Diagnosis of malignant glioma: role of neuropathology

Daniel J. Brat · Richard A. Prayson · Timothy C. Ryken · Jeffrey J. Olson

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Recommendations

Level I: The diagnosis of malignant glioma should be based on the histopathologic review of tissue.

Level II: Both frozen section and cytopathologic evaluation are recommended for the intra-operative diagnosis of malignant glioma. Consultation from a neuropathologist specialized in brain tumor diagnosis is recommended for problematic cases.

Level III: Incorporation of clinical and radiographic information with the final pathologic diagnosis is recommended. The criteria of the WHO classification of brain tumors are internationally recognized and can be utilized for establishing the diagnosis of malignant gliomas. Proliferation studies, such as those based on Ki-67/MIB-1 staining, and molecular genetic tests are recommended as adjuvant studies for classification and prognostication of malignant gliomas.

Rationale

Patients with malignant gliomas require a tissue diagnosis in order to guide further clinical management. Although clinical and neuro-imaging features of malignant glioma can be highly suggestive, the gold standard for diagnosis is based on the histologic examination of appropriately sampled tissue. In order to ensure greatest accuracy, pathologic studies should be performed in a multidisciplinary setting, in conjunction with a patient’s clinical history, the neuro-surgical impression, and the neuroradiologic findings [1–3]. Neuroradiologic features are of particular importance for establishing a diagnosis of a central nervous system disease because they highlight neuro-anatomic locations, generate diagnostic possibilities, direct attention to the most likely entities, and point out discrepancies between clinical and radiologic findings and pathologic assessment [2, 3]. Strong lines of communication across clinical and diagnostic disciplines are recommended both for the most accurate appraisal of disease and to ensure that any diagnostic discrepancies are resolved prior to definitive therapy. Numerous textbooks contain detailed descriptions of the pathologic criteria used for classifying and grading glial neoplasms [4–8]. The purpose here is to evaluate the current literature addressing the diagnosis of malignant glioma focusing on advances in classification and grading, variability and reliability of histopathologic analysis, accuracy of frozen sections and cytopathology, and the roles of immunohistochemistry and molecular diagnostic techniques. This review will address the following questions:

(1) What are the most appropriate diagnostic criteria for establishing a diagnosis of malignant glioma?

(2) What is the best technique for establishing the diagnosis of malignant glioma when a suspicious
lesion is identified on CT or MRI and tissue has been sampled?

(3) What is the reliability and reproducibility of the diagnosis of a malignant glioma?

(4) What is the role of additional testing in the diagnosis of malignant gliomas?

A prospective classification scheme of the pertinent literature was used. For reports that attempted to establish histopathologic and cytopathologic diagnostic criteria for gliomas, the gold standard for categorizing the class of data was based on the strength of the survival analysis. This type of investigation does not generate standard descriptive parameters used for classifying data, such as sensitivity, specificity, positive and negative predictive value, accuracy, likelihood ratio of a positive and negative result, and $\kappa$. Therefore reports were designated Class I that studied the appropriate population with clearly stated inclusion criteria; included diagnostic entities within established histologic categories; compared the diagnostic criteria to survival over an interval that is meaningful for the disease studied; included consensus diagnosis; were blinded to outcome; and were statistically significant on a multivariate analysis of appropriate clinical and pathologic variables [9–11]. Reports were designated Class II that studied the appropriate population, compared the diagnostic criteria to survival over an interval that is meaningful for the disease studied, but fell short of Class I in one of the other criteria. Reports were designated Class III that had more than one shortcoming.

Literature reports that assessed variability between pathologists, or that compared newer diagnostic techniques to more established ones were also categorized as Class I, II and III. Reports designated as providing Class I data were required to: have an appropriate number of cases; include only specific tumor types; present an accepted standard for tumor identification against which the investigative assessment could be compared; be properly blinded and refereed; and provide data for calculation of sensitivity, specificity, positive and negative predictive value, accuracy, likelihood ratio of a positive and negative result, and $\kappa$. Those studies for which a $\kappa$ value could be calculated and the value was greater than 0.6 were designated as Class I. Studies that had a $\kappa$ value was between 0.41 and 0.6, even though all other criteria were met, were designated as Class II. Studies were also designated Class II if the study population was restricted or histologic confirmation was not present on all cases, even if the investigation was performed in blinded fashion and all parameters could be calculated. Those studies that had a $\kappa$ of 0.4 or less were designated as Class III [12]. Class III designation was given to studies that were retrospective, to those that lumped together tumor grades or histologies, and to those that did not include histologic review. Class III data can be of value, but does not provide the same high degree of evidence as Class I and II data. Studies reported as randomized, controlled and/or prospective can vary significantly in quality and need to be reviewed in light of published recommendations for identification of items important in the valid reporting of clinical trials [13, 14]. Though there must be room for special considerations, Level I recommendations are generally based on Class I evidence, Level II recommendations are based on Class II evidence and Level III recommendations are based on Class III evidence [15].

Search criteria

We performed multiple computerized PubMed searches of the National Library of Medicine database of scientific literature published from 1966 to 2007. Keywords that were searched included “glioma”, “astrocytoma”, “glioblastoma”, “oligodendroglioma” “diagnosis”, “pathology”, “cytopathology”, “frozen section”, “immunohistochemistry”, “proliferation”, “genetics”, and “prognosis”. We limited our searches to human studies published in the English language. Key words were searched in multiple combinations. Links to “related articles” from highly relevant studies were utilized to broaden the search. Articles were also identified from the reference lists from references uncovered in initial searches. Reference lists from the most recent editions of standard textbooks of surgical neuropathology were reviewed, including Surgical Pathology of the Nervous System and Its Coverings, Russell and Rubinstein’s Pathology of Tumors of the Nervous System, and WHO Classification of Tumours of the Nervous System [4–8, 16]. Abstracts of all articles related to the pathologic diagnosis of human malignant gliomas were reviewed and relevant articles were retrieved and summarized in evidentiary tables.

Scientific foundation

Diagnostic criteria of malignant gliomas

Pathologic diagnosis of malignant gliomas depends in large part upon the informed application of established histopathologic and cytopathologic criteria to sampled tissue [4, 5, 7, 17]. It relies heavily, and in many instances exclusively, on examination of hematoxylin and eosin (H&E) stained slides from the sampled lesion, but may also incorporate results from ancillary tests as indicated below. A variety of schemes for classifying and grading gliomas have been employed, including those of Kernohan, Zülch, Ringertz, Burger, and Saint Anne/Mayo [4, 5, 7, 18–21]. Each of
these classification schemes uses microscopic morphologic features of the neoplasm that have been demonstrated to correlate with biologic behavior and prognosis. Grading criteria established in this manner are prognostically significant at the population level and can only provide guidelines for the management of a specific individual. One of the more recent outcome-based classification schemes is the St. Anne/Mayo system, which followed from an investigation of prognostic features, grading criteria and reproducibility in a set of 287 patients at the Mayo Clinic with infiltrative astrocytomas [21]. This investigation studied four histologic criteria within astrocytic neoplasms as they related to patient survival, including nuclear atypia, mitoses, endothelial proliferation, and necrosis. Grade 1 was defined as 0 features present; grade 2 with 1 feature; grade 3 with 2 features, and grade 4 with 3 or 4 features. Based on a 15 year follow-up period, a multivariate statistical analysis found that each of the 4 histologic criteria, as well as the tumor grade derived from them, was strongly correlated with survival ($P < 0.0001$). Importantly, these methods of histologic grading showed a concordance of 94% between two double blinded observers. The recent World Health Organization (WHO, 2007) classification is a multi-authored text written by international experts, which uses a grading system largely based on the St. Anne/Mayo criteria [7]. One of the main differences is that the WHO classification does not recognize a grade 1 infiltrating glioma. From a morphologic perspective, nuclear atypia is always present within an infiltrating astrocytoma, which qualifies as a grade 2 tumor by St. Anne/Mayo criteria. From a clinical perspective, even the lowest grade infiltrating astrocytomas will eventually progress and therefore do not warrant a grade 1, a designation which implies a low likelihood of recurrence. Thus, the lowest grade infiltrating glioma recognized by WHO criteria is grade II.

The WHO classification maintains a high level of international acceptance and continuous periodic updates are planned [7]. There has been no direct comparison of the most recent WHO criteria to other classification systems in terms of reliability or prognostic accuracy. However, the related St. Anne/Mayo criteria has been investigated for its predictive value in a comparison to the older Kernohan criteria [22]. In a series of 273 patients with gliomas, histologic grading was performed according to the Kernohan and St. Anne/Mayo criteria and the resulting grades were compared with patient survivals. Specific pathologic features from each grading scheme were examined for their predictive value. In both classification systems, histologic grade was significantly correlated with survival (Cox analysis, $P < 0.0001$). This included 3 grades for the Kernohan classification and 4 grades for the St. Anne/Mayo classification. Mitosis ($P < 0.0001$, $\chi^2 = 17.9$), endothelial proliferation ($P < 0.0001$, $\chi^2 = 39.4$), and necrosis ($P = 0.0007$, $\chi^2 = 11.5$) were all significantly correlated with survival. As noted below, these features form the basis of grading the infiltrative astrocytomas using WHO criteria. Thus, the WHO classification, which incorporates the criteria of the St. Anne/Mayo criteria, can be recommended as a recent and updated international standard for classifying and grading of malignant gliomas.

The WHO classification divides the infiltrating gliomas on the basis of morphologic criteria into astrocytomas, oligodendrogliomas, and oligoastrocytomas and includes criteria for their grading. Infiltrative or “diffuse” astrocytomas represent a spectrum ranging from low grade to highly malignant, and as noted above, the WHO uses a 3-tiered grading system that begins with grade II [7]. The diagnosis of an infiltrative astrocytoma (WHO grade II) is applied when individual tumor cells showing astrocytic differentiation infiltrate CNS parenchyma. Importantly, astrocytic differentiation and tumor grading are determined morphologically and are therefore subject to interpretation [23]. Classification of infiltrating tumors as astrocytic vs. oligodendrogial depends on cell shape, cytoplasmic appearance and the character of the nuclei. In astrocytomas, nuclei are elongate, hyperchromatic and irregular, generally lacking prominent nucleoli and perinuclear halos; some forms have prominent pink cytoplasm (i.e. gemistocytic or granular cell astrocytoma), while others (fibrillary and small cell astrocytomas) display only minimal fibrillar cytoplasm. The histopathologic distinction of grade II (“low grade”) from grade III (anaplastic astrocytoma, “high grade” or “malignant”) has depended on the identification of mitotic activity as the most defining criterion. Prior grading schemes have placed an astrocytoma with one mitotic figure into the grade III category (anaplastic astrocytoma). However, in a recent investigation of proliferative activity in 140 diffuse astrocytomas as it related to prognosis by Giannini et al., it was found that astrocytomas with only one mitotic figure identified (previously considered grade III neoplasms) had significantly longer survivals than astrocytomas with $>1$ mitosis ($P < 0.013$). Astrocytomas with zero or one mitosis had statistically similar survivals [24]. On this basis, current criteria of grade II astrocytomas allow 0 or 1 mitotic figures, but not more. A previous evidence based review has focused on the diagnosis and management of low grade gliomas and they will not be addressed in this review [25].

The malignant gliomas include both anaplastic astrocytoma (AA; WHO grade III) and glioblastoma (GBM; WHO grade IV). Compared to grade II astrocytoma, AA has increased cellularity, more nuclear pleomorphism and atypia, and increased proliferation. As mentioned, the identification of mitotic figures has been used as a strict criterion for the diagnosis of AA based on the finding that this feature identifies a set of tumors with more aggressive
clinical behavior [21, 26]. Since astrocytomas with zero or one mitosis have similar survivals, while those with >1 mitoses have significantly shorter survivals, the diagnosis of AA in the current WHO classification is reserved for infiltrative astrocytomas that have greater than one mitotic figure [5, 7, 24]. The number of mitoses identified within an astrocytoma necessarily depends upon sample size of the tissue, the number of tissue sections examined, and the intensity of the search. In a study of 410 diffuse astrocytoma specimens, Coons et al. [26] found that the examination of fifty 400× fields allowed the detection of mitoses with 92% sensitivity in anaplastic astrocytomas (grade III). This study also concluded that the identification of a single mitotic figure within an infiltrating astrocytoma was strongly associated with shorter survival, confirming the previous studies of Daumas-Duport et al. [21].

Glioblastoma (GBM; WHO Grade IV) is the highest grade of infiltrating astrocytoma. In addition to the histopathologic findings of AA, either microvascular hyperplasia or necrosis, often with pseudopalisading (or both), are required for the diagnosis of GBM [7]. In the past, necrosis within a malignant glioma was often viewed as the sole criterion for the diagnosis of GBM. Recent studies have emphasized that vascular proliferation and necrosis are biologically linked, so that either feature can be used for the diagnosis of GBM [21, 27]. For example, Barker et al. [28] performed a multivariate proportional hazards survival analysis of the prognostic significance of vascular hyperplasia and necrosis in 299 patients with GBM. Among the GBM specimens that contained vascular hyperplasia, 88% also had necrosis. The absence of necrosis was associated with a modest increase in median survival on univariate analysis (12.5 vs. 10.9 months; \( P = 0.02 \)), but was not associated with a difference in 2-year survival rates. Survivals of patients with GBM diagnosed based on the presence of either vascular hyperplasia or necrosis were both much shorter than survivals of patients with AA \( (P < 0.05) \). On this basis, as well as the findings of the St. Anne–Mayo study, the diagnosis of GBM can be established by the presence of either vascular hyperplasia or necrosis within a malignant astrocytoma [21, 28].

High grade astrocytomas are distinguished from other infiltrative gliomas based on morphology, in some instances guided by the use of molecular studies. Most diagnostic difficulty is encountered in distinguishing astrocytomas from oligodendrogliomas and mixed oligoastrocytomas [23]. In contrast to astrocytomas, oligodendroglioma tumor cells have round, regular and monotonous nuclei, with little cell-to-cell variability. The finding of perinuclear cytoplasmic clearing is a helpful but not constant feature that aids in diagnosis of oligodendroglioma. Other common but non-specific features include cortical involvement, microcalcifications, delicate branching capillaries, and microcysts. The current WHO Classification recognizes two grades of oligodendroglial neoplasms: oligodendroglioma, grade II and anaplastic oligodendrogliomas, grade III [7]. Criteria for distinguishing grade II and grade III oligodendrogliomas are not as well defined as they are for astrocytomas. Grade II tumors vary from low to moderate cellularity and can show occasional mitotic figures and cytological atypia, but marked mitotic activity, microvascular proliferation, or necrosis are diagnostic of a WHO grade III, anaplastic oligodendroglioma. A recent investigation of prognostic features in oligodendroglioma by Giannini et al. [29], provided more definitive histologic findings that were associated with aggressive behavior. This study included 124 patients at the Mayo Clinic who had been diagnosed with an oligodendroglial neoplasm, either WHO grade II (81 patients) or III (43 patients). A set of 18 histologic features were assessed by 6 surgical pathologists and 7 neuropathologists from North America and Europe. Consensus ratings for each feature were set at 60% agreement. A Cox proportional hazards model was used to assess association of clinical and pathologic parameters with survival. Histologic features associated with survival on univariate analysis included high cellularity, mitoses, vascular hyperplasia and necrosis (each \( P < 0.05 \)). A threshold of \( \geq 6 \) mitoses per high power field was identified as a critical cutoff in the survival analysis \( (P = 0.0001) \). Thus, the findings of this study suggest that the diagnosis of anaplastic oligodendroglioma should be made when a tumor demonstrates vascular hyperplasia, necrosis, or mitoses \( \geq 6 \) per high power field. Previous multivariate analyses have suggested that necrosis is the single most predictive feature of aggressive clinical behavior in oligodendrogliomas [30, 31].

Two recent studies have investigated whether histopathologic features in high grade oligodendrogliomas could be used to divide these tumors into grade III and IV tumors. Miller et al. studied a group of 216 patients with the diagnosis of anaplastic oligodendrogliomas for the prognostic significance of necrosis and endothelial proliferation. Endothelial proliferation was noted in 60% of these tumors and necrosis (either geographic or pseudopalisading types) was noted in 35%. Neither of feature was found to be prognostically significant in anaplastic oligodendrogliomas on univariate or multivariate analysis [32]. In a separate analysis of 61 anaplastic oligodendrogliomas by Smith et al. [33], 61% of tumors had microvascular hyperplasia, while 62% had necrosis. A Cox multivariate regression reinforced the conclusion that these features were not prognostically significant among pure anaplastic oligodendrogliomas. Thus, there is presently insufficient evidence to subclassify anaplastic oligodendrogliomas into grades III and IV based on histologic criteria and anaplastic oligodendrogliomas are considered grade III neoplasms.
Oligoastrocytomas contain distinct regions of oligodendroglial and astrocytic differentiation. The presence of oligodendroglial differentiation within supratentorial gliomas, including those that are malignant, is associated with a longer survival than astrocytomas of the same grade [34–36]. The minimal percentage of each component required for the diagnosis of a mixed glioma has been debated, resulting in poor inter-observer reproducibility for this group of neoplasms. A recent study of diagnostic criteria of infiltrating gliomas suggested that a single 100× field filled with an oligodendroglioma component could be used as a threshold for mixed oligoastrocytomas. This criterion identifies a subset with a better prognosis than astrocytoma and results in improved inter-observer concordance among pathologists [37]. The WHO criteria for anaplastic oligoastrocytoma (WHO grade III) are not well defined, but suggest that “features of anaplasia” should be present [7]. This list includes nuclear atypia, cellular pleomorphism, high cellularity, high mitotic activity, microvascular proliferation and necrosis. Anaplasia can be present in the astrocytic component, the oligodendroglial component or both.

Among high grade mixed oligoastrocytomas, the currently available evidence suggests that the presence of necrosis divides tumors into prognostically distinct groups. In the above referenced study by Miller et al. [32], the overall survival of 215 patients with anaplastic oligoastrocytomas was investigated as it related to age, gender, type of surgical procedure, necrosis and endothelial hyperplasia. Necrosis was seen in 33% of these tumors and endothelial proliferation was noted in 66%. Median survival was found to be significantly shorter in patients with anaplastic oligoastrocytomas with necrosis (22.8 months; 95% CI, 14.9–33 months) than patients whose tumors lacked necrosis (86.9 months; 95% CI, 48.4–129 months). These findings were statistically significant on univariate and multivariate analysis (P < 0.05). The presence of endothelial proliferation was not prognostically significant among anaplastic mixed tumors. There is debate whether anaplastic oligoastrocytomas with necrosis should be considered “oligoastrocytoma, grade IV” or should be classified as “glioblastoma with an oligodendroglial component, grade IV”. The WHO recognizes the latter designation [7]. The evidence supporting the discussion of pathologic criteria and classification is provided in Table 1.

### Inter-observer and intra-observer variability in the diagnosis of malignant glioma

Numerous studies of inter-observer and intra-observer concordance have confirmed that histopathologic methods lack a high degree of reproducibility for distinguishing between oligodendroglial and astrocytic tumors and for grading these neoplasms. In one of the most comprehensive investigations, Coons et al. [37], reported on the concordance of four neuropathologists in the diagnosis of 244 gliomas that were reviewed in four separate sessions. Each session was separated by a review and discussion of discrepant cases at a multi-headed microscope in an attempt to reach a consensus diagnosis. For the first session, the concordance rates among the 4 neuropathologists were as follows: all 4 reviewers agreeing, 52%; any 3 reviewers, 60%; 2 reviewers, 70%. By the fourth session these rates improved to 69%, 75%, and 80%, respectively. Significant improvement in diagnostic concordance among the neuropathologist was noted with each advancing session (P = 0.02). Kappa values of agreement increased from 0.66 in the first session to 0.82 in the 4th session. The concordance among the neuropathologists was generally high for the diagnosis of glioblastoma (grade IV; 94%) and anaplastic astrocytoma (grade III; 78%). The most common alternative diagnoses for anaplastic astrocytoma were oligodendroglioma and oligoastrocytoma. Using the consensus criteria achieved from this initial study, another 315 cases with established patient outcome were reviewed in order to determine if the consensus classification and grading scheme correlated survival data. The authors concluded from the study of this validation set that gliomas with clear evidence of oligodendroglial differentiation (a single 100× field) should be removed from the astrocytoma category because tumors with oligodendroglial components had longer survivals as a group.

In another study of reproducibility, Mittler et al. [38], had four experienced neuropathologists review and diagnose the same set of 30 stereotactic biopsies of astrocytomas. The biopsies were reviewed twice by each neuropathologist, separated by periods of time that ranged from 5 to 14 weeks. The intra-observer and inter-observer agreements were then calculated for each grade of tumor. Intra-observer agreement was 75% for the diagnosis of GBM and 51% for AA, with a mean intra-observer agreement of 63.9% (κ = 0.50). Inter-observer agreement was 62% for GBM (κ = 0.39) and 36% for AA (κ = 0.06). In this study, a larger degree of variability was seen in the diagnosis of intermediate grade astrocytomas (i.e. AA) than for low grade astrocytomas or GBMs. Overall, there was considerable discrepancy observed between the individual pathologists in the study and also between the interpretations of the same pathologists given at different times.

A subsequent study by Prayson et al. [39] compared the consistency in grading astrocytomas by five neuropathologists to that of five general surgical pathologists. Thirty neoplastic and non-neoplastic lesions were sent to the study participants and each was asked to place the lesion into one
<table>
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<tr>
<th>Author (Reference)</th>
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<tr>
<td>Burger et al. [2]</td>
<td>Correlation of radiologic and pathologic findings in gliomas by diagnostic experts to identify pitfalls in pathologic interpretation that commonly leads to misdiagnosis.</td>
<td>III</td>
<td>Review of the combined radiologic and pathologic features of glial neoplasms can minimize misdiagnosis. Recommendations are based on observation and are Class III evidence.</td>
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<tr>
<td>Burger et al. [1]</td>
<td>Review of clinical, radiologic, and pathologic features of brain lesions by neuropathologists, radiologists, and neuro-oncologists to generate features that should be considered before establishing the diagnosis of malignant glioma.</td>
<td>III</td>
<td>Attention to specific clinical, neuroimaging, and pathologic features in a multidisciplinary setting can minimize misdiagnosis of malignant gliomas. The recommendations are based on the experience of brain tumor authorities rather than investigation and are therefore Class III evidence.</td>
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<td>Daumas-Duport et al. [21]</td>
<td>Study of prognostic features, grading and reproducibility in 287 patients with astrocytomas based on a 4 tier scheme that examined four histologic criteria: nuclear atypia, mitoses, endothelial proliferation, and necrosis. Grade 1 defined as 0 features present; grade 2 with 1 feature; grade 3 with 2 features, and grade 4 with 3 or 4 features.</td>
<td>I</td>
<td>In a 15 year follow-up, each of the 4 histologic criteria as well as the derived grade, were strongly correlated with survival ($P &lt; 0.0001$). The method of histologic grading showed concordance of 94% between two double blinded observers. All criteria for Class I evidence were fulfilled, including a $x^2$ statistic of &gt;0.6. This large study included consensus pathology, meaningful outcome periods, and a multivariate analysis of data qualifying it as Class I data.</td>
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<td>Revesz et al. [22]</td>
<td>Review of 273 patients with gliomas comparing pathological features, histologic grading schemes (Kernohan and St. Anne/Mayo criteria) and survival (data available on 209 (76%))</td>
<td>I</td>
<td>Tumor grade established using either the Kernohan and Daumas-Duport Classification systems was significantly correlated with survival (Cox analysis, $P &lt; 0.0001$). Kernohan grading did not differentiate between Grade III and IV while the Daumas-Duport did. Mitosis ($P &lt; 0.0001$, $x^2 = 17.9$), endothelial proliferation ($P &lt; 0.0001$, $x^2 = 39.4$) and necrosis ($P = 0.0007$, $x^2 = 11.5$) were all significantly correlated with survival. In its analysis of classification systems, this study yields Class I data because all criteria are met for a meaningful outcome study.</td>
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<td>Giannini et al. [24]</td>
<td>Analysis of cellular proliferation (mitotic figures and MIB-1 labeling) in 140 diffuse astrocytomas was related to survival and grading criteria (St. Anne–Mayo scheme).</td>
<td>II</td>
<td>Infiltrative astrocytomas with one mitosis had survivals similar to those with zero mitoses, while astrocytomas with &gt;1 mitoses had significant shorter survivals than those with 1 mitosis ($P &lt; 0.013$). Multivariate analysis did not show histologic features to be predictive of survival within a diagnostic category, resulting in Class II designation.</td>
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<td>Coons et al. [26]</td>
<td>Analysis of the extent of microscopic evaluation in diffuse astrocytomas as related to mitotic counts in order to diagnose a grade III astrocytoma.</td>
<td>II</td>
<td>Histologic review of 410 diffuse astrocytomas revealed that examination of fifty 400x fields yields a 92% sensitivity for identifying a mitotic figure within an anaplastic astrocytoma (grade III). The finding of a mitotic figure within in a diffuse astrocytoma was associated with poor survival. There was a strong correlation between the number of fields examined to identify the first mitosis and survival ($P = 0.02$). A single pathologist performed the mitotic count and diagnosis, resulting in Class II designation.</td>
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<td>Barker et al. [28]</td>
<td>Evaluation of endothelial proliferation and necrosis as prognostic features in the diagnosis of glioblastoma among 299 patients enrolled in clinical trials.</td>
<td>II</td>
<td>88% of GBM specimens that contained vascular hyperplasia also had necrosis. Absence of necrosis was associated with a small increase in median survival (12.5 vs. 10.9 months) on univariate and multivariate analysis, but not with a difference in 2-year survival ($P &gt; 0.05$).The lack of inclusion of patients with AA (with no necrosis or vascular hyperplasia) leads to the designation of this study as Class II data for establishing diagnostic criteria for GBM.</td>
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<td>Study of 124 patients diagnosed with oligodendroglioma, including grade II (81) and grade III (43). A set of 18 histologic features were assessed by 6 surgical pathologists and 7 neuropathologists and consensus features were compared to survival.</td>
<td>III</td>
<td>Consensus ratings for each histologic feature were set at 60% agreement. A Cox proportional hazards model of histologic features associated with survival demonstrated that high cellularity, mitoses, vascular hyperplasia and necrosis were all associated with shorter survival on univariate analysis (each ( P &lt; 0.05 )). A threshold of ( \geq 6 ) mitoses per high power field was identified as a critical cut-off in the survival analysis (( P = 0.0001 )). Consensus ratings were low among reviewers, resulting in a diminished level of evidence for establishing diagnostic criteria for anaplastic oligodendroglioma, resulting in level III data.</td>
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<td>Miller et al. [32]</td>
<td>Study of 1093 adults with new onset high grade glioma for the prognostic significance of age, type of surgery, treatment center, tumor histology, necrosis, endothelial proliferation and molecular markers. Univariate and multivariate analysis was performed.</td>
<td>II</td>
<td>Necrosis was a negative prognostic factor for anaplastic oligo-astrocytoma (AOA) (( P &lt; 0.0001 )), but not for anaplastic oligodendrogliomas (AO) on univariate analysis. Median survivals for patients with AOA with necrosis were 22.8 months (95% CI, 14.9–33.8 months) compared to AOA without necrosis (86.9 months; 95% CI, 48.4–129 months). Multivariate analysis demonstrated the necrosis was an independent predictor of poor prognosis in AOA (( P &lt; 0.028 )). This study lacked a consensus diagnosis or analysis of interobserver variability and is considered Class II data on this basis.</td>
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<td>Smith et al. [33]</td>
<td>Analysis of 98 patients with high grade gliomas with oligodendrogial components for the prognostic significance of age, gender, histology, location, microvascular proliferation and necrosis. Univariate and multivariate analysis was performed.</td>
<td>II</td>
<td>Univariate analysis high grade gliomas with oligodendrogial components demonstrated that necrosis, age over 60 years, and an astrocytic component (i.e. anaplastic mixed oligoastrocytoma) were associated with shorter survivals (( P &lt; 0.05 )). Neither the presence of necrosis nor microvascular proliferation in pure anaplastic oligodendrogliomas was prognostically significant on multivariate analysis (( P &gt; 0.05 )). This study did not have sufficient number of mixed oligoastrocytoma patients to reach conclusions regarding necrosis in this tumor histology and the data is consider as class II.</td>
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<td>Shaw et al. [34]</td>
<td>An analysis of outcome related to histologic type and grade among 196 patients with low grade gliomas and 318 patients with high grade gliomas.</td>
<td>III</td>
<td>Among both low grade and high grade supratentorial gliomas, the presence of oligodendrogial morphology (oligodendroglioma or mixed oligoastrocytomas) was associated with longer survival. For high grade gliomas, survivals of mixed oligoastrocytomas and oligodendrogliomas were similar, but were both longer that high grade astrocytomas (log rank ( P = 0.0008 )). This study did not control for clinical parameters in a multivariate analysis and a consensus diagnosis was not established, resulting in Class III evidence.</td>
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<td>Coons et al. [37]</td>
<td>Analysis of histologic criteria for classification and grading in gliomas as related to interobserver concordance and clinical outcome.</td>
<td>III</td>
<td>For diagnosis of a mixed oligoastrocytoma, identification of a 100× microscopic field (2.5 mm²) of oligodendrogial differentiation is sufficient for identifying a mixed glioma with better prognosis and greater inter-observer concordance among pathologists. The criteria for mixed oligoastrocytoma were set for purposes of reaching consensus diagnosis in this study, resulting in Class III designation for this component of the investigation.</td>
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of 3 histologic grades (grading criteria were given to the participants). Agreement on the diagnoses among the neuropathologists was complete (5 of 5 in agreement) in 12 of 30 cases (40%). Four of the 5 neuropathologists agreed in 26 of 30 cases (87%). In contrast, among the surgical pathologists, there were only 6 cases (20%) for which all 5 agreed and only 13 cases (43%) for which 4 of the 5 agreed. The kappa value for inter-observer concordance among the neuropathologists was higher than that for the surgical pathologists ($\kappa = 0.63$ vs. 0.36). The kappa value for agreement on the diagnosis of GBM was 0.81 for the neuropathologists versus 0.63 for the surgical pathologists. For the diagnosis of AA, the kappa values were 0.88 vs. 0.55, respectively. The authors concluded that training in neuropathology and experience in the diagnosis of grading gliomas are important for reliable diagnosis. In contrast to the study by Mittler et al., this study found less variability in the diagnosis of the malignant tumors (including AA and GBM) than for low grade gliomas. The evidence supporting the discussion of observer variability is provided in Table 2.

**Frozen section**

The role of a frozen section diagnosis is to guide the neurosurgeon at the time of the operation, to ensure that diagnostic tissue has been obtained, and to give the most accurate intra-operative diagnostic interpretation, acknowledging limitations of sampling and of the

<table>
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<td>Mittler et al. [38]</td>
<td>Four neuropathologists reviewed of 30 stereotactic brain biopsies of astrocytomas and the intraobserver and interobserver agreement was analyzed.</td>
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<td>Prayson et al. [39]</td>
<td>The interobserver concordance among five neuropathologists and five surgical pathologists was compared in the grading of 30 neoplastic (astrocytomas) and non-neoplastic lesions.</td>
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</table>
technique. Expert opinion suggests the interpretation of morphology by the pathologist should be performed in the context of clinical history, radiographic features, and neurosurgical findings [1, 2]. Frozen sections are not an optimal technique for detecting the histologic features of an infiltrating glioma, especially those that distinguish oligodendrogliomas from astrocytomas [23]. In particular, the features of oligodendrogliomas, such as perinuclear halos, delicate chromatin pattern, and nuclear regularity, are not as evident in frozen tissue. In most instances, the distinction between oligodendroglial and astrocytic differentiation at frozen section is not critical and the diagnosis of “infiltrating glial neoplasm” is sufficient for guiding intra-operative management. Definitive classification and grading of glial neoplasms is most accurate following examination of all tissue submitted for permanent sections, as tissue examined at frozen section may not represent the entire disease process. Nonetheless, a general degree of histologic differentiation (well-, moderately, or poorly differentiated) or histologic grade can usually be derived by assessing the cellular density, nuclear anaplasia, mitotic activity, microvascular hyperplasia, or necrosis [40].

In addition to the diagnostic limitations of frozen section, the process of freezing tissue introduces artifacts that remain in permanent sections and can limit their interpretation. Most notably, nuclei appear more hyperchromatic and atypical in previously frozen tissue; perinuclear halos of oligodendroglioma are not as evident; and the overall cytologic resolution is lower. Therefore, it is recommended that a portion of sampled tumor tissue be reserved for permanent sections without freezing. If it is not clear at the time of frozen section whether additional tissue will be available for permanent sections, it is prudent to freeze only a portion of the tissue submitted for frozen section [41].

Most studies of intra-operative diagnosis based on evaluation of frozen sections have demonstrated good overall agreement with the final diagnosis rendered following review of permanent sections. Cytologic preparations performed on biopsy material at the time of surgery appear to increase the diagnostic accuracy when used together with frozen sections. Martinez et al. [42], reviewed the intra-operative materials on 100 neurosurgical biopsies and established a diagnosis based examination of (1) cytologic material alone; (2) frozen section material alone and (3) both preparations together. These diagnoses were then compared with the final permanent section diagnosis. The correct intra-operative diagnosis, based on the combination of frozen section and cytopathologic exam, was given in 95% of cases, whereas the diagnosis based on frozen sections alone led to an agreement with the final diagnosis in 88%. A diagnosis based on cytologic examination alone resulted in the correct diagnosis in 76%.

This study concluded that the frozen section diagnosis is superior to cytologic diagnosis at the time of operation, but that the highest diagnostic accuracy is achieved when the techniques are used together.

Reyes et al. [43] performed a comparison of two types of cytologic preparations—imprints and smear preparations—as well as frozen sections and compared the diagnoses established with these techniques to the diagnosis established on permanent sections. A series of 150 brain and spinal cord lesions suspected to be brain tumors were evaluated [43]. Among adequate preparations, agreement with the final permanent section diagnosis was 99% for frozen sections. Among the cytologic preparations, the correct diagnosis was achieved on 82% of imprints and on 92% of smears. Thus, frozen section was more accurate than both cytologic preparations for intra-operative diagnosis, while smear preps were superior to imprints.

In some instances, a diagnosis cannot be established on the first tissue sample that is sent for frozen section evaluation, and in these cases a second, third or fourth biopsy may be required. Colbassani et al. [44], compared the intra-operative frozen section diagnoses with permanent section diagnosis for 100 CT-guided brain biopsies and then further investigated the number of biopsies that were required to establish the diagnosis at the time of frozen section. The initial biopsy that was sent for frozen section was diagnostic in 61% of cases. In an additional 25% of cases, two specimens were needed to establish a frozen section diagnosis; more than two frozen sections were required in the remainder of cases. Overall, the frozen section diagnosis agreed with final diagnosis in 92% of cases in this investigation.

Brainard et al. [45], addressed the same issue by investigating the diagnostic yield of the first and subsequent frozen sections on a series of 188 stereotactic brain biopsies. The cumulative diagnostic accuracy of each consecutive frozen section was compared to the permanent section diagnosis. The first frozen section sent to the neuropathologist was diagnostic in 73% of neoplastic cases; for non-neoplastic conditions, the first frozen section was diagnostic in 50% of cases. With the submission of one additional frozen section, diagnostic yield was 89% for neoplastic cases and 65% for neon-neoplastic cases. For all of the non-neoplastic and neoplastic cases combined, it was found that the diagnostic yield increased from 67% to 89% when the number of biopsies increased from one to four.

Taken together, the evidence suggests that a frozen section diagnosis is an accurate means for establishing an intra-operative diagnosis and correlates with the final diagnosis in greater than 85% of cases. The diagnostic accuracy improves with increased numbers of biopsies and also improves with the use of cytopathologic preparations, such as smear preps and imprints. The evidence supporting
the discussion of frozen section the diagnosis of malignant glioma is provided in Table 3.

### Cytologic preparations

Neurosurgically sampled tissue is often examined cytologically following touch (imprint) or smear preparations. Cytological preparations can be examined quickly and reliably at the time of procedure in order to assess specimen adequacy and to establish a diagnosis. Such examination can be critical because these preparations maintain high cellular detail not present in frozen sections [46, 47]. In particular, the presence of glial processes emerging from neoplastic cells is more evident on smear preparations than frozen sections. Additionally, nuclear features in cytologic preparations show more detail and can assist in establishing the diagnosis of a neoplasm. Finally, macrophages and other inflammatory cells are often best appreciated on cytologic preparations and these findings can be highly valuable in some cases for excluding a neoplastic diagnosis.

The accuracy of cytologic examination alone for establishing a diagnosis of central nervous system tumors has varied. In one comprehensive study, Gaudin et al. [48] analyzed the diagnostic accuracy of a combination of cytopathologic and histopathologic techniques in a series of 74 patients undergoing stereotactic biopsy. Included in this study was a comparison of the crush prep technique with the permanent section diagnosis based on evaluation of the tissue block. Diagnosis based on evaluation of crush prep during the procedure was highly correlated with the final diagnosis following evaluation of the tissue block (76% concordance). The concordance for the diagnosis of GBM was 56% and for AA was 100%. In the cases of GBM that could not be diagnosed at the time of the procedure on cytologic studies, there was insufficient evidence of necrosis to establish the diagnosis. For those cases diagnosed as GBM, the sensitivity and specificity of the cytological technique was 0.54 and 1.0 respectively. For cases of AA, the sensitivity was 0.81 and the specificity was 1.0. In a multivariate survival analysis, three factors correlated with decreased survival: age greater than 55 ($P < 0.001$, hazard ratio 3.58 ($CI = 1.95, 6.57$)), nuclear atypia ($P = 0.004$, hazard ratio 4.30 ($CI = 1.61, 11.46$)), and necrosis ($P = 0.016$, hazard ratio 2.14 ($CI = 1.15, 3.97$)). Thus, a high correlation between the cytologic diagnosis and final diagnosis was reported using these preparations and the presence of nuclear atypia and necrosis were found to be correlated with shorter survival.

### Table 3 Role of frozen section in the diagnosis of malignant glioma

<table>
<thead>
<tr>
<th>Author (Reference)</th>
<th>Description of study</th>
<th>Evidence class</th>
<th>Conclusions</th>
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<tr>
<td>Martinez et al. [42]</td>
<td>Comparison of intra-operative cytologic and frozen section diagnoses, together and alone, with permanent section diagnosis on 100 neurosurgical biopsies.</td>
<td>III</td>
<td>The correct intra-operative diagnosis, based on the combination of frozen section and cytologic exam, was given in 95% of cases. Diagnosis based on frozen section alone agreed with the permanent section diagnosis in 88% of cases. Diagnosis based on cytologic examination alone resulted in the correct diagnosis in 76% of cases. The two techniques used together result in the highest diagnostic accuracy.</td>
</tr>
<tr>
<td>Reyes et al. [43]</td>
<td>Comparison of imprints, smear preparations and frozen section diagnoses with permanent section diagnosis on 150 brain and spinal cord lesions suspected to be brain tumors.</td>
<td>III</td>
<td>Among adequate preparations, agreement with final permanent section diagnosis was 82% for imprints, 92% for smears, and 99% for frozen sections.</td>
</tr>
<tr>
<td>Brainard et al. [45]</td>
<td>Evaluation of the diagnostic yield of the first and subsequent frozen sections on 188 stereotactic brain biopsies. The cumulative diagnostic accuracy of each consecutive frozen section was compared to the permanent section diagnosis.</td>
<td>II</td>
<td>The first frozen section sent to the neuropathologist was diagnostic in 73% of neoplastic cases. For non-neoplastic conditions, the first frozen section was diagnostic in 50% of cases. With the submission of one additional frozen section, diagnostic yield was 89% for neoplastic cases and 65% for non-neoplastic cases. For all cases, four biopsies increased the diagnostic yield from 67% to 89%.</td>
</tr>
<tr>
<td>Colbassani et al. [44]</td>
<td>Comparison of intra-operative cytologic and frozen section diagnoses with permanent section diagnosis for 100 CT-guided brain biopsies.</td>
<td>III</td>
<td>Frozen section diagnosis agreed with final diagnosis in 92% of cases. Initial biopsy sent for frozen section was diagnostic in 61% of cases. In 25% of cases, two specimens were needed to establish a diagnosis; more than two were required in the remainder of cases.</td>
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In one of the largest studies, Roessler et al. [49] retrospectively reviewed 4,172 intra-operative cytologic smears from intracranial procedures (3,541 open procedures and 631 stereotactic biopsies with a wide variety of diagnoses) and compared preliminary versus final pathologic diagnosis. Complete correlation was obtained in 90% and the correlation in the diagnosis of glioblastoma was reported as 95.7% with a calculated sensitivity of 0.97 and specificity of 0.99. Significantly less diagnostic accuracy was achieved with other glial tumors (oligodendroglioma, 81%; ependymoma, 78%). The authors concluded that cytologic smears yielded high diagnostic accuracy when compared to the final diagnosis and were particularly accurate in the diagnosis of malignant gliomas.

Similarly, Firlik et al. [50], compared the intra-operative cytologic diagnosis to final diagnosis on 595 stereotactic biopsies performed for a variety of CNS diseases. Cytologic diagnosis correlated with the final diagnosis in 90% of cases (52% with complete correlation and 38% with partial correlation). For 259 malignant gliomas, complete correlation was found in 58% and partial agreement in 35% of cases. A final diagnosis was established in 91% of the cases. The sensitivity of cytopathologic exam for detecting a diagnostic specimen was 96% and the specificity was 75%. Lastly, Bleggi-Torres et al. [51], performed a retrospective comparison of cytologic diagnosis on smear preparation with the final diagnosis for 650 central nervous system neoplasms that were neurosurgically resected. The diagnosis established by cytologic examination agreed with the final diagnosis in 97% of cases. The sensitivity was 98% and the specificity was 95%. The false-positive rate was 0.9% and false negative rate was 10.4%. Most false positive and false negative diagnoses involved the diagnosis of astrocytic neoplasms. Other investigations have reached similar conclusions on the diagnostic accuracy of cytologic evaluations [52].

Thus, comparisons of cytologic diagnoses to permanent section diagnoses have shown agreements ranging from 80 to 97%. While cytologic preparations are invaluable to the practice of surgical neuropathology, surveys indicate that most neuropathologists prefer the combination of frozen section and cytologic preparation to reach an intra-operative diagnosis [50]. The frozen section was the preferred technique of 86% of neuropathologists surveyed. Among the cytopathologic preparations, smear prep was most preferred (49%), followed by touch prep (34%) and crush prep (14%). Shortcomings of the cytologic preparations for establishing a diagnosis of malignant glioma include: (1) the small amount of tissue that can be examined by cytologic preparations; (2) the infiltrative properties and neoplastic cellularity of glial neoplasms are difficult to assess on cytologic preparations; (3) oligodendroglial vs. astrocytic differentiation is not absolute on cytologic preparations; and (4) features that are used for grading glial neoplasms, such as microvascular proliferation and necrosis with pseudopalisading, are based on histologic examination of tissue and have not been adequately validated in cytologic evaluations. It is therefore recommended that cytopathologic preparations be used in combination with histologic preparations for rendering intra-operative and final diagnoses. The evidence supporting the discussion of cytopathologic preparations in the diagnosis of malignant gliomas is provided in Table 4.

### Immunohistochemistry

The diagnosis of malignant glioma is often aided by the use of immunohistochemical stains. Most importantly, immunohistochemistry can be used to determine the cellular differentiation of individual tumor cells in the setting of an intra-axial, poorly differentiated malignant neoplasm, when the differential diagnosis usually includes GBM, metastatic carcinoma or melanoma, and primary CNS lymphoma. Glial fibrillary acidic protein (GFAP) is an intermediate filament expressed by normal glial cells and by glial neoplasms, which can be identified reliably using immunohistochemistry. Since GFAP can be detected in nearly all malignant gliomas and is negative in nearly all carcinomas, lymphomas, melanomas, and sarcomas, a positive GFAP stain of tumor cells supports the diagnosis of malignant glioma in the setting of a malignant CNS neoplasm. GFAP is nearly 100% sensitive as a marker of glial differentiation. For example, in one retrospective immunohistochemical study by Cosgrove et al. [53], 30 astrocytic neoplasms (6 well-differentiated, 12AAs, and 12 GBMs) were analyzed for GFAP expression and all tumors (100%) demonstrated strong expression. Another study of 23 GBMs by Oh et al. [54], found that 100% of these tumors strongly expressed GFAP. GFAP is expressed more intensely and more frequently in astrocytomas than in oligodendrogliomas, including both their low and high grade forms. Without good evidence, however, GFAP has sometimes been regarded as a “marker” of astrocytic, rather than oligodendroglial, differentiation. More recent investigations have demonstrated that neoplastic oligodendroglia cells, especially “minigemistocytes” and “gliofibrillary” oligodendrocytes, can also show GFAP staining [55]. One retrospective analysis by Herpers et al., examined GFAP expression by immunohistochemistry in 50 oligodendroglomas and in 16 mixed oligoastrocytomas. GFAP expression was demonstrated in 50% of oligodendroglomas, and was most evident in “gliofibrillary” oligodendroglia tumor cells [56]. Dehghani et al. [57], analyzed GFAP expression in 89 oligodendroglia (65 grade II and 24 grade III) and reported that 58% of grade II
oligodendrogliomas and 79% of grade III oligodendro-
gliomas expressed GFAP. In the most comprehensive
analysis, Kros et al. performed a retrospective analysis of
GFAP staining of 111 oligodendrogliomas, (23% grade I,
27% grade 2, 11% grade 3, and 39% grade 4), correlating
the findings to tumor cell morphology and to patient sur-
vival. In this analysis, GFAP staining was found in 68% of
newly diagnosed tumors and in 86% of recurrent tumors.
GFAP staining was noted in 50% of grade 1; 79% of grade
2; 42% of grade 3; and 77% of grade 4 oligodendrogliomas.
There was no significant correlation noted between tumor
grade and GFAP staining and GFAP staining was not cor-
related with survival. Thus, GFAP is not a reliable marker
for distinguishing oligodendrogliomas from astrocytomas
and does not have prognostic significance in these tumors.

Caution must be taken in the interpretation of the immu
nohistochemical identification of cytokeratin expression in
malignant neoplasms involving the CNS. Cytokeratin
expression usually indicates epithelial differentiation and it
might be assumed that its expression would support the
diagnosis of metastatic carcinoma rather than GBM in the
setting of a poorly differentiated malignancy. However,
malignant gliomas often show immunoreactivity to cyto-
keratins, especially AE1/3, which is a commonly used
antibody preparation that recognizes numerous cytokeratin
types (i.e. a pan-cytokeratin marker). This immunoreactivity

Table 4  Role of cytopathologic preparations in the diagnosis of malignant glioma

<table>
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<tr>
<td>Gaudin et al. [48]</td>
<td>Retrospective analysis of 74 patients with gliomas who were diagnosed by cytopathologic (crush preparations) and histopathologic techniques following CT-guided brain biopsy. Cellularity, atypia, vascular proliferation, mitoses, necrosis, and gemistocytes were assessed in each preparation. Lesions were classified as gliosis, astrocytoma (A), anaplastic astrocytoma (AA), or GBM.</td>
<td>II</td>
<td>Based on cytologic exam, 5% were classified as gliosis, 9% as atypical gliosis, 5% as high-grade mixed oligodendroglioma/astrocytoma, 15% as astrocytoma, 28% as AA, and 36% as GBM. Diagnosis based on crush preps was highly correlated with the final diagnosis (76% concordance). Concordance for the diagnosis of GBM was 56% and for AA was 100%. Features associated with shorter survival on multivariate analysis using a Cox proportional hazards model included: age &gt;55 ($P &lt; 0.001$, Hazard ratio 3.58), nuclear atypia ($P = 0.004$, Hazard ratio 4.30), necrosis ($P = 0.016$, Hazard ratio 2.14).</td>
</tr>
<tr>
<td>Roessler et al. [49]</td>
<td>Retrospective analysis of cytologic smears obtained on 4,172 neurosurgical patients (3,541 open procedures and 631 stereotactic biopsies) and correlation of cytologic diagnosis with the final diagnosis.</td>
<td>III</td>
<td>Complete correlation of cytologic diagnosis with final diagnosis was seen in 90% of cases. The correlation was high for the diagnosis of GBM (95.7%; sensitivity, 0.97 and specificity, 0.99) and metastatic neoplasms (96.3%), but lower for oligodendrogliomas (81%) and ependymomas (78%).</td>
</tr>
<tr>
<td>Firlik et al. [50]</td>
<td>Comparison of intra-operative cytologic diagnosis to final diagnosis for 595 stereotactic biopsies.</td>
<td>III</td>
<td>Cytologic diagnosis correlated with the final diagnosis in 90% of cases (52% with complete correlation 38% with partial correlation). For 259 malignant gliomas, complete correlation was found in 58% and partial agreement in 35% of cases. A final diagnosis was established in 91% of the cases. The sensitivity of cytopathologic exam for detecting diagnostic cases was 96% and the specificity was 75%.</td>
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<td>Bleggi-Torres et al. [51]</td>
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<td>III</td>
<td>The diagnosis established following cytologic examination agreed with the final diagnosis in 97% of cases. The sensitivity was 98% and the specificity was 95%. The false positive rate was 0.9% and false negative rate was 10.4%. Most false positive and false negative diagnoses involved astrocytic neoplasms.</td>
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<tr>
<td>Shah et al. [52]</td>
<td>Comparison of intra-operative cytologic and frozen section diagnoses with permanent section diagnosis on 183 central nervous system tumors resected following craniotomy.</td>
<td>III</td>
<td>The cytologic preparation was technically satisfactory in 85.2% of cases and the diagnostic accuracy was 89.7% compared to permanent section diagnosis among those preparations that were technically adequate. Diagnostic accuracy of frozen sections (90.4%) was similar to that achieved by cytologic preparations.</td>
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is thought to represent cross-reactivity of the antibodies with GFAP [53, 58, 59]. One of the first investigations of cytokeratin expression in astrocytic neoplasms was performed by Cosgrove et al., who used AE1/3 to immunostain 30 astrocytomas (6 low grade, 12 AA, and 12 GBMs). They demonstrated that 80% of astrocytomas showed immunoreactivity to cytokeratins (AE1/3) including 66% of low grades, 83% of AAs, and 83% of GBMs. In a more comprehensive study, Oh et al. [54], analyzed 23 GBMs and 22 metastatic carcinomas for immunohistochemical expression of GFAP and a panel of cytokeratins that included antibodies to AE1/3, CAM 5.2, cytokeratin 7 (CK7) and cytokeratin 20 (CK20). 95.7% of GBMs showed keratin expression using AE1/3. Only 4.3% of GBMs showed keratin expression of CAM 5.2, CK7, and CK20. Among the metastatic carcinomas, 100% stained with AE1/3 and CAM 5.2, while 77% stained with CK7 and 41% stained with CK20. Three of 22 metastatic carcinomas showed focal GFAP staining. Therefore, epithelial differentiation in metastatic carcinoma is best documented using cytokeratin antibodies that are not positive in astrocytomas, such a Cam5.2, or other epithelial markers, such as epithelial membrane antigen (EMA).

Similarly, nearly all malignant gliomas express S-100 protein, making it a poor marker for distinguishing them from other S-100 positive tumors such as melanoma [60]. The diagnosis of metastatic melanoma should be supported (if necessary) with other markers of melanocytic differentiation such as HMB-45 or microphthalmia transcription factor [61]. The diagnosis of lymphoma is supported by tumoral expression of leukocyte common antigen (LCA, CD45) as well as markers of B cell and T cell differentiation. The evidence supporting the discussion of the use of immunohistochemistry in the diagnosis of malignant glioma is provided in Table 5.

**Cell proliferation**

A variety of techniques have been utilized to assess cell proliferation in gliomas including tritiated thymidine/bromodeoxyuridine, histone mRNA in situ hybridization, flow cytometry, DNA polymerase alpha, topoisomerase II-alpha, p105, PCNA and Ki-67/MIB-1 [62]. The most reliable and technically feasible method for most pathology laboratories is the Ki-67/MIB-1 antibody. This antibody identifies an antigen present in the nuclei of cells in the G1, S, G2 and M phases of the cell cycle, but is not expressed in the resting phase, G0. The results are usually expressed as a percentage of positive staining tumor cell nuclei.

Importantly, the Ki67/MIB-1 labeling index is not a component of the WHO grading scheme for glial neoplasms, nor is it considered in any other recent grading systems [4, 5, 7, 21]. However, many investigations have demonstrated a significant positive correlation between Ki-67/MIB-1 indices and histologic grade and have shown that higher Ki-67/MIB-1 proliferation indices are associated with shorter survivals. In one of the first comparative analyses of proliferation markers in astrocytic neoplasms, McKeever et al. [63], investigated MIB-1, BrdU and PCNA, as they related to patient survival for 65 astrocytomas (36 grade IV, 15 grade III, and 14 grade II). The MIB-1 proliferation index was found to be more predictive of survival (logrank P = 0.06) than either BrdU or PCNA. In univariate analysis, a low MIB-1 proliferation rate (<2.5%) was associated with a longer survival (P = 0.056). On Cox multivariate analysis, histologic grade, age and Karnofsky performance status were all associated with survival (P < 0.05), but MIB-1 proliferation was not. The authors concluded that MIB-1 is the most predictive proliferation marker and is helpful in cases where clinical or histopathologic factors are ambiguous.

As part of a larger study of proliferation and prognosis, Giannini et al. [24] studied the MIB-1 index as an independent prognostic factor in 140 diffuse astrocytomases, including 45 grade II, 50 grade III and 45 grade IV. MIB-1 indices were higher in grade III than in grade II (P = 0.001) and were higher in grade IV than in grade III (P = 0.014). On a multivariate analysis that included tumors of all grades (grades II, III, and IV), this study found that necrosis, age and mitotic index were independent markers of survival (P < 0.05). MIB-1 proliferation was not an independent marker of prognosis when grade IV tumors were included in the analysis, mostly because the presence of necrosis was such a statistically powerful predictive marker. Among grade II and III astrocytomases, MIB-1 index was highly correlated with survival on multivariate analysis (P < 0.05). The authors concluded that MIB-1 proliferation provides clinically useful information in the categories of grade II and III astrocytomases.

The value of MIB-1 in prognosticating grade II and III astrocytomases was also supported by an investigation by Hsu et al. [64]. The MIB-1 index was investigated as a prognostic marker in 80 diffuse gliomas, including 16 grade II, 31 grade III, and 33 grade IV tumors. The MIB-1 proliferation index was lower in grade II than grade III gliomas (P < 0.0001); however, the grade III and grade IV gliomas did not have statistically different proliferation indices. Univariate analysis that included all tumors showed that a MIB-1 index less than 1.5% was associated with longer survival (P < 0.0005). In multivariate analysis, a MIB-1 index less than 1.5% was prognostically significant only when coupled with histologic grade. Grade II gliomas with MIB-1 indices higher than 1.5% had shorter survivals than grade II or grade III gliomas with MIB-1 indices less than 1.5%; however, these findings were not statistically significant. The authors concluded that the
MIB-1 proliferation index was particularly useful in grade II and III tumors, mostly because it identifies aggressive tumors in the grade II category.

The prognostic utility of MIB-1 indices among the malignant gliomas (i.e., grades III and IV) has been debated. Wakimoto et al. [65], evaluated the MIB-1 labeling index as an independent prognostic marker in 72 supratentorial astrocytomas, including 19 grade II, 25 grade III and 28 grade IV tumors. Proliferation indices in this study correlated with tumor grade: MIB-1 indices were higher in grade IV than in grade II tumors (P < 0.001). Multivariate analysis of factors associated with survival demonstrated that histologic grade, MIB-1 index and Karnofsky performance status before and after treatment were independent statistically significant prognostic factors (P < 0.05). When the analysis was performed only on the high grade tumors (grades III and IV), the MIB-1 index, KPS score after treatment and location (superficial vs. deep) were the only prognostically significant factors (P < 0.05). Thus, this study suggested that MIB-1 studies provide useful prognostic data for high grade gliomas.

A different conclusion was reached in a recent retrospective analysis of MIB-1 proliferation as an independent prognostic marker in a series of 116 GBM patients [66]. Importantly, this study included only newly diagnosed tumors and only grade IV histology (GBM). The mean MIB-1 index was 12.5% and varied from 0 to 76.4%. MIB-1 proliferation was not associated with survival on either univariate or multivariate analysis. Similar to other studies, the multivariate analysis demonstrated that patient age, performance status and extent of resection were each independent markers of survival (P < 0.05). Thus, when tumor histology is restricted to GBM, the MIB-1 proliferation index does not have much utility.

While the histologic grading of astrocytomas has well established criteria, the grading of oligodendrogliomas is more subjective, suggesting that MIB-1 proliferation indices might offer more prognostic utility in this tumor type. With this rationale, Coons et al. [67], evaluated MIB-1 labeling indices as an independent prognostic marker in 55 oligodendrogliomas (grades I–IV) and 26 oligoastrocytomas (grades I–IV). Thirty-one tumors were high grade (grades III and IV) while 50 tumors were low grade (grades I and II). On Cox proportional hazard multivariate analysis, the MIB-1 proliferation index was an independent marker of prognosis, but only after correcting for histologic grade (P = 0.04). Overall, tumors with a MIB-1 index greater than 5% had shorter survivals than those with <5%
Eighty-seven percent of malignant gliomas is this study had MIB-1 indices >5%. Thus, the MIB-1 proliferation index provides useful information on the clinical behavior of oligodendrogliomas.

One potential shortcoming of MIB-1 proliferation studies is the high degree of variability in tissue processing, immunohistochemical staining, and quantitation techniques between laboratories, making it difficult to standardize proliferation indices for prognostic purposes or for use in clinical trials. There is also a well documented variation in proliferation rates within a single tumor. Another variable that has only recently been recognized is the individual variability in cell counting. Marie et al. [68], investigated the interobserver variability of MIB-1 proliferation indices in glial neoplasms. In this study, the authors performed MIB-1 immunohistochemistry on a series of 50 astrocytic gliomas and determined the interobserver variability of MIB-1 labeling indices (LI) calculated by six pathologists using cut-off values of 2.5%, 5.0%, 8.0%, 11.0%, and 15.0%. The interobserver variability was high; the best results were found with a cut-off value of 5.0% yielding a pair wise kappa statistic ranging from 0.52 to 0.80. The authors concluded that the high level of interobserver variability suggests MIB-1 LI prognostic cut-off values may not be clinically useful for predicting outcome in individual patients with primary brain tumors.

The determination of a labeling index is not warranted as a routine part of the evaluation of all gliomas, due to limitations associated with tumor heterogeneity and sampling, as well as differences in staining methodology, index determination, and the degree of inter-observer variability. It may be prognostically helpful, however, in histologically borderline cases, such as those that are at the grade II–III and III–IV border. A high labeling index in this setting may indicate a more aggressive neoplasm. The evidence supporting the discussion of cell proliferation markers in malignant gliomas is provided in Table 6.

Table 6 Role of cell proliferation markers (MIB-1) in malignant glioma

<table>
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<td>McKeever et al. [63]</td>
<td>Analysis of proliferation markers, including MIB-1, BrdU and PCNA, as they relate to survival in 65 astrocytomas (36 grade 4, 15 grade 3, and 14 grade 2).</td>
<td>II</td>
<td>MIB-1 proliferation indices were more predictive of survival (log rank $P = 0.06$) than BrdU or PCNA. In univariate analysis, a low MIB-1 proliferation rate ($&lt;2.5%$) was associated with a longer survival ($P = 0.056$). On Cox multivariate analysis, histologic grade, age and KPS were associated with survival ($P &lt; 0.05$), but not MIB-1 proliferation. The authors concluded that MIB-1 is helpful in cases where clinical or histopathologic factors are ambiguous or where they can not be fully assessed.</td>
</tr>
<tr>
<td>Giannini et al. [24]</td>
<td>MIB-1 index evaluated as an independent prognostic factor in 140 diffuse astrocytomas, including 45 grade 2, 50 grade 3 and 45 grade 4, with assessment of prognostic significance.</td>
<td>II</td>
<td>MIB-1 indices significantly correlated with grade. MIB-1 indices were higher in grade 3 than in grade 2 ($P = 0.001$) and were higher in grade 4 than in grade 3 ($P = 0.014$). Necrosis, age and mitotic index were prognostically significant on multivariate analysis of all astrocytomas (grades 2, 3, and 4; $P &lt; 0.05$) Among grade 2 and 3 astrocytomas, MIB-1 index was highly correlated with survival on multivariate analysis ($P &lt; 0.05$). The authors conclude that MIB-1 proliferation is prognostically useful in grade 2 and 3 astrocytomas.</td>
</tr>
<tr>
<td>Hsu et al. [64]</td>
<td>Evaluation of MIB-1 labeling index as a prognostic marker in 80 diffuse gliomas, including 16 grade 2, 31 grade 3, and 33 grade 4 gliomas.</td>
<td>II</td>
<td>The MIB-1 proliferation index was lower in grade 2 than grade 3 gliomas ($P &lt; 0.0001$). Grade 3 and grade 4 gliomas were not statistically different. Univariate analysis showed MIB-1 index less than 1.5% was associated with a longer survival ($P &lt; 0.0005$). In multivariate analysis, a MIB-1 index less than 1.5% was prognostically significant when coupled with histologic grade. Grade 2 gliomas with MIB-1 higher than 1.5% had shorter survivals than grade 2 or 3 gliomas with MIB-1 indices less than 1.5% (finding not significant). The authors conclude that MIB-1 is useful in identifying aggressive grade 2 tumors.</td>
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<td>Evaluation of the MIB-1 labeling index as an independent prognostic marker in 72 supratentorial astrocytomas (19 grade 2, 25 grade 3 and 28 grade 4).</td>
<td>II</td>
<td>MIB-1 proliferation indices were higher in grade 4 than grade 3 and were higher in grade 3 than in grade 2 astrocytomas ($P &lt; 0.001$) Multivariate analysis showed that histologic grade, MIB-1 index and Karnofsky performance status before and after treatment were independent statistically significant prognostic factors ($P &lt; 0.05$). Among high grade tumors (grades 3 and 4), MIB-1 index, KPS score after treatment and superficial vs. deep location were prognostically significant ($P &lt; 0.05$).</td>
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<td>Moskowitz et al. [66]</td>
<td>Retrospective analysis of MIB-1 proliferation index as an independent prognostic marker in a series of 116 patients with newly diagnosed GBM.</td>
<td>II</td>
<td>Mean MIB-1 index was 12.5% and varied from 0 to 76.4%. MIB-1 proliferation was not associated with survival of patients with GBM on univariate analysis and was not an independent prognostic marker on multivariate analysis. Patient age, performance status and extent of resection were each independent markers of survival ($P &lt; 0.05$).</td>
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<td>Coons et al. [67]</td>
<td>Evaluation of MIB-1 labeling indices in 55 oligodendrogliomas and 26 oligoastrocytomas as an independent prognostic marker. A four-tier grading system was used. 31 tumors were high grade (grades 3 and 4) while 50 tumors were low grade (grades 1 and 2).</td>
<td>II</td>
<td>On Cox proportional hazard multivariate analysis, the MIB-1 proliferation index was an independent marker of prognosis after correcting for histologic grade ($P = 0.04$). Overall, tumors with a MIB-1 index greater than 5% had shorter survivals than those with $&lt;5%$ ($P &lt; 0.001$). 87% of malignant gliomas in this study had MIB-1 indices $&gt;5%$.</td>
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<td>Marie et al. [68]</td>
<td>Investigation of the interobserver variability between six pathologists in the assignment of MIB-1 labeling indices in astrocytomas. Tumors investigated were 10 grade 1-2, 18 grade 3, and 22 grade 4 gliomas. MIB-1 indices were counted for 5000 cells on one slide in the area of highest labeling. Kappa statistics were calculated for pathologist pairs for cut-offs of 2.5%, 5%, 8%, 11%, and 15%.</td>
<td>II</td>
<td>Interobserver variability was high. The best results were with a cut-off value of 5.0% yielding a pair-wise kappa statistic ranging from 0.52 to 0.80. The authors conclude that the high inter-observer variability suggests that MIB-1 LI prognostic cut-off values may not be useful clinically for predicting outcome in individual patients with primary brain tumors. Further prospective studies are needed to investigate the prognostic usefulness of MIB-1 LI ranges that optimize interobserver agreement.</td>
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Genetic testing

It is now clear that multiple genetic alterations are involved with gliomagenesis and that each histologic category of glioma contains a number of distinct molecular genetic subsets. For malignant gliomas, the specific genetic alterations that have been most thoroughly documented include PTEN and TP53 mutations, MDM2 and EGFR amplification, p14ARF and p16(CDKN2A) deletion, and 1p/19q deletions [5, 7, 69, 70]. Some genetic alterations have been used in the diagnostic setting, either to provide assistance with pathologic classification or to provide independent prognostic information [71]. Each technique for genetic testing has its own set of advantages and disadvantages. Most often employed are loss of heterozygosity (LOH, either traditional gel-based assays or capillary electrophoresis), fluorescence in-situ hybridization (FISH) and comparative genomic hybridization (CGH) [72–74]. These tests demonstrate good to excellent concordance (73–99%) and the choice depends largely on the preferences of the pathologist, department, and institution. LOH analysis and FISH have the highest concordance (>93%) and are utilized most frequently for diagnostic purposes on tissue derived from histologic sections [75]. FISH has some advantages from a pathologist’s perspective: (1) analysis is based on the morphologic identification of genetic alterations within tumor cell nuclei; (2) non-neoplastic cells (positive controls) are almost always present within the tissue sections examined (i.e. normal endothelial cells, neurons, etc.); (3) FISH does not require microdissection of normal and tumor before analysis, and (4) genetic gains and losses in infiltrative tumors with a low ratio of neoplastic/normal cells can be analyzed by FISH, whereas these alterations may not be detected by PCR-based analysis (LOH studies) due to overwhelming amounts of normal DNA. One major disadvantage of FISH is that it can be highly labor intensive and automation has not yet reached all of its applications.

Some genetic alterations occur in both astrocytic and oligodendroglial tumors, generally with increasing frequency at
higher grades and are therefore not useful markers for discriminating histologic subtypes. These alterations, including loss of 9p21 (p16/CDKN2A) and losses involving chromosome 10 (PTEN/DMBT1), may provide prognostic information for a given tumor, but are not specific to one histology and will not be further considered. Genetic testing of malignant gliomas is becoming a routine component of pathologic diagnosis and there are some specific examples when such tests can provide diagnostic or prognostic information and are warranted.

1p/19q

One of the best studied relationships between genetic alterations and glioma histology is the strong association of allelic losses on chromosomes 1p and 19q and the oligodendroglioma phenotype [76]. Reifenberger et al. [77], was the first to observe that a high percentage of oligodendroglial tumors contained the specific combination of allelic losses on chromosomes 1p and 19q. This group studied the genetic status at 180 polymorphic loci located throughout the human genome by restriction length polymorphism analysis on 8 oligodendrogliomas, 13 anaplastic oligodendrogliomas, 8 oligoastrocytomas, and 8 anaplastic oligoastrocytomas. Among the pure oligodendrogliomas (not the mixed tumors), they found 19q losses in 81%, which was the most frequent genetic alteration among these neoplasms. In those tumors with 19q loss, 75% also had 1p loss. Subsequent studies by other groups have confirmed that between 60% and 80% of oligodendroglial neoplasms demonstrate combined 1p and 19q losses [73]. Others have suggested that the morphologically pure, or classic, forms of oligodendrogliomas have even higher frequencies of combined 1p/19q loss [78, 79]. On the other hand, gliomas with different or accompanying genetic alterations, such as 10q loss, TP53 mutation, and 9p (p16/CDKN2A) losses, have less classic oligodendroglial features [80, 81].

Enthusiasm for defining genetic subsets of oligodendrogliomas increased substantially with the demonstration of prognostically distinct groups. Cairncross et al. were the first to show that anaplastic oligodendrogliomas with losses of chromosome 1p and 19q were associated with enhanced response to chemotherapy (PCV–procarbazine, CCNU, vincristine) and prolonged survival. In this study, 39 anaplastic oligodendrogliomas were characterized by LOH analysis for 1p and 19q status and for CDKN2A(p16) gene deletion, and for TP53 mutations by sequence analysis. The molecular findings were compared to response to PCV therapy and survival. Sixty-five percent of anaplastic oligodendrogliomas in this study demonstrated combined loss of 1p and 19q and this genetic finding was associated with both chemoresponsiveness (P < 0.01) and longer survival (P < 0.001) on univariate analysis and with improved survival on multivariate modeling (P = 0.05). In contrast, those anaplastic oligodendrogliomas with p16/CDKN2A homozygous deletions (21%) had shorter survivals on univariate (P = 0.0009) and multivariate (P < 0.05) analysis. These molecular subsets were not distinguishable morphologically and had little genetic overlap (i.e. tumors with 1p and 19 q losses did not contain p16/CDKN2A deletions (P = 0.048)). Subsequent studies of therapeutic response in oligodendrogliomas have demonstrated that those tumors with 1p/19q losses are associated with improved responses to other chemotherapies, including temozolomide, and to radiation therapy [82, 83]. It remains unsettled if 1p/19q codeletion is a marker of therapeutic response or a more general marker of favorable prognosis, independent of therapy.

In order to determine whether 1p and 19q losses were specific to the oligodendroglial morphology, Smith et al. [75] investigated the allelic losses of 1p and 19q in 115 diffuse gliomas, including 66 tumors with astrocytic morphology, 33 with oligodendrogial morphology and 16 with mixed oligodendroglial/astrocytic morphology. Eighty-four of the neoplasms in this study were malignant (grades III or IV; 73%) Consensus diagnoses on these gliomas were established by 3 experienced neuropathologists and the kappa statistic for agreement between pairs of neuropathologists were 0.67, 0.61, and 0.66. Tumors were tested for 1p and 19q status by three independent methods: fluorescence in situ hybridization (FISH), comparative genomic hybridization (CGH), and conventional LOH analysis. The correlation coefficients for detecting 1p and 19q alterations were highest between LOH and FISH analyses: 0.98 for 1p loss and 0.87 for 19q loss. In this investigation, combined loss of 1p and 19q was seen in 11% of astrocytomas, 31% of the mixed oligoastrocytomas, and 64% of oligodendrogliomas. The oligodendroglial histology was strongly associated with the finding of 1p loss (P < 0.0001), 19 q loss (P = 0.0017) and the combined loss of 1p and 19 q (P < 0.0001). Taking into consideration only those tumors with pure astrocytic and pure oligodendroglial morphology, this investigation showed that 1p/19q loss had a sensitivity of 64% for oligodendroglial morphology and a specificity of 89%. Thus, while most oligodendrogliomas showed 1p/19q loss, not all did. Moreover, a small percentage of mixed gliomas and astrocytomas also showed similar deletions.

From these investigations, it remained unclear if survival differences among oligodendrogliomas with 1p/19q losses were due to the genetic alterations themselves, the oligodendroglial phenotype, or their combination. This distinction is critical since other histologic types of infiltrating gliomas with 1p and 19q losses, such as GBMs, could have favorable outcomes as well. Smith et al. [72] addressed this question by assessing 1p and 19q status as it
related to survival for 162 patients with diffuse gliomas, including 52 oligodendrogliomas, 79 astrocytomas, and 31 mixed oligoastrocytomas. Within this patient group, 116 (72%) had malignant gliomas. Combined loss of 1p and 19q was found to be predictive of prolonged overall survival for patients with oligodendrogliomas on univariate analysis \( (P = 0.03) \). After adjusting for age and tumor grade, 1p and 19q loss were associated with longer survival on multivariate analysis \( (P < 0.01) \). However, combined 1p and 19q losses were uncommon in tumors with astrocytic morphology (8%) and were not predictive of prolonged survival. Neither were such losses prognostically significant for mixed oligoastrocytomas of any grade. Other investigations have also provided evidence that 1p/19 losses in astrocytic neoplasms are extremely rare and not prognostically significant [73, 84].

In contrast, Schmidt et al. [85] reached a different conclusion about the prognostic significance of 1p/19q loss in GBMs. His investigation of 97 patients with GBM assessed the prognostic significance of genetic alterations, including 1p and 19q loss. This study included 87 primary GBMs, 6 GBMs that had progressed from a lower grade and 4 giant cell GBMs. Similar to other studies, approximately 30% of GBMs demonstrated LOH at 19q and 19% at 1p. The combination of 1p and 19q loss occurred in only 5 of the 97 GBMs. However, the mean survival of these 5 patients was 22.2 months, considerably longer than patients whose tumors retained 1p, 19q, or both (9.0 months) \( (P = 0.053) \). The authors concluded that the frequency of 1p and 19q losses was much lower in GBMs than oligodendroglial tumors, but that these alterations could have prognostic significance. Despite the findings of this study, the cumulative evidence suggests that combined loss of 1p/19q is best viewed as a marker of oligodendroglial differentiation as well as a finding associated with a favorable prognosis in these tumors. Additional studies will be needed to demonstrate the clinical utility, if any, for 1p/19q testing in other primary brain tumors.

**EGFR**

Amplifications of the epidermal growth factor receptor (EGFR) gene occur in approximately 40% of GBMs and 10% of anaplastic astrocytomas and can be detected by FISH, CGH, or PCR-based tests [5, 86, 87]. Amplifications are much less frequent in low grade astrocytomas and are considered a late genetic event in the progression of tumors to GBM. Either wild type or mutated forms of EGFR can be amplified, and in either case, both mRNA and cell surface protein levels are markedly increased. The most common EGFR amplification is a mutated form lacking exons 2–7, which results in a truncated cell surface protein with constitutive tyrosine kinase activity (EGFRvIII) [87–89].

The significance of EGFR gene amplification or EGFR protein over-expression as a prognostic marker in GBM has been debated. Most comprehensive immunohistochemical and molecular genetic studies have concluded that EGFR status is not prognostically significant in patients with GBM [85–87, 90]. Indeed, a recent meta-analysis of seven previously published investigations suggested that EGFR amplification was not associated with a statistically different prognosis [91]. In one of the most recent and most carefully performed investigations, Liu et al. [87] studied 221 astrocytic neoplasms (including 160 GBMs, 41 AA, and 20 grade II) by Southern blot and quantitative PCR for EGFR amplification and EGFRvIII rearrangements and correlated the results with patient survival. This investigation demonstrated that 41% of GBMs, 9.8% of AAs, and 0% of grade II astrocytomas had EGFR amplification and that 54% of the GBMs and 75% of the AAs that had EGFR amplification also had EGFRvIII rearrangements. There was a trend toward shorter survival of patient with AAs that contained EGFR abnormality \( (P = 0.069) \). However, neither EGFR amplification nor EGFRvIII rearrangement was associated with shorter survival in patients with GBM. Other investigations have shown similar frequencies of EGFR amplification in GBMs and reached a similar conclusion that there is no association of these changes with patient outcome [85, 86]. It should be noted that the prognostic significance of EGFR expression may be more complex and that patient age may play a role in the prognostic significance of EGFR expression in GBM [86, 92]. EGFR amplification and overexpression may be a marker of poor prognosis in younger patients and of good prognosis in older patients. Other studies have demonstrated that amplifications and overexpression of EGFR in anaplastic astrocytomas, although less common, might identify those that are further biologically progressed and associated with a poor prognosis [86, 87, 93]. Most analyses of EGFR and EGFRvIII in GBM have been performed at the genetic level using PCR-based techniques or FISH. Immunohistochemical techniques are available for the detection of EGFR and in high grade gliomas, but this application has not yet demonstrated its prognostic significance in GBMs or its ability to distinguish between histologic types of gliomas [93, 94].

**EGFR** amplifications are rare in oligodendroglial tumors and analysis of EGFR status has proven useful for distinguishing high grade astrocytomas from anaplastic oligodendrogliomas in some instances. The majority of GBMs can be diagnosed based on morphology and establishing EGFR status is not generally necessary as a diagnostic test. However, the recently described “small cell astrocytoma” is a form of high grade astrocytoma that has a great deal of morphologic overlap with anaplastic oligodendroglioma and may require ancillary genetic tests for...
correct diagnosis [95, 96]. Both of these tumors contain a high density of neoplastic cells with scant cytoplasm, uniform and deceptively bland nuclei, and a high proliferation index. It is important to recognize small cell astrocytomas because they are biologically aggressive, behaving clinically as GBMs (WHO grade IV) even in the absence of necrosis and microvascular hyperplasia, whereas anaplastic oligodendrogliomas are WHO grade III and have a better prognosis. Small cell astrocytomas are characterized by a high frequency of EGFR amplification (69%) and chromosome 10 losses (97%), but have intact chromosome 1p and 19q (100%) [95]. In contrast, anaplastic oligodendroglialomas show the opposite pattern of genetic alterations, having a high frequency of 1p/19q deletions but only rare EGFR amplifications and chromosome 10 losses. Thus, in current practice, genetic testing for EGFR may be helpful in classification and diagnosis of malignant gliomas in certain circumstances.

Therapies directed at the overexpressed EGFR in GBMs are finding their way into neuro-oncology practice [97–99]. The pharmacologic agents gefitinib and erlotinib are specific, reversible inhibitors of EGFR tyrosine kinase that have been used with variable success in the treatment of GBMs (WHO grade IV) even in the absence of necrosis and microvascular hyperplasia, whereas anaplastic oligodendrogliomas are WHO grade III and have a better prognosis. Small cell astrocytomas are characterized by a high frequency of EGFR amplification (69%) and chromosome 10 losses (97%), but have intact chromosome 1p and 19q (100%) [95]. In contrast, anaplastic oligodendroglialomas show the opposite pattern of genetic alterations, having a high frequency of 1p/19q deletions but only rare EGFR amplifications and chromosome 10 losses. Thus, in current practice, genetic testing for EGFR may be helpful in classification and diagnosis of malignant gliomas in certain circumstances.

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MGMT

Many of the most effective chemotherapies used to treat GBM, including temozolomide and BCNU, are agents that crosslink DNA by alkylating at the O6 of guanine. DNA crosslinking is reversed by the DNA repair enzyme MGMT (O6-methylguanine-DNA methyltransferase). Thus, low levels of MGMT expression in the neoplasm would be expected to be associated with an enhanced response to alkylating agents. The expression level of MGMT is determined in large part by the methylation status of the gene’s promoter. This “epigenetic silencing” of MGMT occurs in 40–50% of GBMs and can be assessed by its promoter methylation status on PCR-based tests of genomic DNA. Epigenetic silencing of MGMT in tumoral tissue is associated with response to BCNU therapy and improved survival in patients with GBM [102].

A recent investigation of temozolamide for the treatment of GBM found that epigenetic gene silencing of MGMT was associated with a longer survival, independent of treatment [103, 104]. The study also demonstrated a survival advantage among those patients treated with temozolomide and radiotherapy whose GBMs had a silenced MGMT gene. Gene silencing in this study was determined by examining the methylation status of the MGMT promoter by methylation-specific polymerase-chain-reaction analysis. These tests are becoming more widely available in molecular diagnostic labs [105]. Antibodies are also available for the detection of the MGMT protein, which would make MGMT testing more techni-
cally feasible for most pathology laboratories [106, 107]. One recent investigation of MGMT promoter status addressed the issue of intratumoral heterogeneity for this marker by testing 3 spatially distinct stereotactic biopsies taken from the same tumor in 18 GBMs and 7 AAs [108]. This study concluded that MGMT promoter methylation, as determined by methylation specific PCR, was homogeneous within a given neoplasm. However, this study also attempted to correlate the findings of MGMT promoter methylation with MGMT protein expression as determined by immunohistochemistry. Importantly, they found no significant correlation between these two tests. Thus, it remains to be seen if immunohistochemistry for MGMT will be of value in diagnostic testing of GBMs. Before it is, correlations of immunohistochemical expression of MGMT with epigenetic status will be necessary as will a validation of a relationship between MGMT immunoe-
xpression and therapeutic response. Since temozolomide has become a standard of care for the treatment of GBM, testing for MGMT status will likely become an important component of a complete diagnostic workup. The evidence supporting the discussion of genetic testing in malignant glioma is provided in Table 7.
Table 7 Role of genetic testing in malignant glioma

<table>
<thead>
<tr>
<th>Author (Reference)</th>
<th>Description of study</th>
<th>Evidence class</th>
<th>Conclusions</th>
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<tr>
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<td>19q losses at 19q13.2-q13.4 were noted in 81% of oligodendrogliomas and were the most frequent genetic alteration among these neoplasms. 31% of mixed gliomas had this loss. Among the oligodendrogliomas with 19q loss, 75% also had 1p loss. No TP53 mutations were uncovered. Losses on chromosome 9p and 10 were seen more frequently in anaplastic oligodendrogliomas.</td>
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<td>Retrospective analysis of genetic alterations in 39 anaplastic oligodendrogliomas, including 1p and 19q status by LOH analysis, CDKN2A(p16) gene deletion, and TP53 mutations. Molecular findings were compared to response to PCV therapy and survival.</td>
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<td>65% of anaplastic oligodendrogliomas had 1p and 19q losses and this genetic finding was associated with both chemoresponsiveness ($P &lt; 0.01$) and longer survival ($P &lt; 0.001$) on univariate analysis and with improved survival on multivariate modeling ($P = 0.05$). Anaplastic oligodendrogliomas with p16(CDKN2A) homozygous deletions (21%) had shorter survivals on univariate ($P = 0.0009$) and multivariate ($P &lt; 0.05$) analysis. Tumors with 1p and 19q losses did not contain p16(CDKN2A) deletions ($P = 0.048$). Consensus diagnoses by 3 neuropathologists gave kappa statistics for agreement between pairs of: 0.67, 0.61, and 0.66. The correlation coefficients for detecting 1p and 19q alterations were highest between LOH and FISH analyses: 0.98 for 1p loss and 0.87 for 19q loss. Combined loss of 1p and 19q was seen in 11% of astrocytomas, 31% of the mixed oligoastrocytomas, and 64% of oligodendrogliomas. The oligodendrogliosthistology was strongly associated with the finding of 1p loss ($P &lt; 0.0001$), 19q loss ($P = 0.0017$) and the combined loss of 1p and 19q ($P &lt; 0.0001$). Combined loss of 1p and 19q was predictive of prolonged overall survival for patients with oligodendrogliomas on univariate analysis ($P = 0.03$). After adjusting for age and tumor grade, 1p and 19q loss were associated with longer survival on multivariate analysis ($P &lt; 0.01$). Combined 1p and 19q losses were uncommon in tumors with astrocytic morphology (8%) and were not predictive of prolonged survival. 1p/19q losses were not prognostically significant for mixed oligoastrocytomas of any grade.</td>
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<td>30% of GBMs demonstrated LOH at 19q and 19% at 1p. The combination of 1p and 19q loss occurred in only 5 of the 97 GBMs. However, the mean survival of these 5 patients was 22.2 months, much longer than patients whose tumors retained 1p, 19q, or both (9.0 months) ($P = 0.053$). TP53 mutations were associated with a longer survival (16.0 vs. 9.1 months, $P = 0.0085$). LOH 10q was associated with a shorter survival (8.8 vs. 18 months, $P = 0.0028$). There was no significant association of survival with PTEN mutation or EGFR amplification.</td>
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<td>Schmidt et al. [85]</td>
<td>Retrospective analysis of 97 consecutive GBM specimens for histologic features and genetic alterations including LOH studies for 1p, 10p, 10q, 17p, and 19q, genetic analysis of TP53, EGFR and PTEN. Features were correlated with patient survival.</td>
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<td>Smith et al. [86]</td>
<td>Study of 174 malignant astrocytomas, including 111 GBMs and 63 AAs, for alterations of EGFR, PTEN and TP53 genes, and for losses or gains of chromosomes 7 and 10 by FISH. Genetic changes were correlated with patient survival.</td>
<td>I</td>
<td>EGFR amplification was seen in 17% of AAs and 41% of GBMs, but was not associated with survival for either tumor type ($P &gt; 0.05$). In patients older than 60-years old, EGFR amplification was associated with prolonged survival ($P &lt; 0.05$). PTEN alterations were noted in 18% of AAs and 34% of GBMs and were associated with a shorter survival in AAs (34.4 months vs. 4.4 months, $P = 0.002$ on univariate analysis), but not in GBMs. TP53 mutations were seen in 36% of AAs and 10% of GBMs and were associated with longer survivals for AAs on univariate analysis (59 vs. 16 months, $P = 0.012$). On multivariate analysis, PTEN mutation was associated with shorter survival for patients with AA (hazard ratio = 4.34; 95% CI = 1.82 to 10.34).</td>
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Summary

The current pathologic diagnosis of malignant gliomas relies heavily on the histopathologic examination of H&E stained slides prepared from tissue received from stereotactic biopsy or neurosurgical resection. A diagnosis should be established in a multidisciplinary setting with knowledge of clinical information, neurosurgical impression, and radiologic findings. Morphologic criteria for classifying and grading gliomas can be found in the WHO Classification of nervous system tumors. Ancillary studies that are frequently utilized to diagnose malignant gliomas include cytopathologic examination, immunohistochemistry for differentiation and proliferation, and genetic studies. Intraoperative frozen sections of suspected glial neoplasms are performed to guide neurosurgical management, assure tissue adequacy, and establish a diagnosis, acknowledging the limitations of the technique and the issue of sampling. Since glial neoplasms are heterogeneous, adequate sampling is critical, especially when tissue is obtained by stereotactic biopsy. Cytopathologic and histopathologic studies should be undertaken together, both for frozen section and permanent section diagnosis. Proliferation studies using Ki-67/MIB-1 staining can provide correlative data that may be beneficial in clinical management, especially for borderline lesions with respect to grade. Genetic determination of chromosome 1p and 19q status can assist in the classification of gliomas and provide an indication of chemosensitivity in oligodendrogliomas, while analysis of EGFR gene status can be diagnostically useful in select circumstances.

Key issues for future investigation

Histologic classification and grading remains a powerful and cost-effective tool for predicting prognostically meaningful diagnoses for glial neoplasms. It has become clear, however, that specific histopathologic entities may include several genetic subtypes, raising the possibility that molecular alterations may be important for predicting survival and responsiveness to therapy within diagnostic groups. A prime example is the finding that oligodendrogliomas with 1p/19q loss are associated with longer survival. Many laboratories now routinely assess the status of 1p and 19q in infiltrative gliomas by PCR-based methods or fluorescence in situ hybridization (FISH). The future of neuro-oncology will likely require an expanded role defining the genetic make-up of gliomas, including the status of 1p, 19q, EGFR, 10q (PTEN), TP53 and MGMT.

Finally, new biologic concepts are emerging from the study of tumor stem cells and new diagnostic techniques have been advanced from gene expression array and proteomic technologies that could revolutionize pathology. It has recently been demonstrated that there is a small subset of tumor cells in GBMs, and presumably other malignant
gliomas, that have stem-cell like properties (CD133+), which are responsible for cellular self-renewal and treatment resistance [109, 110]. The identification and characterization of these cell-types in GBM specimens as a part of diagnosis may prove critical. Proteomic investigations of the CSF from patients with malignant gliomas are uncovering novel proteins that may serve as markers of advanced disease or tumor progression [111, 112]. Gene expression profiling has identified new molecular markers that can be exploited for classification and grading, but has also challenged our conception of tumor classification [113]. Expression profiles coupled with computer modeling algorithms are capable of reliably predicting clinical outcome for glial neoplasms [113–115]. Indeed, computer generated class distinction based on their gene expression profiles may predict clinical outcome better than standard pathologic classifications. These studies are still in their initial stages, but most would agree they hold tremendous potential for improving the diagnostic stratification of glial neoplasms based on biologically meaningful data.

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