Molecular Study of Malignant Gliomas Treated with Epidermal Growth Factor Receptor Inhibitors: Tissue Analysis from North American Brain Tumor Consortium Trials 01-03 and 00-01

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Abstract

Purpose: We investigated the molecular effect of the epidermal growth factor receptor (EGFR) inhibitors erlotinib and gefitinib in vivo on all available tumors from patients treated on North American Brain Tumor Consortium trials 01-03 and 00-01 for recurrent or progressive malignant glioma.

Experimental Design: EGFR expression and signaling during treatment with erlotinib or gefitinib were analyzed by Western blot and compared with pre-erlotinib/gefitinib-exposed tissue or unexposed controls. Tumors were also analyzed for EGFR mutations and for other genomic abnormalities by array-based comparative genomic hybridization. Clinical data were used to associate molecular features with tumor sensitivity to erlotinib or gefitinib.

Results: Erlotinib and gefitinib did not markedly affect EGFR activity in vivo. No lung signature mutations of EGFR exons 18 to 21 were observed. There was no clear association between erlotinib/gefitinib sensitivity and deletion or amplification events on array-based comparative genomic hybridization analysis, although novel genomic changes were identified.

Conclusions: As erlotinib and gefitinib were generally ineffective at markedly inhibiting EGFR phosphorylation in these tumors, other assays may be needed to detect molecular effects. Additionally, the mechanism of erlotinib/gefitinib sensitivity likely differs between brain and lung tumors. Finally, novel genomic changes, including deletions of chromosomes 6, 21, and 22, represent new targets for further research.

The prognosis for patients with high-grade gliomas is poor, with a median survival of 2 to 5 years for anaplastic astrocytomas and 1 year for glioblastomas (1). Unfortunately, glioblastomas are the most common as well as most aggressive subtype (1). New therapies are needed, and small-molecule inhibitors targeting specific molecular abnormalities important in glioma biology may provide benefit (2).

Several types of abnormalities of epidermal growth factor receptor (EGFR), a receptor tyrosine kinase, contribute to the growth and proliferation of tumor cells in the majority of glioblastomas, including EGFR gene amplification, protein overexpression, and constitutively activating mutations (3–9). Normally, EGF and other ligands activate the EGFR, causing dimerization/oligomerization and activation of intrinsic tyrosine kinase activity in the cytosolic domain of the receptor (Fig. 1A; ref. 10). When activated, the receptor both autophosphorylates and initiates downstream signaling through the RAS-MAPK and phosphatidylinositol 3-kinase (PI3K)/AKT signal transduction pathways, which in turn regulate cell proliferation, migration, differentiation, and survival. Patients with glioblastomas in which the EGFR has been amplified or activated have poorer survival (3). EGFR expression and activity are frequently increased in the majority of glioblastomas (1, 4). It has been suggested that EGFR signaling is critical to glioblastoma biology (5–9).

The American Brain Tumor Consortium trials 01-03 and 00-01 for recurrent or progressive malignant gliomas, including glioblastomas, provided material for this investigation. The objectives of this study were to determine the molecular effect of the EGFR tyrosine kinase inhibitors erlotinib and gefitinib in vivo on all available tumors from patients treated on these trials. The effects of erlotinib and gefitinib on EGFR expression and activity were measured by Western blot analysis and compared with pre-erlotinib/gefitinib-exposed tissue or unexposed controls. The clinical data from these trials were used to associate molecular features with tumor sensitivity to erlotinib or gefitinib.
transduction cascades. Activation of EGFR, RAS, and AKT can be detected by analysis of tumor tissue for pEGFR, pERK, and pAKT levels with commercially available antibodies.

The EGFR inhibitors erlotinib (OSI774, Tarceva) and gefitinib (ZD1839, Iressa) are currently under evaluation in clinical trials for gliomas. Seeking to determine whether erlotinib or gefitinib therapy affects EGFR signaling in malignant gliomas in vivo and whether observed effects determined clinical response, we analyzed all available surgically resected malignant glioma tissue from patients who were treated with erlotinib or gefitinib through two multi-institution clinical trials. We also analyzed the available tumors for mutations of the EGFR gene and for other genomic alterations beyond EGFR. Although we did not identify consistent inhibition of EGFR signaling by erlotinib or gefitinib, several new genomic abnormalities were identified as common to malignant gliomas and worthy of further study.

Materials and Methods

Tissue. Patients with recurrent or progressive malignant gliomas were treated with erlotinib through North American Brain Tumor Consortium (NABTC) multicenter phase I/II clinical trial 01-03 or with gefitinib through NABTC trial 00-01. Treatment and correlative analyses were approved by the institutional review board at each center and performed with patient’s informed consent. Patients received daily erlotinib or gefitinib as monotherapy and were followed clinically and radiographically with patient’s informed consent. Patients received daily erlotinib or gefitinib following recovery from surgery. These specimens were analyzed to determine the effect of erlotinib or gefitinib on EGFR signaling at the molecular level in vivo and to determine if molecular effects were associated with clinical response.

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Western blots. Tumors that were flash frozen in liquid nitrogen immediately following surgical resection and stored at −80°C were ground into fine powder in liquid nitrogen and dissolved in T-PER buffer (Pierce, Rockford, IL) containing EDTA-free Complete protease inhibitor cocktail (Roche, Indianapolis, IN) and the phosphatase inhibitors NaVO₃ (1 mmol/L; pH 10) and NaF (30 mmol/L). Protein concentrations were determined by absorption at 595 nm of diluted protein extract (Bio-Rad, Hercules, CA) relative to bovine
Table 1. Features of available tumors and analyses done

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Timing of tumor resection relative to treatment with gefitinib/erlotinib</th>
<th>Histology</th>
<th>Treated with erlotinib (E), gefitinib (G), or neither (N)</th>
<th>Erlotinib/gefitinib sensitive (S), insensitive (I), or not applicable (NA)</th>
<th>EGFR activity assayed</th>
<th>EGFR sequencing</th>
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<td>N</td>
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NOTE: Tumor characteristics, treatment, erlotinib/gefitinib sensitivity, and results. Three sets of paired tumors (1 and 9, 2 and 10, and 3 and 11) were resected from the three patients before and during treatment with erlotinib/gefitinib, respectively. Abbreviations: NA, not applicable; ND, not done.
serum albumin standards. Western blot detection was done using chemiluminescence (Amersham Pharmacia, Piscataway, NJ) with the following antibodies: anti-pEGFR (Tyr(1068) 1:250, anti-total EGFR 1:2,000 (Cell Signaling Technology, Beverly, MA), anti-pAKT (Ser(473) 1:1,000 (Cell Signaling Technology), anti-pERK (Thr(202)/Tyr(204) 1:1,000 (Cell Signaling Technology), anti-glyceraldehyde-3-phosphate dehydrogenase—conjugated anti-rabbit Ig 1:1,000 (Amersham Pharmacia), and horseradish peroxidase—conjugated anti-mouse Ig 1:2,000 (Roche). Anti-pEGFR (Y1068) antibodies were used because pY1068 is a reasonable indicator of EGFR autophosphorylation (14, 15).

Pharmacokinetic analysis. For a subset of patients undergoing surgery as part of NABTC trial 01-03, a separate aliquot of tissue was collected for pharmacokinetic analysis following 7 days of erlotinib. Blood was also drawn at the time of surgery and centrifuged within 60 minutes of collection. Tissue (snap frozen in liquid nitrogen at the time of collection) and the simultaneously collected plasma were stored at or below −20 °C until analysis. The tissue was weighed and homogenized in 1 mL high-performance liquid chromatography analyze-grade methanol. Concentrations of erlotinib and its O-demethylated active metabolite (OSI-420) in plasma and tumor tissue were analyzed using a validated liquid chromatography-mass spectrometry method developed by MDS Pharma Services (Saint-Laurent, Quebec, Canada). Analyte-grade erlotinib and OSI-420 and the internal standard (CP-396,059) were obtained from OSI Pharmaceuticals (Boulder, CO).

Erlotinib and OSI-420 were isolated from plasma and homogenized tissue by liquid/liquid extraction. Briefly, 900 µL plasma (tissue) was added to 100 µL internal standards (500 ng/mL) and vortexed followed by the addition of 4 mL n-butyl methyl ether. After circular rotation for 15 minutes at high speed, the samples were centrifuged (3,000 rpm) at 25 °C for 5 minutes. The samples were flash frozen and the organic layer was evaporated to dryness under a gentle stream of nitrogen in a 35 °C water bath. The dry residue was reconstituted with 200 µL mobile phase and vortexed for 10 seconds. A 20-µL sample was autoinjected at room temperature onto a high-performance liquid chromatography system (HP Series II 1090 high-performance liquid chromatography system, Hewlett Packard, Palo Alto, CA). The mobile phase consisted of 70% methanol:30% ammonium formate (10 mmol/L; pH 4.8) pumped at a flow rate of 0.5 mL/min. Separation of erlotinib and OSI-420 was accomplished using a Waters Symmetry C18 column (50 × 4.6 mm, 3.5 µm; Waters, Milford, MA) preceded by a solvent filter and Waters (2.6 × 10 mm) cartridge guard column. Mass spectrometric detection was done with a Finnigan LCQ spectrometer (San Jose, CA) equipped with an atmospheric pressure chemical ionization probe. The mass spectrometric settings were vaporized at 450 °C, sheath gas (N2) flow rate 62 arb, current 5.0 µA, voltage 0.01 kV, capillary temperature 150 °C, and capillary voltage 22.0 kV.

In the tandem mass spectrometry mode, the collision energy was 41%. For peak identification, full-scan mass spectra were acquired in the positive ion mode. The tandem mass spectrometry scan range was 90 to 450. Selected ion monitoring was used for the determination of the ammonium adducts [M + NH4] and the compound’s respective fragment ion: erlotinib (394.5 → 292.0 m/z), OSI-420 (380.3 → 287.8 m/z), and CP-396,059 (408.4 → 292.0 m/z).

Data acquisition and integration of the chromatograms were done using Xcaliber LCQ program (Finnigan). The chromatographic data were analyzed by linear least-squares regression with a weighting of 1/2 generating a 9- and 7-point calibration curve of area ratios for erlotinib and OSI-420, respectively. The calibration curves were linear (R2 > 0.99) over the range of 1.0 to 3,000 ng/mL for erlotinib and 1.0 to 1,000 ng/mL for OSI-420, respectively. The slope of 15 separate calibration curves used in the analysis of samples over a 2-year span ranged from 0.187 to 0.205 and from 0.036 to 0.045, respectively. Samples were repeated if the independent quality control samples are at the low (3.0 ng/mL erlotinib/OSI-420) exceeded the theoretical value by 20% and the medium (400 ng/mL erlotinib/150 ng/mL OSI-420) or high (2,400 ng/mL erlotinib/800 ng/mL OSI-420) quality control by 15%. Of the 31 analytic runs done, only 2 of the duplicate quality controls failed. The interday precision for erlotinib/OSI-420 was 8.30%/10.66%, 5.47%/6.98%, and 5.85%/8.31% for the low, medium, and high quality-control samples, respectively.

The patients who underwent surgical resection during gefitinib treatment described in this study did not participate in the pharmacokinetic analysis of tissue for NABTC trial 00-01.

Epidural growth factor receptor gene sequencing. Tumor genomic DNA was isolated from fresh-frozen brain tumors by digestion in DNA isolation mix [50 mmol/L Tris-HEC (pH 8), 100 mmol/L EDTA, 100 mmol/L NaCl, 1% SDS, protease K] overnight at 55 °C. After addition of RNase A for 1 hour at 37 °C, samples were serially extracted with phenol, phenol/chloroform (1:1), and phenol/chloroform/isooamy alcohol (25:24:1). After isopropanol precipitation, samples were washed in 70% ethanol, air-dried, resuspended in water, and quantified using a spectrophotometer. If frozen tumor was unavailable, paraffin-embedded sections were used. Both deparaffinization (if required) and PCR amplification, including primer sequences, were done as described previously (15). About 90% of the coding region of EGFR (exons 2-28) was successfully sequenced in all tumors analyzed, except one case in which technical limitations resulting from the quality of the DNA extracted from archival paraffinized tumor precluded sequencing of exons beyond 18 to 21 (lung signature region).

Array-based comparative genomic hybridization. Genomic DNA extracted from tumors was analyzed for chromosome alterations affecting not only EGFR but also the entire genome. We used Roswell Park Cancer Institute custom comparative genomic hybridization (CGH) arrays spotted with Roswell Park Cancer Institute-11 bacterial artificial chromosomes (BAC) as described previously (16). Verification and use

Fig. 2. Two-way hierarchical clustering of aCGH data. Complete linkage clustering of aCGH data from 16 malignant brain tumor samples is shown in the Treemix format. The data are clustered both as tumor groupings (X axis) and BAC groupings (Y axis). The intensity of red signal (amplification) or green signal (deletion) is relative to the log2 ratio (shown in Supplementary Table S1) of a particular BAC for each tumor shown.

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of these BACs for array-based CGH (aCGH) has also been reported elsewhere (17, 18). Briefly, the Roswell Park Cancer Institute array contains ~6,000 Roswell Park Cancer Institute-11 BAC clones that provide an average resolution across the genome of 420 kb. BACs were printed in triplicate on amino-silanated glass slides (Schott NeXterion, type A) using a MicroGrid II TAS arrayer (Apogent Discoveries, Hudson, NH) to generate an array of roughly 18,000 elements. Genomic pooled normal control DNA and tumor DNA were fluorescently labeled by random priming and hybridized as described previously (16). Hybridizations of normal and tumor DNA were done as sex-mismatches to provide an internal hybridization control for chromosome X and Y copy number differences. The hybridized slides were scanned using an Affymetrix 428 scanner to generate high-resolution (10 μm) images for both Cy3 and Cy5 channels, and image analysis was done using ImaGene (version 4.1) software (BioDiscovery, Inc., El Segundo, CA). Mapping information was added for each BAC using the National Center for Biotechnology Information July 2003 build (http://genome.ucsc.edu/cgi-bin/hgGateway). Genes were identified using both University of California-Santa Cruz and National Center for Biotechnology Information May 2003 build (http://genome.ucsc.edu/cgi-bin/hgGateway?org=human), and to the best of our knowledge, clones with ambiguous assignments in the databases were removed.

Hierarchical cluster analysis and in silico mapping. The log2 values of ~5,500 BACs that mapped to autosomal regions of the genome for all 12 tumors were used for hierarchical clustering. The Library of DNA Analysis Cluster 3.0 program (http://bonsai.ims.u-tokyo.ac.jp/~mdehoon/software/clustering/) was used to both filter and cluster the aCGH data (19). An a priori filter was used to exclude BACs with log2 ratios between −0.5 and +0.5 in >25% of the tumors analyzed. In practical terms, a log2 ratio of ~0.5 for a genomic region defined by a BAC is equivalent to a heterozygous deletion of a diploid population of cells. In the same respect, each 0.5 increase in the log2 ratio suggests gain of an additional copy of that region defined by a BAC. Filtered data were hierarchically clustered using the complete linkage setting of the Cluster 3.0 program, and clustered data were viewed using EisenLab TreeView software (http://rana.lbl.gov/EisenSoftware.htm). This method of filtering and clustering allows for the detection of minimal regions of amplification and deletion, thus facilitating the identification of potential target genes. A SD cutoff rule method described previously (20) was also employed to attain independent statistical confirmation of the BACs represented in the cluster analysis. Essentially, the SD cutoff rule is a conservative algorithm that sets a strict threshold to exclude both infrequent and marginal (degree of change <1 SD from the mean) amplifications/deletion events. Therefore, BACs identified in this report include only those meeting numerous criteria for significance, including frequency and amplitude by both supervised clustering and SD cutoff rule methods.

The genomic position of each BAC is defined as the region containing the BAC as well as the flanking sequence extending from the 3' end of the adjacent upstream BAC to the 5' end of the closest downstream BAC. The BACs used for aCGH were mapped using the National Center for Biotechnology Information July 2003 build, and the genomic positions of regions of amplification and deletion were converted to the National Center for Biotechnology Information May 2004 build using the University of California-Santa Cruz Build Converter Browser (http://genome.ucsc.edu/cgi-bin/hgGateway). Genes were identified using both University of California-Santa Cruz and National Center for Biotechnology Information Map Viewer Browser (http://www.ncbi.nlm.nih.gov/mapview/).

Results

Effects of erlotinib and gefitinib on epithelial growth factor receptor activity, expression, and signaling. To determine whether erlotinib/gefitinib inhibited EGFR activity in vivo, the pEGFR levels in tissue resected during erlotinib/gefitinib were compared with the levels in erlotinib/gefitinib unexposed tissue by Western blot. In three patients, tissue was resected both before and during treatment with erlotinib or gefitinib. In the first case (tumor 1 versus tumor 9), the level of pEGFR actually increased substantially during treatment with erlotinib (Fig. 1B), although the levels of downstream signaling through pERK and pAKT were markedly reduced. In the second case (tumor 2 versus tumor 10), pEGFR diminished slightly during treatment with gefitinib, but downstream signaling through pERK and pAKT was unchanged and moderately increased, respectively (Fig. 1B). In the third case, EGFR activity was reduced after exposure to gefitinib (tumor 3 versus tumor 11), but signaling through pERK and pAKT slightly increased and decreased, respectively (Fig. 1B).

There was a wide range of pEGFR levels among all 12 tumors resected during erlotinib or gefitinib (Fig. 1C), and this range did not differ substantially from the range observed among the 12 erlotinib/gefitinib unexposed controls (data from 6 representative controls shown; Fig. 1C). The range of pERK and pAKT levels among the erlotinib/gefitinib–exposed tumors and unexposed controls also did not differ substantially (Fig. 1C). These results suggest that erlotinib and gefitinib did not effectively inhibit either EGFR phosphorylation or signaling in these tumors as a group.

Although the range of pEGFR, pERK, and pAKT among erlotinib/gefitinib–exposed and unexposed tumors overlapped, there were several tumors resected during erlotinib/gefitinib therapy with either low (Fig. 1C; tumors 15 and 19) or high (Fig. 1C; tumors 9, 12, 14, and 16) pEGFR levels. However, there was no obvious association between erlotinib/gefitinib sensitivity (as defined in Materials and Methods) and effects on EGFR activity, EGFR expression, or activation of downstream signaling through RAS/MAPK or PI3K/AKT in these tumors (Fig. 1C). Unfortunately, tissue was not resected during treatment of the patient with the complete and sustained radiographic response to erlotinib; therefore, it is unknown whether erlotinib significantly affected EGFR phosphorylation or signaling in this tumor and whether any such effects were related to control of tumor growth.

We also analyzed pharmacokinetic data to determine drug penetration into tumor tissue. The steady-state trough concentrations of erlotinib and the active metabolite OSI-420 in tumor were 6% to 8% and 3% to 11%, respectively, of the concentrations in plasma drawn during the tumor surgery in four available cases (12, 15, 19, 20). As above, there was no consistent association of erlotinib treatment with either clinical outcome or EGFR signaling effects in these cases. In another case (9), erlotinib and OSI-420 were present in the tumor tissue at 50% and 54% of the concentration in plasma, respectively, suggesting a higher tissue penetration. Surprisingly, this was the case with a marked increase in pEGFR during erlotinib treatment. However, pharmacokinetic results from this case and from another (14) with both high pEGFR during treatment and possibly high drug penetration (erlotinib and OSI-420 present in tumor at 19% and 28%, respectively, of the concentration in plasma) are suspect because the tissue aliquots (different than the aliquots analyzed for EGFR activity) were likely contaminated by a large blood clot. In summary, the pharmacokinetic data suggest that drug penetration was too low in the cases analyzed to consistently achieve inhibition of EGFR phosphorylation, and in one case with higher penetration (if not artifactual), EGFR phosphorylation actually increased during treatment.

Aliquots of the tumors analyzed for EGFR signaling changes during gefitinib treatment (10, 11, 16) were not available for
pharmacokinetic analysis. However, analysis of analogous tumors obtained from other patients receiving gefitinib through NABTC trial 00-01 (n = 2) revealed that the 24-hour trough tissue-to-plasma ratio was 221% to 370%. This suggests that gefitinib is sequestered in, rather than excluded, from glioma tissue. Applying these data to the cases studied for effects on EGFR activity, it is likely that the dose of gefitinib given was sufficient to slightly or moderately inhibit EGFR phosphorylation in the two cases in which tissue resected both before and during gefitinib was available for comparison (2 versus 10 and 3 versus 11; Fig. 1B).

Combining results from both erlotinib and gefitinib exposed tissue, it is of potential interest that pAkt was reduced during treatment in two tumors that were erlotinib/gefitinib sensitive (Fig. 1B, tumor 1 versus tumor 9 and tumor 3 versus tumor 11) and higher in one insensitive tumor (Fig. 1B, tumor 2 versus 10) despite the lack of consistent effects on pEGFR and relatively low erlotinib penetration. However, this pattern was not clearly evident in the other tumors resected during treatment (Fig. 1C), and the interpretation is also limited by the number of cases with both pretreatment and during treatment specimens available for comparison. Therefore, the clinical relevance of the decrease in pEGFR during gefitinib (Fig. 1B) as well as the changes in pERK and/or pAkt remain unclear.

Epidermal growth factor receptor gene sequencing. It was reported recently that several mutations in EGFR exons 18 to 21 are associated with sensitivity of non–small cell lung cancer to erlotinib and gefitinib (15, 21, 22). In light of these results, we first screened genomic DNA from 16 of the gliomas for the lung signature mutations (tumors 1-2, 4-10, 13-15, 17, and 21-23). Although two single nucleotide alterations without a corresponding amino acid change were observed (data not shown), we did not identify any of the mutations reported in non–small cell lung cancer. Therefore, we sequenced the remainder of the EGFR coding sequence in an attempt to identify other mutations, including any that were associated with sensitivity to erlotinib/gefitinib. Ten single nucleotide alterations that did not induce an amino acid change were observed (data not shown). There were also missense mutations in exons 6, 13, and 17 (1 each). In tumor 8, heterozygous C219Y (exon 6; 656 G→A) was observed. This mutation was not found in any other tumor from patients either treated or untreated with gefitinib or erlotinib. In tumor 21, heterozygous R675Q (exon 17; 2,024 G→A) was observed. This mutation was similarly not observed in any other tumor from patients either treated or untreated with gefitinib or erlotinib. Three tumors exhibited heterozygous (13, 14) or homozygous (15) R521K (exon 13; 1,562 G→A) mutations. Notably, this mutation was also observed in both tumors (22, 23) from a patient that was not treated with gefitinib or erlotinib as well as in some brain metastases from non–small cell lung cancer (data not shown). There were no large homozygous deletions that have been reported by others in malignant gliomas (7, 9), such as that of exons 2 to 7 in the mutant form EGFRvIII. Although heterozygous EGFRvIII and similar mutations were not observed, exonic resequencing is not the optimal technique to detect multixon heterozygous deletions. As none of these changes was clearly associated with sensitivity to erlotinib or gefitinib, sequencing of EGFR was not pursued in the remaining tumors.

Array-based comparative genomic hybridization. In addition to assays of EGFR activity and signaling, we also examined chromosome 7p11.2 (containing the EGFR gene) for amplifications or deletions using aCGH. Three tumors (4, 8, 22) exhibited genomic amplification of at least six copies of the Chr7:54860933-55049239 region identified by three contiguous BACs (RP11-14K11, RP11-81B20, and RP11-97P11; Fig. 2). Tumors with high copy EGFR gene amplification determined by aCGH also exhibited high total EGFR protein expression when assayed by Western blot [e.g., tumors 4 and 8 (Fig. 1B) and tumor 22 (data not shown)] serving as an internal control validating our results. However, there was no association between erlotinib sensitivity and EGFR amplification.

Expanding the analysis beyond EGFR, supervised clustering of aCGH identified genetic alterations consistent with malignant brain tumors, such as deletions of the p16 gene (Chr9:21957751-21984357) and loss of chromosome 10q23-26 in six (4, 5, 8, 17, 22, and 23) and seven (1, 4, 5, 7, 15, 22, and 23) of the tumors, respectively (Table 2). Although this analysis focused exclusively on the most frequent changes, events unique to this population of tumors included amplifications of MYCN (Chr2:15,250,313-18,548,231) and GLI1 (Chr12:55,804,692-57,375,460) in tumors 17 and 21, respectively.

In addition to expected losses and gains, we identified recurrent losses of entire copies of chromosomes 13, 14, and 22 as well as loss of a single BAC (RP11-96H121) on chromosome 21. In addition, a region of 7q36.1 containing two contiguous BACs (RP11-236I4 and RP11-43L19) was amplified in tumors 3-5, 10, 17, and 21-23. Amplification of this region may be due to gain of a complete copy of chromosome 7, but in at least one specimen (tumor 17), amplification of this region (Chr7:150340429-152159943) occurred without amplification of EGFR. Although there are 23 known genes mapped to this region of 7q, at least one, Ras homologue enriched in brain (RHEB), may contribute to the malignant phenotype. Similarly, loss of two contiguous BACs on chromosome 13 represent a 437-kb region containing six genes, one of which, forkhead box O1A (FOXO1A), is a known tumor suppressor. Both RHEB and forkhead transcription factors are involved in PI3K/AKT signaling, suggesting that aCGH may be used to identify candidate genes within specific pathways involved in malignant brain tumors.

In addition to RHEB and FOXO1A, deletion of chromosome 14 was prominent in five tumors (5, 7, 15, 17, and 21), and partial loss (Chr14:57910104-58786387, BAC RP11-2122) occurred in one tumor (tumor 4). This region of chromosome 14 contains 11 known genes, one of which, disheveled associated activator of morphogenesis 1 (DAAM1), is the best characterized and has a known role in the Wnt signaling pathway (23). Other highlighted BACs on chromosome 14 include those containing the gene MAX, which is involved in MYC signaling, and PTPN21, which encodes a protein tyrosine phosphatase.

We also identified deletions of chromosomes 6 (tumors 4, 17, 22, and 23), 21 (tumors 4, 5, 7, 8, 22, and 23), and 22 (tumors 4, 5, 17, 22, and 23) that have not been reported previously in brain tumors. Although there was no evidence of an association between deletion or amplification events and sensitivity to erlotinib, the consistency of chromosomal alterations identified in our tumor samples suggests that genes mapped to these regions may be worthy of further study in gliomas.
Discussion

To fulfill the primary molecular aims of NABTC trials 01-03 and 00-01, which involved erlotinib and gefitinib for recurrent or progressive malignant gliomas, 21 available gliomas were analyzed to determine the molecular consequences of EGFR inhibitor treatment in vivo. Comparison of tumor resected during erlotinib or gefitinib with control tissue did not show a consistent effect on EGFR phosphorylation or downstream signaling (Fig. 1B and C). Furthermore, reduced EGFR activity and downstream signaling was also not related to erlotinib/gefitinib sensitivity. Although the strength of our conclusions is limited by the small number of samples available for analysis, other studies have yielded similar results. For example, pEGFR reduction in breast tumors following treatment with gefitinib did not correlate with response (24). Similarly, amplification of chromosome 7p (containing the EGFR gene) was unrelated to erlotinib sensitivity. Others have also reported no relationship between EGFR gene amplification or protein expression and sensitivity of breast, lung, or brain tumors to EGFR inhibitors (25–30).

Taken together, these data suggest that erlotinib and gefitinib did not have a consistent effect on EGFR phosphorylation in the gliomas analyzed. Although a molecularly effective dose of erlotinib/gefitinib (required to achieve consistent pathway inhibition) has not been determined and may differ from a maximally tolerated dose, these data suggest that the concentration of erlotinib in tumor tissue relative to simultaneously collected plasma was too low (steady-state trough levels of ~10% or less for both erlotinib and its active metabolite) to consistently reduce pEGFR, and it is possible that erlotinib was markedly underdosed in this study. In fact, the tissue with the highest penetration by drug actually exhibited a marked increase rather than decrease in EGFR phosphorylation, despite clinical sensitivity to erlotinib, although the high drug concentration in that sample could be artifactual as described above. Of note, the pharmacokinetic results are also limited by the availability of only one tissue specimen per patient representing a trough level 24 hours after dosing. It is possible that levels at time points earlier than 24 hours after drug exposure would have been higher. By contrast, the mean gefitinib concentration in two tumors from other patients treated through NABTC trial 00-01 (not assayed for EGFR signaling changes) was almost triple the concentration in plasma. This may explain the reductions in pEGFR seen in two cases (2 versus 10 and 3 versus 11), although pEGFR remained relatively high in a third case (16).

Although there was no consistent effect of erlotinib or gefitinib on EGFR activity, expression, or signaling and no

<table>
<thead>
<tr>
<th>Tumors</th>
<th>Genomic position</th>
<th>BAC(s)</th>
<th>Event</th>
<th>No. gene(s)</th>
<th>Representative gene(s) symbol and description</th>
</tr>
</thead>
<tbody>
<tr>
<td>4, 10, 17, and 21</td>
<td>Chr10: 106023284-106201712</td>
<td>RP11-101J13</td>
<td>Loss 4</td>
<td>GSTO2, glutathione S-transferase 0β2; C10orf80; KIAA1754; LOC387710</td>
<td></td>
</tr>
<tr>
<td>1, 4, 5, 7, 15, 22, and 23</td>
<td>Chr10: 119134985-120589963</td>
<td>RP11-79A18</td>
<td>Loss 7</td>
<td>CASC2, cancer susceptibility candidate 2</td>
<td></td>
</tr>
<tr>
<td>1, 4, 5, 7, 15, 22, and 23</td>
<td>Chr10: 135111183-135150439</td>
<td>RP11-108K14</td>
<td>Loss 3</td>
<td>Sprn, shadow of prion protein; LOC399832</td>
<td></td>
</tr>
<tr>
<td>1, 4, 5, 7, 15, 22, and 23</td>
<td>Chr10: 106023284-106201712</td>
<td>RP11-101J13</td>
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<td>GSTO2, glutathione S-transferase 0β2; C10orf80; KIAA1754; LOC387710</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>1, 4, 5, 7, 15, 22, and 23</td>
<td>Chr10: 135111183-135150439</td>
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<td>Loss 3</td>
<td>Sprn, shadow of prion protein; LOC399832</td>
<td></td>
</tr>
</tbody>
</table>

(Continued on the following page)
consistent association with clinical outcome (Fig. 1C), it is interesting to note that pAKT was reduced during treatment in two sensitive tumors and increased in one insensitive tumor (Fig. 1B). If confirmed by analysis of more tumors, this could suggest that the PI3K/AKT cascade deserves particular focus during treatment of gliomas with EGFR inhibitors and may be a more important indicator of EGFR inhibition than EGFR phosphorylation status. In fact, there has been conflicting data regarding the importance of RAS/MAPK and PI3K/AKT activity in relationship to sensitivity to upstream inhibition of EGFR (31–36). It is also possible that erlotinib and gefitinib may affect other receptor tyrosine kinases or other signaling cascades not studied in this analysis, as the number of tyrosine kinases studied for activity is limited (37).

It is also notable that the level of phosphorylation mirrored the total EGFR expression in all three cases where both pre–erlotinib/gefitinib and during erlotinib/gefitinib–exposed tissue were analyzed. In one case (1 versus 9; Fig. 1B), it is plausible that the downstream effects of EGFR (pERK and pAKT) were inhibited by erlotinib and the increase in total EGFR expression and pEGFR resulted from stimulation of a feedback loop following the block in downstream AKT phosphorylation status. In fact, there has been conflicting data regarding the importance of RAS/MAPK and PI3K/AKT activity in relationship to sensitivity to upstream inhibition of EGFR (31–36). It is also possible that erlotinib and gefitinib may affect other receptor tyrosine kinases or other signaling cascades not studied in this analysis, as the number of tyrosine kinases studied for activity is limited (37).

Therefore, this mutation may not have functional relevance

Table 2. Results of aCGH analysis (Cont’d)

<table>
<thead>
<tr>
<th>Tumors</th>
<th>Genomic position</th>
<th>BAC(s)</th>
<th>Event</th>
<th>No. gene(s)</th>
<th>Representative gene(s) symbol and description</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-5, 17, and 21-23</td>
<td>Chr13:29592937-30684939</td>
<td>RP11-121019; RP11-64C21</td>
<td>Loss</td>
<td>13</td>
<td>HMGB1, high-mobility group box 1; KATNAL1, katanin p60 subunit A-like 1; LOC387917; FLJ14834</td>
</tr>
<tr>
<td>3-5, 17, and 21-23</td>
<td>Chr13:39103232-40367433</td>
<td>RP11-53F19; RP11-89L15</td>
<td>Loss</td>
<td>6</td>
<td>COG6, component of oligomeric Golgi complex 6; FOXO1A, forkhead box 01A</td>
</tr>
<tr>
<td>3-5, 17, and 21-23</td>
<td>Chr13:41593291-42577676</td>
<td>RP11-21H9; RP11-34K15</td>
<td>Loss</td>
<td>8</td>
<td>D66A1, diacylglycerol kinase; AAK1P1, A kinase (PRKA) anchor protein 11; TNFSF11, tumor necrosis factor (ligand) superfamily member 11; EPST11, epithelial stromal interaction 1 (breast)</td>
</tr>
<tr>
<td>4, 5, 17, and 21-23</td>
<td>Chr13:48242971-50898649</td>
<td>RP11-54G17; RP11-34F20</td>
<td>Loss</td>
<td>22</td>
<td>DLEU1-2, lost set in lymphocytic leukemia, 1 and 2; MLNR, motlin receptor; FDNC3, fibrorectin type III domain 3</td>
</tr>
<tr>
<td>4, 5, 7, 15, 17, and 21</td>
<td>Chr14:57910104-58786387</td>
<td>RP11-2L22</td>
<td>Loss</td>
<td>11</td>
<td>DACT1, dapper homologue 1, antagonist of β-catenin; DAAM1, dishevelled associated activator of morphogenesis 1</td>
</tr>
<tr>
<td>5, 7, 15, 17, and 21</td>
<td>Chr14:69440807-70531028</td>
<td>RP11-18G18</td>
<td>Loss</td>
<td>14</td>
<td>MAP3K9, MAPK kinase kinase 9</td>
</tr>
<tr>
<td>5, 7, 15, 17, and 21</td>
<td>Chr14:77626345-79602555</td>
<td>RP11-46L17</td>
<td>Loss</td>
<td>2</td>
<td>NRPX3, neuroxin 3; LOC388001</td>
</tr>
<tr>
<td>4, 5, 7, 8, 22, and 23</td>
<td>Chr21:29921601-3102834</td>
<td>RP11-96H21</td>
<td>Loss</td>
<td>33</td>
<td>KRTAP, keratin-associated proteins; CLDN8, Claudin 8</td>
</tr>
<tr>
<td>4, 5, 17, 22, and 23</td>
<td>Chr22:31113067-31577786</td>
<td>RP11-70F2</td>
<td>Loss</td>
<td>5</td>
<td>SYN3, synapsin III; TIMP3, tissue inhibitor of metalloproteinase</td>
</tr>
</tbody>
</table>

NOTE: Defining regions frequently amplified or deleted in malignant brain tumors. A total of 36 BACs corresponding to 19 discrete loci were identified by aCGH as either gained or lost in at least 25% of the brain tumors analyzed. Contiguous BACs are shown with a vertical line, and the genomic position for each BAC(s) incorporates the genomic distance between BACs immediately proximal and distal to the known region of amplification or deletion. Each locus is identified as being a region of loss, gain (increase in one or two copies generally of an entire chromosome or chromosomal arm), or amplification (multiple copies of a particular locus). The total number of genes that map to each region is also shown with examples of either known or potential target genes.
and rather represent a polymorphism, a conclusion further supported by the identification of this change in other gliomas as well as in brain metastases from non–small cell lung cancer. Another change (C219Y; exon 6; 656 G→A) was not detected in tumors from other patients treated or untreated with gefitinib or erlotinib. The importance of this alteration is unclear. It should also be noted that EGRF mutations are typically observed only in gliomas with EGRF gene amplifications (9). Therefore, one limitation of our analysis for EGRF gene mutations was that amplified EGRF (at least one extra copy) was observed in only seven of the tumors analyzed (Table 1), and relatively rare mutations could be missed in tumors without amplified EGRF.

We also did not identify homozygous EGRFvIII in any of the tumors in which EGRF was sequenced. However, others have reported that EGRFvIII expression does not correlate with sensitivity of malignant gliomas to gefitinib (29).

Finally, the aCGH analysis also did not identify other areas of change in genomic DNA outside the EGRF region associated with erlotinib/gefitinib sensitivity. However, while investigating genomic DNA changes beyond EGRF, we identified several areas that were consistently amplified or deleted. The genes contained within these areas involve varied aspects of cancer and cellular biology (Table 2). Some of these are already identified as important in glioma biology, but others are novel. Further investigation of these regions may improve our understanding of malignant brain tumors.

In summary, we analyzed malignant gliomas resected during treatment with the EGFR inhibitors erlotinib or gefitinib for changes in EGFR expression, activity, or signaling in vivo. No consistent effects were observed. However, nonsustained stable disease was included in the definition of clinical sensitivity to erlotinib/gefitinib for this analysis, and in the only case with an objective response (complete disappearance of visible tumor), tissue was not resected during exposure to erlotinib precluding analysis of either EGFR signaling or drug penetration during treatment. We also did not identify DNA alterations either in EGRF or elsewhere that associated with tumor sensitivity to erlotinib/gefitinib. The molecular features associated with erlotinib/gefitinib therapy for gliomas remain unclear.

Appendix

The NABTC is dedicated to the development and conduct of innovative clinical trials that will ultimately result in a cure for patients with malignant brain tumors. More information about the NABTC is available by visiting http://www.nabtc.org or by calling 713-792-8519.

Tissue was available for analysis from patients treated at four participating NABTC sites: Memorial Sloan Kettering Cancer Center, Dana-Farber Cancer Institute, University of California-San Francisco, and University of Texas M.D. Anderson Cancer Center. As results reported here are restricted to the analysis of available tissue from these centers, authorship was limited to investigators from these centers and to the multicenter principal investigators. Clinical results will be reported separately, and we recognize and appreciate the contribution to the clinical data made by the site principal investigators for the other NABTC centers, including Drs. Timothy F. Cloughesy (University of California-Los Angeles), Howard A. Fine (Neuro-Oncology Branch, National Cancer Institute, NIH), and Minesh Mehta (University of Wisconsin Hospital and Clinics) and their many colleagues. NABTC 01-03 and 00-01 would not have been possible without the invaluable assistance of multiple research staff assistants, nurses, and data managers.

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Molecular Study of Malignant Gliomas Treated with Epidermal Growth Factor Receptor Inhibitors: Tissue Analysis from North American Brain Tumor Consortium Trials 01-03 and 00-01

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