

North Central Cancer Treatment Group

and

Mayo Clinic

**Diagnostic and Prognostic Markers in High-Grade Glioma**

For any communications regarding this protocol,  
please call the protocol resource person on the following page.

Study Chairs: Jan C. Buckner, M.D. (Mayo Clinic)\*  
Paul L. Schaefer, M.D. (NCCTG)

Laboratory: Robert B. Jenkins, M.D.

Statistician: Wenting Wu, Ph.D. (507/284-8803)

\*Investigator having NCI responsibility for this protocol.

Mayo Clinic  
200 First Street, SW  
Rochester, MN 55905  
507/284-3559

<u>Document History</u>	<u>Effective Date</u>
Activation	November 28, 1999
Addendum 1	June 18, 1997
Addendum 2	April 10, 1998
Addendum 3	July 23, 1999
Addendum 4	December 2, 2005
Addendum 5	April 21, 2006
Addendum 6	February 2, 2007
Update 1	October 17, 2008
Update 2	May 29, 2009

<u>Study Participants</u>	<u>Study Activated</u>
Entire NCCTG	November 28, 1995
Mayo Clinic	November 29, 1995

**Protocol Resource**

Add 6,  
Update 2

Administrative/document: Jack Beranek (507-538-1424)

Pathology Coordinator: Helen Tollefson (507-266-0724)

Update 2

Non-paraffin biospecimens: Roxann M. Neumann, RN, BSN, CCRP (507-538-0602)

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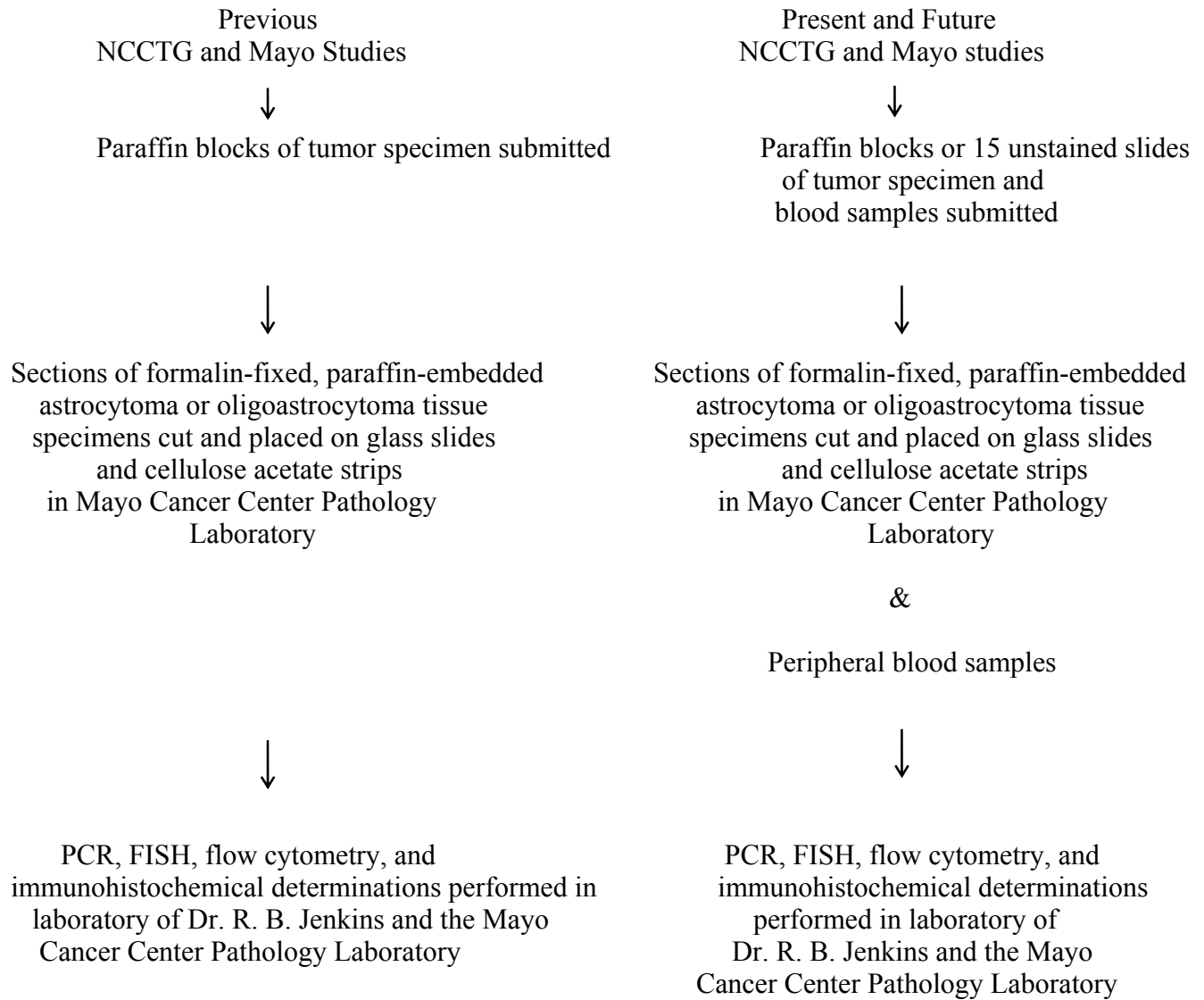
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## 1.0 Background

- 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 IV 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 3 astrocytoma by the WHO classification, hereafter referred to as anaplastic astrocytoma, and in patients with WHO grade 3 or 4 oligoastrocytoma, hereafter referred to as anaplastic oligoastrocytoma.

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 oligodendroglial tumors with alterations of chromosome 10 (a genetic lesion more frequently associated with high-grade astrocytic tumors) behave more aggressively than oligodendroglial 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 allelotyping 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 (101). 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 labelled 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<sub>2</sub>M <6.9; (2) grades 1-3, percent G<sub>2</sub>M ≥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 ≥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.

- 1.9d Patients with Grade 4 astrocytoma (glioblastoma multiforme or GBM) generally have a very poor prognosis. Conversely, patients with Grade 2 astrocytoma have a relatively good prognosis. A crucial question for glioma research is determining the clinical behavior of Grade 3 tumors (anaplastic astrocytoma or AA). While some patients with Grade 3 tumors have a short survival (e.g., <1 year), others may survive for several years. The tumors of such patients cannot be distinguished on a morphologic basis. We hypothesize that the analysis of genetic and other markers may help distinguish these two groups of patients.
- 1.9e Unfortunately, most prior glioma marker studies were performed on specimens collected from patients who were not entered on standardized therapeutic protocols. To overcome these limitations, 94-72-52 was developed to evaluate molecular, cytogenetic, and other markers in a cohort of Grade 3 astrocytomas and Grade 3-4 mixed oligoastrocytomas (anaplastic oligoastrocytoma or AOA) from patients who were treated on NCCTG Grade 3-4 glioma therapeutic protocols 85-72-51, 88-72-52, and 79-72-51.

1.9f Protocol 94-72-52 was approved by NCI on November 1, 1995, and activated by NCCTG on November 28, 1995. We have completed accrual of the eligible patients and have collected paraffin blocks from 110 of them. Each block was audited to determine if tumor was present. Specimens from 94 patients are suitable for further marker analysis. We have cut sections from these blocks, as outlined in the following table, and have begun flow cytometry (completed in 29 patients), immunohistochemical staining for PCNA, MIB-1, and p53 (completed on 33 patients), and plan image cytometry analysis of DNA ploidy as well. In January of 1998, we plan to begin a comprehensive FISH analysis of these specimens and are planning to screen the PTEN gene for mutations in this cohort of patients.

Order	To Be Used For	Section Thickness ( $\mu\text{M}$ )	No. of Sections
1	H&E	4	1
2	Glass (Immunohistochemistry/FISH of thin sections)	6	10
3	MIB-1, PCNA, p53, and Feulgen stains and control	6	5
4	Mictosatellite/gene mutation analysis of thin sections (acetate sheets)	6	10
5	H&E	4	1
6	Glass (Immunohistochemistry/FISH of thin sections)	6	10
7	Mictosatellite/gene mutation analysis of thin sections (acetate sheets)	6	10
8	H&E	4	1
9	Flow cytometry/FISH on isolated nuclei tubes (only if sufficient material available in blocks)	50	3

- 1.9g As written, protocol 94-72-52 did not require the accrual of tumors from patients with GBM. In retrospect, this was an oversight because this group of patients would serve as an appropriate control group for the patients with AA, compared with prior studies of similar patients by our group and others. In addition, the survival of patients with GBM with a specific marker alteration can be compared with the survival of patients with AA and/or AOA with a similar marker alteration. Thus, this addendum requests permission to accrue paraffin-embedded tissue specimens from patients with GBM who were entered into Mayo/NCCTG high-grade glioma clinical trials.

## 2.0 Goals

- 2.1 To evaluate, in paraffin-embedded tissue collected from patients enrolled in the Mayo/NCCTG high-grade glioma trials conducted since 1979, the diagnostic and prognostic relevance of various tumor markers for which there is evidence of diagnostic or prognostic value in high-grade glioma, including:
- 2.11 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.
  - 2.12 DNA ploidy by flow cytometric analysis.
  - 2.13 Various markers of cellular proliferation and cellular function including flow cytometric determination of %S-phase, %G<sub>2</sub>M, and immunohistochemical evaluation of PCNA, Ki-67, and p53.
  - 2.14 Additional markers identified by the Glioma Markers Network.
- 2.2 To compare the incidence of markers in the major histologic subtypes (AA, AOA, GBM) and to assess their correlation in the total group, as well as within each of these subtypes.
- 2.3 To compare the ploidy determinations by FISH and flow cytometry.

### 3.0 Patient Eligibility

Add 6

Paraffin-embedded tumor tissue blocks or 15 unstained slides of patients enrolled in the Mayo/NCCTG clinical trials, conducted since 1979, which were designed to assess specific therapies in patients with newly diagnosed high-grade glioma.

### 4.0 Registration Procedures

Update 2

- 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 46.117 (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 Previous Clinical Trials

- 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 Future and Current NCCTG/Mayo High-Grade Glioma Clinical Trials

### 5.21 NCCTG institutions

- Add 6 5.211 Each patient's block/slides 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.
- Add 6 5.212 Following patient registration, submit the packaged blocks/slides to the NCCTG Operations Office.
- Add 6 5.213 The institutional pathologist must be informed that the blocks may be depleted. At the completion of the study, remaining tissue blocks/slides 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.
- Add 6 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/slides availability, you will be called and may proceed with registration via the eligibility checklist.
- Add 6 5.22 Mayo institutions- Blocks/slides will be routed to Dr. R. B. Jenkins via the study assistant.

## 5.3 GBM Specimens from Previous Clinical Trials

- 5.31 Upon the activation of Addendum 2, a listing of all eligible patients with GBM's who were enrolled in any of the clinical trials listed in Section 3.1 will be sent to each membership. If the blocks are on file at the NCCTG Operations Office, it will be indicated on the listing, and registration via the eligibility checklist may proceed. If the blocks will never be available, the membership will notify the Operations Office by noting this fact on the patient listing and returning it to the Operations Office.

- 5.32 If a patient's surgery was performed at Mayo Clinic Rochester, you may call the pathology coordinator identified on the protocol resource page. If the availability of the block at MCR is confirmed, you will be called and can then proceed with registration via the eligibility checklist.
- 5.33 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 (i.e., 94-72-52), and the number of the study on which the patient was originally registered.
- 5.34 Following patient registration, submit the packaged blocks to the NCCTG Operations Office.
- 5.35 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.

## **6.0 Procedures for Procurement and Handling of Blood Samples for On-Going NCCTG and Mayo High-Grade Glioma Clinical Trials**

### 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 Laboratory (MML). Participating institutions may obtain kits by submitting the MML FAX Supply Request Form (see Forms Packet) to MML (see FAX number on the Supply Request Form). All sections of the form must be completed in order to expedite processing of the request.

Update 1,2

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 salinized-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 appropriate 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

8.2.1 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-labelling 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 aneusomy 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 aneusomy 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 aneusomy 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 mL
dATP (1 to 2000 dilution of stock 100 mmol/L)	4 mL
Digoxigenin 11-dUTP	5 mL
Oligo probe (50 mg/mL)	10 mL
TdT (30,000 U/mL)	<u>1.2 mL</u>
	40 mL
  - Incubate at 37°C, 30 minutes.
  - Add 160 mL H<sub>2</sub>O.
  - Add 30 mL of 3.0M sodium acetate (pH 6.0).
  - Add 1 mL of 20 mg/mL glycogen.
  - Mix, then add 600 mL 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 mL H<sub>2</sub>O (bring to 8 ng/mL), store in 4°C.
- Notes
  - TdT (20-30 U/mL) and TdT buffer (5x) from Promega.
  - Digoxigenin 11-dUTP from Boehringer Mannheim (1 nM/mL).
  - 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 - H <sub>2</sub> O	142.6 mL
TdT buffer (5x)	40 mL
Biotin - dUTP	5 mL
Oligo probe (50 mg/mL)	10 mL
TdT (30,000 U/mL)	<u>2.4 mL</u>
	200 mL
- Incubate at 37°C, 2 hours.
- Add 50 mL TE (Tris-EDTA) buffer. Final volume is 250 mL total (2 ng/mL).
- 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.
  - H<sub>2</sub>O, 3 minutes x 1.
  - 0.2N HCL, 20 minutes.
  - H<sub>2</sub>O, 3 minutes x 2.

- PBS, 3 minutes x 1.
- Proteinase K (25 ug/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 uL (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 uL 125 nM levamisole/4.4 uL NBT/3.2 uL BCIP, in 1 mL buffer C, 300 uL/slide).
  - Stop incubation in buffer C, wash in H<sub>2</sub>O.
  - Dehydrate and coverslip.

## 9.0 Statistical Considerations

- 9.1 Study Design: This is a prognostic factors study using prospectively-collected randomized 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. The primary hypotheses to be tested are:
- 9.11 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
- 9.12 Alterations in chromosome 19q occur more frequently in tumors with oligodendroglial elements than in those with pure fibrillary astrocytoma.
- 9.13 {Added January 1998} 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 Translational Research 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 Translational Research Committee will be incorporated into the master protocol and submitted to NCI for final approval.

- 9.14 {Added January 1998} Our analysis plan is to evaluate as many markers as possible in each of the AA, AOA, and GBM specimens with adequate tissue. Some markers will not be analyzed in all specimens because of the amount of tissue available; for example, there will likely be insufficient material for flow cytometric analysis of DNA ploidy in about 10% of specimens.
- 9.2 Randomization {Revised January 1998} 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 4 phase III clinical trials were calculated using a dynamic allocation procedure (135) which balanced the marginal distributions of the stratification factors between the treatment groups. Similar stratification factors, together with institution, were used in all 4 trials.
- 9.3 Sample Size: {Revised January 1998}
- 9.31 {Revised January 1998} Virtually complete and up-to-date clinical and follow-up data are available for all patients who participated in any of the clinical trials listed in Section 3.1. Recent entries into the ongoing clinical trial (#93-72-52) are still in the pathology review process.
- 9.32 {Revised August 29, 1995} There were 174 with verified WHO Grade 3 astrocytoma (N=139) or WHO Grade 3-4 oligoastrocytoma (N=35) who enrolled in the first 3 NCCTG phase III clinical trials. Deaths have now been recorded for virtually all of the 110 who participated in the 2 earlier trials (N=20 in #79-72-51, N=90 in #85-72-51) and for a majority of the N=64 participants in #88-72-52 with confirmed anaplastic histology to date. The number of additional patients to be accrued from future studies cannot, of course, be accurately estimated at this time. Mixed oligoastrocytomas are expected to constitute approximately 15-20% of the tumors to be studied. The estimated numbers and percentages of all patients who are expected to have the markers specified in 9.11 are summarized in the following table:

MARKER	%	N	MARKER	%	N
Aneuploid DNA	20-30%	35-50	Changes in chromosome: • 7 • 9p • 10	30-40% 20-30% <10%	50-70 35-50 15-17
High %S-phase or %G <sub>2</sub> M-phase cells	15-25%	25-45	Changes in chromosome: • 17 • 22	30-40% <10%	50-70 15-17
High levels of staining for PCNA, ki-67 (MIB-1), or p53	20-30%	35-50	Changes in chromosome: • X • Y	30-40% 30-40%	50-70 50-70

### 9.33 GBM Cohort {Added January 1998}

9.331 We have identified 148 Mayo Clinic patients with GBM's who were entered into 9 of the Mayo/NCCTG high-grade glioma trials listed in Section 3.1. Paraffin blocks are available for 108 of them. In addition, other NCCTG memberships enrolled literally hundreds of patients with GBM's into the 4 phase III trials listed in Section 3.11. The availability of paraffin blocks for these patients is yet to be ascertained.

9.332 We propose to call the Mayo blocks, audit them for the presence of tumor tissue, and then section them as described in Section 1.3. If there are less than 90 Mayo blocks with sufficient tumor for the proposed analyses, additional blocks will be requested from the other NCCTG memberships.

9.333 We plan to evaluate the same markers in this GBM cohort as in the AA/AOA cohort originally selected for 94-72-52. (Note: Analyses were done to compare the distributions of known clinicopathologic prognostic variables, survival, and progression-time between the Mayo GBM's and the GBM's from the other NCCTG memberships enrolled in 85-72-51 and 88-72-52. No differences of even marginal significance were found. Thus, the GBM's enrolled by Mayo appear to be representative of the GBM's enrolled by the other NCCTG memberships.)

9.4 Analysis Plans {Revised January 1998}: In each of the subsets defined by histologic subtype (AA, AOA, GBM), as well as in the total set, frequency distributions of all tumor marker, histologic, and clinical variables will be generated, cross-tabulations of key variables will be produced, and correlation coefficients between pairs of variables will be calculated.

9.41 Binomial 95% confidence intervals will be used to estimate the incidence of each genetic anomaly within histologic subsets.

9.42 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.

9.43 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.

- 9.44 Cox proportional hazards models (138) will be used 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.
- 9.45 CART (Classification and Regression Tree) models (139) will be used to identify possible prognostic groups for (a) time-to-death and (b) time-to-progression based on the available tumor marker, histologic, and clinical variables.
- 9.5 Precision of Estimators: Table A 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 A: 95% Binomial Confidence Intervals Obtained for Various Values of the Sample Size N and the Observed Percentage of Successes

Sample Size N	Width of the 95% Binomial Confidence Interval When the Observed Percentage of Successes is...			
	0%	24% - 26%	49% - 51%	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% - 3.5%	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%

- 9.6 Power of Tests: {Revised January 1998
- 9.61 The 175-200 patients with confirmed anaplastic histology (AA or AOA) who participated in the first 3 phase III clinical trials were accrued at gradually increasing rates over the 15-year period from 1979-1994, and future participants in subsequent studies are expected to be accrued at about the rate observed in the 1990's. Table B shows the sample size N in the smaller of 2 subsets required for a 2-sided 0.05-level logrank test to have 80% power to detect specific differences in survival distributions assuming:

- \* The distributions of (a) time-to-death and (b) time-to-progression for the various subsets of interest have hazard functions that are approximately proportional
- \* The accrual period was 15 years, and the minimum follow-up is 18 months
- \* The median survival for patients with anaplastic tumors is about 80 weeks (i.e., 18.5 months) after onset of radiation therapy.

9.62 The values in Table B, together with the expected sample sizes summarized in 9.32, suggest that there will be approximately 80% power to detect survival differences which result in (a) a reduction by half or more in median survival or (b) an increase by 67% or more in median survival in the patients with the marker compared with patients without the marker.

$M_0 / 80 =$ Ratio of Medians	$M_0$	N	$M_0 / 80 =$ Ratio of Medians	$M_0$	N
0.33	26.4 weeks	14	1.50	120.0 weeks	115
0.40	32.0 weeks	21	1.67	133.6 weeks	73
0.50	40.0 weeks	36	2.00	160.0 weeks	41
0.60	48.0 weeks	67	2.50	200.0 weeks	24
0.67	53.6 weeks	110	3.00	240.0 weeks	18

9.63 {Added January 1998} The patients in the GBM cohort were enrolled in the designated Mayo/NCCTG clinical trials over the same time period as those in the original AA/AOA cohort and satisfy the other assumptions specified in Section 9.61 except, of course, that the median survival is known to be much shorter, approximately 10 months. Consequently, nearly all in the GBM cohort will be dead by the time of data analysis, providing even more power and better precision for the planned analyses.

9.7 Subset Analyses for Women and Minorities {Revised January 1998}:

9.71 All of the Mayo/NCCTG high-grade glioma trials listed in Section 3.1 were open to all eligible patients, regardless of race, ethnic origin, and gender. Women comprised 40% - 45% of the participants in these trials, slightly more than the 40% of women among newly-diagnosed high-grade gliomas in the general population. Information about race/ethnicity was not recorded for the patients enrolled prior to 1993. 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 the women constitute about 40% of the patients in the AA/AOA and GBM cohorts, they will provide a subset of size 80 in which to perform the analyses outlined in Section 9.4, with power and precision of the planned tests and estimators which can be predicted from Tables A and B. It is worth noting that no evidence whatsoever of survival differences between men and women participants, either before or after adjustment for available baseline and treatment variables, was found when Cox Model analyses were performed using the data recorded for the 393 Grade 4 patients with good performance status who were enrolled in the first 3 NCCTG high-grade glioma trials (#79-72-51, #85-72-51, #88-72-52).
- 9.73 The subset of patients known to be accrued into these 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. Jenkins' laboratory, while the flow cytometry raw data are calculated and stored in an IBM personal computer in Dr. Katzmann's laboratory. The pathology variables are recorded in the Glioma Celltypes 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 94-72-52, CA50905. Mayo Medical Laboratory (MML) 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. Israel, M.D. and Robert Jenkins, M.D. Ph.D.  
Project Title: Spontaneous Apoptosis in CNS Tumors

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 (1). 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 wild-type 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 3 gliomas and Grade 3 and 4 mixed oligoastrocytomas 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 3 gliomas and Grade 3 and 4 mixed oligoastrocytomas.

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 3 anaplastic astrocytomas or Grade 3 or Grade 4 oligoastrocytoma 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.

Data Analysis:

The analysis plan described in section 9.4 of the NCCTG 94-72-52 protocol will be carried out using the data collected for all markers measured on the NCCTG Anaplastic Astrocytoma 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 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 4 slides from each tumor being evaluated.

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## Appendix II

**PROTOCOL**

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Date: September 10, 1997  
Investigators: Mark A. Israel, M.D. and Robert Jenkins, M.D., Ph.D.  
Project Title: Superoxide Dismutase Expression in CNS Tumors

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 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 3 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 3 gliomas and Grade 3 and 4 mixed oligoastrocytomas obtained prior to the initiation of cytotoxic therapy.

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 3 gliomas and Grade 3 and 4 mixed oligoastrocytomas.

#### 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 3 anaplastic astrocytomas or Grade 3 or Grade 4 oligoastrocytoma 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 94-72-52 protocol will be carried out using the data collected for all markers measured on the NCCTG Anaplastic Astrocytoma 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 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 2 slides from each tumor being evaluated.

#### References:

1. Oberley TD, Oberley LW. Antioxidant enzyme levels in cancer. *Histology and Histopathology*, 1997 Apr, 12(2):525-35.
2. Slaga TJ. Inhibition of the induction of cancer by antioxidants. *Advances in Experimental Medicine and Biology*, 1995, 369:167-74.
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4. Cobbs CS, Levi DS, Aldape K, Isreal MA. Manganese superoxide dismutase expression in human central nervous system tumors. *Cancer Research*, 1996 Jul 15, 56(14):3192-5.
5. Kurisaka M, Mori K. Immunohistochemical study of medulloblastoma with a monoclonal antibody against human copper and zinc-superoxide dismutase. *Neurologia Medico-Chirurgica*, 1996 Apr, 36(4):220-3.

## Appendix III

**PROTOCOL**

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

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) (1). 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 3 gliomas and Grade 3 and 4 mixed oligoastrocytomas 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 3 gliomas and Grade 3 and 4 mixed oligoastrocytomas.

#### 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 3 anaplastic astrocytomas or Grade 3 or Grade 4 oligoastrocytomas 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 94-72-52 protocol will be carried out using the data collected for all markers measured on the NCCTG Anaplastic Astrocytoma 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 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 4 slides from each tumor being evaluated.

References:

1. Whittle IR, Collins F, Kelly PA, Ritchie I, Ironside JW. Nitric oxide synthase is expressed in experimental malignant glioma and influences tumor blood flow. *Acta Neurochirurgica*, 1996, 138(7):870-5.
2. Cobbs CS, Brenman JE, Aldape KD, Bredt DS, Isreal MA. Expression of nitric oxide synthase in human central nervous system tumors. *Cancer Research*, 1995 Feb 15, 55(4):727-30.
3. Ellie E, Loiseau H, Lafond F, Arsaut J, Demotes-Mainard J. Differential expression of inducible nitric oxide synthase mRNA in human brain tumors. *Neuroreport*, 1995 Dec 29, 7(1):294-6.

## Appendix IV

**PROTOCOL**

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Investigators: Robert Jenkins, M.D., Ph.D., Jan Buckner, M.D., Judith R. O'Fallon, Ph.D.,  
and Dennis A. Carson, M.D.  
Project Title: p16 Deletion Analysis

Specific Aims:

The p16 tumor suppressor gene is homozygously deleted in many patients with gliomas. However, the value of p16 deletion analysis in the diagnosis, staging, and prognostic evaluation of patients is unclear. Exploiting a newly developed PCR-ELISA analysis, we have demonstrated in preliminary experiments (1) that homozygous deletions of the p16 gene are very common in Grade 4 gliomas, and (2) that atypical gliomas, oligodendrogliomas, and gliomas in young people uncommonly display p16 deletions, but frequently have deletions of chromosomal regions just adjacent to the p16 locus. The purpose of the present experiment is to assess the status of the p16 region in a cohort of NCCTG patients with Grade 3 to 4 astrocytomas and Grade 3 to 4 oligoastrocytomas in order to evaluate the prognostic value of alteration of the p16 region in this population compared with other markers identified by members of the glioma marker network.

Background:

The p16 gene encodes an inhibitor of the cyclin-dependent kinases (CDKs) 4 and 6. These kinases phosphorylate the retinoblastoma protein and thus induce cells to enter the growth cycle. Because the p16 gene product is a growth inhibitor, it is not surprising that p16 deletions and mutations are common in malignant disease.

More than two-thirds of all glioma cell lines have homozygous deletions of the region of chromosome 9p encompassing p16. However, cell lines do not necessarily reflect the in vivo situation.

There have been several reports of p16 deletions and mutations in primary gliomas. The data has varied considerably, probably because most investigators have not been able to fully characterize DNA from paraffin-embedded tissues, and hence cannot study a large enough patient population. To overcome this obstacle, we developed a quantitative PCR-ELISA that can rapidly assess gene dosage in DNA extracted from formalin-fixed, paraffin-embedded tissues. The PCR-ELISA was used to determine the presence or absence of deletions of several different sequence-tagged sites (STS) in and around the p16 locus on chromosome 9p. In a recent collaborative pilot study between the Mayo Clinic and UCSD, 18 cases of Grade 4 gliomas were evaluated by this method. Deaths have been observed in 7 of the 10 cases with p16 loss but in only 4 of the 8

cases without p16 loss. Although the survival differences between the p16- and p16+ groups are substantial (median survival = 41 vs. 210 weeks, respectively), they are not statistically significant and may reflect differences in age (median age = 53.5 vs. 40.5, respectively) and histology (0/10 vs. 4/8 with oligo or oligoastro histology, respectively). Nonetheless, the data suggest that p16 homozygous deletion may be a negative prognostic marker. Subsequently, we tested ten gliomas with atypical histologic features that were obtained from Dr. Peter Burger at Johns Hopkins University. Only one of the atypical lesions had a p16 homozygous deletion, but several others had deletions of 9p regions adjacent to the p16 gene. Finally, deletion analysis was performed on 14 glioma xenografts established by Dr. H. Friedman at Duke University. Of the 4 cases from subjects less than 30 years old, 1 had a p16 deletion, while 2 had isolated p15 deletions. In contrast, 9 out of 10 adult cases had homozygous deletions of p16. Collectively, these results suggest that detailed deletion analysis of the p16 locus and adjacent portions of chromosome 9p21 may be useful in the diagnosis, classification, and possibly the prognosis of patients with glioma. Methods are now available to test multiple different formalin-fixed paraffin-embedded sections. Analysis of the NCCTG specimens will enable this goal to be accomplished.

### Description of the Method

#### Tumor Samples:

Specimens for analysis are the available paraffin blocks collected for the newly diagnostic Grade 3 to Grade 4 astrocytomas and oligoastrocytomas enrolled on the NCCTG high-grade glioma trials since 1979. Approximately 5 slides, 4 microns thick, will be cut from each paraffin block. One of the slides will be stained and examined microscopically to determine the tumor-rich region and marked appropriately to permit microdissection. The DNA extraction will be performed in most cases in Dr. Jenkins' laboratory. Microscopically identifiable regions of tumor will be scratched off the slides and placed in a sterile microcentrifuge tube. After melting the paraffin, the samples will be centrifuged and the hardened paraffin rings removed. DNA will be isolated with Qiagen tissue kits and digested with proteinase K and with RNase A. The purity of the DNA will be measured spectrophotometrically, and the samples will be stored in -20°C until used for PCR.

Polymerase Chain Reaction 15 ng of DNA from each sample will be used for amplification. Primers will include p16 exons 1 and 3, p15, MTAP exon 8, and several STS close to the p16 locus. The pseudogene of MTAP, localized to chromosome 3q28, will serve as a reference control. The primers have been designed to amplify PCR products of similar lengths between 197-250 bp, and have been biotinylated at the 5 prime ends. Reaction conditions have been established for each primer pair using DNA from formalin-fixed paraffin-embedded normal placenta. In general, samples are amplified through 25-30 cycles. The reaction mixtures are supplemented with digoxigenin-labeled dUTP. After separation of the biotinylated PCR products from the primers, the reactions are added to microwell plates coated with streptavidin. Bound DNA is detected with anti-digoxigenin antibody conjugated with horseradish peroxidase.

For gene dosage quantification, a regression line for each target sequence is calculated according to the standard curve data obtained in the ELISA from normal placental DNA. The quotient of the particular PCR product absorbance values (OD) divided by that of the reference gene is used to normalize the values. Then, the tumor sample gene dosage ratio is calculated from the equation:

$$\text{sample gene dosage ratio} = \frac{\text{sample OD for test gene/sample OD for reference gene}}{\text{control OD for sample gene/control OD for reference gene.}}$$

A sample is considered to have a homozygous deletion when the normalized gene dosage ratio is  $< 0.3$ . This criterion has been chosen based upon extensive previous analyses using differential PCR to detect p16 in well characterized specimens.

#### Data Analysis:

The analysis plan described in section 9.4 of the NCCTG 94-72-52 protocol will be carried out using the data collected for all markers measured on the NCCTG Anaplastic Astrocytoma Cohort tissues.

Specifically, frequency distribution 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-in-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 hazard 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.