Revised Molecular Testing in Gliomas

A Retrospective Study of 53 Cases

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Key Words: Fluorescence in situ hybridization; FISH; Glioma; Astrocytoma; Oligodendroglioma; Mixed glioma; Glioblastoma; 1p; 19q; Epidermal growth factor receptor; EGFR; Repeated testing

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Abstract

The value of repeated molecular testing in patients with multiple resections of gliomas is unclear. The purpose of this study was to assess for evidence of molecular changes for chromosome 1p/19q deletions and epidermal growth factor receptor (EGFR) amplification by fluorescence in situ hybridization in 53 glioma cases in which repeated testing was done. Paired results for 1p evaluation demonstrated a change in the profile from intact to loss in 1 (2%) of 50 cases; 19q evaluation demonstrated a change in profile in 4 (10%) of 41 cases. There was no change in the EGFR expression in any of the cases tested. There was no change in the clinical management based on the repeated molecular tests in patients with discrepant repeated results. Hence, there seems to be no indication for reflex repeated 1p/19q or EGFR testing in gliomas at the time of repeated biopsy or resection.

An increased understanding of the molecular pathways in gliomas has led to the detection of potential diagnostic, prognostic, and predictive biomarkers, some bearing clinical implications for targeted therapy. Among the most promising genetic alterations that have implications for the clinical management and prognosis to date are large deletions on chromosomes 1p and 19q in oligodendrogliomas and mixed gliomas. Several studies have substantiated the chemoresponsiveness and favorable prognosis associated with the deletions on chromosomes 1p and 19q in oligodendrogial tumors. A subset of glioblastoma multiforme (GBM), particularly tumors arising de novo in elderly people and the small cell variant, is known to demonstrate frequent amplification or overexpression of the epidermal growth factor receptor (EGFR) gene. Various drugs targeting EGFR are being examined for possible therapeutic effect in GBM.

Given the treatment and prognostic implications of such markers, a growing number of laboratories routinely assess the status of these markers, among others, in routine clinical practice. The need for repeated molecular testing in patients with multiple resections is unclear. The purpose of this study was to assess for evidence, frequency, and clinical implications of molecular changes during the natural history or treatment course of gliomas that have undergone repeated molecular testing in our institution.

Materials and Methods

Institutional review board approval was obtained before commencement of the study. The molecular pathology database was searched for cases that had undergone repeated
evaluation of their gliomas for chromosome 1p and 19q deletions or EGFR amplification by fluorescence in situ hybridization (FISH). A total of 53 cases were identified and comprised the study group.

At the time of clinical evaluation of the biopsy and resection specimens, a representative block of tumor was selected and 4-µm-thick sections were cut on positively charged slides. One section was stained with H&E. On this H&E-stained section, the area of tumor targeted for FISH analysis was designated. The unstained slides were deparaffinized in xylene and then rehydrated in graded alcohols and distilled water. Cell conditioning was performed with a target retrieval solution (DAKO, Carpinteria, CA) at 90°C in a water bath for 40 minutes followed by cooling at ambient temperature for 20 minutes and then Proteinase K (Roche, Indianapolis, IN) for 6 minutes at 25°C.

For analysis of chromosome 1p loss, probes specific to the centromeric and telomeric portions of chromosome 1 were applied to the section, and the DNA target was denatured at 90°C for 6 minutes on a heat block. A centromeric probe (SpectrumOrange-labeled CEP1 probe, Vysis, Downers Grove, IL) was applied at a dilution of 1:10 of hybridization buffer and allowed to hybridize to the pericentric region of chromosome 1 (1p12). A digoxigenin-labeled telomeric probe mapping to chromosome 1p36 (p58CLK-1 DNA probe, Ventana, Tucson, AZ) that identifies the cell division cycle 2–like 1 (CDC2L1 locus) gene was also applied. Both probes were hybridized at 37°C for 16 to 18 hours. The coverslip was removed by washing in 2× standard saline citrate (SSC), and excess probe was removed with 0.5× SSC stringency washes, followed by graded SSC stringency washes. The digoxigenin-labeled probe was visualized using fluorescein isothiocyanate (FITC)-antidigoxigenin (Roche). Nucleated cells were identified by using 4′-6-diamidino-2-phenylindole (DAPI) counterstain. A similar procedure was followed for evaluation of loss on chromosome 19q using probes directed to the telomeric portions of 19q and 19p (SpectrumOrange-labeled TelVysion 19q probe and SpectrumGreen-labeled TelVysion 19p probe, Vysis). For EGFR amplification, a dual probe (Vysis) that included a probe specific for EGFR (chromosome 7p12 labeled with SpectrumOrange and centromeric chromosome 7 probe [7p11.1-q11.1] labeled with SpectrumGreen) was used.

After staining, the designated area of interest, as indicated on the H&E-stained section, was evaluated by using an epifluorescent microscope equipped with DAPI/FITC/Texas red band-path filters (Axiophane 2, Zeiss, Göttingen, Germany). We evaluated 40 cells containing a minimum of 2 centromeric signals and determined a telomeric target/reference probe ratio. For 1p/19q evaluation Image 1A, Image 1B, Image 1C, and Image 1D, a target/reference ratio of 0.8 or more was interpreted as representing no evidence of loss on chromosomes 1p and 19q (1p and 19q intact). A ratio of less than 0.7 was interpreted as representing a loss on chromosomes 1p and 19q. For ratios between 0.7 and 0.8, the test was repeated. For interpretation of EGFR FISH results, a ratio of 2 or less was interpreted as representing no evidence of EGFR amplification Image 1E. A target/reference ratio greater than 2 was interpreted as representing evidence of EGFR amplification Image 1F.

Pathology reports were reviewed to obtain information about the patient’s age at the time of initial surgery, sex, location of tumor, tumor diagnosis and grade at the initial biopsy or resection and the subsequent biopsy or resection, and the interval between the 2 surgical procedures. The World Health Organization (WHO) 2007 guidelines for grading and classification of gliomas were used.28

Results

A total of 53 cases in which molecular testing was performed on 2 or more temporally different biopsy or resection specimens formed the study group. The patients included 31 males and 22 females. They ranged in age from 13 to 70 years (mean, 45.4 years) at the time of initial surgery. The most common site of origin was the frontal lobe (n = 25). The remaining tumor locations included temporal lobe (n = 14), parietal lobe (n = 2), insular cortex (n = 2), frontotemporal lobe (n = 1), parietotemporal lobe (n = 1), frontoparietal lobe (n = 1), thalamus (n = 1), and posterior fossa (n = 2). In 4 cases, the precise location was not known.

Original diagnoses included 27 diffuse fibrillary astrocytomas (11 low grade, WHO grade II; 3 anaplastic, WHO grade III; and 13 GBM, WHO grade IV), 16 oligodendrogliomas (11 low grade, WHO grade II; and 5 anaplastic, WHO grade III), 6 mixed gliomas (4 low grade, WHO grade II; and 2 anaplastic, WHO grade III), and 4 gliomas not further classified.

The intervals between the initial biopsy or resection and the subsequent or second biopsy or resection ranged from 1 to 166 months (mean, 23.5 months; median, 5 months). The molecular studies were performed on 3 different biopsy or resection specimens in 5 patients and on 4 different biopsy or resection specimens in 1 patient, with intervals ranging between 1 and 87 months.

Nine tumors progressed to a higher grade during the interval between the initial and subsequent resections. The upgrades occurred in 4 astrocytic neoplasms (2 tumors progressed from grade II to III, 1 from grade II to grade IV, and 1 from grade III to grade IV) and 2 oligodendrogliomas (both progressed from grade II to grade III). Three mixed gliomas upgraded from low-grade mixed glioma to anaplastic mixed glioma.
Paired results for 1p evaluation by FISH were available for assessment in 50 cases. One tumor (2%) demonstrated a change in the profile from intact to 1p loss. This tumor was diagnosed as grade II mixed glioma on the initial biopsy specimen. After the initial biopsy, the patient was treated with chemotherapy and external beam radiotherapy. The tumor upgraded to anaplastic mixed glioma on the subsequent resection (interval, 100 months). Bilateral leg thrombosis and postoperative meningitis developed, and the patient elected to enter hospice. There was no change in the clinical management of the patient based on the change in 1p status. The 19q status and EGFR status were evaluated only on the second resection specimen and showed 19q loss and EGFR not amplified.

Paired results for 19q evaluation by FISH were available in 41 cases. Of the 41 tumors, 4 (10%) demonstrated a change in the profile (Table 1). Two of these tumors demonstrated a change in profile from loss to intact 19q at the subsequent biopsy. Both were diagnosed as GBM on the initial and subsequent subtotal resections. One of these tumors also had areas suggestive of gliosarcoma on the subsequent resection. The EGFR in both cases was not amplified. The 1p status in both cases was intact at the initial and subsequent resections. The interval between the initial and subsequent resection was...
4 months in each case (Table 2). The second resections were performed because the tumor was growing and was causing mass effect. There was no change in the clinical management in either case based on the change of 19q profile.

The remaining 2 tumors (1 low-grade astrocytoma and 1 mixed glioma) initially had intact 19q and changed profile to 19q loss at the subsequent subtotal resection. The patient with low-grade astrocytoma had 1 biopsy followed by 2 subtotal resections; in the initial 2 procedures (9 months apart), the tumor was diagnosed as a low-grade astrocytoma and showed intact 19q. Chemotherapy and proton beam radiation therapy were started. The last subtotal resection demonstrated a recurrent or residual astrocytoma and showed a change in the profile from intact to loss at an interval of 34 months from the initial resection (Table 2). Temozolomide was started based on the histologic diagnosis, not the molecular profile change. The patient with mixed glioma had a total of 4 biopsies and resections. The third and the fourth resections showed a 19q profile change.

**Table 1**

<table>
<thead>
<tr>
<th>Molecular Profile</th>
<th>Change</th>
<th>No Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>1p Status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>19q Status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGFR Status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Change</td>
<td>3</td>
<td>17</td>
</tr>
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<td>0</td>
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<td>1</td>
</tr>
<tr>
<td>Change</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>No Change</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

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**Table 2**

Molecular Profile Change and Histologic Diagnoses in Glioma Cases With Discrepant Results

<table>
<thead>
<tr>
<th>Discrepant Result</th>
<th>Initial</th>
<th>Subsequent</th>
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<tbody>
<tr>
<td>1p Intact</td>
<td></td>
<td>Loss</td>
</tr>
<tr>
<td>19q Loss</td>
<td></td>
<td>Intact</td>
</tr>
<tr>
<td>19q Loss</td>
<td></td>
<td>Intact</td>
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<tr>
<td>19q Intact</td>
<td></td>
<td>Loss</td>
</tr>
<tr>
<td>19q Intact</td>
<td></td>
<td>Loss</td>
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</table>

<table>
<thead>
<tr>
<th>Molecular Profile</th>
<th>Diagnosis</th>
<th>Interval (mo)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1p Intact</td>
<td>Low-grade mixed glioma</td>
<td>100</td>
</tr>
<tr>
<td>19q Loss</td>
<td>Glioblastoma</td>
<td>4</td>
</tr>
<tr>
<td>19q Loss</td>
<td>Glioblastoma</td>
<td>4</td>
</tr>
<tr>
<td>19q Intact</td>
<td>Low-grade mixed glioma</td>
<td>27</td>
</tr>
<tr>
<td>19q Intact</td>
<td>Low-grade astrocytoma</td>
<td>34</td>
</tr>
</tbody>
</table>

| The patient had a total of 4 biopsies and resections. The third and the fourth resections showed a 19q profile change. |

| The patient had a total of 3 resections. The third resection showed a 19q profile change. |

| The interval between the initial biopsy or resection and the third biopsy or resection. |

was done only on the second and the third resection specimens with the upgrade of the tumor (Table 2). Temozolomide was started based on the histologic upgrade. The l1p was intact, and EGFR was not amplified on all 4 occasions.

Paired results for EGFR expression were available in 34 cases; 6 tumors demonstrated amplification, and the remaining 28 did not show any amplification on the initial or subsequent biopsy or resection specimen. In 4 patients whose tumor demonstrated amplification, the tumor was diagnosed as GBM. One case was diagnosed as grade III astrocytoma on the initial specimen, and the tumor progressed to grade IV with areas suggestive of a gliosarcoma on the subsequent resection (interval, 20 months). EGFR analysis showed amplification on the initial and subsequent specimens. The sixth case in which the tumor showed amplification was diagnosed as grade II astrocytoma. The tumor subsequently progressed to a high-grade astrocytoma; the possibility that this tumor was undergraded initially owing to tissue sampling cannot be excluded. There was no change in EGFR expression in any of the cases tested (Table 1).

**Discussion**

Advances in the understanding of molecular genetics have fostered a search for clinical correlates with these findings. In the neuro-oncology arena, such a clinical correlation has been well documented in association with large
deletions on chromosomes 1p and 19q in oligodendrogial neoplasms.\textsuperscript{1-15,29} In 1998, Cairncross et al\textsuperscript{14} reported that allelic losses on chromosomes 1p and 19q were statistically significantly associated with chemotherapeutic response and longer recurrence-free survival in patients with anaplastic oligodendroglioma treated with procarbazine, lomustine (CCNU), and vincristine chemotherapy. Smith et al\textsuperscript{3} extended this finding by reporting that the combined loss of 1p and 19q was a statistically significant univariate and multivariate predictor of overall survival for all patients with oligodendroglioma, independent of tumor grade. Numerous other studies have validated the correlation of these genetic alterations and chemoresponsiveness and favorable prognosis in oligodendrogliomas and mixed gliomas (oligoastrocytomas)\textsuperscript{5-17}; a similar favorable prognosis of these genetic findings with other forms of chemotherapy, most notably temozolomide, and radiotherapy have also been documented.\textsuperscript{3,6,8,15,30}

Based on these data, not surprisingly, many medical centers request ancillary testing for chromosome 1p and 19q status on gliomas. Abrey et al\textsuperscript{3} in their study based on the Web-based survey of members of the Society of Neuro-oncology, reported that molecular genetic testing for allelic loss of 1p and 19q was requested in about 95% of anaplastic oligodendroglioma cases, 92% of mixed glioma cases, 33% of anaplastic astrocytoma cases, 14% of low-grade oligodendroglioma cases, and 7% of GBM cases. They also found that although neuro-oncologists frequently request molecular genetic studies in newly diagnosed anaplastic oligodendroglioma, treatment recommendations vary widely and are often independent of the molecular data.\textsuperscript{3}

Besides 1p and 19q, other molecular genetic alterations are targets of ongoing studies.\textsuperscript{2,14,20,29} EGFR amplification or overexpression is found in about 40% of GBMs, particularly those arising de novo in elderly people and in the small cell variant of GBM, making EGFR-targeted therapy a rational approach for treatment.\textsuperscript{2,18-23} Various drugs targeting EGFR, including the tyrosine kinase inhibitors, monoclonal antibodies, and anti-EGFR vaccines, are being examined for possible therapeutic effect in GBM.\textsuperscript{23-27}

Various methods can be used to detect these genetic alterations including FISH, polymerase chain reaction (PCR), loss of heterozygosity (LOH), and array comparative genomic hybridization (CGH). The advantage of FISH is its considerably higher resolution compared with CGH analysis and its higher sensitivity compared with LOH analysis. FISH can detect a deletion when it is present in as few as 20% to 30% of the cells, does not require additional clinical material, and is technically less complex. FISH has a considerably higher sensitivity for tumors with clonal heterogeneity or mixed populations or in infiltrative areas of gliomas.\textsuperscript{1,3} However, it is more difficult to score than LOH and may not detect all allelic losses. While LOH has the advantage of detecting all allelic losses, including smaller and partial deletions, and is easy to interpret, it is technically more complex, requiring much purer samples and blood for comparative DNA analysis.\textsuperscript{1,3,31}

Repeated molecular testing may be performed at the time of recurrence, although the actual frequency of status change and whether change in the status of these genetic alterations provides any further useful prognostic or therapeutic information are not certain. Fallon et al\textsuperscript{32} characterized 138 (73.0%) of 189 available primary (n = 80) and recurrent (n = 109) oligodendroglial neoplasms (84% pure oligodendrogliomas and 15% mixed oligoastrocytomas) from 80 patients, using paired FISH probes for 1p, 19q, and EGFR. Of the tumors, 64% progressed to a higher grade after 1 or more recurrences, with the remainder staying the same grade throughout their clinical course. Combined 1p/19q deletions were encountered in tumors from 71% of the patients and always retained the deleted or nondeleted status in all tested recurrent specimens from each individual patient (138 of 189 samples; 73.0% of blocks). EGFR amplification was found focally in 1 specimen, a recurrent anaplastic mixed glioma.\textsuperscript{32} The authors suggested that longitudinal samples were genetically stable in the loci investigated and that changes in 1p and 19q arise early in histogenesis and other genetic events might be associated with histologic progression.\textsuperscript{32}

Walker et al\textsuperscript{33} also studied the histologic and molecular profile changes in sequential oligodendrogial neoplasms (oligodendrogliomas and mixed gliomas). They found that 74% of oligodendrogial neoplasms retained their initial histologic differentiation (23/31) in longitudinal samples. However, 4 tumors showed a change in histologic diagnoses between oligodendroglioma and oligoastrocytoma, 3 showed a change between oligoastrocytoma and astrocytoma, and 1 showed a change between oligoastrocytoma and glioblastoma in longitudinal samples. Twelve tumors demonstrated a histologic upgrade. They also found that 29% of the oligodendrogial neoplasms showed new genetic alterations in longitudinal samples (9/31). Six of the cases showed a change in 1p profile, 4 cases showed a 19q status change, and the –1p/–19q genotype was acquired in 3 cases in longitudinal samples.\textsuperscript{33}

In our study consisting of gliomas of oligodendrogial and astrocytic lineages, we found only rare evidence of profile change in 1p and 19q status (5/53 tumors). Theoretically, repeated testing might be performed because new genetic events may be associated with tumor recurrence or tumor upgrade. However, a possibility is that the treatment might target a particular cell population, leading to selection of clones that were initially present in low numbers but now multiply and become detectable, yielding discrepant results. Tumor heterogeneity is another variable that could account for the discrepant results in longitudinal samples. Gliomas are notoriously heterogeneous neoplasms, morphologically and in many other ways; however, it is unclear how homogeneous
they are in terms of cytogenetic alterations. Also, in the present study in which the initial diagnosis was low-grade glioma (3/5 cases with discrepant results on longitudinal studies), it is conceivable that the initial molecular results might represent a false-negative result owing to a possibility that the majority of cells evaluated by FISH analysis were normal resident glial cells. The use of other methods like PCR would have been confirmatory in these cases. PCR could not retrospectively be done in the present study owing to the nonavailability of blood or nonlesional DNA tissue for comparative DNA analysis.

There was no change in the clinical management based on the results of repeated molecular tests in patients with discrepant results in our study. None of the tumors with changes in 1p/19q status were pure oligodendrogliomas. There was no change in EGFR status in any of the cases. Of the 9 tumors showing an upgrade on repeated histologic examination, only 2 (both mixed gliomas) showed a change in molecular status. The discrepant results in these cases could be accounted for by a variety of the aforementioned discussed reasons. None of the other 7 tumors showed a change in the molecular profile with the upgrade of the tumor. The study supports the conclusion that there seems to be no definitive indication for reflex repeated 1p/19q or EGFR FISH testing in gliomas at the time of repeated biopsy or resection.

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References


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