Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Mellinghoff IK, Wang MY, Vivanco I, et al. Molecular determinants of the response of glioblastomas to EGFR kinase inhibitors. N Engl J Med 2005;353:2012-24.

Supplementary Appendix

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SUPPLEMENTARY DETAILED METHODS:

Sequencing of tumor genomic DNA

Each PCR reaction contained 5ng of DNA, 1X HotStar Buffer, 0.8 mM dNTPs, 1 mM MgCl2, 0.2U HotStar Enzyme (Qiagen, Valencia, CA), and 0.2 μM forward and reverse primers in a 10 μL reaction volume. PCR cycling parameters were: one cycle of 95°C for 15 min, 35 cycles of 95°C for 20s, 60°C for 30s and 72°C for 1 min, followed by one cycle of 72°C for 3 min. The resulting PCR products were purified by solid phase reversible immobilization chemistry followed by bi-directional dye-terminator fluorescent sequencing with universal M13 primers. Sequencing fragments were detected via capillary electrophoresis using ABI Prism 3700 DNA Analyzer (Applied Biosystems, Foster City, CA). PCR and sequencing were performed by Agencourt Bioscience Corporation (Beverly, MA). Forward (F) and reverse (R) chromatograms were analyzed in batch by Mutation Surveyor 2.03 (SoftGenetics, State College, PA), followed by manual review. High quality sequence variations found in one or both directions were scored as candidate mutations. Exons harboring candidate mutations were re-amplified from the original DNA sample and re-sequenced as above. Primer sequences have been published elsewhere[1].

RT-PCR: Total RNA was extracted from 50-100 mg of each frozen tumor specimen using TRIzol reagent (Invitrogen) in a tissue grinder. Total RNA was then treated with amplification grade DNase I for 15 minutes at 37°C. For each patient, 1 microgram of total RNA was reverse transcribed using Superscript II (Invitrogen) with oligo(dT) priming according to the manufacturer's protocols. 2 microliters of 1st strand cDNA (1/10th of the reverse transcription reaction volume) was then used as template in a 50 microliter PCR reaction containing 2 mM $MgSO_4$, 0.2 μM of each primer, 0.2 mM dNTPs, 1x High Fidelity PCR Buffer, and 1 unit of Platinum Taq High Fidelity (Invitrogen). Forward and reverse primer sequences to specifically amplify EGFR and EGFRvIII were 5' CTT CGG GGA GCA GCG ATG CGA C 3' (spanning the 5' untranslated region and the beginning of exon 1) and 5' ACC AAT ACC TAT TCC GTT ACA C 3' (within exon 9), respectively. These primers generate a 1044 bp PCR product for the wild type EGFR transcript compared to a 243 bp PCR product for the EGFRvIII transcript. PCR cycling conditions began with an initial denaturation step at 95°C for 2 minutes, followed by 42 cycles of 95°C denaturation for 30 seconds, 56.5°C annealing for 30 seconds, and 68°C extension for 1:20. PCR reactions were analyzed by running 5 μl of product on a 1.5% agarose gel and staining with ethidium bromide. Cloned wild type EGFR and EGFRvIII cDNAs were used as templates in parallel positive control reactions, alongside reverse transcription and PCR negative control reactions. GAPDH was also amplified for each patient sample to assess relative RNA template quality

and amount. Primers for GAPDH were 5’ GTG AAG GTC GGA GTC AAC GG 3’ and 5’ TGA TGA CAA GCT TCC CGT TCT C 3’ (generating a 198 bp product) and the extension time during cycling was reduced to 30 seconds.

Real-time PCR: Real-time PCR was performed using the iCycler thermocycler (Bio-Rad Laboratories). Amplification conditions were: 95°C for 3 min., 40 cycles of 95°C/30sec and 72°C/1 min., and 75 cycles of 63°C + 0.5°C per cycle for 5sec for melt curve analyses. Each amplification reaction contained 10 ng of tumor DNA, 10μM of each primer, Titanium Taq polymerase, 1x Titanium Taq buffer (Clontech), 125 μM dNTP, SYBR™ Green I (Molecular Probes), and Fluorescein (Bio-Rad Laboratories). Normal human genomic DNA (Promega) was used as control DNA template. To control for variations in input DNA between tumor samples, GAPDH amplifications were performed in parallel with EGFR exon 4 and EGFR exon 9 amplifications and used for subsequent normalization. All measurements were collected in triplicates and confirmed by independent experiments. Primers for realtime-PCR included: EGFR exon 4: forward, AAAGAGTGCTCACCGCAGTT, reverse, CACTGGATGCTCTCCACGTT; EGFR exon 9: forward, CTTCAAAAACTGCACCTCCA, reverse, CAAGCAACTGAACCTGTGACT; GAPDH: forward, CAGCAAGAGCACAAGAGGAA, reverse, CAACTGTGAGGAGGGGAGAT.

Immunoblotting. Snap-frozen tissues or cell culture cells were lysed and homogenized in RIPA lysis buffer containing fresh protease inhibitors by standard procedures. Protein concentrations were quantified with the BCA Protein Assay kit (Pierce Chemical Co.), and 30 μg of proteins were separated in 8% SDS-PAGE gel, transferred to nitrocellular membranes, and hybridized with antibodies to the indicated antigens by standard procedures. Signals were detected by chemoluminescence using ECL detection reagents (Amersham Pharmacia Biotech). Primary antibodies to the following antigens were used: EGFR/EGFRvIII cocktail (#AHR5062, Biosource Corp., Camarillo, CA, USA), phospho-Tyr (#9411, Cell Signaling), PTEN (#ABM-2052, Cascade), Akt (#9272, Cell Signaling), phospho-Akt (Ser473/587F11, #4051, Cell Signaling), S6 (#2212, Cell Signaling), phospho-S6 (Ser235/236, #2211, Cell Signaling), and β-tubulin (T4026, Sigma).

Immunohistochemistry: Sections were stained with monoclonal antibodies to PTEN (clone 6H2.1, Cascade Bioscience, Winchester MA) and EGFRvIII (clone L8A4, a generous gift from Dr. Darrell Bigner). L8A4 has been shown to react with EGFRvIII, but not full-length EGFR (Supplementary Figure 2)[2]. Antigen retrieval was performed using 0.01 M citrate buffer, pH 6.0 for 30 minutes in an oven. Peroxidase activity was quenched with 3% hydrogen peroxide in water. Primary antibodies (PTEN at 1:400, EGFRvIII at 1:150) were diluted in phosphate buffered saline with 2% bovine serum albumin and

2% normal horse serum and applied for 16 hours at 4°C, followed by biotinylated secondary antibodies (Vector) at 1:200 dilution for 30 minutes, and avidin-biotin complex (Elite ABC, Vector) for 30 minutes. Negative control slides received blocking serum (phosphate buffered saline with 2% normal horse serum and 2% bovine serum albumin). Vector NovaRed was used as the enzyme substrate to visualize specific antibody localization. Slides were counterstained with Harris hematoxylin.

Pathologist based scoring of immunohistochemistry: PTEN staining was scored according to a previously established scale of 0-2, which has been shown to be highly consistent[3,4]. Tumor cells are graded as 2 if their staining intensity is equal to that of the vascular endothelium, 1 if it is diminished relative to the endothelium, and 0 if it is undetectable in the tumor cells and present in the vascular endothelium. Tumors with PTEN scoring of 0 or 1 are considered PTEN deficient. EGFRvIII staining was scored as positive for tumors demonstrating at least focal moderate to strong immunoreactivity, as previously reported[3]. Tumors were scored for PTEN and EGFRvIII by two independent neuropathologists, blinded to the molecular analyses.

Image analysis-based scoring of immunohistochemistry: Representative images from PTEN and EGFRvIII immunostained sections were photographed using a Colorview II camera mounted on an

Olympus BX61 microscope. Multiple images were captured (at least 3 per slide) from representative regions of the tumor (and adjacent normal brain if present). Borders between individual cells were approximated using a filter function. The amount of reaction product per cell was determined by measuring mean saturation per cell in the red-brown hue range. 1000-1500 cells per case (on average) were measured for EGFRvIII and PTEN. As an internal control, for PTEN analysis, mean saturation was measured in vascular endothelium; for EGFRvIII analysis, mean saturation was measured in adjacent normal brain tissue. For samples in which no adjacent normal brain was present on the slide, a normal reference standard was established by analyzing 9700 cells from 15 normal brain sections. Ratios of mean PTEN staining per tumor cell/ mean PTEN staining per endothelial cell; and mean EGFRvIII staining per tumor cell / mean EGFRvIII staining per normal brain were determined. False color images representing the distribution of such cells were generated. For PTEN staining, a tumor/vessel ratio <0.6 was considered to be PTEN loss. The agreement between traditional semi-quantitative pathologic assessment and image analysis was very high (kappa=0.92; p=0.000006) For EGFRvIII, the correlation between semi-quantitative pathologic assessment and image analysis was also very high (kappa=0.91; p=0.000007).

Statistical Methods: To test the dependence of 2 categorical variables (corresponding to the rows and columns of a contingency table), we used Fisher's exact test. We used a logistic regression model to

estimate the odds ratio (relative risk) and its confidence interval between 2 binary variables. To test whether ordinal variables differed across 2 groups, we used the Wilcoxon or the Kruskal-Wallis test, both of which are non-parametric group comparison tests. To measure agreement between categorical measurements, we used Cohen's kappa statistic, which takes on values smaller than or equal to 1 (=perfect agreement). The asymptotic standard error of the kappa statistic can be used to arrive at an asymptotic p-value, which measures the significance of agreement. We used the Spearman correlation coefficient and the corresponding p-value to determine the correlation between quantitative or ordinal variables. The Kaplan-Meier (KM) method was used to estimate survival distributions. The Cox proportional hazards model was used to estimate hazard rates, their confidence intervals, and corresponding Cox regression p-values. All p-values were two sided and $p<0.05$ was considered significant. All statistical analyses were carried out with the freely available software (http://www.r-project.org/).

REFERENCES FOR SUPPLEMENTARY APPENDIX

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2. Wikstrand CJ, Hale LP, Batra Sk, et al. Monoclonal antibodies against EGFRvIII are tumor specific and react with breast and lung carcinomas and malignant gliomas. Cancer Res 1995; 55: 3140-3148
3. Choe G, Horvath S, Cloughesy TF, et al. Analysis of the phosphatidylinositol 3'-kinase signaling pathway in glioblastoma patients in vivo. Cancer Res 2003; 63:2742-6.
4. Perren A, Weng LP, Boag AH, et al. Immunohistochemical evidence of loss of PTEN expression in primary ductal adenocarcinomas of the breast. Am J Pathol 1999; 155:1253-1260.

Supplementary Table 1. **UCLA Patients with <25% tumor growth or shrinkage on EGFR kinase inhibitor therapy**

Patient No.	Sex	Age	Diagnosis	Drug	Dose (mg)	EIAED	EGFR FISH	EGFR vIII* IHC	PTEN** IHC	TTP (months)	TTS (months)
27	M	58	GBM	Gefitinib	500-750	no	AMP	neg	loss	3.3	7.4
28	M	63	GBM	Gefitinib	500-1000	no	AMP	neg	loss	4.4	8.7
29	M	61	GBM	Erlotinib	150	no	POLY	neg	no loss	2.8	12.4
30	F	45	GBM	Erlotinib	300-500	yes	AMP	pos	loss	3.7	13.7
31	M	61	GBM	Erlotinib	150	no	AMP	pos	loss	4.6	12.9
32	F	67	GBM	Gefitinb	500	yes	POLY	pos	loss	2.5	6.9
33	M	48	GBM	Gefitinib	500-1000	no	NT	neg	loss	3.7	11.3
34	M	57	GBM	Gefitinib	500-1000	no	POLY	neg	loss	2.8	3.3
35	F	43	GBM	Gefitinib	500	no	POLY	neg	loss	4.7	5.5
36	M	47	GBM	Erlotinib	150	no	POLY	neg	no loss	5.6	8.4

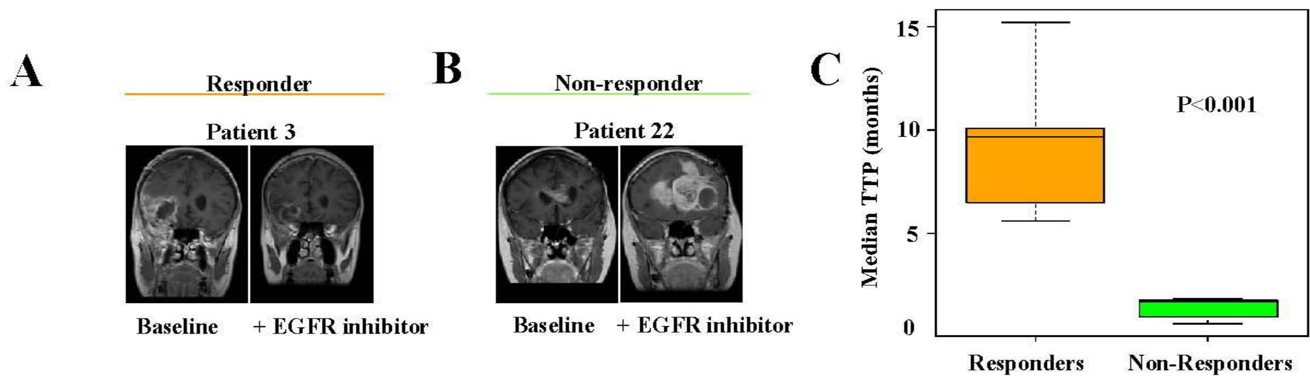
Abbreviations: M=male; F=female; GBM = glioblastoma; TTP = time to progression (months)
TTS = time to survival (months); FISH=fluorescence in situ hybridization; AMP=amplified;
NON-AMP=non-amplified/diploid; POLY=polysomy; EIAED = enzyme inducing anti-epileptic drugs; NT = no tissue available
* EGFRvIII determined by immunohistochemistry. Frozen tissue available for RT-PCR confirmation on patients 29 and 33.
**PTEN loss determined by immunohistochemistry.

Supplementary Table 2. **PTEN and EGFRvIII status in validation set**

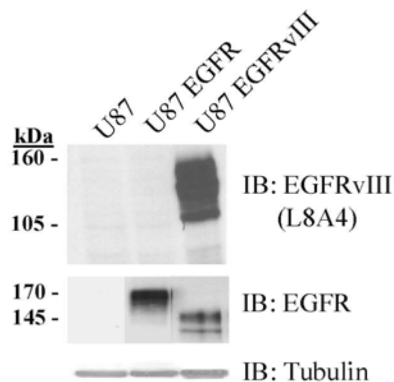
Patient No.	Sex	Age	Diagonsis	Drug	Dose (mg)	Temozolide	EIAED	PTEN IHC	EGFR vIII IHC
RESPONDERS									
1	M	50	GBM	Erlotinib	400	no	yes	no loss	positive
2	M	74	GBM	Erlotinib	150	no	no	no loss	positive
3	M	40	GBM	Erlotinib	350	no	yes	loss	positive
4	M	45	Oligo	Erlotinib	250	yes	yes	no loss	positive
5	F	56	GBM	Erlotinib	300	no	yes	loss	positive
6	M	47	AA	Erlotinib	400	no	yes	no loss	positive
7	F	50	GBM	Erlotinib	200	yes	no	no loss	positive
8	M	60	GBM	Erlotinib	500	no	yes	loss	negative
NON-RESPONDERS									
9	F	53	GBM	Erlotinib	100	yes	no	loss	positive
10	F	53	GBM	Erlotinib	100	no	yes	loss	negative
11	F	44	GBM	Erlotinib	250	no	yes	loss	positive
12	M	64	GBM	Erlotinib	400	no	yes	loss	negative
13	F	58	GBM	Erlotinib	150	no	yes	loss	negative
14	F	47	GBM	Erlotinib	250	no	no	loss	positive
15	M	65	GBM	Erlotinib	300	no	yes	loss	negative
16	F	60	GBM	Erlotinib	150	no	no	loss	positive
17	F	63	GBM	Erlotinib	100	yes	yes	loss	positive
18	F	72	GBM	Erlotinib	200	yes	yes	loss	positive
19	F	58	GBM	Erlotinib	100	no	yes	loss	negative
20	F	28	AO	Erlotinib	250	no	yes	no loss	negative
21	F	68	GBM	Erlotinib	100	no	no	loss	positive
22	F	60	GBM	Erlotinib	200	no	yes	loss	negative
23	M	38	AA	Erlotinib	100	no	no	loss	negative
24	F	42	GBM	Erlotinib	250	yes	yes	loss	negative
25	M	34	GBM	Erlotinib	450	no	yes	loss	negative
26	M	41	Oligo	Erlotinib	400	no	yes	no loss	negative
27	M	58	GBM	Erlotinib	350	no	yes	loss	negative
28	M	50	GBM	Erlotinib	150	no	no	loss	positive
29	M	53	AA	Erlotinib	200	no	no	no loss	negative
30	M	58	AA	Erlotinib	250	no	no	no loss	positive
31	F	35	GBM	Erlotinib	250	no	no	loss	negative
32	F	44	AA	Erlotinib	250	no	no	loss	positive
33	M	66	GBM	Erlotinib	500	no	yes	loss	positive

Abbreviations: M=male; F=female; GBM = glioblastoma; AO=anaplastic oligodendroglioma; AA = anaplastic astrocytoma; Oligo = oligodendroglioma
EIAED = enzyme inducing antiepileptic drugs

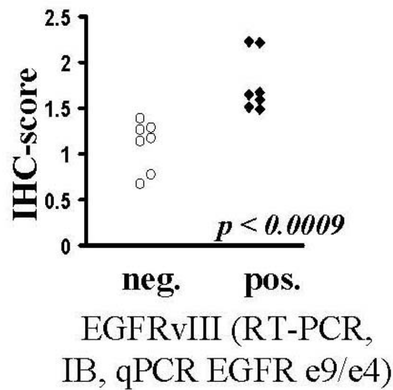
Supplementary Figures:



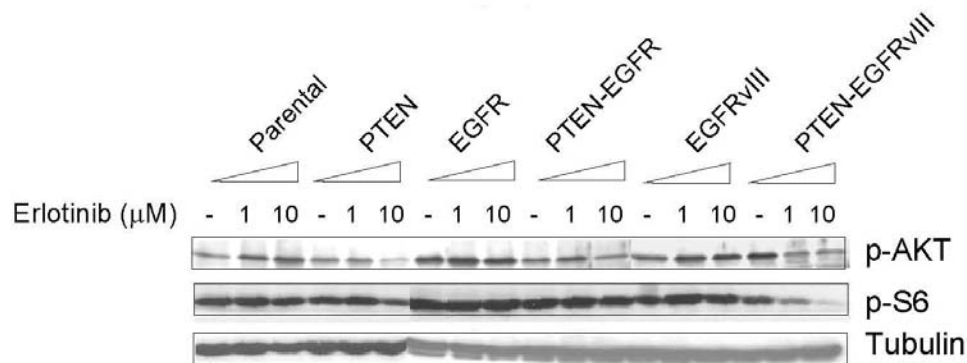
Supplementary Figure 1: Response of glioblastomas to EGFR kinase inhibitors
A. Representative MRI images to assess response to EGFR kinase inhibitors. Patient 3 had tumor shrinkage relative to baseline, as shown in this scan taken 8 weeks after starting EGFR inhibitor therapy. **B.** Patient 22 had considerable tumor growth relative to baseline, as shown in this scan taken after 7 weeks of therapy. **C.** Median time to progression (TTP) (+/- interquartile range) for recurrent malignant glioma treated with erlotinib or gefitinib, stratified by MRI response. Responders demonstrated at least 25% decrease in bi-directional tumor area; non-responders had at least 25% tumor growth while on EGFR kinase inhibitors. Patients classified as responders by MRI criteria had significantly prolonged time to progression relative to non-responder patients (p<0.001). The two anaplastic oligodendroglioma patients (1 responder, 1 non-responder) and three secondary glioblastoma patients (all three non-responders) were not included in these analyses, since these tumors may have different rates of progression than primary glioblastomas.



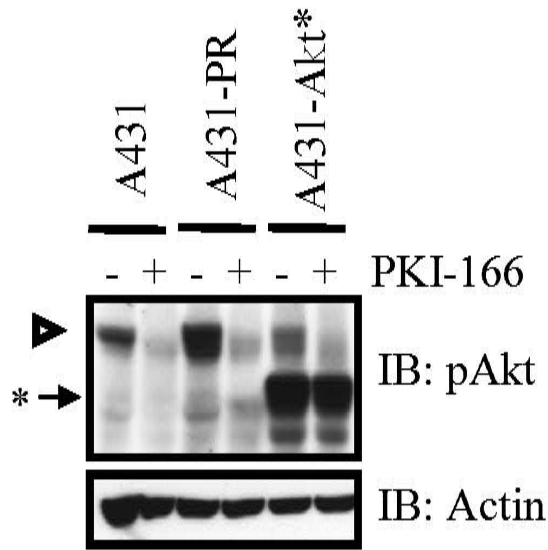
Supplementary Figure 2: Specificity of the EGFRvIII antibody: The EGFR antibody L8A4(46) (a gift from Dr. Darell Bigner) reacts with EGFRvIII, but not with full length EGFR. Whole cell lysates from three isogenic U87 GBM sublines (U87, U87-EGFR, and U87-EGFRvIII) were subjected to SDS-PAGE and probed with L8A4 (top panel), a pan-reactive EGFR/EGFRvIII antibody (middle panel), and anti-tubulin as loading control.



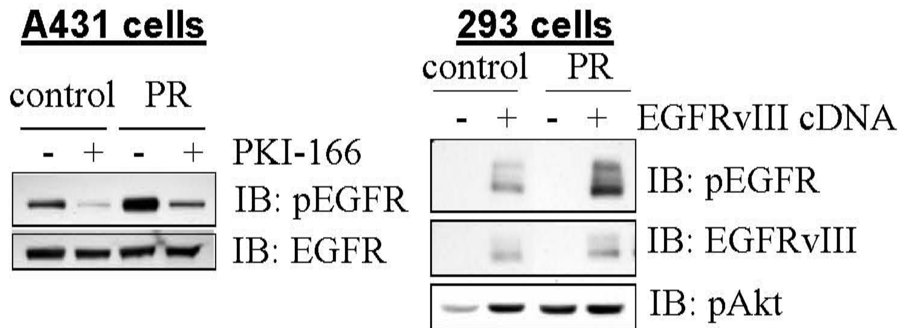
Supplementary Figure 3: Agreement between immunohistochemical and other molecular assays for detection of EGFRvIII. Immunohistochemical scores (IHC scores - Y-axis) were determined by quantitative analysis (Supplementary methods) and compared with non immunohistochemical molecular detection methods (X-axis).



Supplementary Figure 4. Analysis of phosho-Akt and phospo-S6 after Erlotinib treatment. Immunoblot analysis of phospho-Akt and phospho-S6 in U87 sublines after 24 hours of treatment with Erlotinib at a range of doses.



Supplementary Figure 5: Stable overexpression of a constitutively active Akt allele in A431 cells. Phospho$_{Ser473}$ Akt and Actin immunoblots of stable A431 sublines (vector control, PTEN RNAi, myr. Akt) 8 hours after treatment with PKI-166 (5 µM). Endogenous (arrowhead) and myristoylated Akt (asterix) alleles can be distinguished by their differential migration in the SDS-PAGE gel.



Supplementary Figure 6: Increased phosphorylation of EGFR and EGFRvIII in PTEN deficient cells. PTEN knockdown is associated with higher basal levels of EGFR/EGFRvIII phosphorylation and less complete inhibition of phosphorylation in response to equimolar concentrations of PKI-166. Immunoblots of whole cell lysates from A431 (left panel) and 293 cells transfected with EGFRvIII (right panel) were probed with anti tyrosine 1068 EGFR, total EGFR, and phosphoSer473 Akt antibodies. Stable PTEN knockdown was accomplished by PTEN shRNA (PR). Cells were treated with PKI-166 (3µM) or vehicle for 3 hours.