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Molecular Genetics of Malignant Glioma

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Cancer arises as a somatic disorder of genes controlling the cellular processes that contribute to malignancy. Genetic damage to genes critical for such processes as cell division and cell migration leads to the disordered regulation of these processes. The result is that cells proliferate at times in the life of the organism when they should not be dividing, and they migrate at times when they should remain quiescent. The genetic changes in cells that give rise to primary brain tumors have been widely catalogued. In adults, approximately 85 percent of all such tumors are tumors of cells in the astrocytic lineage; these are also the most common tumors of childhood.

Diffuse astrocytic tumors of adults arise most commonly in the cerebral hemispheres and manifest a wide range of histopathologic features that are organized as tumor grades, most frequently according to a classification schema outlined by the World Health Organization (WHO). This classification includes astrocytoma (WHO grades I and II), anaplastic astrocytoma (AA) (WHO grade III), and glioblastoma multiforme (GBM) (WHO grade IV), in order of increasing malignancy.[1] These designations generally reflect the tumor's degree of cellularity, mitotic activity, and cellular anaplasia as marked by nuclear and cytoplasmic atypia and pleomorphism.[2] Additionally, microvascular proliferation and necrosis are important indicators on which current grading is based (Figure 4–1).

A focus of current research elucidating the origins of astrocytic tumors is the determination of how specific genetic alterations contribute to the characteristic pathology of specific grades of tumors.[3] There is increasing evidence that the progression from low-grade astrocytoma to AA and GBM is associated with a cumulative acquisition of multiple genetic alterations (Figure 4–2). The types of molecular alterations that contribute to the development of cancers including primary brain tumors fall into two general classes. One type of genetic change typically results in the loss of cellular activities that operate physiologically to restrain growth. Genes that are altered in this manner are known as *tumor-suppressor genes*. Since each cell contains two copies of all genes that are located on the non-sex chromosomes, loss of both normal alleles generally is necessary to promote neoplasia. The second class of mutations results in the inappropriate activation of genes that typically enhance cellular proliferation or other features of malignancy. These genes are known as *proto-oncogenes*. Proto-oncogenes encode proteins such as growth factors or growth factor receptors, mediators of signaling pathways, or regulators of gene expression. Activating mutations convert proto-oncogenes to oncogenes; oncogenes have a variety of functions promoting the neoplastic phenotype.

PATHOGENESIS OF ASTROCYTIC TUMORS

A key aspect of the development of malignant tumors is the occurrence of genetic alterations in cellular pathways important for the regulation of genetic instabil-

ity. Cells use a variety of different strategies to protect the integrity of their genomes, and it is thought that one or more such safeguards must be compromised for the cell to collect a sufficient number of genetic alterations to grow as a malignant tumor. Astrocytic tumors are an important example of tumors in which it is sometimes possible to observe the evolution of an aggressive malignancy from a less malignant one in association with the accumulation of genetic alterations in the most rapidly growing cells of the tumor[4] (Figure 4–3). Some patients who initially present with low-grade, diffuse, astrocytic tumors are not cured by surgical resection and develop recurrent lesions, which are sometimes of higher pathologic grade than the original lesions. These tumors typically are associated with additional genetic alterations; studies that have surveyed specific genetic alterations in many tumors of different pathologic grades have led to a model that describes the pathogenesis of high-grade astrocytic tumors (see Figure 4–3).[5] Grade IV astrocytic tumors that arise as the result of this pattern of sequential genetic change have been dubbed "sec-

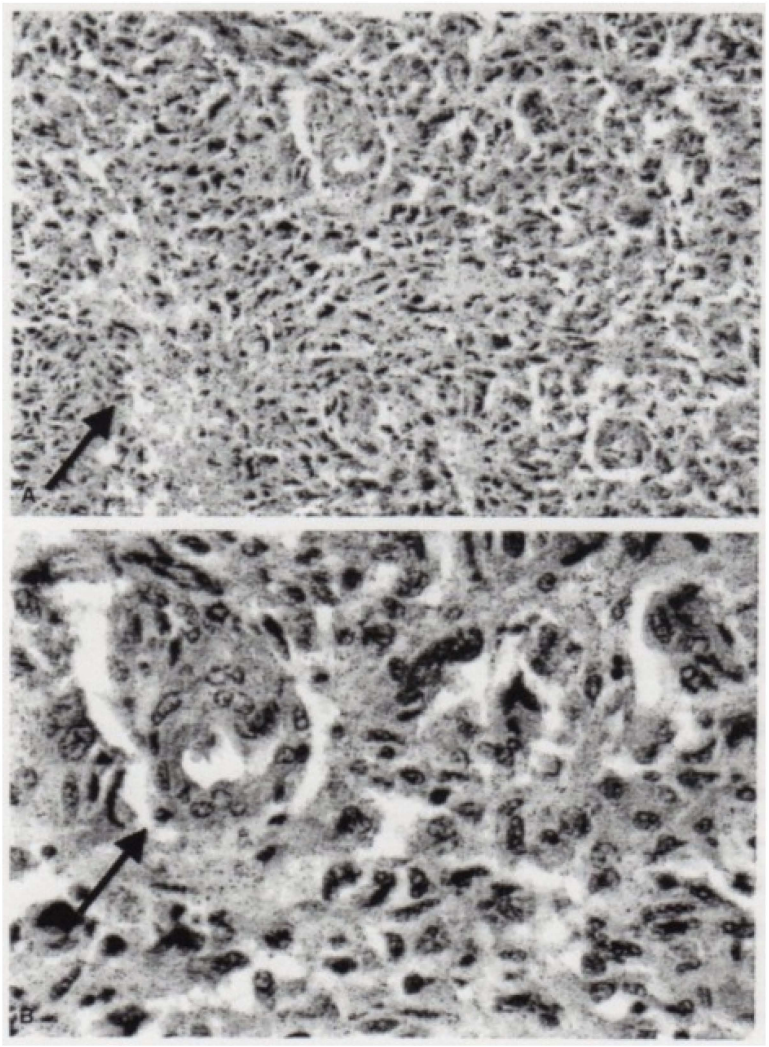

Figure 4–1. Histopathology of glioblastoma multiforme. *A*, Low magnification (×100) of this neoplasm stained with hematoxylin and eosin demonstrates the increased cellularity, necrosis with pseudopalisading, and vascular endothelial proliferation that is typical of advanced-grade astrocytic neoplasms. *B*, Higher magnification (×400) better demonstrates the vascular endothelial proliferation and nuclear atypia that are present.

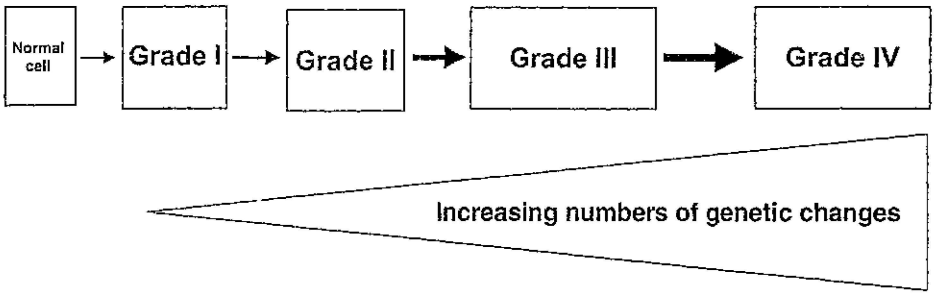

Figure 4–2. Accumulation of genetic alterations is associated with progression from low-grade to high-grade tumors.

ondary GBM." The most prominent genetic alteration in these tumors is the inactivation of *P53*. Secondary GBMs constitute only a small proportion of all GBMs.

Although it is believed that all tumors arise as the result of sequential genetic alterations, which confer increasingly malignant characteristics on tumor behavior, some tumors may not be detected until numerous alterations have occurred. This may occur (1) because the sequential accumulation of the specific genetic alterations that give rise to this subgroup of tumors occurs over a very short period of time or (2) because cells that acquire this particular pattern of genetic alterations grow rapidly. It is of interest that grade IV astrocytic tumors presenting without an evident precursor lesion generally are characterized by a somewhat different group of genetic alterations than those observed in secondary GBM (see Figure 4–3).[6] The most prominent alteration in these tumors is amplification of the epidermal growth factor receptor gene (EGFR). These tumors are sometimes called de novo or primary GBM and constitute a large majority of all grade IV astrocytic tumors.

An important strategy for the identification of genes of importance in the development of brain tumors has been the determination of genes responsible for several different inherited cancer predisposition syndromes, which include brain tumors. Although some genes recognized in this manner, such as *P53*, clearly play a role in the development of sporadically occurring primary brain tumors,[7] the importance of other such genes, for example *NF1*, remains largely unknown.[8] Additionally, genomic surveys have revealed many other putative sites of deoxyribonucleric acid (DNA) rearrangement, which seem likely to include genes of importance for the pathogenesis of these tumors. For astrocytic tumors, these alterations include (among others) the gain of DNA from chromosome 7 and loss of DNA from chromosomes 10, 19, and 22 (see below).[9] Emerging data have made it possible to recognize that some genes altered in the pathogenesis of astrocytic tumors encode functions that are important within the same cell regulatory pathway. We interpret these findings as indicating the particular importance of these pathways, and below

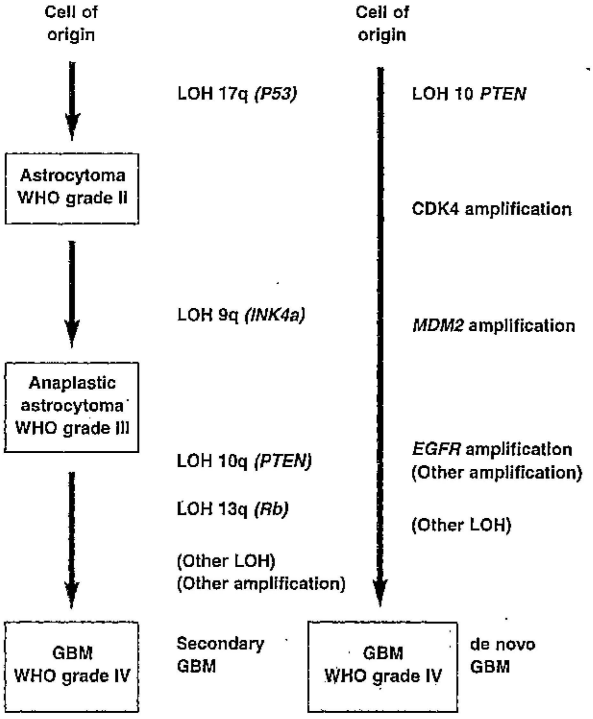

Figure 4–3. Genetic alterations associated with the development of astrocytic tumors. WHO = World Health Association; GBM = glioblastoma multiforme; LOH = loss of heterozygosity; CDK4 = cyclin-dependent kinase 4; EGFR = epidermal growth factor receptor. (Adapted from von Deimling A, Louis DN, Wiestler OD. Molecular pathways in the formation of gliomas. Glia 1995;15:328–38.)

we have outlined pathways that the most commonly altered genes in astrocytic tumors appear to function.

SPECIFIC GENETIC ALTERATIONS IN ASTROCYTIC TUMORS

P53/MDM2/ARF Pathway

The *P53* gene is located on chromosome 17p13.1 and encodes a 53-kDa protein, p53, that plays a role in several key cellular processes, including regulation of the cell cycle, the response of cells to DNA damage, genomic instability, cell death, cell differentiation, and neovascularization.[10] Inheritance of one mutant allele of *P53* can cause a well-described cancer predisposition syndrome, the Li-Fraumeni syndrome. The predominant tumor types in humans who are heterozygous for *P53* mutation are soft tissue and bone sarcomas, carcinomas of the breast, and astrocytic tumors of the brain.[11]

p53 may have multiple functions, but it is best characterized as a transcription factor to induce or repress the transcription of multiple genes through sequence-specific interaction with DNA. Little is known of the precise determinants that regulate p53 function as a transcription factor; however, there is considerable evidence that the loss of *P53*, which has been described in a variety of tumor types, affects the transcription of many different genes, including some recognized as being critical for cellular processes that contribute to the dysregulated growth and malignant characteristics of brain tumors. These include genes that are involved in the cell cycle, tumorigenesis, neoangiogenesis, and apoptosis.

As mentioned, usually the biologic effects of the inactivation of a tumor-suppressor gene are recognizable only after both copies of the gene are inactivated by a mutational mechanism. Inactivation of one copy typically would lead to a loss of 50 percent of the protein encoded by the two copies, and this might not affect its normal, physiologic cellular activities. A prominent exception to this rule is the occurrence of mutations in one copy of a gene whose protein products function as multimers. If the genetic alteration in one copy results in a protein that renders the multimeric complexes in which it functions unstable, mutations in only a single allele can cause a loss of gene function that results in altered cell behavior. Such mutations are called dominant negative mutations, and they have been reported to occur in *P53*. However, inactivation of the *P53* locus more commonly involves inactivating point mutations in the DNA of one allele and loss of the normal allele by deletion of the region of the chromosome on which it is located.

Often, the loss of this second *P53* allele can easily be recognized by studying polymorphic sites on the chromosome in the vicinity of the *P53* gene (Figure 4–4). Silent alterations in primary DNA structure, polymorphisms, occur frequently, and if different polymorphic alleles are inherited from each parent, normal tissue will have both alleles. That individual can be said to be heterozygous at that locus. If a deletion occurs in a chromosome containing one of these polymorphic alleles, the individual will have a loss of

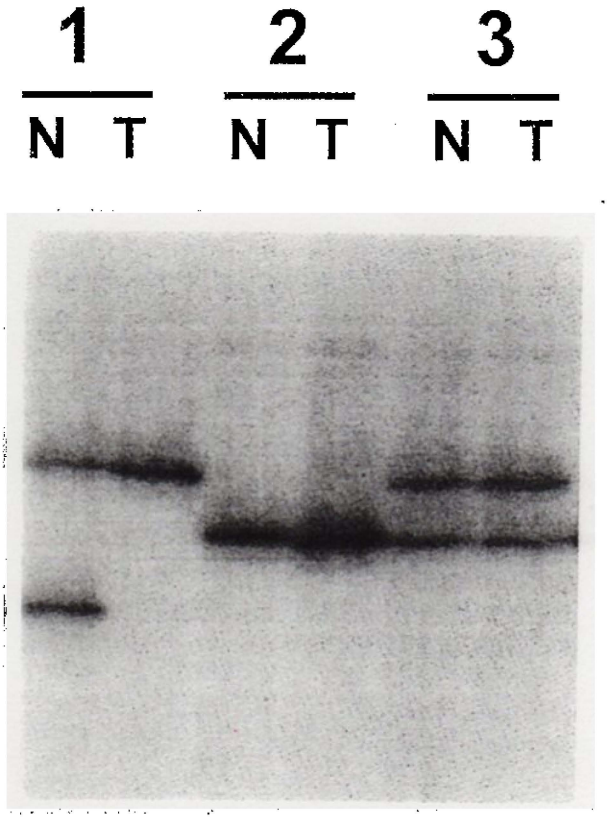

Figure 4–4. Loss of heterozygosity (LOH). Polymerase chain reaction primers flanking simple repeat regions in intron 1 of *P53* were used to amplify normal (N) and tumor (T) DNA pairs from three different glioma patients. Tumor DNA in case 1 had LOH in the *P53* gene, case 2 was non-informative, and tumor DNA in case 3 did not show loss of alleles in the *P53* region.

heterozygosity (LOH) in that particular region. When this loss occurs in the region of a tumor-suppressor gene that has already been inactivated on the paired chromosome, usually by the random occurrence of a mutational event, the locus is totally inactivated and is likely to contribute to the phenotype of the tumor.

A variety of molecular technologies have been developed to identify LOH and point mutations that occur in tumor-suppressor genes. Polymerase chain reaction (PCR)-based strategies (see Figure 4–4), single-strand conformation polymorphism (SSCP)-based approaches (Figure 4–5), and DNA sequencing (Figure 4–6) are among the most popular and have been widely exploited for the evaluation of *P53*, as well as other tumor-suppressor genes important for the pathogenesis of astrocytic tumors. Because *P53* mutation reduces degradation of the abnormal protein product, immunohistochemical assays will detect mutant *P53* accumulation, though this can sometimes be difficult to distinguish from the expression of apparently wild-type *P53* in some tumors. Immunohistochemistry is a standard method available in most diagnostic pathology laboratories and is therefore well suited for clinical research using routinely processed archival tissue. *P53* mutations have been reported in approximately 30 to 40 percent of astrocytic tumors. They occur in approximately 40 percent of grade I and II astrocytoma, in 30 percent of AA, and in 30 percent of GBM. This suggests that *P53* mutations are associated principally with the change from normal tissue to low-grade neoplasia, rather than with the progression from low-grade to high-grade tumors.[12] *P53* mutations are found most commonly in gliomas occurring in young adults. In contrast, *P53* mutations have not been as frequently observed in supratentorial astrocytic tumors of children, although they do occur in childhood brainstem gliomas. The prognostic implication of *P53* mutations has not yet been clearly defined. It remains a strong candidate for being clinically significant, however, since *P53* mediates the response of tumors to irradiation, an important modality of treatment for these tumors.[13] Mice with homozygously deleted *P53* or one deleted allele are phenotypically normal, but they develop spontaneous tumors at a higher frequency than do heterozygotes. Brain tumors occur

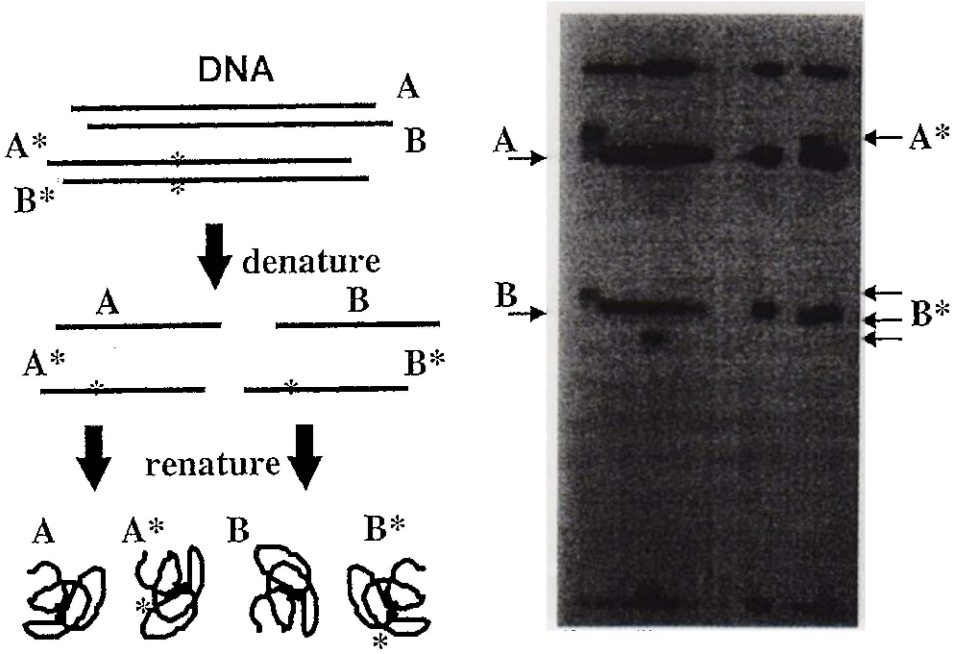

Figure 4–5. Use of single-strand conformation polymorphism to detect small DNA changes (point mutations or small deletions) in the *P53* gene. Single-stranded DNA with mutations (A* and B*) assumes different conformation and migrates differently in nondenaturing gel compared with DNA with wild-type sequences (A and B). *Arrows next to A and B* point to the migration of DNA with wild-type sequences; *arrows next to A* and B** point to aberrant migration as a result of mutations.

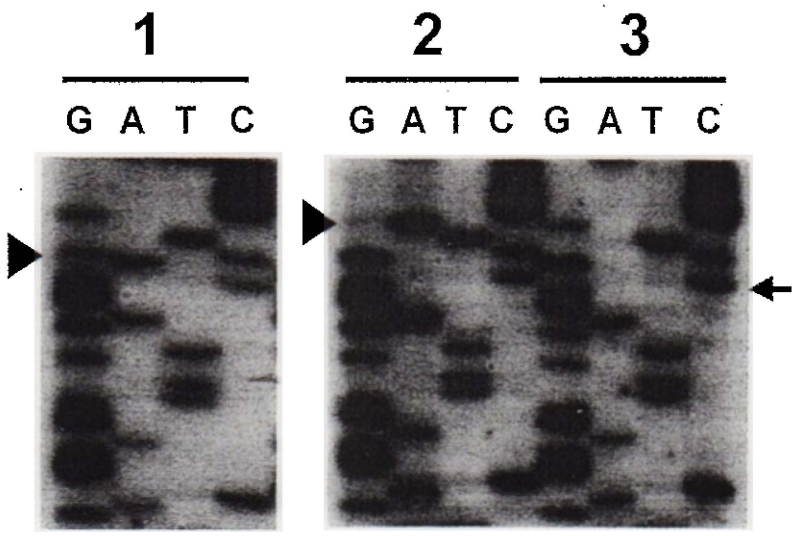

Figure 4–6. DNA sequencing analysis on exon 5 of the *P53* gene. Tumor DNA in case 3 had normal DNA sequences for codons 175 and 176, cgc-tgc (bottom to top starting from location of *arrow*). Tumor DNA in cases 1 and 2 had point mutations (indicated by *solid triangles*). The patient in case 1 had a cgc to c(G/A)c change at codon 175 resulting in an amino acid change of arginine to histidine/arginine. The patient in case 2 had a tgc to tAc change at codon 176 resulting in an amino acid change of cysteine to tyrosine. G = guanosine; A = adenosine; T = thymidine; C = cytosine.

infrequently in such animals, perhaps because they die so early in life from other tumors.

MDM2 is found at chromosome 12q14.3–q15 and encodes a protein with a predicted molecular weight of 54 kDa. *MDM2* was discovered by cloning a highly amplified gene from a spontaneously transformed derivative of mouse 3T3 cells that displayed *m*ultiple *d*ouble *m*inute chromosomes—hence its name. The MDM2 protein binds to the acidic activation domain of P53 and inhibits the ability of *P53* to promote transcription. In addition, MDM2 promotes the degradation of P53. This amplification of *MDM2* and dysregulation of MDM2 function are alternative mechanisms for escaping *P53*-regulated control of cell growth. MDM2 can function as an oncogene, and in contrast to tumor-suppressor gene mutations that result in the loss of function of proteins key for the inhibition of cell proliferation, the activation of oncogenes results in enhanced function leading to increased cell proliferation. Approximately 10 to 15 percent of GBM and AA and 7 percent of glioblastoma cell lines have been reported to display amplification of *MDM2*[14] (Figure 4–7). Overexpression of *MDM2* has been observed immunohistochemically in more than 50 percent of primary glioblastomas, but the fraction of immunoreactive cells varies considerably.

The human *INK4a* locus maps to chromosome 9p21 and contains an alternative reading frame encoding two proteins, human $p19^{ARF}$ and $p16^{INK4a}$ (Figure 4–8). *INK4a* is also known as CDKN2, MTS-1, and CDK4I. Each transcript from the $p16^{INK4a}$ locus has a different promoter and a different specific 5' exon, $E1_\alpha$ or $E1_\beta$, that is spliced into common exons E2 and E3. The E1α-containing transcript encodes $p16^{INK4a}$, and the E1β-containing transcript encodes $p19^{ARF}$ from a different reading frame initiated in *E2*.[15] Recent studies provide evidence that ARF modulates P53 function as a checkpoint in response to proliferative signals but not in response to DNA damage. Overexpression of *MYC, E1A*, or *E2F1* in primary mouse embryo fibroblasts upregulates *ARF* expression and results in apoptosis that is *P53* dependent. Apoptosis does not occur in *ARF*-null or *P53*-null cells. ARF can directly bind to MDM2 and inhibit MDM2-mediated *P53* degradation and transactivational silencing. Deletion of the *ARF* locus as shown in Figure 4–8 (sample 1) can result in the enhanced degradation of p53 and the loss of *P53* function. Interestingly, expression of

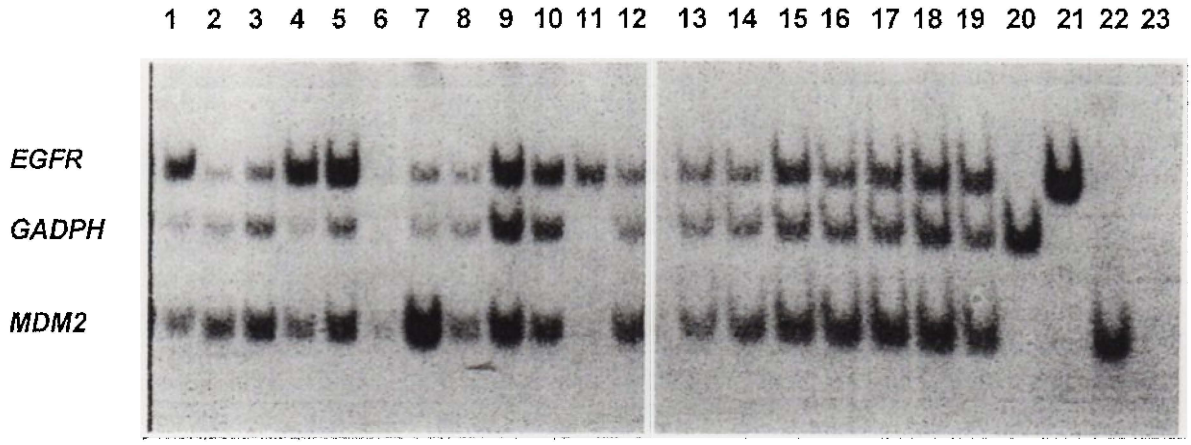

Figure 4–7. Multiplex polymerase chain reaction (PCR) assay for gene amplification of *EGFR* and *MDM2*. Lanes 1–19 and 23: multiplex PCR with primers that simultaneously amplify fragments of *EGFR*, *GAPDH*, and *MDM2* genes. Lanes 20–22 were control PCR with primers used to amplify individual gene fragments (lane 20: *GAPDH*; lane 21: *EGFR*; and lane 22: *MDM2*). Lanes 1–12: tumor DNA samples; lanes 13–19: normal DNA controls; lane 23: water. Lanes 1, 4, 5, and 11 had amplifications of *EGFR*; lane 7 had amplification of the *MDM2* gene.

ARF is negatively regulated by p53 and in some human tumor cell lines correlates inversely with *P53* function.

Ectopic expression of the ARF protein can induce an arrest in both the G1 and G2 phases of the cell cycle in a variety of cells, including those derived from glioblastoma. This arrest is p16 and p53 independent. Thus, the two distinct tumor suppressors, p16 and ARF, encoded by the single genetic $p16^{INK4a}$ locus regulate both the *Rb1* and *P53* pathways. Approximately 25 to 50 percent of AA and 40 to 70 percent of GBM carry homozygous deletions of *INK4a* (Figure 4–9).

Rb1/INK4a/CDK4/CDK6 Pathway

The retinoblastoma gene, *Rb1*, is a well-characterized tumor-suppressor gene located on chromosome 13q14. Patients with hereditary retinoblastoma have a germline mutation in one *Rb1* allele. Both copies of the *Rb1* gene are mutated or deleted in tumor tissue from these patients as well as in sporadic retinoblastomas. The cytogenetic observation that some glioblastomas are associated with loss of 13q and the observation that some hereditary retinoblastoma patients also develop brain tumors have prompted speculation that this gene may be important in the pathogenesis of sporadic glial tumors.

The human *Rb1* gene encodes a 110-kDa protein, pRb. pRb is a nuclear phosphoprotein that contains a bipartite nuclear localization signal. pRb also has two globular domains (A and B) that bind viral oncoproteins, including SV40 T antigen, adenovirus E1A, and human papillomavirus E7, when pRb is in its unphosphorylated form. These regions are also critical for

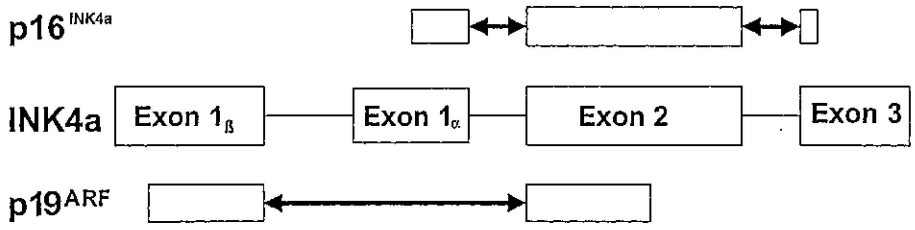

Figure 4–8. Structural organization of the *INK4a* locus. The human *INK4a* locus encodes not only $p16^{INK4a}$, but as the result of alternative splicing and an alternative reading frame, it also encodes the unrelated protein human $p19^{ARF}$. (Adapted from Quelle DE, Zindy F, Ashmun RA, Sherr CJ. Alternative reading frames of the INK4a tumor-suppressor gene encode two unrelated proteins capable of inducing cell cycle arrest. Cell 1995;83:993–1000.)

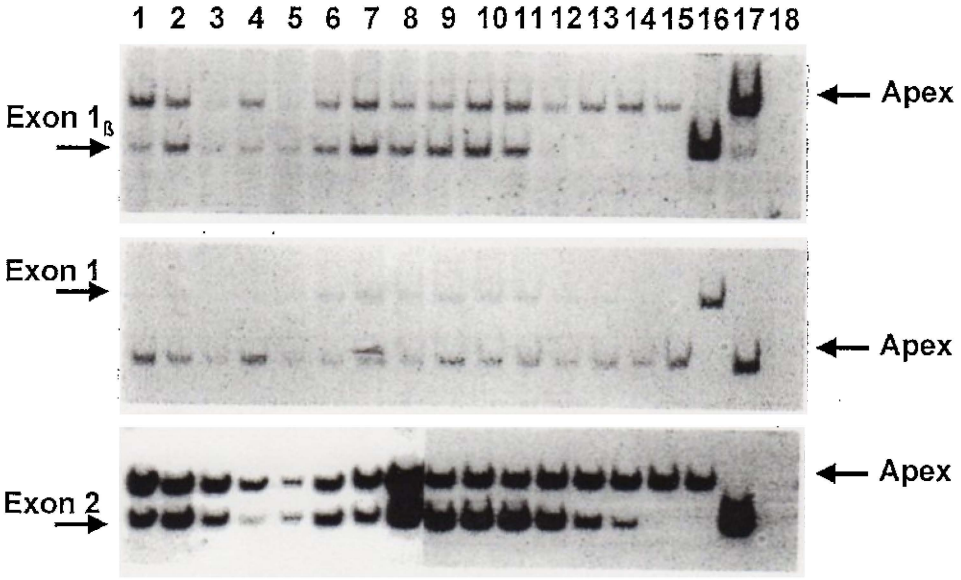

Figure 4–9. Deletion analyses of the *INK4a* gene in glioma; polymerase chain reaction (PCR) amplifications of exon 1β, exon 1, and exon 2 of *INK4a*, respectively. Primers that amplify apex nuclease (apex) were included in each PCR as an internal control for presence of DNA and the PCR reaction. Lanes 1–5: tumor DNA samples; lanes 6–8: normal DNA controls; lanes 9–15: a series of mixed DNA between normal DNA and DNA with *INK4a* deletion at different ratios (lane 9: 100% normal DNA; lane 10: 90% normal DNA; lane 11: 75% normal DNA; lane 12: 50% normal DNA; lane 13: 25% normal DNA; lane 14: 10% normal DNA; lane 15: 0% normal DNA). Deletion (samples with target to apex ratios that are less than that of samples with less than 50% normal DNA) for exon 1β was detected in sample 1; deletions for exon 1 were detected in samples 1, 3, 4, and 5; deletions for exon 2 were detected in samples 1, 3, and 4.

binding to transcription factors such as E2F. The ability of pRb to regulate cell growth is tightly linked to its ability to bind to and inhibit E2F, an important mediator of cellular proliferation (Figure 4–10).

Although the frequency of *Rb1* inactivation in primary astrocytic tumors has not been well defined, other genes that influence *Rb1* function are frequently altered in these tumors (see Figure 4–10). Most prominent among these is the tumor-suppressor gene *INK4a*.[16,17] $p16^{INK4a}$ functions as an inhibitor of CDK4, a cyclin-dependent kinase whose normal action is to promote cell division by mediating the phosphorylation of pRb. Phosphorylation of pRb decreases the inhibitory effects of pRb on the E2F transcription factor with which it complexes and thereby leaves E2F unopposed in stimulating cell proliferation.

The CDK4 gene encodes a 33-kDa protein and maps to chromosome 12q13–14. CDK6, another cyclin-dependent kinase that phosphorylates pRb and is inhibited by $p16^{INK4a}$, encodes a 38-kDa protein and maps to chromosome 7q21–22. Both CDK4 and CDK6 require interaction with their regulatory subunits, members of the cyclin D family, to achieve activation. Overexpression of either of these kinases or deletion of the genes encoding their inhibitors such as $p16^{INK4a}$ leads to inappropriate phosphorylation of pRb, which results in a loss of growth suppression. The CDK4 gene is amplified in nearly 15 percent of high-grade gliomas, particularly in those without *INK4a* alterations. Additionally, a few tumors without *INK4a* mutations or CDK4 amplification have been shown to have CDK6 amplification (see Figure 4–10).

PTEN

PTEN, a tumor-suppressor gene also known as *MMAC* or *TEP1*, is located at 10q23. Deletion of chromosome 10 occurs frequently in astrocytic tumors, and there is considerable evidence for the presence of multiple tumor suppression genes on this chromosome. In addition to its predicted involvement in brain tumors, germline *PTEN* mutations have been

OTHER GENETIC ALTERATIONS IN ASTROCYTIC TUMORS

Regions of chromosomal gain or loss in astrocytic tumors are thought to be strong candidates for marking the presence of currently unknown tumor-suppressor genes and oncogenes. Such regions have been identified in astrocytic tumors using comparative genomic hybridization.[21] Comparative genomic hybridization surveys the entire genome for copy number aberrations. Tumor-specific alterations have been reported from several laboratories for astrocytic tumors, and these include frequent gains on chromosomes 17q, 19, and 20. Frequently involved areas of loss include chromosomes 9q21, 10p, and 10q25. Other chromosomal copy number aberrations include +3q (13.3–29), –4q, +5q, –9q34, +12, –13q, –15, –16p, +17qter, –18, –21, and –22.

PATHOGENESIS OF OLIGODENDROGLIOMA

Oligodendrogliomas occur less commonly than astrocytic tumors and account for only about 5 to 10 percent of primary intracranial tumors. Most frequently oligodendrogliomas occur supratentorially and display various histologic grades of malignancy. Many gliomas have mixtures of cells with both astrocytic and oligodendroglial features, and it is unclear if this reflects the transformation of a common precursor cell or simply the divergent histopathologic appearance of these tumor types. Cytogenetic studies of oligodendrogliomas occasionally show a loss of chromosomes 9p and 22 or a gain of chromosome 7, but the most characteristic cytogenetic findings in oligodendrogliomas are loss of chromosomes 1p36 and 19q13.3. The presence of these alterations is thought to correspond to an enhanced responsiveness to cytotoxic therapies and an improved prognosis.[22] Loss of 1p36 occurs not only in oligodendrogliomas but also in some astrocytomas. Presumably these are the locations of tumor-suppressor genes that have not yet been isolated. Loss of heterozygosity for chromosome 9p loci, observed in some oligodendrogliomas, corresponds to deletions at the *INK4a* locus, and these have been associated with a poor prognosis for patients with this tumor type. Other molecular genetic events common in astrocytic tumors seem to be rare in oligodendrogliomas. *P53* mutations and *EGFR* amplification are almost never present, although high levels of *EGFR* expression have been reported in up to 40 percent of these tumors.

PATHOPHYSIOLOGY OF ANGIOGENESIS IN GLIOMAS

An important event that accompanies glioma development and progression is the formation of a blood vessel system supplying the tumor with oxygen and nutrients. The vasculature of low-grade gliomas closely resembles that of normal brain, whereas high-grade gliomas show prominent microvascular (eg, smooth muscle/pericyte and endothelial cell) proliferation and often contain areas with a much higher vascular density than low-grade gliomas and normal brain (see Figure 4–1). Indeed, GBMs are among the most highly vascularized tumors in humans.

Angiogenesis in gliomas is regulated by endothelial cell receptor tyrosine kinases including vascular endothelial growth factor (VEGF) receptor-1, VEGF receptor-2, Tie-1, Tie-2, PDGF receptor-b, c-met, and integrins such as $a_v\beta_3$. Typically, these receptors are not expressed in quiescent endothelium such as that found in the normal adult brain. Each of these receptors, however, is typically upregulated in proliferating tumor vessels, suggesting a role in tumor progression. The ligands for these receptors typically are expressed in the tumor cells, arguing in favor of a paracrine pathway (glioma cell–endothelial cell) mediating angiogenesis in these tumors. Current evidence suggests that VEGF, which binds to the VEGF receptor-1 and VEGF receptor-2, is the most important regulator of vascular proliferation in glioma-induced angiogenesis. Vascular endothelial growth factor is a secreted dimeric glycoprotein that specifically acts on endothelial cells and induces angiogenesis and vascular permeability in vivo. During glioma progression, VEGF expression is upregulated in tumor cells.[23] Its expression is particularly high in GBM where it is abundantly expressed in the perinecrotic palisading cells. Expression of VEGF is hypoxia inducible, and a major trigger of angiogenesis in gliomas appears to be cellular hypoxia. Functions of VEGF not only mediate tumor neoangiogenesis but also enhance vascular permeability of the

established vasculature. Due to this dual function, VEGF may be responsible for both the microvascular proliferation and the peritumoral edema that is routinely found in malignant gliomas. This model is consistent with the finding that dexamethasone, a drug widely used for the management of peritumoral edema, downregulates the expression of VEGF.

Vascular remodeling, including both proliferation and recruitment of smooth muscle pericytes, is regulated by TGF-β and an additional group of receptors, including receptors for TGF-β, the PDGF receptor-b, and Tie-2. The Tie-2 receptor is specifically upregulated on endothelial cells during glioma progression. The agonist for this receptor, angiopoietin-1, is constitutively expressed in glioma cells. Interestingly, its antagonistic ligand, angiopoietin-2, is also upregulated, suggesting a role for this ligand in some other pathophysiologic pathway.

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