

North Central Cancer Treatment Group

and

Mayo Clinic

**Diagnostic and Prognostic Markers in Low-Grade Gliomas**

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| <b>Document History</b> | <b>(Effective Date)</b> |
|-------------------------|-------------------------|
| Activation              | December 1, 1995        |
| Addendum 1              | June 18, 1997           |
| Addendum 2              | December 2, 2005        |
| Update 1                | May 29, 2009            |

| <b><u>Study Participants</u></b> | <b><u>Date Activated</u></b> |
|----------------------------------|------------------------------|
| Entire NCCTG                     | December 1, 1995             |
| Mayo Clinic                      | December 4, 1995             |

NCI Version Date: May 19, 2009

**Protocol Resource**

Update 1

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Update 1

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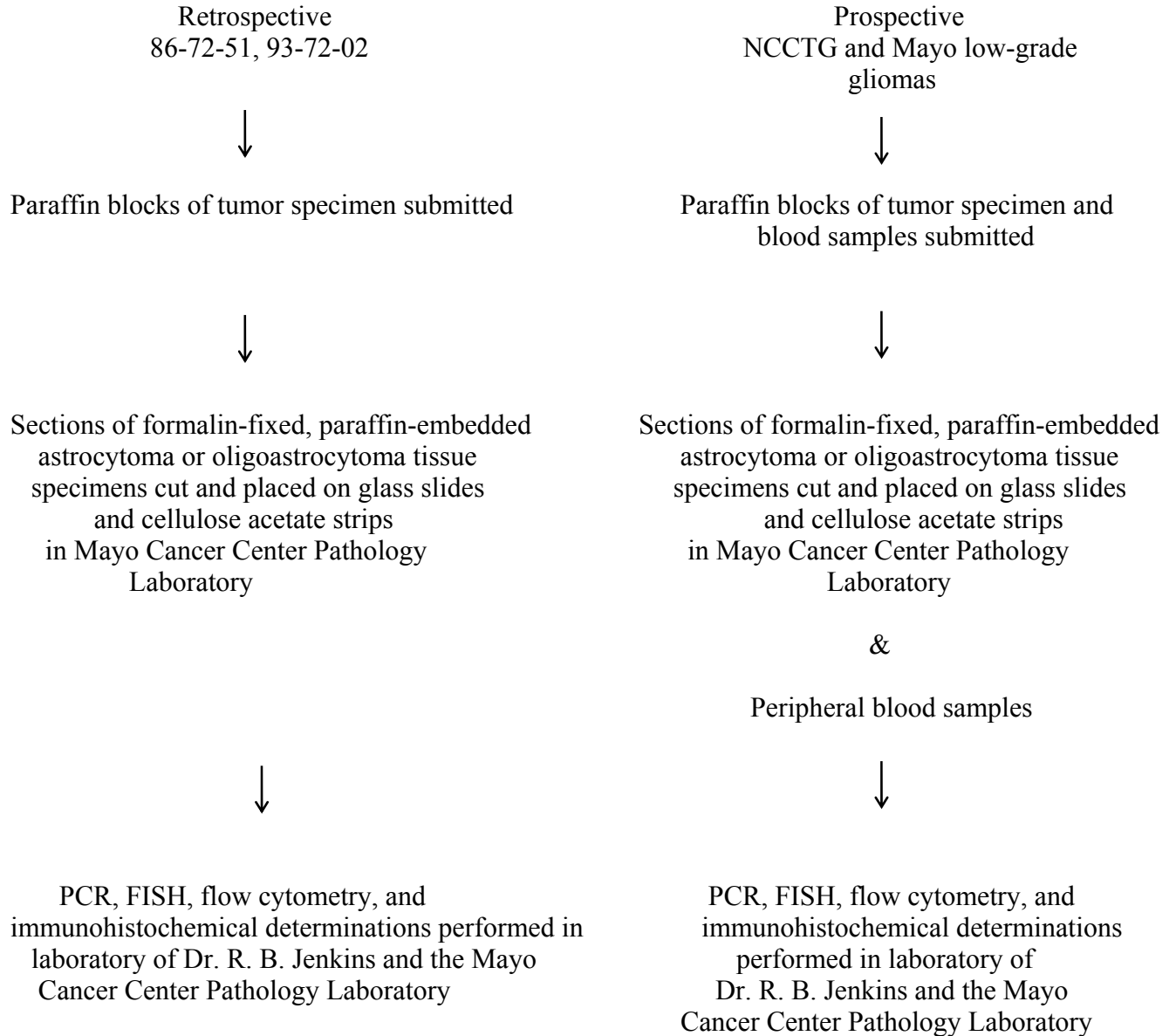
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**Schema**

## 1.0 Background

- 1.1 Clinical relevance. There will have been about 17,500 primary CNS (central nervous system) neoplasms and about 12,100 deaths due to CNS neoplasms in the United States during 1994 (1). Approximately 50% of these tumors will be malignant gliomas. High- and low-grade diffuse fibrillary astrocytomas are the most frequent gliomas. However, oligodendrogliomas, mixed oligoastrocytomas, juvenile pilocytic tumors, and ependymomas comprise a significant proportion of gliomas (2). Accurate classification and grading of gliomas is vitally important for assessing prognosis, prescribing appropriate treatment(s), and evaluating new therapeutic approaches. Current therapies include surgery, radiation, and various chemotherapeutic agents. Patients with varying histologic types and grades of glioma may require differing therapies (2,8-10).
- 1.2 Variability in prognosis. There are clear prognostic differences between glial neoplasms. Median survival is 5-10 years for patients with various types of low-grade glioma, but less than 1 year for those with Grade 4 astrocytomas. Patients with pilocytic astrocytoma have a 90%, 10-year survival rate. Determining diagnosis, assessing prognosis, and assigning appropriate therapy for patients with gliomas has traditionally relied on pathological and clinical factors, including patient age, performance status, and extent of surgical resection as well as tumor histologic type, pathologic grade, and specific location. Some of these are easy to assess while others, especially histologic type and grade, can be difficult and depend upon the interpretation of a skilled neuropathologist. Even within the group of patients with carefully determined pathologic type and grade of glioma, there are patients with differing prognoses.
- 1.3 Pathology issues. Until recently, no consensus had developed regarding astrocytoma classification and grading (3-5). Some pathologists, notably Burger, used a three-tiered grading system: astrocytomas, anaplastic astrocytoma, and glioblastoma multiforme (3). More recently, Daumas-Duport and Scheithauer developed the four-tier St. Anne-Mayo grading scheme for diffuse fibrillary astrocytomas (4). In this system Grades 1 and 2 are roughly equivalent to the Burger astrocytoma group, and Grades 3 and 4 are roughly equivalent to the Burger anaplastic astrocytoma and glioblastoma multiforme (GBM) groups, respectively. Both grading systems have demonstrable clinical relevance (3,4). Although the approaches differ, the distribution of their resultant grades are roughly comparable (5). The simplicity of application and interobserver reproducibility of the St. Anne-Mayo system are particularly appealing. The World Health Organization (WHO) has essentially adopted the histologic criteria and method of grading inherent in the St. Anne-Mayo system in its recently revised classification of tumors of the nervous system (6).

Several histologic problems remain. For example, the significance of atypia and histologic malignancy in pilocytic astrocytoma is unclear. Although a small portion of pilocytic astrocytomas, such as those in the cerebellum (microcytic cerebellar astrocytomas), may appear high-grade, for reasons unclear they have a good prognosis following surgical resection (7). Similarly, mixed oligoastrocytomas and oligodendrogliomas may have a distinctly better prognosis (8) and appear to be significantly more chemosensitive (9) than astrocytic tumors of similar grade. However, it is often difficult to clearly distinguish mixed oligoastrocytomas from astrocytomas or “pure” oligodendrogliomas, especially since many relatively “pure” oligodendrogliomas exhibit focal morphologic evidence of an astrocytic component. Even experienced neuropathologists often have difficulty classifying astrocytic tumors with a variable oligodendroglial component (5,6). If molecular markers that improve the classification and grading of tumors of glial lineage can be found, they would aid in establishing the diagnosis, predicting the prognosis, selecting therapy, evaluating treatment protocols, comparing inter-institutional treatment results, and studying pathobiology of these tumors.

- 1.4 Proposed study. Dr. Robert B. Jenkins, a pathologist and geneticist in the Mayo Clinic Department of Laboratory Medicine and Pathology, and other colleagues at Mayo have been funded by the NCI (National Cancer Institute) for the past five years to assess multiple markers in diffuse fibrillary astrocytomas, “pure” oligodendrogliomas, mixed oligoastrocytomas, and pilocytic astrocytomas. They have identified markers that have important biologic and clinical relevance in gliomas. The biological and clinical associations of these markers need to be defined more precisely and validated in larger, well-characterized, and prospectively obtained sets of patients with gliomas. Dr. Jenkins has recently obtained funding to extend the initial observations to tissues obtained from patients entered in prospective clinical trials of the NCCTG (North Central Cancer Treatment Group) and RTOG (Radiation Treatment Oncology Group). The NCCTG pathology committee chair and group chair have supported his efforts to obtain funding by agreeing to provide tumor tissue and clinical data for patients treated in previous NCCTG clinical trials. In addition, the mechanism of Dr. Jenkin’s funding is through the cooperative group mechanism (U01). Through this instrument, NCCTG has a “gateway” for collaboration with other investigators throughout the United States aimed at discovering more about the biology and outcome of patients with malignant glioma. Through the goals of the cooperative group, we propose to define more precisely and validate the biological and clinical relevance of specific molecular markers identified by Jenkins’ group and others. The overall goal of this project is to assess and utilize molecular markers to more accurately classify and grade gliomas, and thus, improve the prediction of prognosis, assign optimal therapy, and interpret the results of clinical trials. This particular trial is designed to assess those markers in patients with Grade 2 astrocytoma, oligoastrocytoma, or oligodendroglioma by the WHO classification.

Molecular genetic, cytogenetic, flow cytometric, and immunohistochemical factors have been used to assess many solid tumors including gliomas. During the initial grant period at Mayo, several potential factors, which may be useful in the classification of glial tumors, have been identified. The overall goals of the next study period are (1) to determine if such factors can be used to classify astrocytic tumors of favorable and poor prognosis and (2) to develop factors which will reliably differentiate astrocytomas, oligodendrogliomas, and mixed oligoastrocytomas.

- 1.5 Genetic observations: Cytogenetic and molecular genetic studies have elucidated many, if not most, of the physically large genetic alterations found in gliomas (11,12). With few exceptions, the cytogenetic alterations have been numeric anomalies and chromosomal deletions, including +7, -10, -22, 9p-, 13q-, and structural alterations of chromosomes 17 and 19 (11-16). Molecular genetic evaluations of allelic loss have confirmed and extended these observations (11,12,15-21). However, only a few genes have been implicated in glioma pathogenesis including p53 (17,22-28) and NFI (29) (both mapped to chromosome 17), EGFR (30,31) (on chromosome 7), gli (32) and MDM2 (33) (both amplified in a very small fraction of tumors). Several groups, including our own, are actively attempting to identify the genes on chromosomes 9 (34), 10 (16,35,36), 19 (15,19), and 22 (an excellent candidate gene is NF2 [37,38]) involved in glioma pathogenesis.

Many of these genetic alterations are thought to be associated with particular grades of glial malignancy and/or morphologic subtypes. For example, chromosome 10 loss and 9p deletion appear to be associated with the transition of Grade 3 or 4 astrocytomas (12,16,18,31,39). Alterations of 17p and/or mutations of the p53 gene have been observed to occur in approximately 50% of fibrillary astrocytomas, even in tumors of low grade, suggesting that these genetic changes occur early in the development of about half of such tumors (12,18,22-24). Loss of 17q alleles has also been recently associated with juvenile pilocytic astrocytomas (40). Loss of alleles mapped to 19q occur in a significant fraction of gliomas, especially oligodendrogliomas (15,19,41). Do the small fraction of high-grade apparently diffuse astrocytomas with loss of 19q alleles (15) behave like oligodendrogliomas in terms of improved survival and increased responsiveness to chemotherapy? Conversely, do high-grade oligodendroglioma tumors with alterations of chromosome 10 (a genetic lesion more frequently associated with high-grade astrocytic tumors) behave more aggressively than oligodendroglioma tumors that lack such an anomaly? Interestingly, a high-grade oligodendroglioma with chromosome 10 loss and especially aggressive behavior has been recently reported (42).

Within defined morphologic subtypes, genetic markers have been shown to have prognostic relevance for patients with some solid tumors (e.g., neuroblastoma) (43,44). Few such studies have been performed for gliomas (14,16,45,46), although some have been reported from the Mayo Clinic as a result of the initial

grant period (14,16,46), but rarely with multivariate analysis (16,46). Notably, these markers are often highly correlated with other clinical factors such as patient age and tumor grade. There are at least three other reasons why such markers might not have been identified as independent prognostic factors in gliomas, and these must be excluded before it can confidently be stated that specific genetic markers do not have significant prognostic utility.

First, routine cytogenetic studies of fresh tumor tissue often miss chromosome anomalies (15,16,47). This lack of sensitivity is a result of both biologic and technical factors and has been reviewed (16,47). Chromosome anomalies may be found as a result of culture artifact (47,48). In addition, cytogenetic studies require highly trained personnel to perform and interpret, thus making transfer of the technology to general pathology practice difficult. However, the new method of FISH (fluorescence in situ hybridization) analysis (49,50), can potentially assess specific chromosome centromeres and chromosomal regions within nearly every paraffin-embedded tumor specimen. Thus, one of the goals of this project is to evaluate FISH detection of chromosomal anomalies within interphase nuclei of large groups of frozen paraffin-embedded tumor specimens collected for ongoing and previous clinical trials.

Second, until 1992, the assessment of some molecular genetic markers, especially those detecting allelic loss, was based on technology (primarily Southern blotting) that was time-consuming. Thus, it has been difficult to analyze a large number of patients for allelic loss. In addition, the markers tested were often genetically uninformative in specific patients. For these technical and biological reasons, significant numbers of patients with adequate follow-up have not been reported for molecular genetic allelic loss. The advent of highly informative microsatellite markers (51) evaluable by the polymerase chain reaction (PCR) has significantly reduced the technical and biological problems associated with allelic loss detection. Most, if not all, patients will be informative for a particular chromosomal region. One of the goals of this project is to assess chromosomal loss in glioma specimens.

Third, measurements of allelic loss are only indirect evidence that a putative gene in the region of loss is involved in tumor pathogenesis, detecting only chromosomal deletion mutations, but not other mutational mechanisms such as microdeletion, point mutation, and translocation. Only direct assessment of the gene itself (once isolated) by genomic or cDNA sequencing, immunohistochemistry, and perhaps functional assay (52) will determine the clinical relevance of a specific molecular marker. We have already undertaken such studies of the p53 gene in the first grant period. One of the goals of this project is to use our frozen tumor resources from Mayo as a “training set” to assess the pattern of mutations in new genes relevant for glioma pathogenesis as they are cloned. We intend to validate such studies (using appropriate methodology) in the NCCTG and RTOG specimens to establish their prognostic relevance.

- 1.6 Genetic studies: preliminary observations. During the first two years of the previous grant period, efforts were focused on performing a molecular allelotype using Southern blotting methods on human gliomas (15,16,21). Briefly, we investigated loss of alleles mapped to all autosome arms and to the X and Y chromosomes. In diffuse astrocytomas, approximately 70% demonstrate loss of chromosome 10 alleles (10p and/or 10q), 33% lost 7q alleles, and about 40% have evidence of 17p allele loss. The interferon genes have been deleted from chromosome 9p in one third of gliomas, primarily diffuse astrocytomas. Alleles on 13q (primarily within the retinoblastoma locus) have been deleted in approximately one-third of gliomas. Our results were generally consistent with other data concerning the relative order of genetic events in glioma tumorigenesis (11,12,18). For example, 17p is altered in approximately 50% of diffuse astrocytic tumors of Grade 2-4, often in the presence of other genetic events but sometimes as the sole detectable genetic event. Such data strongly support the hypothesis that a gene or genes on 17p are important for the initiation and/or early progression of gliomas (11,12). The loss of chromosome 10 in high-grade tumors, often in the presence of many other genetic events, suggests that this event is often late in glial pathogenesis. However, in a small but significant number of tumors chromosome 10 loss is the sole detectable genetic event, without detectable chromosome 17 alterations. Although we have not completely excluded other genetic events (p53 sequencing on these tumors is currently being performed) these data suggest that early chromosome 10 loss might result in rapid progression to high-grade.

We observed evidence of specific chromosomal losses associated with specific morphologic subtypes. For example, 4 of 4 (100%) informative oligodendrogliomas had alterations of 19q (15).

With the number of available chromosome 10 probes suitable for Southern blotting, we initially attempted to determine the region of the chromosome necessary for minimal deletion in gliomas. Based on these results and our cytogenetic data (14,16), we hypothesized that there are at least two putative regions on chromosome 10 that may contain tumor suppressor genes: one on the short arm near 10p13 and one on the long arm near 10q22-q26. The presence of two or more important genes may explain why a whole chromosome 10 is lost so frequently (two or more tumor suppressor genes can be deleted by a simple single nondisjunction event).

Using pulsed-field gel electrophoresis (PFGE) blocks prepared at Mayo from four Mayo-derived glioma cell lines, a group at University of Chicago began to map the deleted region on 9p involved in the pathogenesis of several solid tumors including gliomas (34).

During the third year we performed multivariate survival analyses of cytogenetic and flow cytometric parameters associated with chromosomes 7, 10, 13, 17, 19, 22, X, and Y in a series in the first 207 Mayo gliomas obtained for this project. These studies were designed to begin to assess the prognostic relevance of genetic markers on gliomas.

During the fourth year of the grant, the methodology evolved from the use of conventional RFLP markers toward the use of microsatellite markers (51) to detect LOH. The highly informative nature of these microsatellite probes has provided more complete LOH data for use in our current clinical correlative studies and those planned for this proposal. Briefly, a library of over 150 microsatellite polymorphism-detecting PCR primer pairs representing the entire human genome has been assembled. In a comparison of data from 40 informative cases, RFLP and CA repeat analysis were found to be nearly equivalent for the detection of chromosome 10 loss, indicating that microsatellite markers give comparable results to those of RFLPs. Fifteen new chromosome 10 markers applied to the 10 recombinant patients previously identified through RFLP analysis further narrowed the boundaries of the common deletion region on the long arm to 10q23-24. Thirteen new chromosome 19 markers applied to 18 oligodendrogliomas, 8 mixed oligoastrocytomas, and 75 astrocytomas (41) revealed abnormalities in 61% of oligodendrogliomas, 62% of mixed oligoastrocytomas, and 32% of astrocytomas (primarily glioblastomas), providing further evidence that this anomaly is associated with tumors of oligodendroglial lineage. Importantly, while 19q alleles were lost in oligodendrogliomas and mixed oligoastrocytomas, 19p alleles were lost in the astrocytomas (41). The renewal will ascertain if the astrocytomas with chromosome 19q anomalies have a significant oligodendroglial component.

Because of the recent publication from Dr. Stephen S. Thibodeau of the Mayo Clinic describing genomic instability in colorectal cancer (100), we analyzed a large number of Mayo Clinic gliomas for the presence of similar genomic instability. No Type I genomic instability (100) was observed in any primary human glioma. Because of the probable involvement of the p53 gene in the development of diffuse fibrillary astrocytomas, we initially sent gliomas to Dr. Bernd Seizinger at Massachusetts General Hospital (MGH) for point mutation analysis (17). We have now developed our own protocol for screening and sequencing p53 mutations (28). Briefly, with appropriate PCR primers, we have screened and sequenced multiple introns, exons 1-10 as well as the 5' portion of exon 11. We use RNA-single-stranded conformational polymorphism (rSSCP) to screen for mutations (102). rSSCP, a modification of the single-stranded conformation polymorphism (SSCP) technique now commonly used to screen for DNA mutations, detects mutational differences in tertiary structures in single-stranded RNA. rSSCP is more sensitive than SSCP (101). Putative mutations are subjected to direct DNA sequence analysis using the dideoxy-chain termination method.

We observed 87 migration shifts among 107 Mayo Clinic glioma or gliosis specimens screened for p53 mutations by rSSCP. We used a conservative approach in our interpretation of rSSCP data. Briefly, we believe that any questionable gel migration anomaly should be subjected to direct DNA-sequencing. This conservative approach should result in a relatively low false-positive rate: an apparent rSSCP gel migration shift without a demonstrable mutation upon DNA-sequencing. However, we are aware that there may be situations where rSSCP may detect a mutation and direct-sequencing may not. For example, the percentage of tumor cells may be too low to result in a DNA-sequencing anomaly but high enough to result in an rSSCP gel migration shift. Since this probably occurs relatively rarely, we plan to evaluate its incidence by comparing the screening/sequencing p53 data with immunohistochemistry results. Our conservative approach to rSSCP data should also result in a low false-negative rate: There should be a very small number of tumors which contain DNA-sequence alterations but have no apparent rSSCP gel migration anomaly. Indeed, the false-negative rate of our p53 rSSCP screening/DNA-sequencing strategy is very low. When we directly sequenced all 11 p53 exons from 40 paired blood/tumor DNA specimens (80 specimens total) which had no apparent rSSCP anomalies, we observed no DNA-sequence alterations. This low false-negative (0%) rate provides us confidence that we will be able to detect most if not all DNA mutations by just sequencing those exons which have an rSSCP gel migration anomaly.

We have completed p53 sequencing on 48 gliomas and gliosis specimens, focusing our attention on Grade 2-3 astrocytomas and nonastrocytic tumors. We plan to direct sequence the remaining tumors. Grade 2-3 astrocytomas have a significant number of acquired, biologically relevant mutations. Six mutations were found in five Grade 2 and 3 astrocytomas. Five occurred within exons 5-8 (the “hot spots” for p53 mutation) and involved an amino acid substitution. The false-positive rSSCP screening rate is high as expected, given our conservative interpretation strategy, with only one mutation found upon direct sequencing of the 11 rSSCP gel shifts observed among 10 gliosis specimens. That mutation was within intron 4A, and its biologic relevance is uncertain. We are currently immunostaining these specimens (and other apparent false-positive specimens) to ascertain if there is a low percentage of p53 overexpressing cells within them. In the near future, we should have information comparing the loss of chromosome 17, alterations of p53 expression, and alterations of p53 DNA sequence in a large number of astrocytomas.

- 1.7 Ploidy studies. For nearly 15 years, flow cytometry has been used to measure DNA content on fresh or fixed paraffin-embedded tumors. One aim of this project is to assess the prognostic relevance of ploidy for glial tumors. Compared to other solid tumors, there have been relatively few reports in the literature of ploidy in patients with gliomas. In a review of 16 series of astrocytomas of all grades (53-68), 52% were DNA-diploid and 48% were DNA-aneuploid, with increasing DNA aneuploidy observed in higher grade tumors. Five assessed the correlation of DNA-ploidy with survival among astrocytomas of all grades. In two series patients with DNA-aneuploid tumors had significantly poorer survival than patients with DNA-diploidy (54,61). Two series had opposite results (60,62). In the fifth series, there was no significant association with ploidy and survival (55). Results have been comparably mixed in a few series that have attempted to correlate survival with ploidy within a given grade of astrocytoma. Among patients with anaplastic astrocytomas, one series observed that patients with DNA-diploid tumors had better survival than patients with DNA-aneuploid tumors (64), while two series (55,60) noted the reverse. Thus, while DNA-ploidy may correlate with tumor grade, DNA-diploidy/DNA-aneuploidy has not had a consistent association with better/poorer survival. However, in only one of these series (69) was multivariate analysis employed.

In a review of nine series of oligodendrogliomas studied by flow cytometry (65-73), overall 35% were DNA-diploid and 65% were DNA-aneuploid. Six of the nine series had <10 patients, making meaningful conclusions about the influence of ploidy on survival difficult. In the largest series (73), patients with DNA-diploid tumors had a better, but not significantly different survival than patients with DNA-aneuploid tumors. There is a greater likelihood of DNA-aneuploidy in higher grade tumors; one series found ploidy correlated better with survival than grade (73). No study attempted to correlate survival with ploidy within a given grade of tumor, or employed multivariate analysis.

- 1.8 Cellular proliferation studies. Identification of proliferating cells in tissue sections of CNS tumors is of fundamental importance given the central role of abnormal cell proliferation to the malignant phenotype and the cell cycle specificity of therapeutic regimens. One ongoing aim of this project is to assess and compare the relevance and the prognostic value of markers of cellular proliferation, including, %S, %G<sub>2</sub>M, Ki-67, and a p53 marker of cellular function associated with abnormal cell cycle regulation.

%S and %G<sub>2</sub>M determined by flow cytometry, have shown strong associations with survival and tumor grade. Tumors with increased %S or %G<sub>2</sub>M are more likely to be DNA-aneuploid than DNA-diploid (54,63). Similarly, astrocytomas with increased %S are more likely to be high-grade than low-grade (59,69). Poorer patient survival has been associated with increased %S and %G<sub>2</sub>M within the tumor (53,54,56,64).

Ki-67 has long been used as a marker of cellular proliferation despite the antibody (Ki-67) not being effective in formalin-fixed, paraffin-embedded sections. Ki-67 immunoreactivity is tightly associated with the cell cycle with expression appearing late in G<sub>1</sub>, rising through S and G<sub>2</sub> to maximal at mitosis. Recently, a fusion protein containing a cloned Ki-67 gene DNA fragment was used to raise novel Ki-67 specific antibodies. One of these, MIB-1, is effective in formalin-fixed, paraffin-embedded material (86-88). All studies of Ki-67 in astrocytomas have used the Ki-67 antibody. These studies have shown highly variable results (81,83). Ki-67 LI tends to increase with increasing grade in astrocytomas (89-92), but has not been associated with a poorer survival (89,90). In one study, however, Ki-67 LI emerged as an independent indicator of survival in multivariate analysis of 78 patients with brain tumors (92).

In its wild-type state, the p53 nuclear phosphoprotein suppresses the growth of neoplastic cells through, as yet, not clearly defined means. It binds to DNA and is capable of activating transcription. Mutations in the p53 gene are the most commonly detected lesions in a wide variety of human tumors, including gliomas (17,18,22,24-27), and most result in p53 molecules that have lost the functional properties of the wild-type protein. The most common mutations in p53, missense changes, result in an abnormal over-expression of p53 which is detectable by immunohistochemistry. Wild-type p53, while present in all cells, is not at a concentration sufficient to allow immunohistochemical detection. Over-expression of p53 correlates well with the presence of missense mutations. It is important to remember, however, that changes in p53 that result in protein instability have been reported and, for breast carcinomas, may comprise up to 36% of the mutations detected (93). Immunohistochemical studies of the over-expression of p53 have been reported in gliomas (26,85,92,94-96). Most studies were restricted to frozen tissue, since the available antibodies were optimal in such material. Recently, a series of anti-p53 monoclonal antibodies

have been developed for use in archival material (the DO series, 98), and the past year has seen an impressive increase in studies aimed at determining the clinical relevance of p53 staining. In astrocytoma patients, p53-positive tumors have been associated with a significantly reduced survival compared with p53-negative tumors, but p53 failed to emerge as an independent prognostic variable upon multivariate analysis (92).

Increased expression of wild-type, but not mutant, p53 is evoked in response to agents that damage DNA, resulting in a transient stop in the cell cycle at G1/S. It has been hypothesized that this allows time for DNA repair and proposed that mutations in p53 would result in the development of aneuploidy (98). Since p53 functions in suppressing the cell cycle, one might predict a relationship between markers of cellular proliferation and abnormal p53 expression. Mercer et al. (99) demonstrated that PCNA was down-regulated in a GBM-cell line whose growth was suppressed by wild-type p53. Barbareschi et al. (85) were unable to show any relationship between PCNA and p53 alterations in a series of 86 CNS tumors. A significant increase in Ki-67 LI, but not p53 expression, was observed with increasing astrocytoma grade (92), while Haapasalo et al. (98) found p53 staining to be significantly associated with PCNA LI in 102 astrocytomas.

The ultimate question is whether tumor ploidy and cellular proliferation are independent predictors of survival in relation to other known patient/tumor prognostic factors. Of the series reviewed, only a few (61,82,92) have attempted multivariate analysis. Importantly, multivariate analysis showed DNA-ploidy and %G<sub>2</sub>M variables to be significant predictors of survival in the group of gliomas studied during years 1-4 of Dr. Jenkin's grant (46). Because of these initial results, we intend to validate our DNA-ploidy observations (46) in NCCTG specimens to establish their prognostic relevance. We plan to perform a multivariate analysis of Ki-67 and p53 immunostaining from previous specimens to learn if alterations of these markers are predictors of survival. Since we suspect that one or more of these markers will also be a significant predictor of survival, we plan to validate such observations in NCCTG materials.

- 1.9a Flow cytometry studies: preliminary investigations. We completed DNA ploidy, %S-phase, and %G<sub>2</sub>M analysis on 281 specimens and included these parameters in the clinical correlative studies described below. For a small percentage of gliomas (<5%) the residual tumor within the paraffin-embedded block was not sufficient for ploidy analysis. By the end of the first grant period, we expect to compare DNA ploidy, %S-phase, and %G<sub>2</sub>M with immunohistochemical studies of cellular proliferation (Ki-67 and p53).

- 1.9b Immunohistochemical staining: preliminary observations: Dr. Julie Cunningham, a research associate in the Division of Oncology Research, has been responsible for the immunohistochemical portions of the project. Her initial experiments utilized frozen sections for p53 and Ki-67 and paraffin-sections for PCNA. The initial p53 data were analyzed with respect to cytogenetic and molecular genetic data and flow cytometry data, and have been presented (103). Briefly, p53 expression was not associated with any specific or general genetic changes, notably there was no association with either loss on chromosomal 17p or with DNA-aneuploidy.

The focus has now shifted to the use of formalin-fixed, paraffin-embedded samples to directly compare PCNA, Ki-67, and p53, and flow cytometry parameters. To date, p53 expression (antibodies DO-7, PAb1801 and CM1) has been assessed in 154, PCNA (PC10) in 179 and Ki-67 (MIB-1) in 77 glioma specimens using a sensitive streptavidin method (104). A proliferative index (PI) was obtained for PCNA and MIB-1 by reading ten separate fields of highly cellular areas on a CAS Image Analyzer (Cell Analysis Systems, Inc.), the PI value being the mean percentage of positive nuclear area out of total nuclear area. PCNA positivity was restricted to strongly, definitively labeled cells, as there is no clear consensus on how to treat lightly stained cells and concern over the persistence of PCNA beyond the active cell cycle. The MIB-1 staining has been completed on over 160 additional tumors, but the MIB-1 PI is in the process of being determined on the CAS instrument. Gliomas were deemed p53-positive when greater than 10% of neoplastic nuclei were stained. Tumors with less than 10% were noted and will be analyzed in the future.

In agreement with two previous reports (85,92), we found no association between p53 over-expression and PCNA PI: mean PCNA-PI was  $22.6 \pm 17.8$  for p53-positive astrocytomas and  $21.4 \pm 17.6$  for p53-negative astrocytomas. In the Grade 4 astrocytomas, mean PCNA PIs for p53-positive and negative tumors were  $16.1 \pm 18.7$  and  $27.5 \pm 18.7$ , respectively.

- 1.9c Preliminary clinical correlation studies. The primary goal of our project has been to assess the prognostic value of flow cytometric (DNA ploidy, S-phase), cytogenetic, molecular genetic, immunohistochemical, and clinical variables in our series of gliomas. Cytogenetic results were correlated with survival data in an initial group of 117 gliomas (99 astrocytomas, 16 mixed oligoastrocytomas, and 2 gliosarcomas) (14). Briefly, in a multivariate analysis using Cox models, survival was significantly better in patients whose tumor had normal or nonclonal cytogenetic analyses than in those whose tumors had clonal abnormalities.

More recently, a preliminary investigation of the association of survival with cytogenetic, molecular genetic, flow cytometric, and clinical variables in the first 207 new astrocytomas obtained for this project, identified patient age, tumor grade, and diffuse fibrillary astrocytoma histology as prognostically relevant (46). Univariate analyses also showed significant associations between survival and many molecular pathologic parameters, including genetic alterations of chromosomes 7 and 10. To identify a subset of parameters that are strongly associated with survival individually after adjustment for the effects of the others, multivariate analyses using classification and regression tree (CART, also known as recursive partitioning) (105,106) models were performed. CART divided the 207 patients into five prognostic groups based on patient survival. In order of increasingly poor prognosis they were: (1) Grades 1-3, percent  $G_2M < 6.9$ ; (2) Grades 1-3, percent  $G_2M \geq 6.9$ ; (3) Grade 4, age  $< 66$  years, DNA-aneuploid; (4) Grade 4, age  $< 66$  years, DNA-diploid or tetraploid; and (5) Grade 4, age  $\geq 66$  years. We then compared the distributions of various cytogenetic and molecular genetic allelic loss data among these five survival groups. Interestingly, anomalies of chromosomes 7, 10, and 13 are associated with the groups 3-5 while anomalies of chromosomes 17 and 19 are more equally distributed across all groups. This observation is consistent with the putative order of genetic alterations in gliomas. We anticipate that we will be able to perform these analyses more readily using the larger sample sizes available from NCCTG studies.

## 2.0 Goals

- 2.1 Evaluate the diagnostic and prognostic relevance of alterations of specific chromosomes and chromosomal regions including 7, 9p, 10p, 10q, 13q, 17p, 17q, 19q, 22q, X, and Y, using PCR analysis of microsatellite repeats and FISH.
- 2.2 Evaluate the diagnostic and prognostic relevance of DNA ploidy by flow cytometric analysis. Compare with ploidy determination by FISH.
- 2.3 Assess the diagnostic and prognostic relevance of various markers of cellular proliferation and cellular function including flow cytometric determination of %S-phase, % $G_2M$ , and immunohistochemical evaluation of PCNA, Ki-67, and p53.

## 3.0 Patient Eligibility

- 3.1 Paraffin-embedded tumor tissue blocks of patients enrolled in NCCTG 86-72-51 or 93-72-02 and who had the diagnosis of low-grade glioma.
- 3.2 Patients who have the diagnosis of low-grade glioma with an available paraffin-embedded tumor tissue block enrolled in prospective NCCTG and Mayo studies.

#### 4.0 Registration Procedures

Update 1

- 4.1 To register a patient, fax (507/284-0885) a completed eligibility checklist to the Registration Office between 8 a.m. and 4:30 p.m. central time Monday through Friday.
- 4.2 A signed HHS 310 form is to be on file at the Registration Office before patient entry. *Note: This study meets the criteria for expedited institutional review board procedure.*
- 4.3 It is at the discretion of each institutional review board whether a consent form is required. [Code of Federal Regulations 45 CFR 46, Section 46117 (c) (1) and (2)].
- 4.4 Patient eligibility will be checked by Registration Office personnel before a patient will be registered into this study.

#### 5.0 Procedures for Procurement and Handling of Paraffin Blocks

- 5.1 Retrospective studies 86-72-51 or 93-72-02
  - 5.11 A patient listing will be sent to each membership with the activation of this study. If the blocks are on file at the Operations Office, it will be indicated on the listing, and you may proceed with registration via the eligibility checklist. If the blocks will **never** be available, please let the Operations Office know by indicating so on the patient listing and returning the list to the Operations Office.
  - 5.12 If a patient's surgery was at Mayo Clinic Rochester, you may call the pathology coordinator listed on the protocol resource page. With confirmation of block availability, you will be called and may proceed with registration via the eligibility checklist.
  - 5.13 Each patient's block should be placed in a plastic bag and the bag labeled with the accession number, NCCTG patient number, patient's initials, this protocol number, and the number of the study on which the patient was originally registered.
  - 5.14 Following patient registration, submit the packaged blocks to the NCCTG Operations Office.
  - 5.15 The institutional pathologist must be informed that the blocks may be depleted. At the completion of the study, remaining tissue blocks will be retained in the NCCTG Operations Office for possible future laboratory studies. The blocks will be returned promptly to the institutional pathologist upon request at any time. Should the block be depleted, we will let you know.

## 5.2 Prospective NCCTG and Mayo low-grade glioma studies

### 5.21 NCCTG institutions

5.211 Each patient's block should be placed in a plastic bag and the bag labeled with the accession number, NCCTG patient number, patient's initials, this protocol number, and the number of the study on which the patient was originally registered.

5.212 Following patient registration, submit the packaged blocks to the NCCTG Operations Office.

5.213 The institutional pathologist must be informed that the blocks may be depleted. At the completion of the study, remaining tissue blocks will be retained in the NCCTG Operations Office for possible future laboratory studies. The blocks will be returned promptly to the institutional pathologist upon request at any time. Should the block be depleted, we will let you know.

5.214 If a patient's surgery was at Mayo Clinic Rochester, you may call the pathology coordinator listed on the protocol resource page. With confirmation of block availability, you will be called and may proceed with registration via the eligibility checklist.

5.22 Mayo institutions - Blocks will be routed to Dr. R. B. Jenkins via the study assistant.

## 6.0 Procedures for Procurement and Handling of Blood Samples for Prospective NCCTG and Mayo Low-Grade Glioma Studies

### 6.1 NCCTG institutions

6.11 The institution entering the patient will be required to draw 30 cc of peripheral blood into six tubes (three in EDTA and three in Heparin) at the time of the next scheduled blood draw per protocol.

6.12 Kits will be supplied through Mayo Medical Laboratories (MML). Participating institutions may obtain kits by submitting the attached MML FAX Supply Request Form (see Forms Packet) and fax to the FAX number on the Supply Request Form. All sections of the form must be completed in order to expedite processing of the request.

A small, but sufficient, supply of the specimen collection kits should be ordered prior to patient entry. Allow at least two weeks to receive the kits. MML will **not** be able to forward kits to you by express mail.

### 6.2 Mayo institutions

6.21 The institution entering the patient will be required to draw 30 cc of peripheral blood into six tubes (three in EDTA and three in Heparin) at the time of the next scheduled blood draw per protocol.

6.22 Samples will be routed to Dr. R. B. Jenkins via the study assistant.

## **7.0 Preparation of Tissue Specimens on Glass Slides and Cellulose Acetate Strips**

- 7.1 Appropriate blocks will be cut and stained for PCNA, Ki67 (MIB-1), and p53. Micro-satellite marker and FISH analysis will also be performed on materials derived from the section.
- 7.2 Up to 80 5-micron sections of each block will be cut and placed on sialinized-coated glass slides and cellulose acetate strips in the Pathology Core Laboratory of the Mayo Cancer Center. The number of slides will be reduced if the block is deemed by the pathologist to contain insufficient material.
- 7.3 Five 5-micron hematoxylin and eosin-stained slides will be prepared for each tissue block processed.

## **8.0 Methodology**

### **8.1 PCR**

8.11 We have recently adapted and developed methods for reproducibly analyzing the loss of heterozygosity of specific chromosomal regions using PCR analysis of microsatellite repeats (41,118-120) on DNA extracted from freshly frozen tumors and from paraffin-embedded archival specimens. These microsatellite loci have several advantages for loss of heterozygosity analysis.

- They are highly informative. Only rare patients are found to be homozygous for all markers within a broad chromosomal region. Some interesting genetic mechanisms (e.g., uniparental disomy and/or genomic imprinting) may explain these rare patients.
- Because they are PCR-based, very small pieces of tissue are necessary for analysis. Multiple microsatellite markers can be easily evaluated using ten 5 mM paraffin sections. The same section or adjacent sections can be used for histologic, flow cytometric, or FISH evaluation.
- Once the set of PCR primers has been synthesized and the amplification/analysis conditions optimized, the experiments can be performed rapidly using a 96-well thermal cycler.
- Although this genetic phenomenon appears to be absent among gliomas, these markers can be used to detect genomic instability as revealed by expansion or contraction in the size of one or more microsatellite repeats (100).

- 8.12 PCR analysis of microsatellite repeats will be performed as previously described (118,119). When appropriate, DNA from peripheral leukocytes will be used with normal controls. Briefly, the paraffin will be solubilized within the snap cap tubes containing the tissue slices. The resultant suspension will be aliquoted into several tubes, and appropriate PCR primers and reaction mixture added. Tubes will be placed (with other specimens and appropriate controls, e.g., tubes containing no template DNA and tubes with DNA of known genotype) in a 96-well thermal cycler, and the appropriate cycling temperature program utilized for the set(s) of primers selected. The PCR products will be end-labeled with <sup>32</sup>P and subjected to electrophoresis on DNA sequencing gels. The resultant gels will be dried and autoradiography performed. The resulting autoradiograms will be visually inspected quantitatively for loss of heterozygosity. When appropriate, densitometric scanning will be performed to ascertain quantitatively if loss is present. Primers have been synthesized, and the PCR conditions optimized for multiple loci mapped to the chromosomal regions of interest. As a standard for the densitometric studies, a control chromosome (e.g. chromosome 16) will be analyzed that is rarely lost in glioma (13,16).
- 8.13 Several potential problems need to be addressed when using microsatellite markers to assess chromosome (allelic) loss:
- Resected brain tumor specimens submitted for paraffin-block embedding may not contain sufficient residual normal tissue for the assessment of the constitutional genotype. There are multiple approaches to determine if LOH has occurred in such specimens.
  - Microsatellite alleles often differ by only one to two repeats in size. Thus, shadow bands (due to strand slippage during the DNA-polymerization portion of each cycle) are frequently observed. These shadow bands may simulate or obscure a heterozygous genotype. In the absence of family studies, it can be difficult to interpret such microsatellite patterns. We have elected to classify many of such results as indeterminate and in such cases will utilize other microsatellite markers in this region.
  - Normal glial and neuronal cells are often present within glioma specimens. Extensive contamination by these cells may obscure potential allelic losses. Our proposed microdissection methods and densitometric studies provide a partial solution to this difficult issue. In addition, we intend to restrict analysis to those tumor specimens with  $\geq 50\%$  tumor cells.
  - PCR analysis of microsatellite markers is insensitive to homozygous deletion (the deletion of all copies of a particular locus). The DNA of normal cells within the tumor specimen will still contain both alleles, and thus, be amplified. The tumor specimen will appear heterozygous albeit at reduced signal

intensity. Multiplex analysis with appropriable control microsatellite markers will assist in the ascertainment of homozygous deletion. Also the presence of a “return to heterozygosity” within a region known to contain LOH will be suggestive of homozygous deletion.

- Some PCR primers do not amplify DNA derived from paraffin-embedded material. When this occurs, we will utilize other microsatellite markers in the region.
- Some archival specimens are resistant to DNA amplification by any PCR primer; primarily due to subtle (and not so subtle) differences in initial tissue fixation, processing, and embedding. From our pilot experiments, we conservatively estimate that we will be unable to amplify appropriate microsatellite alleles from approximately 5-10% of paraffin-embedded archival specimens. All of the above problems tend to result in an under-determination of allelic loss. The use of an independent method will allow us to estimate this under-determination.

## 8.2 FISH

- 8.21 In addition to microsatellite analysis of chromosomal regions, FISH can be used to assess chromosomal loss of deletion. Multiple probes of various types are available for FISH. These probes include probes for a-satellite sequences (which often map to chromosome centromeres), b-satellite sequences (which map to the acrocentric chromosome knobs), whole chromosome painting probes, and region- as well as gene-specific probes. Through an independently-funded and nonoverlapping grant, the Mayo Clinic Molecular Cytogenetics Laboratory has access to the full-line of directly-labeled fluorescent probes available from Imagenetics, Inc. (Naperville, IL) as well as the Imagenetics reagents for directly-labeling DNA probes (121). Both Boehringer Mannheim and Amersham also supply fluorescently-labeled nucleotides for incorporation into DNA probes. The availability of fluorescently-labeled nucleotides will allow us to label probes we develop at Mayo as well as those obtained through collaborative efforts. Furthermore, a method for preparing specific biotinylated-a-satellite probes utilizing human-hamster somatic cell hybrids has recently been described (122). Using the nucleotides from the above sources we have modified this procedure to develop specific directly-labeled fluorescent a-satellite probes (47). Finally, biotinylated centromere-specific probes can be purchased from Oncor, Inc. (Gaithersburg, MD).
- 8.22 The first portion of our strategy will be to complete development and then standardize the detection of aneusomy of chromosomes 7, 8, 9, 10, 12, 17,

X, and within paraffin tissue. Chromosomes 7, 10, X, and Y are frequently aneusomic in gliomas (13-16,116,123-125). Chromosomes 8 and 12 are rarely aneusomic in gliomas; however, we have extensive experience in the use of these two probes (112-116). Chromosomes 9 and 17 are rarely aneusomic in gliomas but frequently undergo genetic rearrangement resulting in alterations in the IFNA and p53 loci, respectively. We will use probe mixtures which contain two (or more) directly-labeled probes, each labeled with a different fluorophore. One probe will hybridize with a chromosome that is rarely aneusomic (8 or 12) in gliomas and is an important internal hybridization control. For example, a mixture containing probes for the centromeres of chromosomes 10 and 12 will be hybridized to touch preparations of normal brain. The number of chromosome 10-specific and chromosome 12-specific dots will be enumerated for each nucleus. Since aneuploidy of chromosome 12 is rare in gliomas and normal brain, the incidence of nuclei with two chromosome 12 signals and one chromosome 10 signal will give us an estimate of the incidence of monosomy 10 in normal brain. We have recently submitted for publication an initial set of experiments utilizing a hybridization mixture of probes specific for chromosomes 10 and 12 in normal brain (47), gliosis (47), and gliomas (116). Similar combinations will be developed which contain a mixture of probes specific for other commonly abnormal chromosomes in gliomas (e.g. 7) and control chromosomes. When we have completed development and standardization of the detection of aneuploidy of chromosomes 7, 8, 9, 10, 12, 17, X, and Y, we will follow the same strategy for chromosomes 19 and 22.

- 8.23 We have already established the best procedure for detection of aneuploidy in paraffin-embedded material (114). We will establish a normal range for the number of hybridization dots per interphase nucleus by hybridizing a mixture of probes to isolated nuclei from 20 paraffin-embedded normal brain specimens derived from autopsy material for all probes. Such an analysis was critical to determine the background frequency of specific chromosomal monosomy or trisomy within normal tissue (see reference 116 for detailed discussion of normal range determination).
- 8.24 Once we have established a normal range for the documentation of interest, we will apply FISH analysis to a series of isolated nuclei from the series of tumors analyzed by microsatellite analysis. We have already completed considerable development (data not shown) to determine which specimen preparations give predictable hybridization with little background. Ongoing evaluations of hybridization conditions and specimen preparations will continue.

- 8.25 The statistics of population sampling and the degree and type of aneusomy will help us determine how many nuclei will need to be analyzed by FISH (115). For example, normal brain should have a low rate of aneusomy (except perhaps for the Y chromosome; see reference 47). To obtain an accurate estimate of apparent aneusomy we will enumerate the dots in at least 500 normal brain nuclei. In tumor specimens, depending on the percent of abnormal cells, we may enumerate 200, 500, or 1000 nuclei. If the percent aneusomy after 200 tumor nuclei are enumerated is significantly higher than that of the upper limit of normal we may not count further nuclei. If the percent aneusomy is not significantly different than the upper limit of normal, then we may enumerate an additional 300 or 800 nuclei (if they are available), in a two-stage sampling strategy.
- 8.26 We will next use DNA probes specific for chromosomal regions relevant for glioma pathogenesis. For example, since 9p is frequently deleted without whole chromosomal aneusomy we have obtained YAC clones specific for the relevant chromosome regions for the IFNA gene cluster. Using a recently published labeling procedure (117), we have directly labeled these probes and hybridized them to paraffin-embedded tumor preparations. In addition, region-specific probes for the RBI region and the p53 region will soon be available from Imagenetics and others. These region-specific probes may be used in combination with other control probes for the same chromosome. For example, we will use a probe specific for the centromere of chromosome 9 in combination with the region-specific probe for the IFN cluster. A similar experiment described in reference 113 illustrates the use of a chromosome 17 centromere probe in combination with a HER/2neu probe. The appropriate control experiments on normal brain will be performed to determine the background rate of regional monosomy.
- 8.27 There are at least five major problems that need to be addressed and understood when using FISH to assess chromosomal alterations.
- Autofluorescence is often quite prevalent in human tissue especially brain tissue. The broad emission spectrum, intensity, and distribution of autofluorescence is often quite prevalent in human tissue especially brain tissue. The broad emission spectrum, intensity, and distribution of the autofluorescence often results in the appearance of fluorescent signals not unlike nuclear FISH signals, making nuclear "spot" counting difficult. We have tried multiple strategies to circumvent this problem. The use of multiple-band pass filters to simultaneously visualize the hybridization of two probes and the DAPI-nuclear counterstain, significantly reduces the autofluorescence problem. Our current procedure, which is performing well, is described in references 47 and 116.

- The detection of chromosomal monosomy can be difficult by FISH. Biologically relevant aneusomy, incomplete hybridization, and statistical sampling error all result in a significant rate of FISH aneusomy in normal tissues. This rate is especially significant for monosomy. Most of the strategy described above is designed to address this issue, especially our planned extensive analysis of paraffin-embedded normal brain tissues.
  - Anatomic co-localization of two chromosomal homologues within a nucleus (126), may also increase the apparent monosomy level, unless the observer is especially sensitive to signal intensity. For brain this has been amply demonstrated for chromosomes 8 and 17 (47,127). We will routinely examine each tumor for apparently nonrandom co-localization of FISH signals by comparing the intensity of the experimental probe with that of the internal control probe.
  - The identification of tumor cells within the smear and touch preparations can be difficult. We will explore other means to identify tumor cells, including the use of immunohistochemical markers such as PCNA, Ki-67, and GFAP. Through the current collaborative grant with Dr. Allan Yates of OSU, we have access to new glycolipid antibodies which may be specific for tumor cells of the astrocytic lineage. We will attempt to use these markers to identify tumor cells, then score the chromosome constitution using FISH. The use of multiple single, double, and triple band pass filters (many of which are available in the molecular cytogenetics laboratory) should allow us to perform dual FISH/immunocytochemical procedures on the same slide if necessary (128).
  - There are multiple problems associated with FISH analysis of thin sections. In particular, transection and overlapping nuclei results in significant false aneusomy rates making normal range development crucial. Thus, we plan to analyze isolated nuclei for our prognostic and diagnostic studies. We have expertise in the analysis of paraffin-embedded sections and it will be important to compare the isolated nuclei results with those of thin sections on selected groups of patients.
- 8.3 Flow cytometry - The FISH data obtained in Section 7.2 can also be used to assess DNA ploidy and compared with ploidy determination by flow cytometry (46).

## 8.4 Immunohistochemistry Determinations

### 8.41 Immunohistochemical technique

- Air dry paraffin slides overnight.
- Deparaffinize in 2 changes of xylene (includes one change of 1% iodine in xylene) or histoclear - 5 minutes each, 10 dips in absolute ETOH (alcohol), and 10 dips in 95% ETOH.
- Block endogenous peroxidase activity in H<sub>2</sub>O<sub>2</sub>/MeOH (methyl alcohol) at room temperature in one of the following solutions (made fresh daily):
 

|  |           |  |
|--|-----------|--|
| <u>30 minutes</u>                      | <b>OR</b> | <u>10 minutes</u>                      |
| 20 mL 3% H <sub>2</sub> O <sub>2</sub> |           | 50 mL 3% H <sub>2</sub> O <sub>2</sub> |
| 80 mL absolute MeOH (HPLC grade)       |           | 50 mL absolute methanol                |
- Rinse in tap water - 1 to 2 minutes.
- Rinse in gentle running tap water - 30 seconds.
- Block nonspecific PBS (protein binding sites) by incubation at room temperature for 15 minutes in 5% normal goat serum/PBS/0.05% Tween 20, pH 7.4.
- Add primary antibody appropriately diluted in 1% normal goat serum/PBS/Tween 20, pH 7.4 and incubate for 1 hour at room temperature. (MIB-1 1:60; PCNA 1:500; DO-7 1:100).
- Drain off primary antibody and rinse twice in tap water - 2 minutes each, then once for 2 minutes in PBS/Tween 20.
- Add secondary antibody-biotin conjugate diluted 1:200 in 1% normal goat serum/PBS/Tween 20 incubate for 30 minutes at room temperature.
- Drain off secondary antibody and rinse twice in tap water - 2 minutes each, then once for 2 minutes in PBS/Tween 20.
- Add Streptavidin appropriately diluted 1:500 in 1% normal goat serum PBS/Tween 20 and incubate for 30 minutes.
- Remove slides from Streptavidin and rinse twice in tap water - 2 minutes each, then once for 2 minutes in PBS/Tween 20.
- Rinse 2 minutes in tap water.

- Incubate 2 minutes in 0.1M sodium acetate buffer, pH 5.2.
- Place slides in AEC (3-amino-9-ethylcarbazole) substrate solution and incubate for 15 minutes at room temperature.
- Rinse in tap water 1-2 minutes.
- Counterstain with 0.2% methyl green or hematoxylin according to current protocol.
- Rinse in running tap water for 5 minutes.
- Coverslip with glycerin-jelly (Kaiser's).

8.42 Labeling oligonucleotide probes with digoxigenin - UTP: To label 500 ng oligo-probe

- Day 1
  - Add the following reagents in 1.5 mL centrifuge tube
 

|  |                              |
|--|------------------------------|
| TdT Buffer (5x)                                  | 19 $\mu$ L                   |
| dATP (1 to 2000 dilution<br>of stock 100 mmol/L) | 4 $\mu$ L                    |
| Digoxigenin 11-dUTP                              | 5 $\mu$ L                    |
| Oligo probe (50 mg/mL)                           | 10 $\mu$ L                   |
| TdT (30,000 U/mL)                                | <u>1.2 <math>\mu</math>L</u> |
|  | 40 $\mu$ L                   |
  - Incubate at 37°C, 30 minutes.
  - Add 160  $\mu$ L H<sub>2</sub>O.
  - Add 30  $\mu$ L of 3.0M sodium acetate (pH 6.0).
  - Add 1  $\mu$ L of 20 mg/mL glycogen.
  - Mix, then add 600  $\mu$ L 100% ETOH.
  - Precipitate at -20°C overnight.
- Day 2
  - Centrifuge 1 hour, 10,000 rpm, 4°C.
  - Remove supernatant, wash the pellet with 300 mL cold 80% ETOH.

- Centrifuge 30 minutes, 10,000 rpm, 4°C.
- Remove supernatant, dry under vacuum.
- Dissolve in 62  $\mu\text{L}$   $\text{H}_2\text{O}$  (bring to 8 ng/ $\mu\text{L}$ ), store in 4°C.
- Notes
  - TdT (20-30 U/ $\mu\text{L}$ ) and TdT buffer (5x) from Promega.
  - Digoxigenin 11-dUTP from Boehringer Mannheim (1 nM/ $\mu\text{L}$ ).
  - dATP lithium salt (100 mmol/L) from Boehringer Mannheim.

#### 8.43 Labeling oligonucleotide probes with biotin

- Add the following reagents in 1.5 mL centrifuge tubes:
 

|  |                                     |
|--|-------------------------------------|
| DEPC - $\text{H}_2\text{O}$                | 142.6 $\mu\text{L}$                 |
| TdT buffer (5x)                            | 40 $\mu\text{L}$                    |
| Biotin - dUTP                              | 5 $\mu\text{L}$                     |
| Oligoprobe (50 $\mu\text{g}/\mu\text{L}$ ) | 10 $\mu\text{L}$                    |
| TdT (30,000 U/mL)                          | <u>2.4 <math>\mu\text{L}</math></u> |
|  | 200 $\mu\text{L}$                   |
- Incubate at 37°C, 2 hours.
- Add 50 mL TE (Tris-EDTA) buffer. Final volume is 250  $\mu\text{L}$  total (2 ng/ $\mu\text{L}$ ).
- Store at 4°C.

#### 8.44 In situ hybridization with digoxigenin labeled probes: For paraffin section

- Day 1
  - Xylene, 10 minutes x 2.
  - 100% ETOH, 5 minutes x 2.
  - 95% ETOH, 3 minutes x 2.
  - $\text{H}_2\text{O}$ , 3 minutes x 1.
  - 0.2N HCL, 20 minutes.
  - $\text{H}_2\text{O}$ , 3 minutes x 2.

- PBS, 3 minutes x 1.
- Proteinase K (25 µg/mL in PBS), 15 to 20 minutes, 37°C.
- PBS, 3 minutes x 2.
- 2 x SSC (chloride sodium citrate), 3 minutes x 1.
- 0.1M triethanolamine 200 mL/acetic anhydride 500 µL (freshly made up), 10 minutes.
- 2 x SSC, 30 minutes, 70°C.
- 2 x SSC, 3 minutes, room temperature.
- Prehybridization buffer, 1 hour, room temperature.
- Hybridization overnight in 50°C oven.
- Day 2
  - Wash in 2 x SSC, 10 minutes, 42°C.
  - Wash in 1 x SSC, 10 minutes, 42°C.
  - Wash in 0.5 x SSC, 10 minutes, 42°C.
  - Rinse in buffer A, 1 minute, room temperature.
  - Incubate in buffer A with 1% normal sheep (or swine) serum and 0.3% triton 100, 30 minutes, room temperature.
  - Anti-digoxigenin (1:200 in buffer A containing normal serum and triton as above), 2 hours, 37°C.
  - Buffer A, 5 minutes x 2.
  - Buffer C, 5 minutes x 1.
  - NBT/BCIP (nitroblue tetrazolium/Bromochloroindol phosphate), in the dark for 2 hours to overnight (8 µL 125 nM levamisole/4.4 µL NBT/3.2 µL BCIP, in 1 mL buffer C, 300 µL/slide).
  - Stop incubation in buffer C, wash in H<sub>2</sub>O.
  - Dehydrate and coverslip.

## 9.0 Statistical Considerations

### 9.1 Study design:

9.11 This is a prognostic factors study using prospectively-collected clinical trials data and associated baseline tumor marker data measured on paraffin-embedded tissue collected from each of the clinical trials participants at the time she/he enrolled in the trial. Each subject of this prognostic factors study participated in 1 of the following 3 trials for patients with histologically-proven low-grade gliomas or will participate in a future trial developed for this patient population:

- randomized phase III trial comparing two doses of radiation therapy in patients with oligodendrogliomas, astrocytomas, or mixed oligoastrocytomas (86-72-51);
- a natural history study of patients with pilocytic astrocytomas (86-72-51);
- a 2-stage phase II trial of preirradiation combined chemotherapy in patients with oligodendrogliomas, astrocytomas, or mixed oligoastrocytomas (93-72-02).

9.12 The primary hypotheses to be tested are:

- Alterations in chromosome 19q occur more frequently in tumors with oligodendroglial elements than in those with pure fibrillary astrocytoma.
- Patients whose tumors exhibit alterations of any of the following tumor markers experience a worse clinical outcome (time-to-progression or survival) compared with patients whose tumors do not contain alterations in that marker:
  - alterations in chromosomes 7, 9p, 10, 17, X, or Y as determined by FISH
  - loss of chromosomal materials in chromosomes 9p, 10, 13, 17, or 22 by PCR analysis
  - high levels of immunostaining with monoclonal antibodies for PCNA, Ki-67 (MIB-1), or mutated p53
  - aneuploid DNA by flow cytometry or FISH
  - high %S-phase or %G<sub>2</sub>M-phase cells as measured by flow cytometry

9.13 While the blocks were being collected from the NCCTG member institutions, several new potential markers that could be measured in paraffin-fixed tissue were identified by participants in the NCI-sponsored Glioma Markers Network (GMN) to which Mayo belongs. In order to enhance the likelihood of finding the most prognostic markers associated with this disease, the NCCTG Laboratory Science Committee and the GMN participants jointly agreed to include in the univariate and multivariate analyses outlined in Section 9.4 those promising GMN markers that were approved by both of them. The peer review process is as follows:

\* For each promising marker identified by GMN investigators and approved for this study by majority vote of the principal investigators of the 6 GMN sites, a brief addendum to the master protocol will be prepared to provide scientific evidence of the marker's potential prognostic value.

\* Addenda reviewed and approved by the NCCTG Laboratory Science Committee will be incorporated into the master protocol and submitted to NCI for final approval.

9.2 Randomization: Because this is not a treatment-evaluation study, randomization is not relevant. However, it is worth noting that the treatment assignments of the participants in the phase III clinical trial portion of 82-72-51 were calculated using a dynamic allocation procedure (135) which balanced the marginal distributions of the stratification factors between the treatment groups.

9.3 Sample size: Accrual has been completed for 86-72-51 and is currently suspended between "batches" of patients in 93-72-02. The numbers of eligible participants in each of the trials are: 207 in the randomized phase III trial, 21 in the pilocytic natural history trial, and 9 (currently) in 93-72-02. The number of additional patients to be accrued from future studies cannot, of course, be accurately estimated at this time.

9.31 Virtually complete and up-to-date clinical and follow-up data are available for all 237 eligible participants in the clinical trials listed above..

9.32 The numbers of tumor specimens which have been re-read by reference neuropathologist, Bernd Scheithauer, are (see Table A): 175 in the randomized phase III trial, 14 in the pilocytic natural history trial, and 6 (currently) in 93-72-02. Tissue review is still in progress for several of the final entries (both studies).

- 9.33 Deaths have now been recorded for 35 of the 207 eligible participants in the phase III clinical trial portion of 86-72-51. No deaths have yet been recorded for any of the 21 participants in the observational study portion of 86-72-51 or for any of the 9 participants in 93-72-02.

Table A  
Number of Tumor Specimens Re-Read by Reference  
Neuropathologist by Cell Type and Clinical Trial Participation

| STUDY NUMBER | CELL TYPE |            |             |           | TOTAL |
|--------------|-----------|------------|-------------|-----------|-------|
|              | OLIGO     | OLIGOASTRO | ASTROCYTOMA | PILOCYTIC |       |
| 86-72-51     | 97        | 43         | 35          | 14        | 189   |
| 93-72-02     | 4         | 1          | 1           | 0         | 6     |
| TOTAL        | 101       | 44         | 36          | 14        | 195   |

- 9.34 The estimated numbers and percentages of all patients who are expected to have the markers specified in Section 9.12 are summarized in the following table:

Table B

| MARKER   | %         | N     | MARKER   | %                         | N                       |
|--|-----------|-------|--|---------------------------|-------------------------|
| Aneuploid DNA  | 10-15%    | 24-35 | Changes in chromosome:<br>• 7<br>• 9p<br>• 10  | 15-20%<br>10-15%<br><5%   | 36-47<br>24-35<br>10-12 |
| High %S-phase or %G <sub>2</sub> M-phase cells             | 7.5-12.5% | 18-30 | Changes in chromosome:<br>• 17<br>• 19<br>• 22 | 15-20%<br>12.5-15%<br><5% | 36-47<br>30-35<br>10-12 |
| High proportions of cells with PCNA, Ki-67 (MIB-1), or p53 | 10-15%    | 24-35 | Changes in chromosome:<br>• X<br>• Y           | 15-20%<br>15-20%          | 36-47<br>36-47          |

- 9.4 Analysis plans: Because (a) low-grade glioma is an indolent disease (median survival = 10-15 years), (b) the work involved in performing the marker measurements on more than 230 tissue blocks will take more than a year to complete, and (c) the minimum follow-up on all currently 237 available subjects is about 12 months, it is anticipated that the final analysis will not be done until there is a minimum follow-up of at least 4 years on these subjects.

- 9.41 Frequency distributions of all tumor marker, histologic, and clinical variables will be generated, and cross-tabulations of key variables by specific patient subsets will be produced. Correlation coefficients between pairs of variables will be calculated.
  - 9.42 Binomial 95% confidence intervals will be used to estimate the incidence of each genetic anomaly within subsets defined by histologic type and grade.
  - 9.43 0.05-level chi-squared tests will be used to estimate survival distributions (both time-to-death and time-to-progression) for various subsets of interest.
  - 9.44 Kaplan-Meier survival curves (136) will be used to estimate survival distributions (both time-to-death and time-to-progression) for various subsets of interest.
  - 9.45 Two-sided logrank tests (137) will be used to compare the (a) time-to-death and (b) time-to-progression distributions of patients with and without each genetic anomaly within each of the various subsets of interest.
  - 9.46 Multivariate Cox proportional hazards models (138) and CART (Classification and Regression Tree) models (139) may be generated in an attempt to identify the variables most strongly associated with the distributions of (a) time-to-death and (b) time-to-progression after adjustment for the effects of other potential prognostic factors among the available tumor marker, histologic, and clinical variables, including gender and race.
- 9.5 Precision of estimators: Table C (below) shows the 95% binomial confidence intervals that are obtained for various values of the sample size and the observed percentage of successes, e.g., the observed incidence rate for anomalies of interest.

Table C

| SAMPLE<br>SIZE<br>N | WIDTH OF THE 95% BINOMIAL CONFIDENCE INTERVAL WHEN<br>THE OBSERVED PERCENTAGE OF SUCCESSES IS... |               |               |              |
|---------------------|--|---------------|---------------|--------------|
|                     | 0%   | 24%-26%       | 49%-51%       | 100%         |
| 20                  | 0% - 16.8%   | 8.7% - 49.1%  | 27.2% - 72.8% | 83.2% - 100% |
| 35                  | 0% - 10.0%   | 12.5% - 43.3% | 34.0% - 68.6% | 90.0% - 100% |
| 50                  | 0% - 7.1%  | 13.1% - 38.2% | 35.5% - 64.5% | 92.9% - 100% |
| 75                  | 0% - 4.8%  | 16.0% - 36.7% | 38.9% - 62.4% | 95.2% - 100% |
| 100                 | 0% - 3.6%  | 16.9% - 34.7% | 39.8% - 60.2% | 96.4% - 100% |
| 120                 | 0% - 3.0%  | 17.6% - 33.8% | 40.8% - 59.2% | 97.0% - 100% |
| 140                 | 0% - 2.6%  | 18.1% - 33.0% | 41.5% - 58.5% | 97.4% - 100% |
| 170                 | 0% - 2.2%  | 19.0% - 32.5% | 42.3% - 57.7% | 97.8% - 100% |
| 200                 | 0% - 1.8%  | 19.2% - 31.6% | 42.9% - 57.1% | 98.2% - 100% |

#### 9.6 Power of tests:

9.61 Table D (below) shows the sample size N in the *smaller* of 2 subsets required for a 2-sided 0.05-level binomial test to have 80% power to detect specific differences in incidence percentages between subsets. These values, together with those in Tables A and B (above) suggest that with about 100 tumors classified as pure oligodendrogliomas, 45 classified as mixed oligoastrocytomas, and 35 classified as astrocytomas with no oligo component, there will be approximately 80% power to detect differences in the percentages of tumors with chromosome 19q alterations of the magnitude of, for example, (a) 2.5% in the non-oligo group and  $\geq 25\%$  in those with oligo components, (b) 5% in the non-oligo group and  $\geq 30\%$  in those with oligo components, or (c) 10% in the non-oligo group and  $\geq 40\%$  in the oligos. Since there are expected to be a total of only 30-35 tumors with chromosome 19q alterations, however, only differences of about 2.5% vs. 25% are likely to be seen with these samples sizes.

Table D

| INCIDENCE<br>IN GROUP-1 | INCIDENCE<br>IN GROUP-2 | N  | INCIDENCE<br>IN GROUP-1 | INCIDENCE<br>IN GROUP-2 | N  | INCIDENCE<br>IN GROUP-<br>1 | INCIDENCE<br>IN GROUP-<br>2 | N  |
|-------------------------|-------------------------|----|-------------------------|-------------------------|----|-----------------------------|-----------------------------|----|
| 2.5%                    | 10%                     | 14 | 5%                      | 20%                     | 69 | 10%                         | 25%                         | 96 |
|                         | 15%                     | 8  |                         | 25%                     | 44 |                             | 30%                         | 59 |
|                         | 20%                     | 69 |                         | 30%                     | 31 |                             | 35%                         | 40 |
|                         | 25%                     | 42 |                         | 35%                     | 24 |                             | 40%                         | 30 |
|                         | 30%                     | 29 |                         | 40%                     | 19 |                             | 45%                         | 23 |
|                         | 35%                     | 22 |                         | 45%                     | 15 |                             | 50%                         | 18 |
|                         | 40%                     | 17 |                         | 50%                     | 13 |                             | 55%                         | 15 |
|                         |                         | 14 |                         |                         |    |                             |                             |    |

9.62 Since the total group were accumulated over a period of 7.75 years, the number of deaths expected to be observed for various values of the median survival in the total group after a minimum follow-up of 4 years is approximately: (a) 69 deaths if the median survival is 8 years, (b) 58 deaths if the median survival is 10 years, (c) 50 deaths if the median survival is 12 years, and (d) 43 deaths if the median survival is 14 years. With such numbers, only 2-4 prognostic factors are likely to be identified by Cox models.

#### 9.7 Subset analyses for women and minorities

9.71 The two low-grade glioma trials in which the subjects of this investigation participated were open to all eligible patients, regardless of race, ethnic origin, and gender. Women comprised 82 (41%) of the 212 participants with oligodendrogliomas, astrocytomas, or mixed oligoastrocytomas in these trials and 13 (62%) of the 21 participants with pilocytic astrocytomas. Information about race/ethnicity was not recorded for the patients enrolled prior to 1990. Subsequently, about 2.5% of the patients enrolled in all NCCTG glioma protocols during 1993-94 were classified as ethnic minorities.

9.72 The planned analyses will look for gender differences in the incidence and prognostic effects of the various tumor markers. If women constitute about 40% of the patients with available tissue, they will provide a subset

of about 80 subjects in which to perform the analyses outlined in Section 9.4. The precision of the corresponding estimates can be predicted from Table B.

9.73 The subset of patients known to be accrued into these two trials from racial/ethnic minority populations is, regrettably, too small to provide the basis for analyses of racial/ethnic differences in the incidence and prognostic effects of the various tumor markers.

#### 9.8 Data management

9.81 The basic raw data for the cytogenetic, molecular genetic, and immunohistochemical testing are entered into Macintosh computers in Dr. R. B. Jenkins' laboratory while the flow cytometry raw data are calculated and stored in an IBM personal computer in Dr. J. A. Katzmann's laboratory. The pathology variables are recorded in the glioma cell types databank on the IBM mainframe. The prospectively collected clinical data are in the NCCTG clinical trials data files on the IBM mainframe.

9.82 A master analysis dataset will be created in SAS by merging all databanks to generate a set of key variables for each participant. When the master file has been created, various quality control programs will be run to identify logically inconsistent or out-of-range values.

### **10.0 Human Studies Evaluation**

This study involves no medical risk to the patient.

### **11.0 Budget**

NCCTG institutions: The cost of shipping will be reimbursed. Forward any bills to the NCCTG Operations Office and include the following on the bills: NCCTG 94-72-53, CA50905. Mayo Medical Laboratories will bill NCCTG directly for costs incurred with the submission of the blood samples. Limited funds may also be available upon request for additional handling fees.

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## Appendix I

**PROTOCOL**

---

Date: September 12, 1997  
Investigators: Mark A. Isreal, M.D. and Robert Jenkins, Ph.D.  
Project Title: MnSOD Expression in CNS Tumors: Grade 2 Gliomas

Background:

Gliomas, the most common primary brain tumors of both children and adults, arise from the glial cells of the brain, rather than from neuronal cells. Gliomas may be more common than tumors arising from neuronal cells because some glial cells (astrocytes, oligodendrocytes, and other less well-defined glial cells) apparently retain the capacity to divide throughout adult life, whereas neurons stop dividing before or soon after birth. Tumors arising from astrocytes are the most common gliomas accounting for about 60% of primary brain tumors in adults. Astrocytic tumors occurring in adults are graded by histologic appearance based on criteria such as the presence of mitoses, necrosis, and endothelial proliferation within the tumor. Their neuropathological appearance is highly variable, and numerous attempts have been made to devise histological grading systems that accurately predict their clinical course. The most widely used of these is the WHO grading system, which is also a four-tiered system. Grade 1 is reserved for special histological variants of astrocytoma that have an excellent prognosis following surgical excision. At the other extreme is Grade 4, glioblastoma multiforme, which has multiple features of clinical aggressiveness. In between are astrocytoma (Grade 2) and anaplastic astrocytoma (Grade 3). The defining features of aggressive behavior are hypercellularity, nuclear and cytoplasmic atypia, endothelial proliferation, mitotic activity and necrosis. The presence of endothelial proliferation and necrosis are widely regarded as important predictors of a tumor's potential for rapid growth and aggressive invasion of normal, surrounding tissue.

The overall prognosis for patients with astrocytoma is poor. In a representative Finnish population, employing the WHO grading system, the median survival was 93.5 months for patients with Grade 1 or 2 astrocytomas, 12.4 months for patients with Grade 3 (anaplastic astrocytoma), and 5.1 months for patients with Grade 4 (glioblastoma) tumors. In America, the median survival of patients with high-grade brain tumors is approximately 12 months. Besides histopathology, other clinical features that correlate with poor prognosis include age over 65 and a poor functional status, as defined by the Karnofsky performance scale, at the time of presentation. Oligodendrogliomas have a more benign course and respond better to chemotherapy than astrocytomas. The five-year survival of patients with oligodendroglioma is greater than 50% and the 10-year survival is 25-34%. The WHO classification includes parallel grading systems for oligodendroglial tumors.

Although ionizing radiation is the most effective treatment modality for malignant gliomas, many of these tumors are resistant to radiation therapy. One important mechanism by which ionizing radiation is thought to kill tumor cells is by the generation of hydroxyl ( $\text{OH}^-$ ) and superoxide ( $\text{O}_2^-$ ) free radicals. Low levels of MnSOD in some tissues may be associated with oncogenesis arising as a result of the inability of the cell to inactivate endogenously arising free radicals (for review see Ref. 1,2). Less frequently tumor express high levels of MnSOD (1,3) which might be associated with therapeutic resistance resulting from its inactivation of radiation-induced toxic superoxide radicals. Cytokines such as TNF $\alpha$  and IL-1 produced in response to radiation injury induce mitochondrial  $\text{O}_2^-$  production that contributes to cell killing following radiation. Mitochondrial  $\text{O}_2^-$  toxicity is also implicated in the mechanism of action of redox-active chemotherapeutic drugs (e.g., vincristine). Thus,  $\text{O}_2^-$  production is critical in the cytotoxic pathways of TNF $\alpha$ , ionizing radiation and some chemotherapeutic agents used in the treatment of brain tumors and other cancers. MnSOD is a mitochondrial enzyme which catalyzes the conversion of  $\text{O}_2^-$  to oxygen ( $\text{O}_2$ ) and hydrogen peroxide ( $\text{H}_2\text{O}_2$ ). Increased expression of mitochondrial MnSOD has been found to play a central role in protecting various cell types, including tumor cells, from the lethal effects of IL-1 and TNF, cell-mediated immune responses, certain anticancer drugs, and ionizing radiation. Increased levels of MnSOD expression have been reported in some human cancers including ovarian cancer, and serum MnSOD levels were found to correlate with the malignant potential of some leukemias and neuroblastoma. The role of MnSOD in the pathogenesis of these cancers is not known, although these tumors may have an enhanced resistance to the  $\text{O}_2^-$  mediated anti-tumor effects of TNF, ionizing radiation, and redox-active chemotherapeutic drugs.

#### Preliminary Data:

We recently examined a selection of different brain tumors for the expression of MnSOD (4). Although MnSOD is not readily detected in normal brain, malignant central nervous system (CNS) tumors, including tumors metastatic to the brain, displayed marked immunoreactivity to MnSOD. The pattern of immunostaining in the tumor sections was heterogeneous and diffuse. The cytoplasm was the most intense cellular area of staining in malignant tumor cells. Tumor endothelial cells were also strongly positive for MnSOD immunoreactivity. Glioblastomas and Grade 3 astrocytomas generally exhibited more intense MnSOD immunoreactivity than low-grade (Grade 2) astrocytomas. Two recurrent anaplastic astrocytomas, from patients who had undergone previous surgery and radiation therapy, showed very intense MnSOD immunoreactivity. Strong MnSOD immunostaining was also observed in the extracellular interstitial tissue of these tumors. Other types of brain tumors, including ependymoma, meningioma, schwannoma, and medulloblastoma also had a high level of MnSOD immunostaining. Reactive gliosis, obtained from tissue surrounding a cerebral vascular malformation, exhibited only minimal immunoreactivity to MnSOD in one of two specimens. These findings are consistent with a more recent study in which high levels of MnSOD were found in some medulloblastoma tumor specimens (5). We also used an ELISA to analyze the CSF of brain tumor patients for the presence of MnSOD and demonstrated that most patients with high-grade CNS tumors had dramatically increased levels of MnSOD in their CSF. The

mean MnSOD levels in CSF from high-grade CNS tumors and from high-grade tumor cyst fluid were 836.9 ng/ml and 780.3 ng/ml respectively. MnSOD mean concentrations from those patients without CNS pathology and from patients with low-grade CNS neoplasms were 85.7 ng/ml and 70 ng/ml respectively. Kruskal-Wallis analysis showed that significant differences between these groups of MnSOD measurements did exist ( $p < 0.006$ ). Dunn analysis revealed a significant ( $p < 0.05$ ) increase in CSF MnSOD in patients with high-grade CNS neoplasms compared to normal controls. The highest level of MnSOD was found in the CSF from a patient who had previously undergone radiation treatment for an anaplastic astrocytoma which recurred.

To examine the potential usefulness of MnSOD as a predictor of treatment outcome, we propose to evaluate the expression of this gene in Grade 2 tumors from patients treated on protocol. The identification of predictive markers may be of clinical relevance for the management of current patients and is likely to be of importance for the development of future treatment strategies.

#### Specific Aims:

To determine the extent and frequency of MnSOD expression in pathologic specimens from patients with Grade 2 moderately anaplastic astrocytomas and oligodendroglioma.

To determine if MnSOD expression observable in surgical specimens obtained prior to the initiation of cytotoxic therapy correlates with the outcome of patients with Grade 2 moderately anaplastic astrocytomas and oligodendroglioma.

#### Experimental Procedures:

Fixed, histologic sections from a group of patients entered onto NCCTG protocols 79-72-51, 85-72-51, and 88-72-52 and diagnosed as having Grade 2 moderately anaplastic astrocytomas and oligodendroglioma will be examined in MnSOD expression. Paraffin sections will be baked for 30 minutes at 60°C. Sections will then be deparaffinized in xylene and hydrated with graded alcohols. Endogenous peroxidases will be blocked by soaking in 3% hydrogen peroxide with 0.2% Tween-20 in PBS for 15 minutes at room temperature. Sections will be washed 3 times for 2 minutes each in PBS containing 0.05% Tween-20 (PBS/Tween). Sheep polyclonal antibody to human MnSOD was diluted 1:200 in 10% rabbit serum, placed on sections, and these will be incubated at 4°C overnight. Adjacent sections of all specimens will be used as negative controls. These control sections will be treated exactly the same as the study sections except they were incubated with non-immune sheep IgG (2 ug/ml). The sections will then be washed in PBS/Tween as before and incubated with biotinylated rabbit anti-sheep secondary antibody for 30 minutes at room temperature. We will again wash the sections 3 times in PBS/Tween, and streptavidin-horseradish peroxidase conjugate (Zymed Laboratories, South San Francisco, CA) will be applied to 10 minutes at room temperature. After being washed 3 times with PBS/Tween, the sections will be incubated with diaminobenzadine tetrahydrochloride

(DAB) for 3 minutes at room temperature. We will then rinse the sections in distilled H<sub>2</sub>O, stained with hematoxylin, dehydrated with graded alcohols, cleared with xylene, and mounted.

The MnSOD immunoreactivity of all specimens will be evaluated blindly by two independent observers. A grade of 0 will be assigned to tumors with no detectable signal and Grades of 1, 2, and 3 to tumors with light, moderate and intense reactivity, respectively. Normal brain tissues will be graded as 0.

#### Data Analysis:

The analysis plan described in Section 9.4 of the NCCTG 94-72-53 protocol will be carried out using the data collected for all markers measured on the NCCTG Low-Grade Glioma Cohort tissues.

Specifically, frequency distributions of all tumor marker, histologic, and clinical variables will be generated, and cross-tabulations of key variables by specific patient subsets will be produced. Correlation coefficients between pairs of variables will be calculated. Kaplan-Meier survival curves will be used to estimate survival distributions (both time-to-death and time-to-progression) for various subsets of interest, and 2-sided logrank tests will be used to compare time-to-death and time-to-progression distributions of patients with and without each marker within each of the various subsets of interest. Cox proportional hazards models will be used to identify the variables most strongly associated with the distributions of time-to-death and time-to-progression after adjustment for the effects of other potential prognostic factors among the available tumor marker, histologic, and clinical variables, including gender and race.

#### Reagent Requirements:

For these studies it is necessary to have 3 slides from each tumor being evaluated.

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## Appendix II

**PROTOCOL**

---

Date: September 12, 1997  
Investigators: Mark A. Isreal, M.D. and Robert Jenkins, Ph.D.  
Project Title: Nitric Oxide Synthase in CNS Tumors: Grade 2 Gliomas

Description:

Gliomas, the most common primary brain tumors of both children and adults, arise from the glial cells of the brain, rather than from neuronal cells. Gliomas may be more common than tumors arising from neuronal cells because some glial cells (astrocytes, oligodendrocytes, and other less well-defined glial cells) apparently retain the capacity to divide throughout adult life, whereas neurons stop dividing before or soon after birth. Tumors arising from astrocytes are the most common gliomas accounting for about 60% of primary brain tumors in adults. Astrocytic tumors occurring in adults are graded by histologic appearance based on criteria such as the presence of mitoses, necrosis, and endothelial proliferation within the tumor. Their neuropathological appearance is highly variable, and numerous attempts have been made to devise histological grading systems that accurately predict their clinical course. The most widely used of these is the WHO grading system, which is also a four-tiered system. Grade 1 is reserved for special histological variants of astrocytoma that have an excellent prognosis following surgical excision. At the other extreme is Grade 4, glioblastoma multiforme, which has multiple features of clinical aggressiveness. In between are astrocytoma (Grade 2) and anaplastic astrocytoma (Grade 3). The defining features of aggressive behavior are hypercellularity, nuclear and cytoplasmic atypia, endothelial proliferation, mitotic activity and necrosis. The presence of endothelial proliferation and necrosis are widely regarded as important predictors of a tumor's potential for rapid growth and aggressive invasion of normal, surrounding tissue.

The overall prognosis for patients with astrocytoma is poor. In a representative Finnish population, employing the WHO grading system, the median survival was 93.5 months for patients with Grade 1 or 2 astrocytomas, 12.4 months for patients with Grade 3 (anaplastic astrocytoma), and 5.1 months for patients with Grade 4 (glioblastoma) tumors. In America, the median survival of patients with high-grade brain tumors is approximately 12 months. Besides histopathology, other clinical features that correlate with poor prognosis include age over 65 and a poor functional status, as defined by the Karnofsky performance scale, at the time of presentation. Oligodendrogliomas have a more benign course and respond better to chemotherapy than astrocytomas. The five-year survival of patients with oligodendroglioma is greater than 50% and the 10-year survival is 25-34%. The WHO classification includes parallel grading systems for oligodendroglial tumors.

Several pathophysiological properties important for tumor cell survival and tumor pathology may be mediated by nitric oxide (NO). Recent studies have suggested a role to NO in causing increased tumor blood flow, edema, and vascular permeability. These features of tumors are

particularly prominent in pathologically high-grade tumors of the central nervous system. Furthermore, cytokines found in brain tumors such as IL-1, TNF, and gamma interferon induce NOS activity in vitro.

Preliminary Data:

We recently evaluated specimens of human CNS tumors for NOS expression by immunohistochemistry, NADPH diaphorase (NADPHd) histochemistry, and Western blot analysis (2). We detected increased expression of the brain and endothelial forms of NOS (NOS I and NOS II) respectively in astrocytic tumors. The highest levels of NOS I and NOS II immunoreactivity were found in high-grade gliomas, some juvenile pilocytic astrocytomas, medulloblastomas, and in a mixed malignant glioma. Of interest in this regard is the distinctively higher level of NOS expression in high-grade astrocytic tumors compared to WHO Grade 2 tumors and normal brain tissue. Immunohistochemical analysis of normal brain revealed NOS I reactivity only in rare neurons, while NOS II and NOS III were not detected (data not shown). Glioblastomas, which are characterized by rapid growth, vascular proliferation, and edema, expressed readily detectable NOS I and NOS II. We used adjacent sections of glioblastoma shown to illustrate marked tumor cell immunoreactivity with both NOS I and NOS II, and intense NOS II reactivity of endothelial cells within the tumor vasculature. NOS III Immunoreactivity was rarely detectable in tumor cells, though moderate staining was seen in tumor endothelial cells. Examination of these specimens at higher magnification revealed that cells which were strongly immunoreactive for NOS I and NOS II were often adjacent to cells with no reactivity. The macrophage isoform of NOS (NOS III) was less frequently detected and expressed at a lower level, predominantly in tumor endothelial cells. Western blot analysis of tumor tissues for these NOS isoforms confirmed these observations.

These data and more recent data from other labs (3) indicate that malignant CNS neoplasms express unexpectedly high levels of NOS and suggest that NO production may be associated with pathophysiological processes important to these tumors. Other experiments evaluating NADPH desphorase and NOS expression by Western blotting were compatible with this interpretation. We propose to examine the potential usefulness of NOS as a predictor of treatment outcome. The identification of predictive markers may be of clinical relevance for the management of current patients and is likely to be of importance for the development of future treatment strategies.

Specific Aims:

To determine the extent and frequency of NOS isoform expression in surgical specimens from patients with Grade 2 moderately anaplastic astrocytomas and oligodendrogliomas obtained prior to the initiation of cytotoxic therapy.

To determine if NOS expression observable in surgical specimens obtained prior to the initiation of cytotoxic therapy correlates with the outcome of patients with Grade 2 moderately anaplastic astrocytomas and oligodendrogliomas.

Experimental Procedures:

Fixed, histologic sections from a group of patients entered onto NCCTG protocols 79-72-51, 85-72-51, and 88-72-52 and diagnosed as having Grade 2 moderately anaplastic astrocytomas or oligodendroglioma will be examined for immunohistochemical evidence of NOS gene expression. Histologic sections (6  $\mu$ m) of paraffin wax-embedded, fixed tissues were dewaxed in xylene and hydrated through graded alcohols to PBS. After a wash in PBS containing 0.05% Tween-20 (PBS/Tween), endogenous peroxidases will be blocked by incubation in PBS containing 3% hydrogen peroxide and 0.2% Tween-20 at room temperature for 15 minutes. The sections to be stained for NOS III and the controls for NOS III will be washed in PBS/Tween for 5 minutes and then boiled in 10 mM citrate buffer, pH 6.0, for 10 minutes to enhance antigen retrieval. After further washing in PBS/Tween, the sections will be blocked and immunolabeled. For NOS detection, the samples will be incubated with mouse monoclonal antibodies against NOS I, NOS II, or NOS III, diluted 1:250, 1:100, or 1:50 respectively in 10% normal rabbit serum. After incubation overnight at 4°C, these sections were washed in PBS/Tween (3 times x 2 minutes each) and incubated with rabbit anti-mouse biotinylated IgG (Zymed Laboratories, South San Francisco, CA). Mouse IgG will be used at identical concentrations as a control primary antibody. Biotinylated conjugates will be detected with avidin-peroxidase conjugate (Zymed Laboratories). Immunolabeling will be detected with the chromogen diaminobenzadine tetrahydrochloride (DAB) after which the slides were washed in water, stained with hematoxylin, dehydrated, and mounted for examination.

The NOS immunoreactivity of all specimens will be evaluated blindly by two independent observers. A grade of 0 was assigned to tumors with no detectable signal and Grades of 1, 2, and 3 to tumors with light, moderate and intense reactivity, respectively. Normal brain tissues, when only the expected staining of rare neurons was observed, were graded as 0.

Data Analysis:

The analysis plan described in Section 9.4 of the NCCTG 94-72-53 protocol will be carried out using the data collected for all markers measured on the NCCTG Low-Grade Glioma Cohort tissues.

Specifically, frequency distributions of all tumor marker, histologic, and clinical variables will be generated, and cross-tabulations of key variables by specific patient subsets will be produced. Correlation coefficients between pairs of variables will be calculated. Kaplan-Meier survival curves will be used to estimate survival distributions (both time-to-death and time-to-progression) for various subsets of interest, and 2-sided logrank tests will be used to compare time-to-death and time-to-progression distributions of patients with and without each marker within each of the various subsets of interest. Cox proportional hazards models will be used to identify the variables most strongly associated with the distributions of time-to-death and time-to-progression after adjustment for the effects of other potential prognostic factors among the available tumor marker, histologic, and clinical variables, including gender and race.

Reagent Requirements:

For these studies, it is necessary to have 10 slides from each tumor being evaluated.

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## Appendix III

**PROTOCOL**

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Date: September 12, 1997  
Investigators: Mark A. Isreal, M.D. and Robert Jenkins, Ph.D.  
Project Title: Spontaneous Apoptosis in CNS Tumors: Grade 2 Gliomas

Description:

Gliomas, the most common primary brain tumors of both children and adults, arise from the glial cells of the brain, rather than from neuronal cells. Gliomas may be more common than tumors arising from neuronal cells because some glial cells (astrocytes, oligodendrocytes, and other less well-defined glial cells) apparently retain the capacity to divide throughout adult life, whereas neurons stop dividing before or soon after birth. Tumors arising from astrocytes are the most common gliomas accounting for about 60% of primary brain tumors in adults. Astrocytic tumors occurring in adults are graded by histologic appearance based on criteria such as the presence of mitoses, necrosis, and endothelial proliferation within the tumor. Their neuropathological appearance is highly variable, and numerous attempts have been made to devise histological grading systems that accurately predict their clinical course. The most widely used of these is the WHO grading system, which is also a four-tiered system. Grade 1 is reserved for special histological variants of astrocytoma that have an excellent prognosis following surgical excision. At the other extreme is Grade 4, glioblastoma multiforme, which has multiple features of clinical aggressiveness. In between are astrocytoma (Grade 2) and anaplastic astrocytoma (Grade 3). The defining features of aggressive behavior are hypercellularity, nuclear and cytoplasmic atypia, endothelial proliferation, mitotic activity and necrosis. The presence of endothelial proliferation and necrosis are widely regarded as important predictors of a tumor's potential for rapid growth and aggressive invasion of normal, surrounding tissue.

The overall prognosis for patients with astrocytoma is poor. In a representative Finnish population, employing the WHO grading system, the median survival was 93.5 months for patients with Grade 1 or 2 astrocytomas, 12.4 months for patients with Grade 3 (anaplastic astrocytoma), and 5.1 months for patients with Grade 4 (glioblastoma) tumors. In America, the median survival of patients with high-grade brain tumors is approximately 12 months. Besides histopathology, other clinical features that correlate with poor prognosis include age over 65 and a poor functional status, as defined by the Karnofsky performance scale, at the time of presentation. Oligodendrogliomas have a more benign course and respond better to chemotherapy than astrocytomas. The five-year survival of patients with oligodendroglioma is greater than 50% and the 10-year survival is 25-34%. The WHO classification includes parallel grading systems for oligodendroglial tumors.

There are no widely recognized biological markers predictive of outcome for brain tumors, although various molecular biological and genetic strategies have been pursued to identify candidates. More recently, the opportunity to study the actual mechanisms by which cells respond to cytotoxic therapy has identified novel strategies for the development of predictive markers. The evaluation of cell death induced by either radiation or cytotoxic therapy has been dominated by extensive experimental analyses demonstrating the ability of such agents to stop cells from replicating. Such cellular reproductive failure is closely associated with the damage of genetic material and the aberrant segregation of chromosomes that occurs following the exposure of cells to radiation. More recently, a second cellular response to cytotoxic therapy, apoptosis, has been recognized to contribute to the sensitivity of various cell types. Apoptosis, or programmed cell death, is an important biological consequence of cellular exposure to ionizing radiation and other DNA-damaging agents. Apoptosis plays a role in physiological processes such as immune and nervous system development and contributes to defense mechanisms important for the prevention of infectious illnesses and cancer. Initial reports of radiation-induced apoptosis emphasized the p53-dependence of this cellular response. Recently, however, we have found that cells derived from gliomas undergo apoptosis in a wt p53-independent fashion following irradiation (2,3). Because of the likelihood that the ability to undergo apoptosis might predict therapeutic response, attempts have been made to correlate treatment outcome with the occurrence of spontaneous apoptosis that is detectable in tumors at the time of diagnosis (4-12). Spontaneous apoptosis has been detected in brain tumors (13), and we have recently pursued the feasibility of examining apoptosis in archived brain tumor specimens and correlating the level of apoptosis with treatment outcome (14).

#### Preliminary studies:

We examined archived, routinely fixed tumor specimens from a cohort of brain tumor patients consisting of 43 children diagnosed with medulloblastoma between 1984 and 1995: 29 high-risk (HR) patients treated with radiation and chemotherapy, and 14 low-risk (LR) children treated with radiation alone. A terminal deoxynucleotidyl transferase (TdT) end-labeling assay was used to detect apoptosis in paraffin-embedded tissue sections prepared at diagnosis. Following a determination of the degree of apoptosis (AL, apoptosis index) present in each pretreatment tumor specimen, progression-free survival was examined in cohorts of children divided into quartiles based on the AL of their tumor. A comparison of these 4 groups of children revealed an association between AL and outcome ( $p=0.03$ ) and was the result of patients in the highest AL quartile having substantially improved outcome compared to all other patients combined ( $p=0.02$ ). In this cohort of patients, assignment at the time of diagnosis to low- and high-risk groups based on widely accepted clinical criteria was not closely associated with outcome ( $p=0.47$ ). These results suggest that the degree of apoptosis is a strong indicator of treatment outcome for children with medulloblastoma following treatment with cytotoxic therapy

independent of risk group. Since high- and low-risk patients included in this study received different modalities of cytotoxic therapy, it is possible that AL predicts outcome independent of the precise anti-neoplastic therapy a patient receives.

We chose to study patients with medulloblastoma in these initial studies because of the observation that these tumors are very sensitive to currently available modalities of cytotoxic therapy. In the proposed studies, we seek to examine the potential usefulness of apoptosis and molecules in the apoptotic pathway to serve as predictors of treatment outcome for patients with glioma since radiation plays such a prominent role in the treatment of these patients and the determinants of radiosensitivity and resistance are unknown. The identification of predictive markers may be of clinical relevance for the management of current patients and is likely to be of importance for the development of future treatment strategies.

#### Specific Aims:

To determine the extent and frequency of spontaneous apoptosis in surgical specimens from patients with Grade 2 moderately anaplastic astrocytomas and oligodendrogliomas obtained prior to the initiation of cytotoxic therapy.

To determine if spontaneous apoptosis observable in surgical specimens obtained prior to the initiation of cytotoxic therapy correlates with the outcome of patients with Grade 2 moderately anaplastic astrocytomas and oligodendrogliomas.

To determine if the expression of genes important for the apoptotic response of cells to anti-neoplastic therapy correlates with the outcome of patients with Grade 2 moderately anaplastic astrocytomas and oligodendrogliomas.

#### Experimental Procedures:

Fixed, histologic sections from a group of patients entered onto NCCTG protocols 79-72-51, 85-72-51, and 88-72-52 and diagnosed as having Grade 2 tumors including moderately anaplastic astrocytomas and oligodendrogliomas will be examined for apoptosis as measured by the presence of DNA fragmentation. Histologic sections on glass slides will be heated for 5 minutes in a microwave oven (Quasar, Thomas, Swedesboro, NJ) at 370 W, followed by 1 1/2 hours incubation at 65°C in an oven (Thermolyne, Dubuque, Iowa). We will deparaffinize the sections by incubation in xylene and rehydration in a series of graded ethanol concentrations. Apoptosis will be detected by an in situ end-labeling technique which recognized DNA breaks resulting from endonuclease activity (FragEL-Klenow DNA fragmentation detection kit, Oncogene Research Products, Cambridge, MA). Diaminobenzidine (DAB) will be used to detect apoptotic cells, which in preliminary studies have stained dark brown. Control sections will be evaluated simultaneously with the substitution of buffered saline for active enzyme. All slides were counter-stained with hematoxylin, Gill No. 2 (Sigma Chemical Co., St. Louis, MO) and mounted with Permount (Fisher Scientific, Pittsburgh, PA). To characterize the extent of apoptosis, we will determine an apoptosis index (AL) by averaging

the number of apoptotic cells in 20 blindly selected high-power fields (hpf) in a histologic tumor section from each patient. A Leica DMLS microscope with an attached grid will be used to count the cells at a magnification of x400. The tumor histology and clinical status of each patient will be unknown to the examiner. To examine the uniformity of apoptosis throughout the tumor, we determined the AL in several different tissue blocks from each of 4 patients.

To examine the expression of various molecules important in the pathways over which apoptosis is mediated and resistance to apoptosis can arise, histologic sections of paraffin wax-embedded, fixed tissues dewaxed in xylene and hydrated through graded alcohols to PBS. After a wash in PBS containing 0.05% Tween-20 (PBS/Tween), endogenous peroxidases were blocked by incubation in PBS containing 3% hydrogen peroxide and 0.2% Tween-20 at room temperature for 15 minutes. Section examined for the expression of some antigens may require incubation in PBS/Tween for 5 minutes and then boiling in 10 mM citrate buffer, pH 6.0, for 10 minutes to enhance antigen retrieval. After further washing in PBS/Tween, the sections will be blocked and immunolabeled. The samples will be in molecules such as BCL2 and BAX which are known to be important in the apoptotic response. The antibodies will be diluted in 10% normal rabbit serum. After incubation overnight at 4°C, these sections will be washed in PBS/Tween (3 times x 2 minutes each) and incubated with rabbit anti-mouse and anti-rabbit biotinylated IgG (Zymed Laboratories, South San Francisco, CA). Mouse or rabbit IgG will be used at identical concentrations as a control primary antibody. Biotinylated conjugates were detected with avidin-peroxidase conjugate (Zymed Laboratories). Immunolabeling was detected with the chromogen diaminobenzadine tetrahydrochloride (DAB) after which the slides were washed in water, stained with hematoxylin, dehydrated, and mounted for examination.

#### Data Analysis:

The analysis plan described in Section 9.4 of the NCCTG 94-72-53 protocol will be carried out using the data collected for all markers measured on the NCCTG Low-Grade Glioma Cohort tissues.

Specifically, frequency distributions of all tumor marker, histologic, and clinical variables will be generated, and cross-tabulations of key variables by specific patient subsets will be produced. Correlation coefficients between pairs of variables will be calculated. Kaplan-Meier survival curves will be used to estimate survival distributions (both time-to-death and time-to-progression) for various subsets of interest, and 2-sided logrank tests will be used to compare time-to-death and time-to-progression distributions of patients with and without each marker

within each of the various subsets of interest. Cox proportional hazards models will be used to identify the variables most strongly associated with the distributions of time-to-death and time-to-progression after adjustment for the effects of other potential prognostic factors among the available tumor marker, histologic, and clinical variables, including gender and race.

Reagent Requirements:

For these studies, it is necessary to have 10 slides from each tumor being evaluated.

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