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CASE SERIES

Genomic Analysis of Tumors from Patients with Glioblastoma with Long-Term Response to Afatinib

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Abstract: Glioblastoma is an aggressive form of central nervous system tumor. Recurrence rates following primary therapy are high, and few second-line treatment options provide durable clinical benefit. Aberrations of the epidermal growth factor receptor (*EGFR*) gene are observed in up to 57% of glioblastoma cases and EGFR overexpression has been identified in approximately 60% of primary glioblastomas. In preclinical studies, afatinib, a second-generation ErbB blocker, inhibited cell proliferation in cells harboring mutations commonly found in glioblastoma. In two previous Phase I/II studies of afatinib plus temozolomide in patients with glioblastoma, limited efficacy was observed; however, there was notable benefit in patients with the *EGFR* variant III (*EGFRvIII)* mutation, *EGFR* amplification, and those with loss of phosphatase and tensin homolog (PTEN). This case series report details treatment histories of three long-term responders from these trials. Next-generation sequencing of tumor samples identified alterations in a number of cancer-related genes, including mutations in, and amplification of, *EGFR*. Tumor samples from all three patients shared favorable prognostic factors, eg O^6-methylguanine-DNA methyltransferase (*MGMT*) gene promoter methylation; however, negative prognostic factors were also observed, suggesting that these shared genetic features did not completely account for the favorable responses. The genetic profile of the tumor from Patient 1 showed clear differences from the other two tumors: lack of involvement of *EGFR* aberrations but with a mutation occurring in *PTPN11*. Preclinical studies showed that single-agent afatinib and temozolomide both separately inhibit the growth of tumors with a C-terminal EGFR truncation, thus providing further rationale for combining these two agents in the treatment of glioblastomas harboring EGFR aberrations. These findings suggest that afatinib may provide treatment benefit in patients with glioblastomas that harbor ErbB family aberrations and, potentially, other genetic aberrations. Further studies are needed to establish which patients with newly diagnosed/recurrent glioblastomas may potentially benefit from treatment with afatinib.

Keywords: next-generation sequencing, long-term response, *EGFR*, genetic aberrations

Introduction

Gliomas represent the most common type of malignant central nervous system tumor, among which glioblastoma is the most frequent and most aggressive form.[1,2] Recurrence rates are high in glioblastoma, despite standard treatment with surgery followed by temozolomide plus radiotherapy, and few second-line options have been identified that provide durable clinical benefit.[3] Glioblastomas are molecularly heterogeneous, with frequent genetic alterations including mutations in *TP53*, phosphatase and tensin homolog (*PTEN*), and isocitrate dehydrogenase (*IDH*), as well as methylation of the O^6-methylguanine-DNA methyltransferase (*MGMT*) promoter.[1,4] Although the precise role of these aberrations is

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unconfirmed, some associations with a poorer prognosis and/or differential responses to standard treatment have been observed.[4,5]

ErbB pathway dysregulation is also believed to play a role in glioblastoma. Indeed, mutation, rearrangement, altered splicing and/or focal amplification of the epidermal growth factor receptor (*EGFR*) gene is observed in up to 57% of glioblastoma cases.[6,7] EGFR overexpression is also identified in approximately 60% of primary glioblastomas.[8] The *EGFR* variant III (*EGFRvIII*) mutation may worsen patient prognosis.[9,10] Accordingly, various therapeutic approaches to inhibit ErbB pathway signaling have been investigated, including targeted inhibition of EGFR using small molecule tyrosine kinase inhibitors (TKIs).[7] Unfortunately, the first-generation reversible EGFR TKIs, gefitinib and erlotinib, are not clinically active as monotherapy or in combination with temozolomide or chemoradiotherapy in patients with recurrent or newly diagnosed glioblastoma.[7,11] One of the mechanisms proposed for this lack of activity is an inability of most TKIs to penetrate the blood–brain barrier, limiting effective drug delivery.[12] In one study, gefitinib reached the tumor tissue in sufficiently high concentrations to dephosphorylate EGFR, but not to regulate activation of downstream signal transducers.[13]

Afatinib is a second-generation ErbB family blocker that irreversibly inhibits EGFR, HER2, and HER4, and blocks transphosphorylation of HER3; thus, it has the capacity to block signaling via all ErbB family homo- and heterodimers.[14,15] In preclinical studies, afatinib inhibited the proliferation of cells harboring mutations that are commonly found in glioblastoma, including *EGFRvIII* and *R108K*.[15] Moreover, preclinical and clinical evidence in non-small cell lung cancer (NSCLC) suggests that afatinib can penetrate the blood–brain barrier at concentrations that are sufficient to inhibit tumor growth.[16] Afatinib has been assessed in two clinical trials in patients with glioblastoma: a Phase I/II study of afatinib with or without temozolomide in recurrent glioblastoma (NCT00727506; Study 1200.36), and a Phase I study of afatinib plus radiotherapy, with or without temozolomide in newly diagnosed glioblastoma (NCT00977431; Study 1200.38). Overall results from both studies have been published previously.[17,18] In the recurrent glioblastoma trial, limited efficacy was observed with afatinib, either alone or in combination with temozolomide, in unselected patients;[17] however, there was evidence that certain patient populations may show better responses. Progression-free survival (PFS) with afatinib plus temozolomide was longer in patients with tumors that were highly immunoreactive for the *EGFRvIII* mutation, compared with those with *EGFRvIII*-negative tumors (median: 3.65 vs 1.05 months). Median PFS associated with afatinib plus temozolomide in patients with *EGFR* amplification or loss of PTEN was 2.73 months for both subgroups; in the same subgroups, median PFS associated with temozolomide monotherapy was 1.02 and 1.87 months, respectively.[17] In the 1200.38 study, the median time to disease progression among patients who received afatinib plus radiotherapy with and without temozolomide was 14.3 months and 6.9 months, respectively.[18]

One particular case of a 58-year-old female patient with multifocal primary glioblastoma who experienced prolonged benefit with afatinib plus temozolomide in Study 1200.36 has been published previously: this patient had several *EGFR* aberrations, including *EGFR* gene amplification and *EGFRvIII* mutation positivity, and, at the time of publication, remained on treatment after 63 cycles.[19]

Given the observed heterogeneity in responses to afatinib in glioblastoma, we used next-generation sequencing (NGS) to investigate potential links between cancer gene mutation profiles and long-term response to afatinib treatment in selected patients from the 1200.36 and 1200.38 trials.[17,18] In this report, we describe two cases from the newly diagnosed glioblastoma trial (Study 1200.38) with long-term responses to afatinib,[18] and provide an update of over 2 years of additional follow-up to the previously reported case from Study 1200.36.[19] In addition, we present preclinical data supporting the anti-tumor activity of afatinib in a patient-derived xenograft model of glioblastoma.

Materials and Methods

Among 36 patients enrolled in Study 1200.38, five patients with a long-term response (> 12 months) to afatinib were identified; of these, tumor samples were available from two patients, taken at the time of diagnosis. Additionally, tumor samples were available from the patient previously described as having a sustained response to afatinib plus temozolomide in Study 1200.36.[19] For this patient, tumor samples were obtained at diagnosis and upon surgical resection following subsequent progressive disease after many cycles of afatinib treatment. FoundationOne™ NGS was performed on DNA isolated from the specimens available at diagnosis. The second sample from the patient involved in Study

1200.36 was analyzed by whole-exome sequencing. Both Study 1200.36 and Study 1200.38 were conducted in accordance with the Declaration of Helsinki. All three patients provided written informed consent for the subsequent molecular profiling of their tumors and agreed to the publication of the data.

Afatinib was also evaluated preclinically in nude mice carrying subcutaneously implanted glioblastoma patient-derived GB136 xenografts, characterized by a C-terminal EGFR truncation (provided by V. Peter Collins).[20] Mice were treated with single-agent afatinib (10 mg/kg orally [P.O.] once daily [QD]), temozolomide (50 mg/kg P.O. on Days 1–5) or vehicle control for 30 days. Afatinib was dissolved in 2% HP-ß-CD, 5% acetic acid (10%), and Natrosol (0.5%). Dose levels are given in terms of the free base of the active ingredient. Temozolomide was dissolved in Natrosol (0.5%) and administered five times per week. Each group included 10 animals, each with one subcutaneously implanted tumor piece (2x2x2 mm^3). Mice were inspected daily for abnormalities. Body weight and tumor growth were monitored three times per week (Mon, Weds, Fri) by bilateral caliper measurements. The tumour volume (V) was calculated using the formula: $V = (\text{length} \times \text{width}^2) \times \pi/6$. Median tumor volume was plotted over time. Animals were euthanized at the end of the study when control tumors reached a size of approximately 1200 mm^3 on average. Tumor growth inhibition (TGI) values were calculated as follows: $TGI = 100 \times (1 - (\text{Volume}_{\text{Treated Day 30}} - \text{Volume}_{\text{Treated Day1}})/(\text{Volume}_{\text{Control Day 30}} - \text{Control}_{\text{day 1}})$. For statistical analysis, pairwise comparisons between the groups were performed by Kruskal–Wallis test followed by Dunn's multiple comparisons test. $P < 0.05$ was considered as significant. GraphPad Prism software was used for statistical analyses.

Case 1 (Study 1200.38)

Presentation

A 62-year-old male initially presented with a seizure, and imaging revealed a left frontal lesion. Over a 3-month period, the observed lesion initially regressed without treatment, and later progressed, leading to a generalized tonic-clonic seizure. He underwent craniotomy with intraoperative smear cytology in February 2011, at which time the diagnosis of glioblastoma was made. *MGMT* promoter methylation was detected. No mutations affecting *IDH1/2* were detected (Table 1). Further details of mutational analyses in all patients are provided in the next-generation sequencing analysis section of this report.

Treatment and Response

Scans at time-points throughout treatment are shown in Figure 1A-G. The patient received afatinib (P.O. 20 mg QD) plus radiotherapy (60 Grays [Gy] in 30 fractions over 6 weeks) and temozolomide (P.O. 75 mg/m^2 QD) for 6 weeks, starting in March 2011, followed by afatinib (40 mg QD) monotherapy for approximately 4 weeks from 06 May 2011. He then received treatment with afatinib (40 mg QD) plus temozolomide (150 [Cycle 1] to 200 mg/m^2 [Cycle 2]; Days 1–5 of each 28-day cycle) from 31 May 2011. The temozolomide dose was reduced to 100 mg/m^2 after 2 cycles due to Grade 3 thrombocytopenia, and he continued on afatinib (40 mg QD) plus temozolomide (100 mg/m^2; Days 1–5 of each 28-day cycle) from Cycles 3 to 6 until November 2011. Subsequently, the patient received afatinib (40 mg QD) monotherapy, which was continued beyond the end of the 1200.38 trial in August 2017, as part of a Named Patient Use program.

The patient had tumor shrinkage ≥ 50%, which was achieved in January 2012. At the patient's last scan during the trial (August 2017), the patient showed a complete response and remained on afatinib 40 mg after the trial. The patient discontinued the trial in August 2017. As of July 2020, the patient was well and continued to show a complete response based on local neurology review.

Most adverse events (AEs) experienced by this patient during the 1200.38 trial were Grade 1 or 2 in intensity, with the exception of Grade 3 seizure, lower respiratory tract infection, and thrombocytopenia, which were unrelated to study treatment. Treatment-related rash and diarrhea resolved with supportive care.

Table 1 Next-Generation Sequencing Analysis of Primary Tumors from Patients with Long-Term Responses to Afatinib

	Patient 1 (First-Line, ≥2340 Days[a])	Patient 2 (First-Line 664 Days)	Patient 3 (Second-Line, ≈2300 Days)
EGFR gene amplification	No	Yes (estimated copy number = 115)	Yes (estimated copy number = 60)
EGFRvIII deletion	No	Yes, extensive	Yes, low level
EGFR point mutations	No	No	Yes (D247Y amplified, P596L, G598V non-amplified)
PTPN11/SHP2 mutation	Yes (S502L)	No	No
CDKN2A/2B loss	Yes	Yes	Yes
PTEN	No	No	Yes, R130 premature stop mutation
PIK3CA mutation	Yes, Q546E	No	No
BCOR/BCORL1	Yes (BCOR: G1588fs)	No	Yes (BCORL1: T1111M)
RPTOR	Yes (A862T)	Yes (A953T)	No
Other point mutations	NF1 ARID1A GNAS KDR RAD52 SH2B3 ZNF703	AR DDR2 EPHB1 FLT1 MAP3K13 PRKDC	BAP1 C17orf39 CDH1 EPHA5 ESR1 GRIN2A MAP3K1 NOTCH3 STAG2

Notes: [a]Patient remained on afatinib treatment after the 2340 days of recorded treatment during the 1200.38 trial.

Abbreviations: AR, androgen receptor; ARID1A, AT-rich interactive domain-containing protein 1A; BAP1, breast cancer type 1-associated protein-1; BCOR, BCL6 co-repressor; BCORL1, BCL6 co-repressor-like 1; C17orf39, chromosome 17 open reading frame 39; CDH1, cadherin 1; CDKN2A/2B, cyclin-dependent kinase inhibitor 2A/2B; DDR2, discoidin domain-containing receptor tyrosine kinase 2; EGFR, epidermal growth factor receptor; EGFRvIII, epidermal growth factor receptor variant III; EPHA5, ephrin type-A receptor 5; EPHB1, ephrin type-B receptor 1; ESR1, estrogen receptor 1; FLT1, Fms related tyrosine kinase 1; GNAS, guanine nucleotide-binding protein, alpha stimulating; GRIN2A, glutamate receptor ionotropic, NMDA 2A; KDR, knock down resistance gene; MAP3K13, mitogen-activated protein kinase kinase kinase 13; MAP3K1, mitogen-activated protein kinase kinase kinase 1; NF1, neurofibromatosis type 1; NOTCH3, notch receptor 3; PIK3CA, phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha; PRKDC, protein kinase, DNA-activated, catalytic subunit; PTEN, phosphatase and tensin homolog; PTPN11/SHP2, protein tyrosine phosphatase non-receptor type 11; RAD52, DNA repair protein RAD52 homolog; RPTOR, regulatory-associated protein of mammalian target of rapamycin; SH2B3, SH2B adapter protein 3; STAG2, stromal antigen 2; ZNF703, zinc finger protein 703.

Case 2 (Study 1200.38)

Presentation

A 60-year-old female, presenting with a 3-week history of word-finding difficulties and dysphasia, was diagnosed with left parietal glioblastoma in August 2012. On enrollment to the 1200.38 trial, she had symptoms of Grade 1 dysphasia, hemianopia, and short-term memory impairment. *MGMT* promoter methylation was detected. No *IDH1/2* mutations were detected (Table 1).

Treatment and Response

Scans at time-points throughout treatment are shown in Figure 2A-E. The patient underwent total resection in August 2012 and received afatinib (30 mg QD) plus radiotherapy (60 Gy/30 fractions over 6 weeks) and temozolomide (75 mg/m^2 QD) for 6 weeks, starting in September 2012, followed by afatinib (30 mg QD) monotherapy for approximately 4 weeks starting in October 2012. She then received afatinib (30 mg QD) plus temozolomide (150 mg/m^2

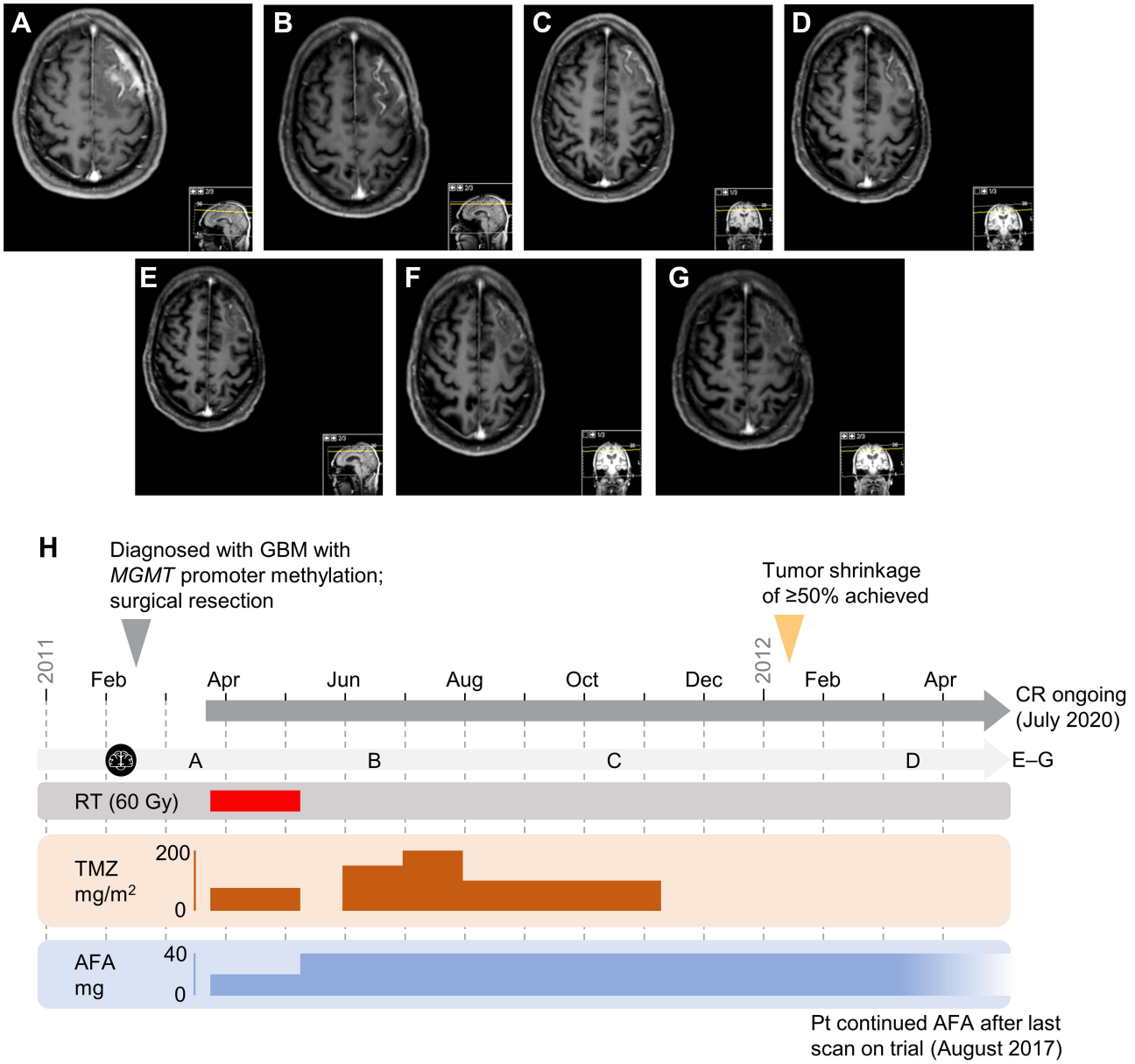
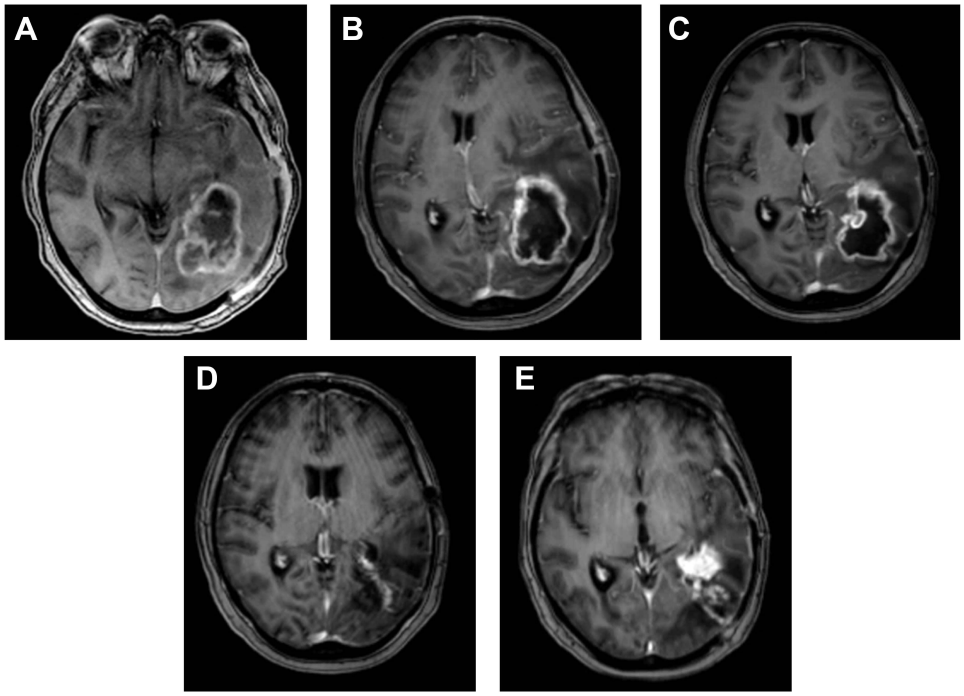


Figure 1 Radiological Disease Assessment in Patient 1. (**A**) March 2011: Scan taken at entry (baseline) for trial 1200.38. Treatment with afatinib (20 mg QD), RT (60 Gy in 30 fractions over 6 weeks) and TMZ (75 mg/m^2 QD) commenced in the same month. From 06 May 2011, the patient received afatinib (40 mg QD) monotherapy for approximately 4 weeks. The patient then began six cycles of afatinib (40 mg QD) with TMZ (100–200 mg/m^2 for Days 1–5 of each 28-day cycle) on May 31, 2011. (**B**) June 2011: Scan taken during Cycle 1, after 10 weeks of treatment with afatinib ± TMZ. (**C**) October 2011: Scan taken during Cycle 5 of 6 with afatinib with TMZ QD (Days 1–5 per cycle). The patient then received afatinib monotherapy from November 2011. (**D**) March 2012: Scan taken during Cycle 10, after approximately one year of treatment. Tumor shrinkage ≥ 50% was achieved in January 2012 and maintained beyond March 2015. (**E**) April 2013 (Cycle 24). (**F**) March 2014 (Cycle 36). **(G)** March 2015 (Cycle 48). Patient continued afatinib monotherapy as part of a Named Patient Use program after August 2017. As of July 2020, the patient was well and continued to show a complete response based on local neurological review. (**H)** Timeline of treatment and response. Horizontal dark gray arrow represents time on treatment before disease progression. The dates of the scans shown in panels (**A-G)** are illustrated on the light gray horizontal arrow, which is labelled with the black, circular scan icon.
Abbreviations: AFA; afatinib; CR, complete response; GBM, glioblastoma; Gy, Grays; *MGMT*, O^6-methylguanine-DNA methyltransferase (use of italics denotes reference to a gene); Pt, Patient; RT, radiotherapy; TMZ, temozolomide.

[Cycle 1]; Days 1–5), followed by afatinib (40 mg QD) plus temozolomide (200 mg/m^2 [Cycles 2–6]; Days 1–5). The patient then continued on afatinib monotherapy (40 mg QD) from May 2013 until progression.

The patient had tumor shrinkage of ≥ 50%, which was achieved in August 2013 and maintained until July 2014; overall, the patient benefited from afatinib for a total of 22 months. At recurrence, she underwent repeat resection and commenced postoperative lomustine (CCNU).



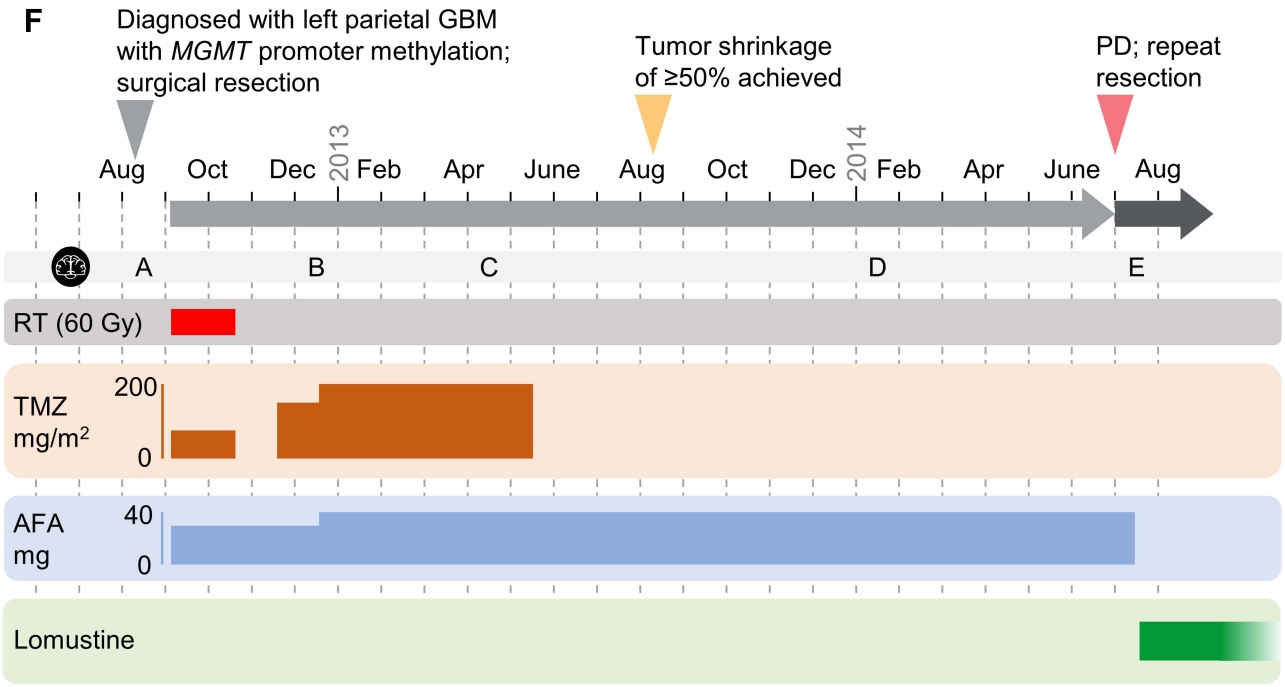


Figure 2 Radiological Disease Assessment in Patient 2. (**A**) August 2012: Scan taken at entry (baseline) for trial 1200.38, following surgical resection in the same month. From September 2012, the patient received 6 weeks of treatment with afatinib (30 mg QD) with RT (60 Gy in 30 fractions over 6 weeks) and TMZ (75 mg/m^2 QD), then four weeks with afatinib (30 mg QD) monotherapy. (**B**) December 2012: patient imaged during Cycle 1 of 6 of treatment with afatinib (30–40 mg QD) with TMZ (150–200 mg/m^2 on Days 1–5 of each 28 day cycle). (**C**) April 2013 (Cycle 5). Following Cycle 6, the patient received afatinib 40 mg QD monotherapy from May 2013. Patient achieved tumor shrinkage ≥ 50% in August 2013. (**D**) January 2014 (Cycle 15). (**E**) July 2014 (Cycle 21): after 22 months of benefit from afatinib, the patient experienced recurrence. Subsequently, the patient underwent repeat resection and commenced post-operative treatment with lomustine (CCNU). (**F**) Timeline of treatment and response. Horizontal gray arrows represent time on treatment before disease progression. The dates of the scans shown in panels (A-E) are illustrated on the light gray horizontal bar (with black, circular scan icon).

Abbreviations: AFA; afatinib; GBM, glioblastoma; Gy, Grays; *MGMT*, O^6-methylguanine-DNA methyltransferase (use of italics denotes reference to a gene); PD, progressive disease; RT, radiotherapy; TMZ, temozolomide.

AEs experienced during the afatinib plus temozolomide trial were all Grade 1 or 2 in intensity and consisted mainly of gastrointestinal and skin toxicities, and mild infections, which in some cases required supportive therapy (diarrhea) or treatment (infections).

Case 3 (Study 1200.36)

Presentation

Patient 3 was a 58-year-old female who presented with constant right frontal headache, mild weakness of the left side, gait disturbance, and behavioral changes. She was diagnosed with multifocal primary glioblastoma in October 2009. Radiological assessment indicated three lesions; a right frontal lesion, a subcortical left basal frontal area lesion, and a left inferior frontal gyrus lesion. *MGMT* promoter methylation was detected. No *IDH1/2* mutations were detected (Table 1).

Treatment and Response

Scans at time-points throughout treatment are shown in Figure 3A–G. Following surgical resection of the right frontal lesion in October 2009, the patient received first-line treatment beginning 10 November 2009, consisting of radiotherapy (60 Gy/30 fractions over 6 weeks) plus oral temozolomide (75 mg/m^2 QD) for 6 weeks, followed by adjuvant temozolomide (150–200 mg/m^2; Days 1–5 of each 28-day cycle).

The patient experienced disease progression (in left frontal lesion) in February 2010, after 3 cycles of adjuvant temozolomide. In April 2010, she was enrolled in Study 1200.36, in which she received afatinib (20–40 mg QD) plus temozolomide (50 mg/m^2 QD for 21 days of every 28-day cycle). After one cycle of second-line treatment, magnetic resonance imaging (MRI) revealed a minimal decrease in lesion size. Significant disease regression was observed after 5 cycles and was maintained for approximately 5.5 years.

AEs experienced by this patient during second-line treatment have been reported previously;[19] drug-related AEs consisted mainly of skin toxicities and diarrhea, which were managed effectively with dose reductions/interruptions and standard medications. The patient's neurologic and physical status were stable (Karnofsky Performance Status > 90) throughout second-line treatment.

Temozolomide was discontinued in March 2015 after a total of 63 cycles, and the patient received afatinib monotherapy (20 mg QD) for a further 10 months (total 74 cycles) until it was discontinued due to skin toxicity in January 2016. In March 2016, she developed progressive disease and withdrew from the trial. MRI showed evidence of a larger lesion in the right frontal lobe, which was characterized by increased enhancement and a significant increase in signal in the T2 sequence, without any evidence of significant mass effect. The lesion had not been present on the previous examination, suggesting a recurrent tumor and associated pseudo-progression. No distant metastases or neurologic symptoms were evident, and the patient was neurologically asymptomatic at follow-up on 30 March 2016.

In April 2016, the patient underwent further surgical resection, during which the second tumor sample was collected, followed by adjuvant radiotherapy (35 Gy/10 fractions) for 14 days. Executive function declined significantly after this surgery and worsened after radiotherapy. Afatinib monotherapy (30 mg QD) was resumed in June 2016 within a compassionate use program, and, between July 2016 and February 2017, MRIs showed controlled disease with radionecrosis in the opinion of the treating physician.

By February 2017, the patient had extremely limited cognitive function and was almost bedridden. She was hospitalized from early February until March 2017 due to seizures, and afatinib was continued during hospital admission. The last known afatinib dose (30 mg QD) was in March 2017, at the time of discharge. The last MRI was conducted in April 2017 and showed a progression of the pathological enhancement involving the right frontal lobe. The perfusion analysis obtained from the right frontal enhancing lesion at two different sites demonstrated low cerebral blood volume (CBV) or decreased CBV values. There was no significant mass effect. The findings were compatible with significant, asymptomatic pseudo-progression. Additionally, there was evidence of a 4 mm lesion affecting the posterior aspect of the medulla, raising the possibility of a secondary tumor localization. The patient died in June 2017 due to progressive disease, having survived for approximately 7.5 years since diagnosis.

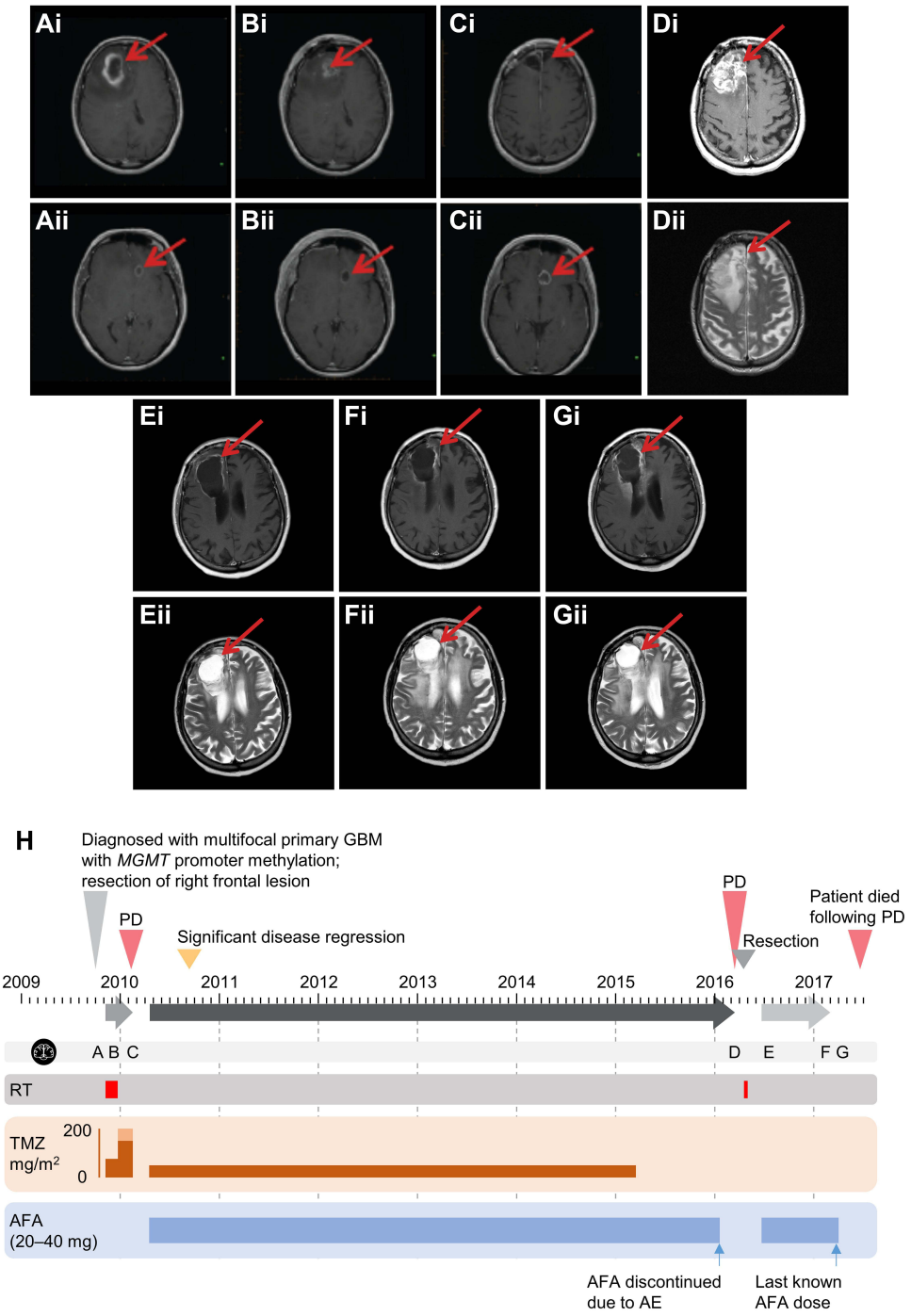


Figure 3 Radiological Disease Assessment in Patient 3. (**A**) October 11, 2009: Preoperative assessment. (**B**) October 16, 2009: Postoperative assessment. Following resection of right frontal lesion, the patient began first-line treatment with RT (60 Gy in 30 fractions over 6 weeks) with TMZ (75 mg/m^2 QD) for 6 weeks, followed by adjuvant TMZ (150–200 mg/m^2; Days 1–5 of each 28-day cycle). (**C**) February 2010: the patient experienced disease progression in February 2010 after three cycles of adjuvant TMZ. In April 2010, the patient enrolled in trial 1200.36 and began afatinib (20–40 mg QD), plus TMZ (50 mg/m^2 QD for 21 days of every 28-day cycle). Significant disease regression was observed after 5 cycles and maintained for approximately 5.5 years. TMZ was discontinued in March 2015. Afatinib was discontinued in January 2016 due to skin toxicity. (**D**) March 2016: the patient experienced disease progression, and underwent surgical resection in April 2016, followed by adjuvant RT. Afatinib monotherapy (30 mg QD) resumed in June 2016. (**E**) July 2016, and (**F**) February 2017: the patient showed controlled disease, with radionecrosis in the opinion of the treating physician. Last known afatinib dose was received in March 2017. (**G**) April 2017: final assessment. Progression was observed involving the right frontal lobe. The patient died in June 2017. (**A**–**C**) i and ii: scans at different positions. (**D**–**G**) i and ii: T1-weighted, gadolinium enhanced and T2-weighted scans, respectively, at the same position. **H** Timeline of treatment and response. Horizontal gray arrows represent time on treatment before disease progression. Red arrows indicate lesion location. The dates of the scans shown in panels (**A**–**G**) are illustrated on the light gray horizontal bar (with black, circular scan icon).

Abbreviations: AE, adverse event; AFA; afatinib; GBM, glioblastoma; Gy, Grays; *MGMT*, O^6-methylguanine-DNA methyltransferase (use of italics denotes reference to a gene); PD, progressive disease; RT, radiotherapy; TMZ, temozolomide.

Next-Generation Sequencing Analysis

Table 1 shows the key findings from the NGS analysis. The tumors from all three patients shared certain features, including *MGMT* promoter methylation, loss of cyclin-dependent kinase inhibitor 2A/B (*CDKN2A/B*), and a lack of mutation in key genes, including *TP53, IDH1/2, ErbB2*, and platelet-derived growth factor receptor A (*PDGFRA*). Tumors from Patients 1 and 2 carried point mutations in the regulatory-associated protein of mammalian target of rapamycin (*RPTOR*) gene. Patients 1 and 3 carried point mutations in the BCL6 co-repressor (BCOR)/BCL6 co-repressor-like 1 (*BCORL1*) gene. However, the complex *EGFR* genotype of the tumors from Patients 2 and 3 distinguished them from Patient 1, with these tumors showing focal amplification of *EGFR*, with concomitant deletion and amplification of the *EGFRvIII* and *D247Y* alleles, respectively. By contrast, the tumor from Patient 1 showed no evidence of *EGFR* involvement via amplification or mutation, and the key finding appeared to be the mutation in protein tyrosine phosphatase, non-receptor type, 11 (*PTPN11*).

For Patient 3, in addition to FoundationOne NGS analysis of the primary tumor, whole-exome sequencing was performed on DNA from the secondary surgical resection conducted in April 2016. Molecular analyses showed that samples from the primary and secondary surgical resections were largely genetically identical (Table 2). Minor clone(s) carrying the *EGFR* alleles *EGFRvIII, P596L*, and *G598V* identified in the primary tumor were not detectable in tumor tissue from the secondary surgical resection, but all of the remaining alleles in the original tumor were detected in the secondary sample. Of note, fewer copies of the *EGFR* gene appeared to be present in the recurrent compared with the primary tumor (20 vs 60). The secondary sample from Patient 3 was analyzed by whole-exome sequencing in order to detect novel alleles that could have led to acquired drug resistance. However, no additional mutations were detected in this sample that were considered to be clinically relevant; and as such, no novel genetic alterations that could have led to acquired resistance to afatinib could be detected.

Afatinib in a Mouse Xenograft Model of Glioblastoma

While ErbB pathway aberrations are common in glioblastoma and are thought to contribute to transformation, there are few models with proven ErbB deregulation available. The primary explant in vivo glioblastoma models GB138, GB218, and GB136 were initially established by researchers at the Ludwig Institute.[21] It was shown previously that treatment of mice carrying either GB138 (*EGFR* wild type amplification) or GB218 (*EGFRvIII* mutant) tumors with afatinib results in anti-tumor efficacy.[19,21] In this report, we demonstrate that afatinib and temozolomide both suppressed tumor growth compared with vehicle control in the GB136 xenograft model with C-terminal EGFR truncation, which is a rare *EGFR* mutation in patients with glioblastoma.[21,22] Compared to control, tumor growth inhibition was similarly significant with both treatments and involved tumor regressions (afatinib 7/10 mice; temozolomide 10/10 mice) with overall treatment/control ratios of 8% (afatinib) and 2% (temozolomide) at the end of the experiment on Day 30 (Figure 4A). Both treatments were equally well-tolerated by the mice, and there were no apparent differences in tolerability based on body weight changes (2–5% body weight loss, respectively, by the end of the experiment; Figure 4B).

Discussion and Conclusion

NGS analyses of tumor samples from these three patients with long-term responses to afatinib plus temozolomide showed alterations in a number of cancer-related genes, including mutations in, and amplification of, *EGFR*. Although all three tumors had *MGMT* promoter methylation (a favorable prognostic factor), they also had negative prognostic factors (eg, absence of *IDH* mutation, and loss of *PTEN* in one case), suggesting that these shared genetic features did not account for the favorable responses to afatinib plus temozolomide.

In Patients 2 and 3, both tumors were *EGFR*-amplified and carried an additional allele on the amplicon, namely the *EGFRvIII* mutation (Patient 2) or a point mutation in the *D247Y* allele (Patient 3), which may underlie the responses seen. *EGFRvIII* arises from an in-frame deletion, leading to translation of a truncated protein that lacks parts of the extracellular domain and is constitutively active.[23] Approximately 50% of *EGFR*-amplified glioblastomas carry the *EGFRvIII* mutation, which rarely occurs in the absence of *EGFR* amplification.[23] Although the prognostic significance of *EGFR* amplification and *EGFRvIII* mutation is controversial, *EGFRvIII*-mediated activation of cMET and other receptor

Table 2 Next-Generation Sequencing Analysis of Primary and Recurrent Tumors from Patient 3

Gene	Allele in Tumor Obtained at First Diagnosis	Evidence in Tumor Obtained at First Diagnosis	Evidence in Recurrent Tumor
EGFR	Amplification of full gene	Estimated gene copy number = 60	Amplification of full gene, estimated gene copy number = 20
EGFR	Known somatic mutation *P596L* [c.1787C>T]	2% of 6153 reads	c.1787C>T = 0.1% of 4067 reads[b]
EGFR	Known somatic mutation *G598V* [c.1793G>T]	2% of 6108 reads	c.1793G>T = 0.1% of 3788 reads[b]
EGFR	Known somatic mutation *EGFRvIII*	5 supporting reads	No evidence for *EGFRvIII*[b]
EGFR	*D247Y* [c.739G>T][a]	89% of 3206 reads	c.739G>T = 90% of 1209 reads
PTEN	Known somatic mutation *R130** [c.388C>T]	35% of 369 reads	c.388C>T = 52% of 386 reads
CDKN2A (p16)	Homozygous deletion of full gene		Homozygous deletion of full gene
CDKN2B (p15)	Homozygous deletion of full gene		Homozygous deletion of full gene
BAP1	*V447I* [c.1339G>A][a]	54% of 466 reads	c.1339G>A = 45% of 185 reads
BCORL1	*T1111M* [c.3332C>T][a]	44% of 325 reads	c.3332C>T = 48% of 121 reads
CDH1	*P30T* [c.88C>A][a]	46% of 296 reads	c.88C>A = 38% of 50 reads
EPHA5	*M987T* [c.2960T>C][a]	50% of 579 reads	c.2960T>C 48% of 462 reads
ESR1	*H6Y* [c.16C>T][a]	50% of 301 reads	c.16C>T 43% of 130 reads
GRIN2A	*C800**[c.2400C>A][a]	26% of 263 reads	C.2400C>A 32% of 149 reads
MAP3K1	*S939C* [c.2816C>G][a]	50% of 240 reads	c.2816C>G 46% of 615 reads
NOTCH3	*R1669H* [c.5006G>A][a]	19% of 409 reads	C.5006G>A 15% of 79 reads
STAG2	Splice [c.2026–1G>C][a]	32% of 457 reads	c.2026–1G>C 34% of 210 reads
IKZF1	Amplification of full gene	Estimated gene copy number = 51	Amplification of full gene, estimated gene copy number = 20

Notes: [a]Variant of unknown significance. [b]Allele not detected in recurrent tumor.

Abbreviations: BAP1, breast cancer 1-associated protein-1; BCORL1, BCL6 co-repressor-like 1; CDH1, cadherin 1; CDKN2A/2B, cyclin-dependent kinase inhibitor 2A/2B; EGFR, epidermal growth factor receptor; EGFRvIII, epidermal growth factor receptor variant III; EPHA5, ephrin type-A receptor 5; ESR1, estrogen receptor 1; GRIN2A, glutamate receptor ionotropic, NMDA 2A; IKZF1, IKAROS family zinc finger 1; MAP3K1, mitogen-activated protein kinase kinase kinase 1; NOTCH3, notch receptor 3; PTEN, phosphatase and tensin homolog; STAG2, stromal antigen 2.

tyrosine kinase signaling pathways can lead to tumor recurrence and resistance to chemoradiation.[23,24] Notably, a recent preclinical study showed that the combination of afatinib and temozolomide synergistically inhibited proliferation, survival, and invasion of *EGFRvIII*-amplified glioblastoma cells in vitro, and significantly reduced tumor growth in vivo,[24] thus supporting evaluation of afatinib plus temozolomide in *EGFR* amplified and/or *EGFRvIII*-mutant glioblastoma. Mechanistically, afatinib was shown to inhibit EGFRvIII-mediated cMET and Janus kinase/signal transducer and activator of transcription 3 (JAK2/STAT3) pathway activation, leading to enhanced temozolomide-induced cytotoxicity.[24] The *D247Y* mutation also affects the extracellular domain of EGFR, which is notable as preclinical evidence suggests that extracellular missense mutations could be a mechanism for oncogenic EGFR activation and may sensitize cells to EGFR TKIs.[25] Overall, therefore, it is likely that the *EGFR* amplification together with the *EGFRvIII* mutation or other extracellular aberrations underlie the observed sensitivity to afatinib and temozolomide in Patients 2

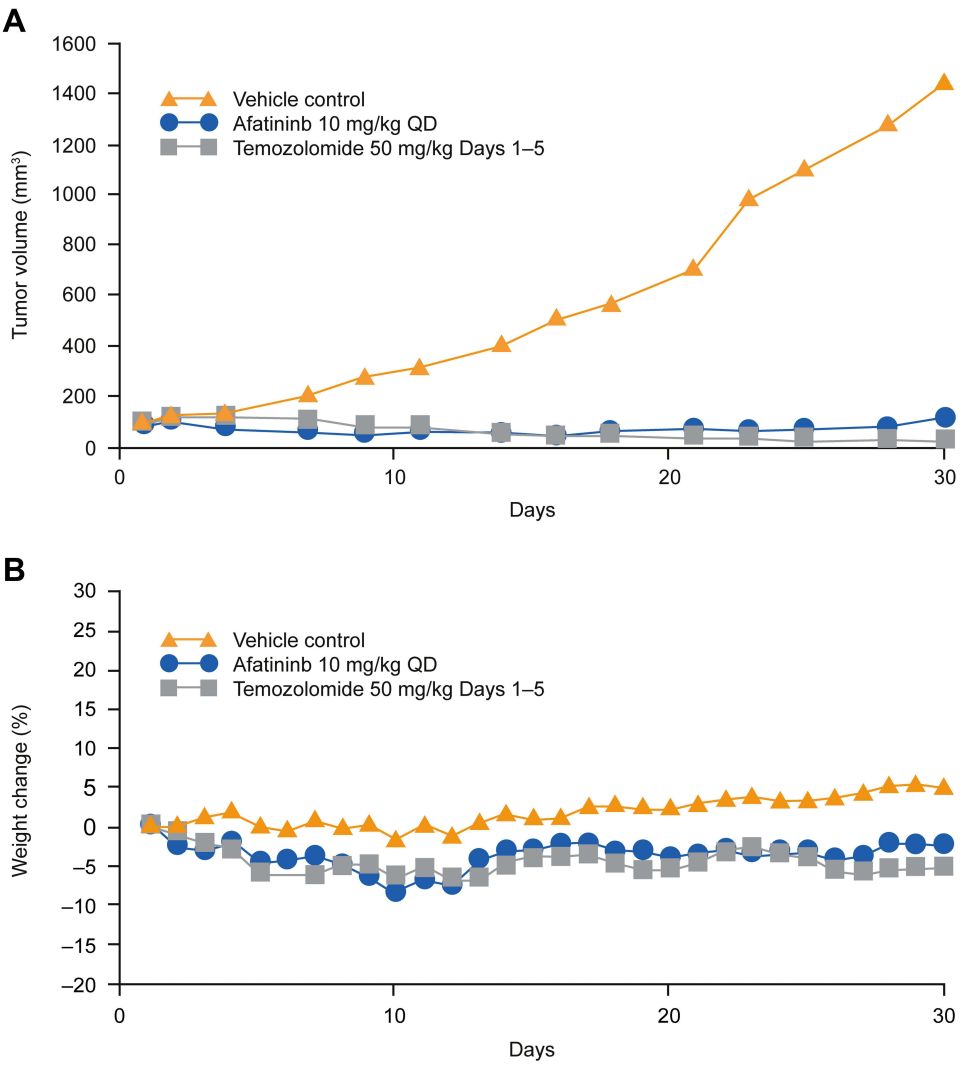


Figure 4 Tumor growth kinetics in an EGFR-mutated human patient-derived xenograft model of glioblastoma (GB136) nude mice. (**A**) Median relative tumor volume over time. (**B**) Weight change over time.
Abbreviations: EGFR, epidermal growth factor receptor (use of italics denotes reference to a gene); QD, once daily.

and 3. For one of these patients (Patient 3), the surgery and radiotherapy received prior to second-line afatinib may also have contributed to the prolonged disease-free period.

Our preclinical studies showed that single-agent afatinib and temozolomide both separately inhibited the growth of tumors with a C-terminal EGFR truncation. The deletion of exons 25 to 27 in *EGFR* observed in this model results in oncogenic isoforms of EGFR known to cause transformation.[22] Experiments in which mice were treated with both afatinib and temozolomide were not performed here. However, experiments in other preclinical models of glioblastoma indicate a possible synergy resulting from combination treatment with afatinib and temozolomide.[24]

The genetic profile of the tumor from Patient 1 showed clear differences from the other two tumors in the lack of *EGFR* aberrations but with a mutation occurring in *PTPN11*. SHP2, the protein encoded by the *PTPN11* gene is known to regulate a variety of cellular processes, including oncogenic transformation.[26,27] Gain-of-function *PTPN11* mutations promote glioblastoma cell proliferation, metastasis, and tumor growth via the extracellular signal-regulated kinase/cAMP response element binding protein (ERK/CREB) pathway.[28] Of note, SHP2 has the ability to simultaneously drive ERK1/2 and antagonize STAT3 pathways in glioma cells, thereby influencing both proliferation and resistance to EGFR/c-MET co-inhibition.[29] The extent of these SHP2 functions is diminished in glioblastoma cells that express sufficiently high levels of the *EGFRvIII* mutant, while SHP2 appears to negatively regulate EGFRvIII phosphorylation in these cells.[29] In the case of

Patient 1, however, the tumor was *EGFRvIII*-negative and was responsive to afatinib, suggesting that the *PTPN11* mutation may be contributing to this patient's sensitivity to afatinib via an *EGFRvIII*-independent mechanism.

For Patient 3, brain tumor samples from the primary and secondary surgical resections were largely genetically identical, and no new genetic alterations that could have led to acquired resistance to afatinib were detected in the recurrent sample. Interestingly, some minor clones carrying *EGFR* alleles, including *EGFRvIII*, were identified in the primary tumor but not in the recurrent tumor sample, although this may be due to the lower sensitivity of the exome analysis performed in the recurrent tumor sample in detecting these low-level variants. Nevertheless, this finding is consistent with reports that *EGFR* amplification is typically maintained between primary and recurrent glioblastomas, but that *EGFRvIII* status may change upon recurrence in a small subset of patients.[23,30]

AEs in each of the three patients were consistent with the known safety profile of afatinib, consisting mainly of skin reactions and diarrhea, and no unexpected AEs were observed. The safety profile of afatinib in combination with temozolomide was also in line with the main safety findings from the 1200.36 trial, in which the most common AEs included EGFR-TKI class-related toxicities of diarrhea and rash/acne.[17] Consistent with clinical trials and real-world studies of afatinib monotherapy in NSCLC,[31–33] drug-related AEs were generally managed effectively with dose modification and/or supportive care in each of the three patients, allowing them to remain on, and benefit from, afatinib treatment for longer.

In summary, these three patients with glioblastomas harboring certain genetic aberrations (that may contribute to EGFR addiction) achieved substantial clinical benefit from afatinib treatment. The findings suggest that afatinib may be a promising treatment for patients with glioblastomas that harbor ErbB family aberrations and, potentially, other genetic aberrations such as the *PTPN11* mutation. Further studies are needed to confirm the criteria for identifying patients with newly diagnosed and recurrent glioblastomas who may potentially benefit from treatment with afatinib.

Data Sharing Statement

To ensure independent interpretation of clinical study results and enable authors to fulfill their role and obligations under the ICMJE criteria, Boehringer Ingelheim grants all external authors access to relevant clinical study data. In adherence with the Boehringer Ingelheim Policy on Transparency and Publication of Clinical Study Data, scientific and medical researchers can request access to clinical study data after publication of the primary manuscript in a peer-reviewed journal, regulatory activities are complete and other criteria are met. Researchers should use the https://vivli.org/ link to request access to study data and visit https://www.mystudywindow.com/msw/datasharing for further information.

Ethics Approval and Consent to Participate

Study 1200.38 was approved by the Ethics Committee of the NHS Health Research Authority London (West London and Gene Therapy Advisory Committee). Study 1200.36 received approval or favorable opinion from the local or central Institutional Review Boards prior to the start of the study. Both studies were conducted in accordance with the Declaration of Helsinki. All patients provided written informed consent according to International Conference on Harmonization Good Clinical Practice and local legal regulations. All animal studies were conducted in an AAALACaccredited animal facility at Boehringer Ingelheim, Austria in accordance with institutional guidelines and EU legislation, and were approved by Austrian Authorities.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Disclosure

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