allow for appropriate tissue collection and storage. Tissues also need to be carefully screened by an experienced pathologist for composition and purity prior to analysis. In the second step, cDNA or oligonucleotides representing a large number of genes are deposited onto a solid support in an ordered array (Southern et al., 1992; Schena et al., 1995). The total complement of mRNA from a cell or tissue sample is used to create a radioactive or fluorescent probe that is then hybridized to the array of DNA fragments. After washing, the bound signal is quantitated and the signal for each gene is used to determine the relative abundance of each gene. Three microarray formats are currently available: filter based arrays of cDNAs (Pietu et al., 1996), oligonucleotides synthesized on solid supports using photolithography techniques (Lockhardt et al., 1996), and glass slide microarrays of cDNAs (DeRisi et al., 1996; Schena et al., 1996). High-density arrays contain ESTs of unknown genes and can therefore be used for gene discovery purposes, while low-density arrays comprised of pre-selected known genes that are grouped according to their involvement in specific processes can be very useful for examination of specific cellular pathways. A typical array experiment generates thousands of data points and creates special, serious challenges for data analysis. Databases need to be constructed specifically for managing the vast information generated for the genes represented on the array. In addition, strong statistical support is also needed for analysis and interpretation of these massive

data files.

Most recently, an array-based high-throughput technique has been introduced that allows molecular profiling of a large number of tissue samples in a single experiment. Tissue microarrays are constructed by incorporating minute cylindrical tissue samples (0.6 mm in diameter) from hundreds of different tissues into a single paraffin block. Microtome-cut tissue sections from these microarray blocks make possible the parallel detection of DNA (by fluorescence *in situ* hybridization), RNA, and protein (by immunohistochemistry). Using this technique, consecutive analyses of a large number of molecular markers can be accomplished with relative ease (Kononen et al., 1998).

Applications of gene expression technologies

Techniques used to generate gene expression profiles have the potential for a wide variety of applications, and it seems likely that they will ultimately become standard tools for both molecular biologic research and clinical diagnostics. For example, these new techniques can be used to identify gene mutations and polymorphisms, to analyze differential expression of genes in normal versus malignant cells, to reveal genes that may prove useful as diagnostic or prognostic markers, and to investigate mechanisms of drug action. It is highly likely that novel insights into the complex relationships between gene expression and the many different cancer phenotypes, previously unattainable, will be forthcoming.

Gene amplification is one mechanism utilized by tumor cells for increasing the expression of a protein whose activity promotes cell proliferation. To better understand the contribution of amplifications to tumor progression, it is necessary to have a detailed analysis of the genetic composition of the amplified regions. Because amplification of a mutated *EGFR* allele is found in fully one-third of glioblastomas (Hunter et al., 1995), EST sequencing was used to examine the *EGFR* locus for novel gene amplifications in 28 primary glioblastoma tumor samples and in two cell lines (Wang et al., 1998). Southern analysis revealed sequence amplification for 11 of 13 probes in one or more of the tumor samples tested. Northern blot analysis with the same probes indicated that three ESTs identified transcripts that were overexpressed in cell lines having corresponding sequence amplification. Identification of increased transcript levels for multiple expressed sequences raises the possibility that there are genes in addition to *EGFR* that are involved in the amplification of this region.

Serial analysis of gene expression (SAGE) is another technique used to simultaneously analyze a large number of transcripts in tumor cells as compared with their normal cell counterparts. At present, no studies have been published using SAGE to analyze glioma tissue samples. However, SAGE has been used to study gene expression patterns in gastrointestinal tumors (Zhang et al., 1997). Transcripts isolated from at least 45,000 different genes from human colorectal (CR) epithelium,

CR cancers, and pancreatic cancers were analyzed. Although the gene expression profiles were similar between normal colon epithelium and primary colon cancers, expression profiles revealed 289 transcripts that were expressed at significantly different levels. SAGE was also performed on mRNA derived from pancreatic cancers and 404 transcripts were expressed at higher levels compared to transcripts isolated from normal colon epithelium.

Recently, SAGE has been used in the study of non-small cell lung cancer (NSCLC) (Hibi et al., 1998). SAGE analysis was performed on primary squamous cell lung cancer samples and normal lung large airway epithelial cells. Comparison of the tags present in the tumor samples with those identified in the normal tissue allowed construction of a comprehensive profile of gene expression patterns in the lung. Analysis of the most frequently expressed SAGE tags led to the identification of three genes that were consistently expressed in primary NSCLC: PGP 9.5, *B-myb*, and human *mutT*. These results suggest that several of the transcripts identified by SAGE are frequently associated with lung cancer and that their overexpression may contribute to lung tumorigenesis.

cDNA microarrays are another powerful tool that can be used to discern whether specific patterns of gene expression can be reliably and reproducibly associated with specific tumor types and thereby provide information for molecular classification. They can also be used to facilitate the identification of cellular pathways associated with chemotherapeutic agents. For example, the molecular response of gliomas to the DNA-alkylating agent 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) has been investigated using a cDNA array (Rhee et al., 1999a). The study found major differences between gene expression in a BCNU-resistant glioblastoma cell line compared to a BCNU-resistant subline before and after treatment with BCNU. Overall, more genes were expressed in the sensitive cells. More specifically, BCNU treatment decreased the expression of six DNA repair genes in the sensitive cell line but not in the resistant cell line. Moreover, marked induction of tumor necrosis factor was detected only in the sensitive cells. These results demonstrate that the use of gene expression profiling can facilitate the investigation and understanding of tumor response to anticancer chemotherapies.

cDNA arrays can also be used to identify candidate genes involved in tumor development, invasion, or progression. For example, in a parallel screening study comparing gene expression among primary gliomas of different grades, cDNA array technology was used to search for genes associated with glioma progression (Fuller et al., 1999). The *IGFBP2* gene was found to be specifically overexpressed in glioblastomas but not in lower grade gliomas, thus providing novel insight into a potential signal transduction pathway involved in glioma progression.

cDNA array technology has recently been used to

assess the fidelity of models used in glioma research. Established cell lines are commonly employed as *in vitro* models of different tumor categories based on the assumption that they are representative their primary diseases. cDNA arrays were used to determine the extent to which cell lines reflect the molecular features of the primary tumors from which they were derived (Rhee et al., 1999b). Gene expression profiling was performed on ten glioblastoma multiforme (GBM) primary tissue samples and on three GBM cell lines. Hierarchical statistical analysis showed that the three cell lines map as one clustered group and the ten tissue samples map as a completely separate cluster. Multidimensional scaling and principal component analysis provided additional supportive evidence that the cell lines are clearly distinct from the tissue samples and also that the cell lines exhibit considerably more internal variation among themselves than do the primary tissue samples (Hess et al., submitted). Thus, the validity of using derivative cell lines as representative substitutes for primary tissues must be strongly qualified. It is possible that cell lines of early passage may more faithfully emulate their corresponding tumor types than do the established multipassage lines examined in this study; further investigation is required to answer this question.

While cDNA arrays make possible the expression analysis of thousands of genes in a single tumor specimen, another array-based technology has been developed that can be used to simultaneously analyze hundreds of tumor specimens in a single experiment, the tissue microarray (Kononen et al., 1998). Bubendorf et al. (1999) used fluorescence in situ hybridization on tissue microarrays to obtain a comprehensive survey of gene amplifications in different stages of prostate cancer progression. They found that high-level (>3X) amplifications were very rare (<2%) in primary prostate cancers. However, in metastases from patients with hormone-refractory disease, amplification of the androgen receptor gene was seen in 22%, MYC in 11%, and Cyclin D-1 in 5% of cases. These results suggest that androgen receptor amplification is more closely associated with the development of hormone-refractory cell growth, whereas MYC amplification is associated with metastatic progression. This study illustrates that tissue microarray technology is a powerful tool suited for the molecular profiling of large numbers of tumors. In another recent study, combined cDNA array and tissue microarray analysis was used to evaluate gene expression in renal cell carcinoma, a disease in which genetic markers have not yet been successfully applied for diagnosis or prognostication (Moch et al., 1999). cDNA analysis was performed on a renal cell cancer line and on normal kidney tissue. Eighty-nine differentially expressed genes were identified in the cancer cell line, including vimentin, a cytoplasmic intermediate filament. Next, a tissue array composed of renal cell carcinoma specimens of different grades was used to determine the incidence of vimentin expression as detected by immunohistochemistry. Vimentin expression was found

to be more frequent in grade II (44%) and grade III (42%) tumors than in grade I (13%) tumors. Vimentin expression was also correlated with tumor stage, being more common in the higher stages. The results of this study illustrate the power of combining cDNA microarray and tissue array technologies to identify and characterize cancer genes. The application of tissue array technology to the study of gliomas has not yet been reported but is likely in the near future.

Conclusions

Classification and grading systems for the gliomas are continually evolving. Historically, the gliomas have been categorized based on pattern recognition of various cellular and tissue architectural features that are sometimes ill-defined, with the ultimate assignment to a particular rubric often dependent on the individual neuropathologist's relative weighting of particular features. Despite the generally proven benefit and utility of stratifying glioma patients based on current morphologic schemes, classification and grading criteria are nonetheless too often subjective, and consensus agreement on specific objective criteria for many of the glioma subtypes has been difficult to achieve. Extensive

immunohistochemical studies using newly introduced proliferation markers have significantly advanced the assessment of tumor growth potential and are beginning to impact grading criteria for some tumor subtypes. Recent preliminary studies indicate that the identification and characterization of relevant genetic alterations show considerable promise for subdividing and subclassifying the gliomas. In the future, gene expression profiling may serve as a tool for use in the molecular subclassification of the gliomas and also may contribute to the development of new treatment strategies through the identification of novel therapeutic targets and by facilitating the genetic parsing of the complex tumor response to chemotherapeutic agents.

A montage of the classical histopathology, genetic alterations, and cDNA array images of low grade (oligodendroglioma, O), intermediate grade (anaplastic oligodendroglioma, AO; anaplastic astrocytoma, AA), and high grade (glioblastoma multiforme, GBM) gliomas is provided in Figure 1. It may soon be possible to develop clinically and biologically relevant classification and grading systems for the gliomas that are based on a synthesis of traditional histology, molecular genetics, and gene expression profile data. It is hoped that such systems will offer improved

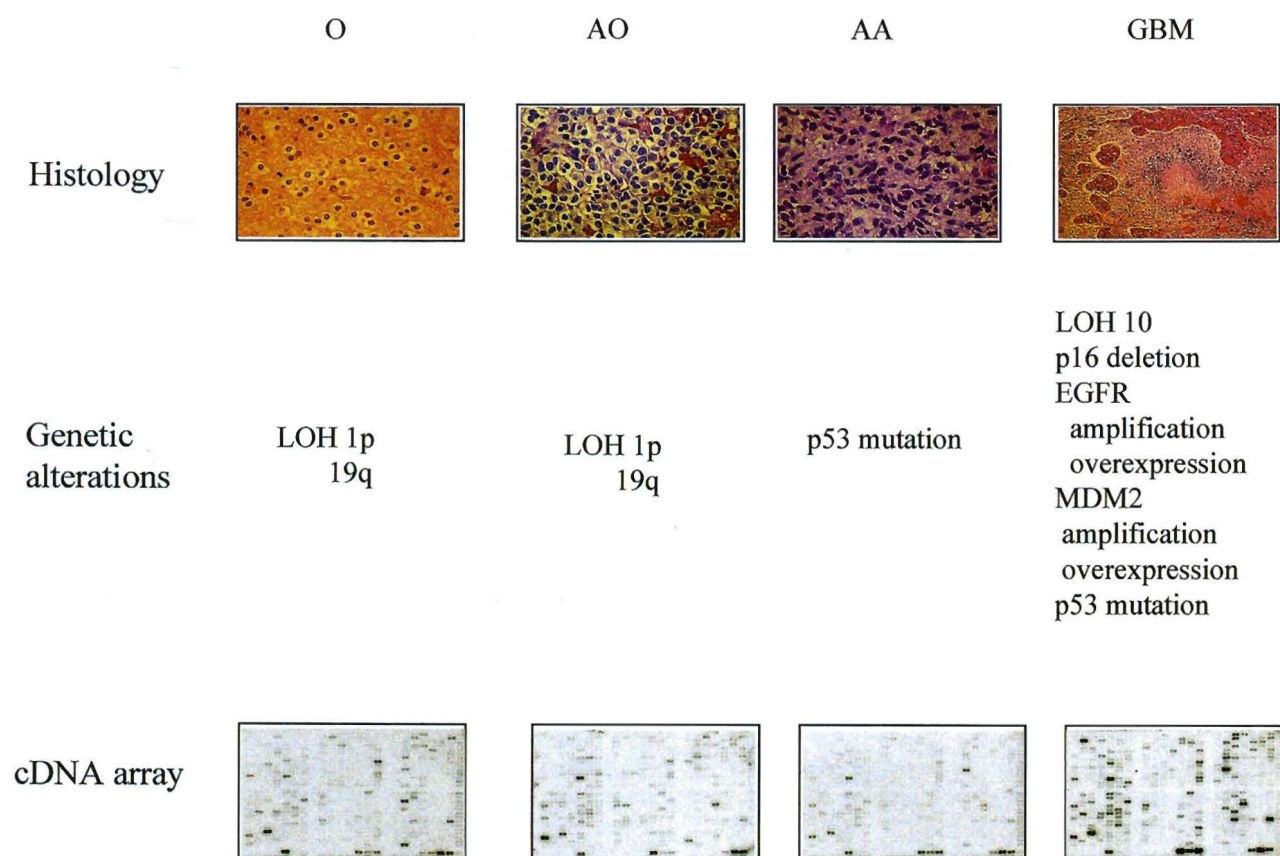


Fig. 1. Summary of the principal histologic features, genetic alterations, and cDNA array images characteristic of oligodendroglioma (O), anaplastic oligodendroglioma (AO), anaplastic astrocytoma (AA), and glioblastoma multiforme (GBM).

diagnostic accuracy, prognostic reliability, and therapeutic interventions.

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Accepted March 14, 2000