Clinical Utility of Fluorescence In Situ Hybridization (FISH) in Morphologically Ambiguous Gliomas with Hybrid Oligodendroglial/Astrocytic Features

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Abstract. Gliomas with hybrid oligodendroglial/astrocytic features are diagnostically problematic, and our ability to predict tumor behavior is limited. Some likely represent intermingled mixed oligoastrocytomas (MOAs), though precise diagnostic criteria and specific markers for this lesion are lacking. From the files at Washington University (1987–2000), 155 “ambiguous” glioma/intermingled MOA candidates were independently classified and graded by 5 neuropathologists, with consensus-derived pure oligodendrogliomas and astrocytomas excluded from further study. The 90 remaining cases (grades II = 29, III = 44, IV = 17) were analyzed by FISH on formalin-fixed, paraffin-embedded sections. Detectable deletions included combined 1p/19q (9%), solitary 19q (22%), PTEN/DMBT1 (26%), and p16 (32%). EGFR amplification was found in 11%. Patients were followed until death (47%) or a median of 3.3 years. Similar to prior glioma series, patient age (p < 0.0001) and tumor grade (p < 0.0001) were strongly associated with survival times. EGFR amplification (p = 0.0007) and deletions of PTEN/DMBT1 (p = 0.016) or p16 (p = 0.014), either individually or as a group (p = 0.04), portended a shorter median survival compared with tumors lacking these alterations. We conclude that 1) distinct genetic subsets are identifiable by FISH in morphologically ambiguous gliomas, and 2) both histological grading and molecular analysis yield prognostically useful information.

Key Words: Chromosome 1; Chromosome 19; EGFR; In situ hybridization; Oligoastrocytoma; p16; PTEN.

INTRODUCTION

Three distinct subtypes of diffuse gliomas are recognized in the current WHO classification of tumors of the nervous system: astrocytoma, oligodendroglioma, and mixed oligoastrocytoma (MOA) (1). Accurate identification of oligodendroglial tumors is of paramount concern, given their enhanced overall survival and response to chemo- and radiotherapy compared with astrocytomas (2–5). Unfortunately, there is considerable morphologic overlap between glial cell types. Furthermore, concepts have changed over time and definitional shifts have led to a broadening of the accepted morphologic spectrum of the oligodendroglial phenotype (i.e. microgemistocytes and gliofibrillary oligodendrocytes) (1, 6, 7).

Comprising 10% to 20% of diffuse gliomas, MOAs represent the most controversial and diagnostically challenging subset of gliomas. They can be further subclassified as “biphasic” when geographically distinct areas of astrocytoma and oligodendrogloma are present, or “intermingled” with a mixing of these 2 elements (1). Due to divergent diagnostic approaches and subjective morphologic criteria, there has been considerable interobserver variability and low diagnostic reproducibility among neuropathologists asked to classify such lesions, particularly the intermingled subtype (8). Not surprisingly, these tumors vary greatly in terms of biologic behavior, likely representing a heterogeneous group of “indeterminate-type gliomas,” potentially including pure astrocytomas, oligodendrogliomas, and true mixed gliomas. However, since no immunohistochemical markers are currently available to reliably distinguish between glial cell types, alternate means of classification are needed for gliomas with “non-classical” morphology. Thus, we hypothesized that genetic phenotyping might discriminate biologically meaningful subsets, further enhancing the prognostic accuracy and therapeutic management for patients with intermingled MOAs or morphologically ambiguous gliomas in general.

Recent efforts at defining glioma-associated molecular markers have shown promise in the stratification of gliomas. For example, oligodendrogliomas typically lose chromosomal arms 1p and 19q (9–13). Not only has combined 1p/19q deletion been implicated as a marker of the “oligodendrogial phenotype,” it has been found to correlate with prolonged survival and increased chemoresponsiveness (4, 5). Several studies have shown that a subset of MOAs harbor combined 1p/19q deletions, whereas others harbor “astrocytoma-associated” and/or “progression-associated” alterations (14–16). For instance, abnormalities involving the p16/CDK4 cell-cycle regulatory pathway have been detected in a high percentage of grade III–IV astrocytomas, with 1 large study of 96 gliomas demonstrating highly significant associations between p16/CDK4/RB pathway alterations...
and astrocytic morphology (15). Half of the MOAs in that study also showed alterations involving this pathway. Allelic loss of chromosome 10q is another frequent genetic alteration in gliomas and may represent an independent prognostic factor in astrocytomas (10, 17, 18). Candidate chromosome 10-associated tumor suppressors have included PTEN (10q23) and DMBT1 (10q25.3–26.1); deletions involving both of these regions have been observed in all glioma subtypes, though with highest frequency in high-grade astrocytic tumors, usually with loss of an entire chromosome 10 (19–21). PTEN inactivation has also been associated with poor prognosis in both astrocytic and less commonly, oligodendrogial lesions (19, 21, 22). Additionally, amplification of the epidermal growth factor receptor (EGFR) gene, once thought to be limited to high-grade astrocytomas, has recently been reported in rare oligodendrogliomas (23–25); the incidence in MOAs is not known. Prognostic roles for these genetic alterations in gliomas outside the confines of pure oligodendroglioma and astrocytoma have yet to be determined. Lastly, although biphasic MOAs have been genetically characterized, studies focusing on intermingled MOAs or gliomas with hybrid oligodendrogial/astrocytic features are generally lacking.

Given the continued difficulties of histopathologic classification, our work focused on the most morphologically ambiguous (i.e. diagnostically challenging) diffuse gliomas, including gliomas with “intermingled” and/or hybrid astrocytic/oligodendrogial features. Fluorescence in situ hybridization (FISH) was utilized for genetic analysis using the various oligodendroglioma, astrocytoma, and progression-associated markers depicted above. Associations were then sought between genetic data, cellular morphology, and previously established prognostic parameters such as tumor grade, patient demographics, and surgical intervention. Our data suggest that this rapid and sensitive method of genetically stratifying morphologically ambiguous gliomas provides prognostically useful information, which could potentially enhance the quality of patient care.

MATERIALS AND METHODS

Patient/Tumor Cohort

From roughly 900 diffuse gliomas from the files at Washington University School of Medicine (1987–2000), all cases bearing the terms “oligastrocytoma,” “mixed glioma,” “glioma not otherwise specified,” or “glioblastoma with oligodendrogial features” were retrieved in an attempt to identify specimens representing intermingled MOA candidates and otherwise ambiguous gliomas of indeterminate lineage. Those with insufficient tissue (e.g. minimal tumor) were excluded. The remaining 155 cases were then independently reviewed by 5 neuropathologists (RES, KAR, PCB, BWS, AP). A concerted effort was made to accurately reflect the diagnostic realities of routine daily surgical neuropathology. Therefore, each participant was simply asked to classify the cell type(s) and grade for each individual tumor, applying current World Health Organization (WHO) criteria exactly as they would normally do for standard clinical cases (1). Further instructions were not given and no attempt was made to create a state of enhanced diagnostic conformity beyond that which already exists in current practice. The 4 possible diagnostic categories included astrocytoma, oligodendroglioma, MOA, or glioma not otherwise specified (NOS). The available choices for grade were II, III, IV, or indeterminate. Consensus-derived (at least 3/5 pathologists in agreement) pure oligodendrogliomas and astrocytomas were excluded from further study.

Subsequently, 90 tumor specimens from 78 patients were identified for molecular characterization. These included 29 grade II (32%), 44 grade III (49%), and 17 grade IV (19%) tumors. One or 2 representative paraffin blocks were selected per case, and 5-μm-thick sections were cut and mounted on poly-L-lysine-coated slides for FISH analysis. Clinical data and follow-up was obtained by chart review in accordance with institutional IRB approval.

Fluorescence In Situ Hybridization

Dual-color FISH assays were performed on 5-μm-thick tissue sections as previously reported (26). Target retrieval included steam cooking in citrate buffer (20 min) followed by pepsin (4 mg/ml) digestion at 37°C for 30 min. Commercial locus specific fluorochrome-labeled probes included chromosome enumeration probe CEPI, a 1q telomere probe, and a CEP9/p16 dual probe cocktail (Vysis, Inc., Downers Grove, IL). Bacterial artificial chromosome (BAC)-derived probes targeting DMBT1 (10q25.3–26.1) (gift from Dr. Robert Jenkins, Mayo Clinic, Rochester, MN) and EGFR (7p12) (Human BAC library clone CIT-HSP-343B1, Research Genetics, Huntsville, AL), and 1q (BAC clone CIT978SK-A 309C5, Research Genetics) were labeled with rhodamine, while those targeting PTEN (10q23) (gift from Dr. Robert Jenkins, Mayo Clinic, Rochester, MN) and p16 (human BAC clone RPCI-11 260I23, Research Genetics), and 19p (BAC clone RPCI-11 575H1) were labeled with FITC. Probe pairings were as follows: 1p1 q, 1p/19q, CEP7/EGFR, PTEN/DMBT1, and CEP9/p16. All probes were diluted 1:50 in DenHyb buffer (Insitus, Albuquerque, NM) for dual target hybridizations. The hybridization mix (10 μl per slide) was applied to the sections, followed by simultaneous probe/target denaturation at 90°C for 13 min. The slides were then incubated overnight at 37°C in a humidified chamber with subsequent washes including 50% formamide/1× SSC (5 min) and 2× SSC (2 min ×2). Nuclei were counterstained with DAPI (0.5 μM/ml) (Insitus Laboratories).

The sections were viewed under an Olympus BX60 fluorescent microscope with appropriate filters (Olympus, Melville, NY). Samples with >90% nuclei showing signals were considered informative and 100 non-overlapping nuclei were scored for the number of fluorescent signals. Non-neoplastic brain from 5 to 10 temporal lobectomy specimens served as the controls for each probe pair. Deletions for 1p, 19q, PTEN, or DMBT1 were subsequently defined as >38% to >44% of tumor nuclei containing 1 signal (mean ± 3 standard deviations in non-neoplastic controls), depending on which probe was being used. Probes targeting 1p and 19p were used as reference probes for

by the simultaneous lack of p16 signals and presence of CEP9 signal (mean 1.8) defined by the presence of 1 CEP9 signal per cell in controls). Loss of an entire chromosome 9 (i.e. monosomy 9) was defined by 3 standard deviations in non-neoplastic control. The vast majority of cases interpreted as harboring relative deletions when the test to reference probe signal ratios were <0.8. Additionally, specimens were considered amplified for EGFR when they demonstrated nuclei containing innumerable red (EGFR) signals and an EGFR:CEP7 ratio >2. Homozygous p16 deletions were defined by the simultaneous lack of p16 signals and presence of CEP9 signals in >20% of tumor cells. In order to rule out the possibility of partial hybridization failure, p16 and CEP9 signals had to be seen within vascular endothelial cells (i.e. internal non-neoplastic control). The vast majority of cases interpreted as harboring homozygous p16 deletion had >50% tumor nuclei lacking detectable p16 probe signals. Hemizygous p16 deletions were defined by >44% tumor nuclei containing a single p16 signal (mean +3 standard deviations in non-neoplastic controls). Loss of an entire chromosome 9 (i.e. monosomy 9) was defined by the presence of 1 CEP9 signal per cell in >45% (mean +3 standard deviations in controls). Polysomies (gains) were arbitrarily defined as >5% nuclei containing 3 or more signals, as no such findings were seen in the control specimens.

Images were captured using a high resolution black and white COHU CCD camera. A Z-stack motor enabled sequential DAPI (1 level), FITC (5 levels), and rhodamine (5 levels) filter settings to be captured, and the resulting images were reconstructed with blue, green, and red pseudocolors using the CytoVisionTM basic workstation (Applied Imaging, Santa Clara, CA).

### Statistical Methods

Survival time was measured from date of diagnosis and censored at the time of last follow-up. Survival distributions were estimated using Kaplan-Meier curves. Patient age at diagnosis, tumor grade, type of initial surgery, Karnofsky performance score, and tumor status for 1p, 19q, PTEN, DMBT1, EGFR, and p16 were included in Cox proportional hazards univariate analyses. Multivariate modeling was performed to identify independent predictors of overall survival. All reported p values were 2-sided and values of <0.05 were considered statistically significant.

### RESULTS

#### Diagnostic Concordance

A summary of the diagnoses rendered by the 5 reviewers for the 90 study set cases is shown in Table 1. At least 3/5 reviewers assigned a diagnosis of MOA or glioma NOS in 40 (44%) and 5 (6%) cases, respectively. In 45 cases (50%), fewer than 3 of the reviewers were in diagnostic agreement regarding tumor type. Although most cases prompted 2 to 3 different diagnoses amongst the reviewers, in 15 cases (17%), all 4 of the possible diagnostic categories were proposed for the same tumor (data not shown). In none of the study cases was there complete agreement for cell type among all 5 neuropathologists. Therefore, the 90 cases identified within this study were felt to represent the most diagnostically challenging 10% of diffuse gliomas encountered at Washington University. For the purposes of this study, all these cases were collectively considered “morphologically ambiguous” diffuse gliomas, rather than classic examples of astrocytomas or oligodendrogliomas. In contrast to cell type, only a minor degree of disagreement was encountered for WHO tumor grade; in only 2 cases did they differ by more than 1 grade (data not shown).

#### Clinicopathologic and Molecular Findings

Demographic and treatment data for the patients in the study group are summarized in Table 2. The 78 patients ranged in age from 12 to 78 (median 37 years) and included 42 males and 36 females. The frontal lobe was the site of tumor occurrence in 41% of patients, while...
59% had lesions centered upon other locations including the temporal (36%), parietal (20%), or occipital lobes (3%). Of the patients for which detailed clinical history was available, the initial surgical procedure was biopsy in 26 (37%), partial resection in 20 (29%), and gross total resection in 24 (34%). Biopsy was immediately followed by definitive surgical procedure in 4 cases, consisting of partial (2 cases) or gross total resection (2 cases). All but 4 patients received adjuvant radiotherapy, and 62% additionally received chemotherapy. Karnofsky performance scores ranged from 90 to 70, the single person receiving the lowest score unfortunately being lost to follow-up.

As expected, the study cases displayed a wide array of histologic appearances. The majority contained intermixed populations of cells, some with elongated or irregular-shaped hyperchromatic nuclei and others bearing round regular nuclei, sometimes with discernible perinuclear halos. Also frequently encountered were cells having hybrid oligodendrogial/astrocytic features including enlarged, slightly elongated or irregular nuclei, mild hyperchromasia, and occasional clear perinuclear halos. Many tumors also displayed microcalcifications, a mucin-rich microcystic growth pattern and cortical involvement. GFAP-immunoreactive microgemistocytes, gliofibrillary oligodendrocytes, and/or conventional gemistocytes were identified in a subset of cases. Tumors designated as grade III by the reviewers typically had an elevated mitotic rate and at least focal evidence of vascular proliferation, while grade IV lesions additionally contained areas of pseudopalisading necrosis. The nomenclature for the latter remains controversial, though they are often designated as “glioblastomas with oligodendrogial components” (1).

Representative FISH hybridizations and corresponding histologic images are illustrated in Figure 1. Of the 360 total paired hybridizations, 353 (98%) yielded interpretable results. Non-informative cases resulted from weak hybridization (3 cases) or tissue loss (4 cases). Of note, deletions of chromosome 1p were always accompanied by concomitant loss of 19q, and similarly PTEN and DMBT1 were always co-deleted. The most frequently encountered alteration was deletion of p16, which was seen in 32% of our cases. Other detectable abnormalities, in descending order of frequency, included co-deletion of PTEN/DMBT1 (26%) (Fig. 1B), solitary 19q loss (22%), and amplification of EGFR (11%) (Fig. 1D). Combined loss of 1p and 19q (Fig. 1F) was encountered in only 8 cases (9%) from this study group, 6 of which were centered in the frontal lobe; no temporal lobe lesions were found to harbor this alteration, however deletion of PTEN/DMBT1 and p16 were frequently encountered at this site. Multiple molecular alterations were detected in 28%, with combined loss of 19q and PTEN/DMBT1 (8%) or p16 (8%) and combined EGFR amplification with losses of p16 and PTEN/DMBT1 (6%) the most frequently encountered. Codeletion 1p/19q was unaccompanied by additional alterations in all cases except one, in which there was deletion of p16 (data not shown).

Table 3 summarizes the frequencies of detected alterations in the study group relative to histologic tumor grade. With the exception of 1p/19q loss, the frequencies of each of these abnormalities increased proportionally with histologic grade (IV > III > II). Combined 1p/19q loss was not seen in grade IV lesions, whereas solitary deletion of 19q was seen predominantly in grade III and IV tumors. Interestingly, the most common finding in grade II lesions was a total lack of the aforementioned alterations (69%), these cases instead exhibiting either normal copy numbers or polysomies at one or more sites. Only 13% of grade IV tumors demonstrated similar findings, having instead much more frequent occurrence of progression-related alterations, including amplification EGFR and deletions of p16 and/or PTEN/DMBT1. In the 9 patients in which multiple specimens were examined, 3 retained identical molecular abnormalities, and 3 had gain of an additional detectable alteration (deletions of 19q, PTEN/DMBT1, or amplification of EGFR). In the remaining 3 patients, tumors samples subsequent to that obtained at initial surgery harbored fewer detectable alterations then the original tumor (deletions PTEN/DMBT1, 19q and p16, and amplification of EGFR) (data not shown).

Following acquisition of the above molecular data, 2 of the authors (CF, AP) re-reviewed all 90 cases to look for potential morphologic/genetic associations. All cases had rounded nuclei representing varying proportions of the tumor. Each case was additionally examined for the presence or absence of nuclear irregularity, hyperchromasia, multinucleation, clear perinuclear halos, microcalcifications, microcysts, perineuronal satellitosis, gemistocytes, and minigemistocytes. We were unable to clearly predict any specific genetic patterns using individual morphologic features. In fact, most individual morphologic features were present in well over half the cases and virtually all our cases had some mixture of features normally attributed to astrocytomas and those attributed to oligodendrogliomas. The few cases harboring 1p and 19q codeletion were not more obviously oligodendroglial in appearance than the rest of the cohort. Cases with EGFR amplification and/or 10q deletions tended to have a greater number of “astrocytic” features, often containing bland, mitotically active oval nuclei that would justify as the small cell variant (45). However, other cell types were also encountered and there remained a great deal of overlap with cases lacking these genetic alterations. The only histologic parameter upon which any clear correlations could be drawn with regard to genetic findings was tumor grade, as noted above.
Fig. 1. Representative morphology (A, C, E) and corresponding FISH hybridizations (B, D, F, G) from 3 study set cases with similar nuclear features, but differing genetic patterns. The first case (A, B) is a grade III tumor with PTEN (green) and DMBT1 (red) deletions (patient dead at 12 months). The second (C, D) is a grade IV tumor with EGFR (red) amplification (patient dead at 18 months). The third (E, F) is a grade III tumor with combined 1p (F) and 19q (G) deletion (tumor is stable at 96 months of follow-up). Note that in these 2 figures, green signals correspond to probes targeting 1p (F) and 19p (G), while red signals represent 1q (F) and 19q (G) probe targets, respectively.
Associations with Overall Patient Survival

Patients were followed until death (47%) or a median of 3.3 years (range = 1 to 251 months); follow-up data was available for all but 8 patients. Table 4 summarizes the comparisons of survival with the various clinicopathologic factors and FISH markers used in the current study. The overall median survival was 78 months. By univariate analysis, overall patient survival was strongly (p < 0.0001) associated with the following variables: 1) histologic grade (Fig. 2A), with median survival not estimable for grade II, 73 months for grade III, and 12 months for grade IV; and 2) patient age, with median survival or 96 months for age younger than 40 years, 73 months for 40 to 60 years, and 4 months for older than 60 years. Although prolonged survival time tended to coincide with extent of initial resection and higher Karnofsky performance score, neither of these variables reached statistical significance (p = 0.076 and p = 0.079, respectively). We were unable to draw any clear association between chemotherapy/radiotherapy and survival as this information was lacking on too many patients and individual therapies were quite varied for both modalities (data not shown).

Overall survival was likewise significantly associated with several of the molecular alterations detected by FISH. Markedly shortened survival was encountered in those patients whose lesions harbored either 1) EGFR amplification (median survival, 16 months with amplification versus 96 months without; p = 0.0007); 2) PTEN/DMBT1 deletion (median survival, 31 months with deletion versus 96 months without; p = 0.016); or 3) p16 deletion (median survival, 18 months with deletion versus 96 months without; p = 0.014). Codeletion 1p/19q and solitary 19q deletions were fairly infrequent, and no statistically significant conclusion could be drawn for either variable regarding survival. On the other hand, as a group those patients whose tumors had deletions of either EGFR amplifications or deletions of PTEN/DMBT1 or p16 had a significantly shorter survival time (median, 16 months, p = 0.040) than those whose lesions had either 1p or 19q deletions (median, 100 months) or had none of the above alterations (median, 193 months) (Fig. 2B).

By multivariate analysis, the clinical/demographic variables with the strongest effects on overall survival time were patient age (Hazard ratio 1.06; p = 0.0001) and tumor grade (II versus III: Hazard ratio 1.18, p = 0.040; II versus IV: Hazard ratio 5.584, p = 0.0019). When deletions of 1p or 19q were incorporated into the model, a trend towards shorter survival (Hazard ratio 6.764) was encountered when either of these alterations were absent, however, this did not quite reach independent significance (p = 0.0637). This may be due to either too small a sample or insufficient follow-up, particularly in the grade II tumors. Not surprisingly, the progression-associated genetic alterations were encountered most frequently in older patients with high-grade gliomas, and therefore there is some prognostically overlapping data provided by each of these variables. However, clinicopathologic and genetic parameters certainly did not provide the same type of information in every case and models using these genetic alterations alone proved approximately as informative as those based on patient age and histologic grade. When modeled in this fashion, amplification of EGFR (Hazard ratio 3.46, p = 0.0063) and deletions of PTEN/DMBT1 (Hazard ratio 2.44, p = 0.014) and p16 (Hazard ratio 2.33, p = 0.012) all represented unfavorable variables as each may have an independent, deleterious effect on overall survival.

DISCUSSION

Morphologically Ambiguous Gliomas and Diagnostic Biases

Mixed oligoastrocytomas (MOAs) are among the most difficult gliomas to objectively define and diagnose. Therefore, they potentially represent the gliomas most likely to benefit from ancillary genetic characterization. Prior genetic studies of MOA have understandably focused on the biphasic pattern, where microdissection of the 2 separate elements is possible (9, 11, 12, 14–16, 29–35). Unfortunately, the biphasic pattern is quite uncommon in comparison to the intermingled or hybrid pattern, which in the current study, was estimated to represent

<table>
<thead>
<tr>
<th>Grade II (n = 29)</th>
<th>Deletion 1p/19q</th>
<th>Deletion 19q only</th>
<th>Amplification EGFR</th>
<th>Deletion PTEN/DMBT1</th>
<th>Deletion p16</th>
<th>None of these alterations</th>
</tr>
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<tr>
<td></td>
<td>2/29 (7)</td>
<td>3/29 (10)</td>
<td>1/29 (3)</td>
<td>2/29 (7)</td>
<td>4/26 (15)</td>
<td>18/26 (69)</td>
</tr>
<tr>
<td>Grade III (n = 44)</td>
<td>6/43 (14)</td>
<td>11/43 (26)</td>
<td>5/44 (11)</td>
<td>12/43 (28)</td>
<td>13/42 (31)</td>
<td>14/41 (34)</td>
</tr>
<tr>
<td>Grade IV (n = 17)</td>
<td>0/17 (0)</td>
<td>6/17 (35)</td>
<td>4/17 (24)</td>
<td>9/16 (56)</td>
<td>10/17 (59)</td>
<td>2/16 (13)</td>
</tr>
<tr>
<td>Total (n = 90)</td>
<td>8/89 (9)</td>
<td>20/89 (22)</td>
<td>10/90 (11)</td>
<td>23/89 (26)</td>
<td>27/85 (32)</td>
<td>34/83 (41)</td>
</tr>
</tbody>
</table>

* Data are given as number of specimens with specific abnormality/total number of informative cases for that abnormality (percentage).
TABLE 4
Kaplan-Meier Estimates for Overall Survival

<table>
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<tr>
<th>Variable</th>
<th>Value</th>
<th>No.</th>
<th>No. Dead</th>
<th>Median Survival (months)</th>
<th>95% Confidence Interval</th>
<th>Log-Rank p Value</th>
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<tbody>
<tr>
<td>Tumor Grade</td>
<td>II</td>
<td>25</td>
<td>5</td>
<td>no estimate</td>
<td>(85, no estimate)</td>
<td>&lt;0.0001</td>
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<tr>
<td></td>
<td>III</td>
<td>34</td>
<td>21</td>
<td>73</td>
<td>(41, 100)</td>
<td>0.85</td>
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<tr>
<td></td>
<td>IV</td>
<td>11</td>
<td>11</td>
<td>12</td>
<td>(7, 18)</td>
<td>0.85</td>
</tr>
<tr>
<td>Gender</td>
<td>female</td>
<td>32</td>
<td>18</td>
<td>78</td>
<td>(31, 115)</td>
<td>0.58</td>
</tr>
<tr>
<td></td>
<td>male</td>
<td>38</td>
<td>19</td>
<td>96</td>
<td>(41, 108)</td>
<td>0.58</td>
</tr>
<tr>
<td>Age (years)</td>
<td>12–39</td>
<td>40</td>
<td>16</td>
<td>96</td>
<td>(70, 108)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>40–59</td>
<td>23</td>
<td>14</td>
<td>73</td>
<td>(30, 115)</td>
<td>0.58</td>
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<td></td>
<td>60–78</td>
<td>7</td>
<td>7</td>
<td>4</td>
<td>(3, 16)</td>
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<tr>
<td>Karnofsky performance score</td>
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<td>0</td>
<td>—</td>
<td>—</td>
<td>0.079</td>
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<td></td>
<td>80</td>
<td>13</td>
<td>10</td>
<td>85</td>
<td>(15, 96)</td>
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<td></td>
<td>90</td>
<td>50</td>
<td>23</td>
<td>100</td>
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<td>Surgery</td>
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<td>12</td>
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<td>73</td>
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<td></td>
<td>gross total</td>
<td>25</td>
<td>9</td>
<td>108</td>
<td>(61, no estimate)</td>
<td>0.14</td>
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<tr>
<td>Deletion 1p/19q</td>
<td>no</td>
<td>62</td>
<td>35</td>
<td>76</td>
<td>(41, 106)</td>
<td>0.14</td>
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<td></td>
<td>yes</td>
<td>8</td>
<td>2</td>
<td>101</td>
<td>(100, no estimate)</td>
<td>0.14</td>
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<tr>
<td>Deletion 19q</td>
<td>no</td>
<td>57</td>
<td>28</td>
<td>84</td>
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<tr>
<td></td>
<td>yes</td>
<td>13</td>
<td>9</td>
<td>95</td>
<td>(62, 106)</td>
<td>0.97</td>
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<tr>
<td>Deletion PTEN/DMBT1</td>
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<td>53</td>
<td>25</td>
<td>96</td>
<td>(61, 108)</td>
<td>0.016</td>
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<tr>
<td></td>
<td>yes</td>
<td>17</td>
<td>12</td>
<td>31</td>
<td>(15, 78)</td>
<td>0.016</td>
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<tr>
<td>EGFR Amplification</td>
<td>no</td>
<td>63</td>
<td>30</td>
<td>96</td>
<td>(61, 108)</td>
<td>0.0007</td>
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<tr>
<td></td>
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<td>7</td>
<td>7</td>
<td>16</td>
<td>(15, 38)</td>
<td>0.0007</td>
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<tr>
<td>Deletion p16</td>
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<td>49</td>
<td>21</td>
<td>96</td>
<td>(73, 115)</td>
<td>0.014</td>
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roughly 10% of diffuse gliomas at Washington University. We therefore chose to focus on such morphologically ambiguous gliomas and the case selection strategy was employed specifically to capture the clinically important group of diffuse gliomas most likely to receive multiple interpretations by expert neuropathologists.

When one carefully examines the diagnoses rendered by the 5 neuropathologists in this study (Table 1), it becomes clear that each has a particular diagnostic bias. Reviewer 4, for example, rendered the diagnosis of pure astrocytoma most frequently (78%), based on his belief that anything short of textbook classic oligodendroglioma is astrocytic in nature. Reviewers 1 and 5 diagnosed MOA most often (80% and 74%, respectively), reflecting their belief that tumors with hybrid nuclear features include both astrocytic and oligodendroglial elements. Similarly, reviewers 2 and 3 rendered the greatest number of “glioma, NOS” diagnoses, reflecting their philosophy that ambiguous cytologic features warrant a descriptive, rather than a definitive diagnosis. This diverse range of philosophical approaches is not uncommon and accurately reflects the diagnostic diversity of the neuropathology community in general.

Despite the shortcomings, the classification of diffuse gliomas is of paramount importance. Oligodendrogliomas repeatedly have been shown to be considerably less aggressive and more responsive to therapy than astrocytomas of comparable grade. The biologic behavior of oligoastrocytomas is reportedly intermediate between the 2 pure forms (2, 3, 5, 28, 36–41). Clearly, the histologic classification schema remain subjective, with considerable differences of opinion as to what constitutes minimal criteria for the “oligodendroglial phenotype” and the intermingled MOA (8, 12). Recent genetic studies have
shown that deletions of chromosomes 1p and 19q are highly associated with both classic oligodendroglioma morphology (up to 80% of cases) and enhanced survival and therapeutic responsiveness to both PCV chemotherapy and radiotherapy (4, 5, 12, 13, 25, 40). Regarding biphasic MOAs, previous groups have demonstrated codeletion 1p/19q in 30% to 50%, with another 30% of these lesions instead harboring astrocytoma-associated genetic abnormalities involving p53, EGFR, p16, and/or chromosome 10. Interestingly, these “oligodendroglioma-associated” and “astrocytoma-associated” alterations overlap minimally, suggesting at least 2 genetically distinct subgroups (9, 11, 12, 14, 16, 29, 30, 34, 35).

**Genetic Classification of Morphologically Ambiguous Gliomas**

Given the limitations discussed above, we assessed chromosome 1p, 19q, EGFR, PTEN, DMBT1, and p16 copy numbers to determine whether they provide additional prognostic information in morphologically ambiguous gliomas. Although a variety of techniques have been successfully applied, we chose FISH, not only for its relative simplicity, our laboratory’s familiarity with the technique, and its morphologic basis, but particularly for its applicability to formalin-fixed, paraffin-embedded tissue without the need for matching non-neoplastic tissue (42). For example, the latter loss of heterozygosity (LOH) study requirement would have necessitated either microdissection of relatively pure non-neoplastic tissue from the same paraffin-embedded specimens or blood from those patients still alive at the time of the study. Since both of these are significantly more difficult to achieve and the latter is significantly more intrusive to patients, FISH allowed for a much larger study cohort than would have been possible using LOH as the technique of choice.

As depicted in Table 3, we detected 1p/19q codeletion in only 9% of our cases, supporting the notion that such codeletions are seen primarily in the most morphologically classic oligodendrogliomas (12, 13, 43). Nevertheless, the few cases in our cohort with detectable 1p/19q deletion were clearly not “textbook” oligodendrogliomas (Fig. 1E), emphasizing the fact that genetic alterations are not 100% correlative with tumor morphology. Similar to 2 recent studies, we found the majority of our 1p/19q codeleted tumors to reside at sites outside the temporal lobes, all but one involving the frontal lobe (35, 43). None of the other alterations showed such site-specific correlations (data not shown).

When tumor grade was taken into account, distinct genetic patterns were observed, with EGFR amplification and deletions of p16 and PTEN/DMBT1 predominantly seen in high-grade (III–IV) lesions, often with 2 or more coexisting alterations. Although solitary 19q deletion was seen in a subset of 17 grade IV tumors (35%), codeletion 1p/19q was not. This suggests that some of our grade IV tumors were genetically equivalent to conventional glioblastomas, where the occurrence of 19q deletions has been associated with tumor progression (11, 44). Furthermore, several cases with EGFR amplification likely represent small cell glioblastomas, a variant with surprisingly bland monomorphous nuclei and substantial morphologic overlap with high-grade oligodendroglial tumors (45). Nonetheless, 3 (27%) of our patients presenting with grade IV gliomas had long survival times (>2 years), with 1 patient surviving over 9 years. This supports the notion that “oligodendroglial features,” despite the subjective nature, may still be predictive of better outcome in a subset of glioblastomas (see survival section below).

Another benefit of the FISH data is that it provides some clues regarding clonality. If one poses the hypothesis that MOAs are collision tumors, with separate astrocytoma and oligodendroglioma components, then they
should be polyclonal, with 1p/19q alterations perhaps limited to the oligodendroglial-appearing cells and the astrocytoma-associated alterations limited to astrocytoma-like nuclei. This did not seem likely in our study, since the alterations were usually widespread, with the vast majority of tumor nuclei involved, regardless of whether they were rounded or irregular in contour. Therefore, despite the intermingling of “astrocytoma-like” and “oligodendroglia-like” nuclei, most of these tumors appeared genetically monoclonal. Whether they are truly differentiating along both astrocytic and oligodendroglial pathways (e.g. dual lineage glioma arising from a multipotent glial precursor cell) awaits more specific glial markers.

Lastly, we re-reviewed our tumors to see whether, in retrospect, any of the genetic patterns could have been predicted by morphology. Unfortunately, other than the associations with grade, there were not any clear correlations with individual morphologic features. In fact, virtually every case had some combination of “astrocytic” and “oligodendroglial” features, which is not surprising since it was the original basis for inclusion in this study. Even so, our data support the notion that considerable genetic stratification can be achieved within this group of diagnostically challenging gliomas with overlapping morphologic features.

Patient Survival Times

Despite the relatively poor concordance between the 5 reviewers regarding tumor type in this series, survival times for our patients generally corresponded to those of previously reported MOAs and fell between those typically encountered in pure astrocytomas and oligodendrogliomas of similar grade (1, 27, 37). Similar to other diffuse glioma series, both histologic grade and patient age were found to be strongly associated with overall survival, with median survival ranging from 96 months for age <40 years to only 4 months for those older than 60 years. At present, MOAs are graded in a 2-tiered WHO system analogous to pure oligodendrogliomas; however, some pathologists retain the designation “grade IV MOA” or “GBM with oligodendroglial components” for cases with brisk mitotic activity, endothelial proliferation, and foci of pseudopalisading necrosis. We identified several such cases in our study. This distinction is relevant due to reports of prolonged survival in comparison to conventional GBMs (46, 47). Although the median survival for our grade IV tumors was similar to standard cases (1 year), the long survival (>2 years) in 3 patients (18%) suggests that at least a subset of such tumors behave more favorably. Likewise, the 6-year median survival encountered in grade III lesions is substantially longer than that expected for pure anaplastic astrocytomas, again suggesting a favorable prognostic role for more subtle oligodendroglial features, even in the absence of 1p/19q deletions.

We also found associations between genetic patterns and survival. Despite the association with tumor progression noted above, solitary 19q loss was frequently coupled with prolonged patient survival, especially taking into account the fact that these were predominantly high-grade tumors (median survival of 96 months). Similarly, in a separate study at Washington University, we encountered a subset of solitary 19q deleted gliomas (predominantly anaplastic MOAs) that accounted for 11% and 6% of our patients with >5-year and >10-year survival rates respectively (43). This is analogous to data recently published by Burton et al, wherein solitary 19q deletion was statistically associated with their long-term survival group of glioblastoma patients (48). Unfortunately, in our cohort, neither 1p/19q codeletions nor solitary 19q deletions were independently associated with survival on univariate or multivariate analysis, most likely due to their relatively low frequencies in this tumor set. Further experience with more extensive follow-up will be required to confirm or refute these associations.

In contrast, statistically significant associations were established between EGFR amplification, deletions of p16 and/or PTEN/DMBT1 losses, and shortened survival times. The largest concentration of these 3 genetic alterations was encountered in our high-grade tumors, particularly grade IV lesions. Although all 3 of these alterations have been documented in oligodendrogliomas, they are much more frequently detected in high-grade astrocytomas (25, 49). In any event, these findings strongly indicate that incorporation of these genetic markers could enhance prognostic accuracy. Interestingly, our relatively large group of cases with no detectable genetic alterations and prolonged survival suggest that other “genetically favorable” patterns have yet to be identified. Some may have p53 mutation as reported by Ino et al, where it was found to represent an intermediate prognosis group (50). Since FISH is not a reliable method for identifying these alterations and mutation analysis is substantially more difficult in archival tissue, this was not part of our panel. Immunohistochemical analysis of the p53 protein was performed, though no associations were found with survival or other genetic alterations (data not shown).

Summary

Morphologically equivocal gliomas with hybrid oligodendroglial/astrocytic features vary greatly in terms of biologic behavior and genetic patterns. They appear to represent a heterogeneous group of tumors that are among the most difficult to classify and most likely to receive multiple diagnostic opinions. Some of these likely represent true examples of intermingled MOAs, but more specific glial markers will be needed to prove or disprove the presence of dual lineages in these challenging tumors. To our knowledge, there have not been any prior large genetic series specifically focusing on morphologically
ambiguous gliomas, as in our current study. Although we were unable to associate individual genetic patterns with specific morphologic features in such tumors, we have established that similar to biphasic MOAs, distinct genetic subsets exist and may be identified using FISH on routine paraffin-embedded tissue, including 1) tumors with deletions of 1p and/or 19q, generally associated with prolonged survival, 2) tumors with EGFR amplifications, p16 deletions, and/or PTEN/DMBT1 losses, generally associated with shorter survival, and 3) a large cohort of primarily grade II gliomas with none of the alterations noted above, exhibiting a similarly prolonged survival to those in group 1. Patient age and histologic grade remain the most powerful prognostic variables, although genetic data provides additionally useful information. Our data suggest that a combined histologic and genotypic approach yields prognostically superior data to that obtainable by either method alone.

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