

North Central Cancer Treatment Group

Optimizing EGFR Inhibitor-based Therapies for GBM

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Document History	(Effective Date)
Activation	November 24, 2006

<u>Study Participants</u>	<u>Date Activated</u>
Entire NCCTG	November 24, 2006

NCI Version Date: October 5, 2006

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Index

- 1.0 Background
- 2.0 Goals
- 3.0 Patient Eligibility
- 4.0 Registration Procedures
- 5.0 Procedures for Procurement and Handling of Paraffin Blocks
- 6.0 Preparation of Tissue Specimens
- 7.0 Methodology
- 8.0 Statistical Considerations
- 9.0 Human Studies Evaluation
- 10.0 Budget
- 11.0 References

1.0 Background

Constitutive activation of the epidermal growth factor receptor (EGFR) and its downstream signaling network plays central roles in the pathogenesis of several malignancies including glioblastoma multiforme (GBM). Signaling from EGFR through the PI3K-Akt, STAT3, PLC-gamma and Ras-Raf pathways regulates multiple processes important for malignant progression of GBM tumors including proliferation, angiogenesis, invasion, evasion of apoptosis and response to hypoxia. These same pro-tumorigenic processes may contribute significantly to the resistance of GBM tumors to radiation therapy, and this may explain the link between EGFR overexpression and poor outcome in patients treated with radiation (1-3). With the development of EGFR-targeted therapeutics (cetuximab, gefitinib, erlotinib, EKB-569), several laboratories have demonstrated that pharmacologic inhibition of EGFR signaling significantly enhances the efficacy of radiation therapy in animal models for various types of human cancers. These observations have led to the development of a number of clinical trials that are testing the combination of EGFR inhibitors with radiation therapy (Reviewed in 4). Unfortunately, despite intensive laboratory and clinical research, little is known about the molecular tumor characteristics associated with sensitivity to EGFR-based therapies.

EGFR amplification or activating mutations occurs in 40% of primary GBM tumors, and in at least this subset of tumors, elevated signaling from EGFR is presumed to play a central role in gliomagenesis. Based on this assumption, 2 clinical trials have been initiated by Mayo investigators within the North Central Cancer Treatment Group (NCCTG) cooperative trials mechanism to evaluate either sequential or concomitant therapy with radiation and small-molecule EGFR inhibitors in patients with newly diagnosed GBM. As an integral part of these clinical trials, paraffin-embedded tumor specimens are collected and stored at Mayo Clinic for translational studies. Using the collected patient specimens, the proposed studies will focus on identifying potential molecular characteristics that will predict for response to EGFR inhibitor-based therapies.

2.0 Goals

Primary Aim: Identify molecular characteristics that predict for overall survival and progression-free survival endpoints for patients treated with erlotinib, temozolomide and radiation for patients enrolled on the NCCTG trial N0177.

Secondary Aim: Identify molecular characteristics that predict for overall survival and progression-free survival endpoints for patients treated with gefitinib following radiation for patients enrolled on the NCCTG trial N0074.

3.0 Patient Eligibility

All patients entered onto N0177 and N0074 who have appropriate archived clinical specimens.

4.0 Registration Procedures

- 4.1 To register a patient, fax (507-284-0885) a completed eligibility checklist to the Randomization Center between 8 a.m. and 4:30 p.m. central time Monday through Friday.
- 4.2 IRB approval(s) is required for each treating site. A signed Cancer Trials Support Unit (CTSU) IRB Certification Form is to be on file at the CTSU Regulatory Office (fax 215-569-0206). This form can be found at the following Web site: www.ctsu.org/rss2_page.asp. Guidelines can be found under Quick Fact Sheets.

In addition to submitting initial IRB approval documents, ongoing IRB approval documentation must be on file (no less than annually) at the NCCTG Randomization Office (fax 507-284-0885). If the necessary documentation is not submitted in advance of attempting patient registration, the registration will not be accepted and the patient may not be enrolled in the protocol until the situation is resolved.

When the study has been permanently closed to patient enrollment, submission of annual IRB approvals to the NCCTG Randomization Office is no longer necessary.

- 4.3 N0177 and N0074 consents do provide language regarding the storage of samples for future research. According to that language, if the patient signed the consent they also agreed to the use of their samples for future research. Therefore, a consent is not needed for the use of these samples.

5.0 Procedures for Procurement and Handling of Paraffin Blocks

- 5.1 Retrospectively enrolled patients on studies N0177 and N0074
 - 5.11 A Master Patient listing of those patients who agreed to provide samples for future research will be sent to each membership with the activation of this study.
 - 5.12 Blocks are already on file at the NCCTG Operations Office and consent was obtained to use this material in future research when the patient was registered on N0177 and N0074.
 - 5.13 The patients indicated on the Master Patient Listing who indicated that they agreed to provide samples for future research will be registered by the NCCTG Randomization Office when each membership has IRB approval.
 - 5.14 If a patient's surgery was at Mayo Clinic Rochester, the NCCTG pathology coordinator will confirm block availability.

6.0 Preparation of Tissue Specimens

- 6.1 Appropriate blocks will be used to construct a tissue microarray. Sections of the microarray will be stained by immunohistochemistry for EGFR expression, vIII mutant, p53, P-Akt, total Akt, P-p70S6K, total p70S6K, P-S6, total S6, P-4EBP1, total 4EBP1, P-STAT3, total STAT3, P-PLC-g, total PLC-g, P-Erk, total Erk, ErbB2, ErbB3, ErbB4, PDGFR, IGFR1, IL-6, FADD and phospho-FADD, and MGMT.
- 6.2 Individual blocks will be processed to assess MGMT promotor methylation status.

7.0 Methodology

Clinical specimens will be available for our analysis from both N0074 (RT → GEFITINIB) and N0177 (RT + ERLOTINIB → ERLOTINIB). The drugs used in these trials are both quinazoline derivatives, and both are selective, reversible inhibitors of EGFR kinase activity with similar mechanisms of action (5). Thus, many investigators believe that these two drugs are essentially interchangeable. However, within the context of our 2 clinical trials, there are 2 important differences in how these drugs were used. First, only a relatively favorable subset of patients with stable disease following definitive RT was enrolled on N0074, while an unselected population of patients is being enrolled on N0177. Second, N0074 evaluates sequential therapy with RT followed by GEFITINIB, while N0177 is evaluating concomitant therapy with ERLOTINIB and RT followed by ERLOTINIB alone. Thus, a favorable response on N0074 could be attributable either to intrinsic radiosensitivity or sensitivity to GEFITINIB alone, while response on N0177 may be attributable to: a) intrinsic radiosensitivity, b) the radio-enhancing effects of ERLOTINIB, and/or c) intrinsic tumor sensitivity to ERLOTINIB. However, because all the published animal studies to date suggest that intrinsic sensitivity to EGFR inhibition is necessary for the radio-enhancing effects of EGFR inhibitors, we believe that predictors of intrinsic sensitivity to ERLOTINIB will be the same as those that predict for the radio-enhancing effects on N0177 (6-10). Thus, we would expect that predictors of response for N0074 and N0177 should be similar as they relate to sensitivity to EGFR inhibition.

EGFR modulates multiple downstream signaling pathways that potentially contribute to the malignant phenotype in GBM. However, based on the importance of the PI3K/Akt and Ras/MAPK pathways in gliomagenesis, we hypothesize that the extent of EGFR-independent signaling through one or both of these pathways is important for the efficacy of EGFR inhibitor therapies. Alternatively, STAT3 and PLC- γ 1 also modulate proliferation, apoptosis and invasion, and also may influence the efficacy of radiation. Signaling in these pathways can be driven not only by EGFR but also by other mitogenic signaling receptors like PDGFR, IGFR1, IL-6, and other ErbB family members. For example, IGFR1 over-expression has been linked to *in vitro* resistance to EGFR inhibitor therapy in a GBM tumor cell line (11). Thus, even in the setting of EGFR amplification, signaling through parallel mitogenic pathways could render tumors resistant to EGFR inhibitor therapy. P53 over-expression also has been linked to prolonged survival following radiotherapy for GBM patients by several groups (12-14), including a collaboration between UCSF, Mayo Clinic and the University of Calgary (15). N0177 also uses temozolomide in combination with radiation and erlotinib. Response to temozolomide has been linked to expression levels of the DNA repair enzyme MGMT and the methylation status of the *MGMT* promotor. On the basis of these observations, we will evaluate the following signaling molecules/pathways:

- A) EGFR expression, vIII mutant, and p53 expression
- B) EGFR signaling mediator activation: the expression and activation states of STAT3, PLC- γ 1, FADD and the PI3K and Ras signaling pathways will be examined.
- C) Other mitogenic receptors: ErbB2, ErbB3, ErbB4, PDGFR, IGFR1, and IL-6.
- D) MGMT expression and promoter methylation.

All of these assays will be performed using immunohistochemistry (IHC) analyses on paraffin-embedded tissue microarrays. The microarray construction and IHC staining and analysis will be supervised by Dr. Caterina Giannini. Dr. Giannini is the chair of the Pathology committee for the NCCTG and will be responsible for coordinating the accrual and processing of patient specimens for the N0074 and N0177 clinical trials. The microarray construction and IHC staining will be performed by the Mayo Cancer Center Tissue and Cell Molecular Analysis (TACMA) Shared Resource and supervised by the core director, Dr. Wilma Lingle. Many of the IHC analyses described below are performed as part of the routine clinical practice at the Mayo Medical Center, and protocols for stains that are not routine will be developed on the basis of published procedures and commercially available antibodies. These techniques will be developed in a collaboration between Drs. Sarkaria, Giannini and Lingle and performed in the TACMA core facility. The extensive experience of the Mayo Medical Laboratories will be available as assistance is needed in the development of any specific assay.

Microarrays are constructed using a custom fabrication device that consists of a 0.6 mm tissue corer and a mold for the 216 capacity recipient block. Tissue microarrays incorporate three to five cores extracted from each patient tumor block into a recipient block. Four to thirty cores of liver or tonsil tissue are included in the recipient block as fiducial markers and controls for immunohistochemistry reactions. The design of the array is laid out on a spreadsheet, indicating column and row placement of each core, along with relevant tumor information. The technician making the array removes the cores from the patient block corresponding to areas marked by Dr. Giannini on an H&E slide corresponding to the block. Depending on the thickness of the tissue in the patient block, 50 to 200 sections can be cut from the array block. An H&E section of the master block is used to verify adequacy of core tissue specimens. Consecutive sections of the master block allow for rapid analysis of multiple molecular markers in the same set of specimens. For any given analysis, the staining intensity for each tissue sample will be scored as present or absent as well as evaluated for intensity of staining and/or fraction of staining cells when appropriate. This analysis will be performed by Dr. Giannini, who will be blinded to patient identification and outcome. Tissue microarrays constructed from N0177, N0074, and the tissue archive will be stained and evaluated independently, and all of the assays proposed in sections 1A through 1C will be performed on all specimens. These combined analyses of patients from N0074, N0177 and historical radiation only patients will allow us to distinguish generic markers of response to radiation from specific markers of response to EGFR inhibition. These studies will provide a basis for a future clinical trial that will select patients for combined EGFR inhibitor/radiation therapy using the predictive molecular features identified.

EGFR IHC: IHC for EGFR expression is routinely performed in clinical practice at the Mayo Clinic. Tissue samples for our studies will be processed for EGFR IHC by the TACMA core facility. IHC for the EGFR vIII mutant was recently developed by the James laboratory. The methods for this assay are quite similar to those used for total EGFR IHC, and these assays also will be performed by the TACMA core laboratory.

P53 IHC: p53 overexpression correlates with improved survival following radiation, and there is an inverse relationship between the presence of EGFR amplification and p53 overexpression. Therefore, we will evaluate p53 expression by IHC using established methods (DO-7, Dako) (15).

PTEN analysis: PTEN expression levels will be evaluated as described by others using a monoclonal antibody (clone 6H2.1, Cascade Bioscience) (16). Staining in the vascular endothelium will be used as an internal control for staining intensity. We have extensive experience in characterizing genetic alterations of the *PTEN* gene in patient samples (17-24), and we are prepared to use these same techniques to clarify the PTEN status in our patient samples if necessary.

Akt: Phosphorylation on Ser473 of Akt will be assessed by IHC using a commercially available rabbit polyclonal antibody (Cell Signaling, cat.#9277) and previously published methods (16, 25, 26). Specificity of the antibody for phospho-Akt will be confirmed with a specific blocking peptide (Cell Signaling, cat.#1140). Expression of Akt protein will be evaluated by IHC using commercially available antibodies (Cell Signaling, Cat#2967).

MAPK: Phosphorylation on Thr202/Tyr204 of p44/42 MAPK will be assessed by IHC using a commercially available mouse monoclonal antibody (E10, Cell Signaling, cat.#9106) and published methods (16, 27, 28). Overall expression of p44/42 will be evaluated using the MAP Kinase (3A7) Monoclonal Antibody (Cell Signaling, cat.#9107).

STAT3: Tissue specimens will be probed with a STAT3 phospho-Tyr705 specific antibody (Cell Signaling, 3E2 mAb) and a total STAT3-specific antibody (Cell Signaling, Cat# 9132) using published techniques (29-31).

PLC- γ 1: Expression levels of PLC- γ 1 will be assessed using commercially available antibodies (BD Biosciences, cat# 610027) according to manufacturers instructions. A phospho-specific Tyr783 antibody is available from Biosource, and we will develop an IHC staining protocol using this antibody for use in our paraffin-embedded micro-tissue array.

FADD: Phosphorylation of FADD on Ser194 and total levels of FADD will be evaluated by IHC using commercially available antibodies (Cell Signaling, cat.#2781 and cat.#2782).

PDGFR: Expression of PDGFRA will be assessed by IHC using a commercially available antibody (Upstate Biotechnology, cat.# 06-495). This technique is routinely done in the Mayo Core IHC laboratory.

IL-6: Expression of IL-6 will be assessed by IHC with a commercially available antibody (R&D Systems, AF-206-NA) according to the manufactures directions.

IGFR-1: Expression of IGFR-1 will be assessed by IHC with a commercially available antibody (Biosource International, Cat.# AHR0321) using previously published methods (32, 33).

HER2: Expression of Her2 will be evaluated using the FDA-approved Hercept-Test assay that is routinely used in clinical practice at Mayo Clinic.

ErbB3 and ErbB4: Commercial antibodies and published protocols will be used to evaluate expression of ErbB3 [RTJ-1, Novocastra Laboratories, UK] (34, 35) and ErbB4 [HFR-1, Calbiochem] (36, 37).

MGMT expression and promoter methylation: These assays currently are under development in the laboratories of Drs. Steven Thibodeau and Robert Jenkins. Assays will be performed in their laboratories.

8.0 Statistical Considerations

Our goal is to relate the overall survival of patients treated with EGFR inhibitor therapy to the specified set of 14 genetic/molecular markers. Each marker will be examined singly as well as in a multiple variable fashion. Two distinct multiple variable models will be examined, corresponding to two different ways in which the markers may interact. One hypothesis is that the survival advantage depends on the interdependent status of a particular subset of markers,

e.g. patients with strong EGFR overexpression, absence of STAT 3, and either Akt or MAPK positive. Subset selection such as this is well adapted to Classification and Regression Tree (CART) models. We will use the proportional hazards CART survival model proposed by LeBlanc and Crowley (38), and implemented in the `rpart` routines of `Splus`. The second multiple variable approach is to assume additive effects for a set of pathways, which can be assessed using a standard Cox proportional hazards model. We will use the lasso technique for variable selection (39); with the relatively small number of subjects in the study, this method is more stable and reliable than stepwise variable selection.

Univariable analysis for each of the predictors will include Kaplan-Meier survival curve estimates of each subset, along with standard tabulation of the frequency of each genetic/molecular variant. In addition, the association among the genetic/molecular variants will be described. The methods of Therneau and Grambsch (40) will be used to examine each predictor for functional form (a steady gradient of survival for levels 0, 1, 2, 3+) and for proportional hazards.

Evaluation of the multiple variable models will be done by the separation of the data into a training and evaluation set: tissue from N0177 patients will comprise the training set and tissue from N0074 patients will comprise the evaluation set. Specifically, the subset of genetic/molecular characteristics that best predicts survival in patients treated with EGFR inhibitor therapy will be done in three steps. In the first step, subsets will be selected of constrained sizes because the decision regarding what subset of genetic/molecular characteristics to use as an eligibility criterion for a clinical trial will depend on the number of individuals expected to meet the criterion. In particular, the best subsets of sizes one, two, and three, as well as the best overall subset (regardless of size) will be selected using the multiple variable techniques described above. Step one will be applied to the training set (ie. tissue from N0177).

Step two uses the evaluation tissue set (tissue from N0074 patients). The models corresponding to the subsets identified in step one will be used to compute the predicted survival for patients in this dataset. The predicted survival experience will be compared to the observed survival. Subsets will be carried forward to step three if the predicted survival for N0074 patients is similar to the observed survival. Step three will be to determine what subset of genetic/molecular characteristics to use as an eligibility criterion for a Phase II trial for GBM patients consisting of a EGFR inhibitor and radiation regimen. The decision regarding what set of genetic/molecular characteristics to propose as an eligibility criterion will be based on the difference in observed survival between patients who meet the proposed criterion and those who do not in the pooled group of N0074 and N0177 patients. Depending on the results of these planned analyses, additional testing and analyses in archived tissue from patients treated on prior radiation studies may be performed if necessary.

There are 60 patients with available tissue from N0074 and we anticipate obtaining at least 60 from N0177. Assuming we obtain 120 tissue samples from patients in N0074 and N0177 combined, we will be able to detect a difference between a 24-month survival rate of 0.05 in the group who does not meet the proposed eligibility criterion and a 24-month survival rate of 0.18 (constant hazard ratio of 1.75) in the group that meets the proposed criterion with approximately 80% power. This is based on a two-sided logrank test with 0.05 level of significance and equal numbers of patients in both groups for step three of our analysis. The general nature of the analysis will be exploratory and the final decision of whether to perform a phase II using the identified set of tumor genetic/molecular characteristics as an eligibility criterion will be based on (1) estimates of the potential difference in survival between patients who do and do not meet the proposed criterion, and (2) an estimate of the potential improvement above our historical 12-month survival rate of 0.50 for GBM patients.

9.0 Human Studies Evaluation

There is no risk to patients as this is a tissue specimen study that does not require any additional specimens to be collected.

10.0 Budget

This study will be conducted and funded by the Mayo Brain Tumor SPORE grant from the NIH.

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