Epidermal Growth Factor Receptor Variant III (EGFRvIII) Positivity in EGFR-Amplified Glioblastomas: Prognostic Role and Comparison between Primary and Recurrent Tumors

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Abstract

Purpose: Approximately 40% of all glioblastomas have amplified the EGFR gene, and about half of these tumors express the EGFRvIII variant. The prognostic role of EGFRvIII in EGFR-amplified glioblastoma patients and changes in EGFRvIII expression in recurrent versus primary glioblastomas remain controversial, but such data are highly relevant for EGFRvIII-targeted therapies.

Experimental Design: EGFR-amplified glioblastomas from 106 patients were assessed for EGFRvIII positivity. Changes in EGFR amplification and EGFRvIII status from primary to recurrent glioblastomas were evaluated in 40 patients with EGFR-amplified tumors and 33 patients with EGFR–nonamplified tumors. EGFR single-nucleotide variants (SNV) were assessed in 27 patients. Data were correlated with outcome and validated in 150 glioblastoma patients from The Cancer Genome Atlas (TCGA) consortium.

Results: Sixty of 106 EGFR-amplified glioblastomas were EGFRvIII-positive (56.6%). EGFRvIII positivity was not associat-
ed with different progression-free or overall survival. EGFRvIII status was unchanged at recurrence in 35 of 40 patients with EGFR-amplified primary tumors (87.5%). Four patients lost and one patient gained EGFRvIII positivity at recurrence. None of 33 EGFR-nonamplified glioblastomas acquired EGFR amplification or EGFRvIII at recurrence for EGFR SNVs were frequent in EGFR-amplified tumors, but were not linked to survival.

Conclusions: EGFRvIII and EGFR SNVs are not prognostic in EGFR-amplified glioblastoma patients. EGFR amplification is retained in recurrent glioblastomas. Most EGFRvIII-positive glioblastomas maintain EGFRvIII positivity at recurrence. However, EGFRvIII expression may change in a subset of patients at recurrence, thus repeated biopsy with reassessment of EGFRvIII status is recommended for patients with recurrent glioblastoma to receive EGFRvIII-targeting agents. Clin Cancer Res; 23(22); 6846–55. ©2017 AACR.
The EGFR gene is amplified in approximately 40% of glioblastomas. About half of the EGFR-amplified tumors are positive for the tumor-specific EGFRvIII deletion variant, and EGFR single-nucleotide variants (SNV) are also commonly associated with EGFR amplification. Various novel therapeutic agents targeting overexpressed EGFR or EGFRvIII proteins are currently being developed. This study indicates that positivity for EGFRvIII and presence of one or more EGFR SNVs are not prognostic in patients with EGFR-amplified glioblastomas. In addition, we show that EGFR amplification is generally maintained between primary and recurrent glioblastomas. However, the EGFRvIII status in EGFR-amplified glioblastomas may change upon tumor recurrence in a subset of patients, suggesting a role for reassessment of the EGFRvIII status in patients with recurrent glioblastoma to receive an EGFRvIII-targeting treatment.

**Patients and Methods**

**Patients**

This study was based on 106 patients with newly diagnosed EGFR-amplified, IDH-wild-type glioblastomas. A total of 52 patients underwent second surgery for recurrent tumors. From 40 of these patients, matched tissue specimens were available from primary and recurrent tumors (Supplementary Table S1). In addition, 33 primary and recurrent tumor pairs from patients with newly diagnosed, IDH-wild-type and EGFR-nonamplified tumors were studied (Supplementary Table S2). The patients were identified in the central database of the German Glioma Network (GGN) or the database of the Central Nervous System (CNS) tumor tissue bank at the Department of Neuropathology, Heinrich Heine University (Düsseldorf, Germany). They included 74 patients from the previous GGN study on the assessment of EGFRvIII expression in glioblastoma tissues obtained at first operation (13). The EGFRvIII status of the 106 primary tumors with EGFR amplification was determined by immunohistochemistry (IHC) in 97 (91.5%) patients, by RT-PCR analysis in 88 (83.0%) patients, and by both methods in 79 (74.5%) patients. For determination of changes in EGFR amplification and protein expression as well as EGFRvIII positivity in glioblastoma patients with recurrent glioblastomas, we additionally investigated recurrent glioblastoma tissue samples from 73 of the 85 patients who had second surgery (median interval between primary and recurrent resections in the 85 patients: 9.1 months, range, 3.3–42.7 months). In 12 patients, tissue from second surgery was either not available or not sufficient for further analyses. The other 73 patients included 25 patients with EGFR-amplified and EGFRvIII-positive primary tumors, 15 patients with EGFR-amplified but EGFRvIII-negative primary tumors, and 33 patients with EGFR-nonamplified tumors. Histology of the tumors was centrally reviewed and confirmed to correspond to glioblastoma World Health Organization (WHO) grade IV, originally based on the WHO classification of central nervous system tumors 2007 (25). All cases were later on shown to correspond to glioblastoma, IDH-wild type, WHO grade IV according to the WHO Classification of Central Nervous System Tumors 2016 (26). Patients gave their written informed consent for participating in the German Glioma Network and the use of their tissue samples and clinical data for research purposes. This study was approved by the institutional review board of the Medical Faculty, Heinrich Heine University, Düsseldorf, Germany (study number 4700).

**Extraction of nucleic acids**

DNA and RNA were extracted from fresh frozen tumor tissue samples either by ultracentrifugation (27, 28) or by using the JETQUICK Tissue DNA Spin Kit (Genomed) and the RNasy Mini Kit (Qia- gen). DNA and RNA preparation from formalin-fixed and paraffin-embedded (FFPE) samples was performed with the QIAamp DNA FFPE Tissue Kit (Qiagen) and the RNasy FFPE Kit (Qiagen).

**PCR-based detection of EGFR amplification and EGFRvIII rearrangement**

Detection of EGFR gene amplification by real-time PCR was performed as reported (29–31). The following primers were used for **EGFR**: EGFR-F (5’-cactgctcatctcctcactc-3’) and EGFR-R (5’-cccagatcattctcattc-3’).
(5’-actctagctgctcagc-3’). Primers for the WI-3306 locus on 2q that served as reference locus were: WI-3306-F (5’-ctctcctgccctcagc-3’) and WI-3306-R (5’-ttgcagcagctcagc-3’). For each tumor, the target/reference gene ratio was normalized to the target/reference gene ratio of human normal brain DNA using the comparative ΔΔCt method. As positive control for EGFR gene amplification, we used tumor DNA extracted from a glioblastoma with known EGFR amplification. Only tumors showing a normalized target/reference gene ratio ≥ 3 were considered as showing EGFR gene amplification.

Detection of EGFRvIII positivity by qualitative RT-PCR has been reported elsewhere (13). The following primers located in EGFR exons 1 and 2 were used to generate products of 92 bp for the EGFRvIII and 893 bp for EGFR wild type (wt) mRNA sequences: EGFR-Ex1-F: CAGTTGGCCCTCTCCAGGAAA; EGFR-Ex5-R: CCACTCTCATAGCGTCTCGGCC. The EGFR wt product of 893 bp, however, was not obtained in each case when high molecular weight RNA extracted from frozen tissue samples was used for cDNA generation. In case of RNA extracted from FFPE tissue samples, this fragment was usually not detectable due to RNA degradation. Therefore, we used additional primers located in exon 1 (as described above) and exon 2 (EGFR-Ex2-R: CAGTTATTTGACATCTCCGGAG) generating a product of 111 bp representing EGFR wild-type sequences (Fig. 1). PCR was performed with HotStarTaq-Polymerase (Qiagen) for 15 minutes at 94°C, followed by 40 cycles for 30 seconds at 95°C, 30 seconds at 60°C (EGFR-Ex1-F, EGFR-Ex8-R), or 58°C (EGFR-Ex1-F, EGFR-Ex2-R) and 1 minute at 72°C (ref. 13; Fig. 1).

IHC for EGFR and EGFRvIII protein

IHC was performed as reported previously (13). For antigen retrieval, rehydrated sections were treated in 10 mmol/L citrate buffer at pH 6.0 (for staining with E30 or 6549 antibodies) or at pH 9.0 (for staining with DAK-H1-WT) for 20 minutes in a steamer. Sections were immunostained either with the mouse mAb DAK-H1-WT (Dako) that detects only the wild type EGFR protein (antibody dilution: 1:200), a rabbit polyclonal antisera (lot #6549, Celldes) that exclusively detects the EGFRvIII protein (antibody dilution: 1:5,000), or the monoclonal mouse antibody E30 (Dako) that detects both wild type EGFR and EGFRvIII proteins (antibody dilution: 1:200). Immunoreactivity for wild type EGFR (DAK-H1-WT, E30) was semiquantitatively scored as follows: −, negative; +, weakly positive; ++, moderately positive; +++, strongly positive (32). EGFRvIII immunoreactivity was classified as positive when immunoreactive tumor cells were detectable (irrespective of the fraction of positive tumor cells) or as negative when immunoreactive tumor cells were absent (Fig. 2). Evaluation of the IHC stainings was jointly performed by two experienced neuropathologists (I. Felsberg and G. Reifenberger).

Analysis for IDH mutation

All tumors were screened for IDH1-R132H mutation using IHC with the mAb clone H09 (Dianova; ref. 33). Tumors from patients younger than 55 years of age were additionally investigated for other IDH1 or IDH2 mutations using Sanger sequencing or pyrosequencing as reported (29, 34) and recommended in the WHO classification 2016 (26).

O6-methylguanine-DNA methyltransferase promoter methylation analysis

The O6-methylguanine-DNA methyltransferase (MGMT) promoter methylation status was determined for all tumor samples using methylation-specific PCR (MSP) analysis as reported previously (35). Tumor DNA was treated with sodium bisulfite using the EZ DNA Methylation-Gold Kit (H1SS Diagnostics). DNA from the A172 glioma cell line (obtained from the ATCC) was used as positive (methylated) control while peripheral blood leukocyte DNA served as negative (unmethylated) control. In addition, a no template DNA control was run with each experiment.

Targeted sequencing of the EGFR coding sequence

In a subset of 27 primary and recurrent paired glioblastoma samples, including 13 pairs with and 14 pairs without EGFR amplification, we performed targeted next-generation sequencing (NGS) of the EGFR coding sequence employing an amplicon-based approach for a predefined glioma gene panel and the Ion Proton sequencing platform as reported (31). In total, 59 amplions covering the entire EGFR coding region from exon 1 to exon 28 were amplified from tumor DNA and sequenced. Evaluation of the NGS data for sequence variations and copy number alterations was performed as described in detail elsewhere (31).

Evaluations involving The Cancer Genome Atlas data sets

We retrieved data from 150 IDH-wild-type glioblastoma patients with available information on EGFR amplification and EGFR single-nucleotide variants (SNV) in The Cancer Genome Atlas (TCGA) glioblastoma data set (https://portal.gdc.cancer.gov/projects/TCGA-GBM). For a subset of these patients (n = 66), the EGFRvIII status was additionally available. Information concerning age at diagnosis, gender, MGMT promoter methylation status, IDH mutation status, temozolomide therapy, and OS were retrieved from the respective TCGA publications (14, 15). EGFR copy number data (GISTIC-scores) and EGFR mutation calls (SNVs) from the TCGA-GBM data set were downloaded via the
cBio Cancer Genomics Portal (www.cbioportal.org) using the open-source R package "cgsdr" (version 1.2.5) and the statistical computing language R (version 3.3.2). Each case with at least one SNV in \textit{EGFR} was classified as "SNV in \textit{EGFR}". EGFR amplification was assumed if the GISTIC score was 2. Cases with EGFRvIII allele frequencies (AF) > 0.01 as reported by Brennan and colleagues (14) were regarded as EGFRvIII positive.

Statistical analyses
In the GGN cohort, progression-free survival (PFS) was calculated from the day of first surgery until tumor progression, death, or end of follow-up. OS was calculated from the day of first surgery until death or end of follow-up. Kaplan–Meier survival curves and log-rank test as well as Cox regression analyses were used for univariate and multivariate analyses of survival data. Statistical analyses were performed with IBM SPSS Statistics version 24.0 or the R-package "survival" (version 2.40-1). To test for associations between EGFR-SNV status and \textit{EGFR} amplification or EGFRvIII positivity, we used the Fisher exact test from the basic R-package "stats" (version 3.3.2).

Results
Prognostic significance of EGFRvIII in EGFR-amplified glioblastoma
This study is based on 106 patients with \textit{EGFR}-amplified, IDH-wild type glioblastomas documented in the GGN central database. Clinical characteristics, treatment, and outcome of this patient cohort are summarized in Table 1 according to EGFRvIII status. EGFRvIII positivity was detected in 60 of the 106 tumors (56.6%). EGFRvIII expression was detected by IHC in 49 of 97 tumors investigated (50.5%), while RT-PCR for EGFRvIII was positive in 50 of 88 tumors investigated (56.8%; Supplementary Table S1). Among the 79 tumors evaluated for EGFRvIII by IHC

Figure 2.
Representative IHC results obtained in four glioblastoma patients (A–D). Shown are histologic features in hematoxylin and eosin (H&E)-stained sections and immunostainings for pairs of primary tumor (PT) and recurrent tumor (RT) from each patient. IHC stainings were done using antibodies detecting wild-type \textit{EGFR} (EGFRwt), wild type \textit{EGFR} and EGFRvIII (EGFRwt/vIII, E30), or EGFRvIII. All four depicted tumor pairs showed \textit{EGFR} gene amplification (as demonstrated by real-time PCR) as well strong immunoreactivity for EGFRwt and EGFRwt/vIII (E30). All immunostained sections are counterstained with hemalum. Although EGFRwt expression remained similar between primary and recurrent tumors, EGFRvIII expression changed in two of the depicted pairs. A, Strong immunoreactivity for EGFRwt and EGFRvIII in the primary and recurrent tumor (patient 97). B, Expression of EGFRwt but not EGFRvIII in the primary and recurrent tumor (patient 81). C, Expression of EGFRwt and EGFRvIII in large areas of the primary tumor but loss of positivity for EGFRvIII in the recurrent tumor (patient 99). D, Only a subpopulation of EGFRvIII-positive tumor cells in the primary tumor but widespread EGFRvIII positivity in the recurrent tumor (patient 100). Scale bars, 100 \mu m.
and RT-PCR, a total of 39 tumors were EGFRVIII positive by both methods (49.4%; Figs. 1 and 2). All tumors with IHC positivity for EGFRVIII were also EGFRVIII-positive by RT-PCR, whereas 9 tumors lacked IHC EGFRVIII positivity but showed positive results by RT-PCR, thus suggesting a higher sensitivity of RT-PCR analysis (Figs. 1 and 2; Supplementary Table S1). EGFR-amplified glioblastomas generally showed strong and widespread immunopositivity with antibodies detecting wild-type EGFR proteins or both wild type EGFR and EGFRVIII (Fig. 2). In contrast, EGFRVIII immunopositivity was frequently restricted to subpopulations of tumor cells, with sometimes striking regional distribution (Supplementary Fig. S1). Patients with EGFRVIII-positive tumors were slightly older (P = 0.081) and less often had a high KPS (P = 0.253) than patients with EGFR-amplified but EGFRVIII-negative glioblastomas (Table 1). However, PFS and OS did not differ in patients with EGFR-amplified glioblastomas stratified according to EGFRVIII status (Fig. 3A and B). HR with regard to PFS and OS were determined

| Table 1. Overview of the 106 patients with newly diagnosed EGFR-amplified glioblastoma, IDH-wild-type |
|-----------------------------------------------|-----------------------------------------------|----------------|----------------|-----------------------------------------------|-----------------------------------------------|
| EGFRVIII-negative, all, n = 46 | EGFRVIII-positive, all, n = 60 | EGFRVIII-negative, no second surgery, n = 25 | EGFRVIII-positive, no second surgery, n = 29 | EGFRVIII-negative, second surgery, n = 21 | EGFRVIII-positive, second surgery, n = 31 |
| Age at diagnosis | | | | | |
| Median (years) | 59 | 63 | 60 | 63 | 58 | 60 |
| Gender | | | | | | |
| Male | 28 (60.9%) | 40 (66.7%) | 13 (52.0%) | 20 (69.0%) | 15 (71.4%) | 20 (64.5%) |
| Female | 18 (39.1%) | 20 (33.3%) | 12 (48.0%) | 9 (31.0%) | 6 (28.6%) | 11 (35.5%) |
| KPS at diagnosis | | | | | | |
| 90–100 | 21 (50.0%) | 17 (33.3%) | 12 (48.0%) | 7 (25.0%) | 9 (52.9%) | 10 (43.5%) |
| 70–80 | 18 (42.9%) | 28 (54.9%) | 11 (44.0%) | 16 (57.1%) | 7 (41.2%) | 12 (52.2%) |
| <70 | 3 (7.1%) | 6 (11.8%) | 2 (8.0%) | 5 (17.9%) | 1 (5.9%) | 1 (4.3%) |
| No data | 4 | 9 | 1 | 4 | 8 |
| Tumor location | | | | | | |
| Fetal | 12 (26.7%) | 14 (23.7%) | 5 (20.0%) | 7 (24.1%) | 7 (33.3%) | 7 (23.3%) |
| Temporal | 11 (23.9%) | 12 (20.3%) | 6 (24.0%) | 6 (20.7%) | 5 (23.8%) | 6 (20.0%) |
| Parietal | 6 (13.0%) | 9 (15.3%) | 3 (12.0%) | 5 (17.2%) | 3 (14.3%) | 4 (13.3%) |
| Occipital | — | 4 (6.8%) | — | 1 (3.4%) | — | 3 (10.0%) |
| Not localized to one site | 14 (30.4%) | 14 (23.7%) | 8 (32.0%) | 6 (20.7%) | 6 (28.6%) | 8 (26.7%) |
| Multifocal | — | 1 (1.7%) | — | 1 (3.4%) | — | — |
| Others | 3 (6.5%) | 5 (8.5%) | 3 (12.0%) | 3 (10.0%) | — | 2 (6.7%) |
| No data | 1 | | | | | |
| Surgery | | | | | | |
| Gross total resection | 26 (60.5%) | 21 (43.8%) | 12 (50.0%) | 7 (30.4%) | 14 (73.7%) | 14 (56.0%) |
| Subtotal resection (50%–99%) | 12 (27.9%) | 22 (45.8%) | 9 (37.5%) | 13 (56.0%) | 5 (25.6%) | 9 (36.0%) |
| Partial resection (<50%) | 3 (7.0%) | 5 (10.4%) | 2 (8.3%) | 3 (15.0%) | 1 (5.3%) | 2 (8.0%) |
| Biopsy | 2 (4.7%) | — | 1 (4.2%) | — | 1 (5.3%) | — |
| No data | 3 | 12 | 1 | 6 | 2 | 6 |
| Histologic subtype | | | | | | |
| Glioblastoma, IDH-wild-type | 46 (100%) | 59 (98.3%) | 25 (100.0%) | 28 (96.6%) | 21 (100%) | 31 (100%) |
| Gliosarcoma, IDH-wild-type | — | 1 (1.7%) | — | 1 (3.4%) | — | — |
| MGMT promoter methylation status | | | | | | |
| Methylated | 18 (39.1%) | 30 (50.0%) | 9 (36.0%) | 18 (62.1%) | 9 (42.9%) | 12 (38.7%) |
| Unmethylated | 28 (60.9%) | 30 (50.0%) | 16 (64.0%) | 11 (37.9%) | 12 (57.1%) | 19 (61.3%) |
| First-line treatment | | | | | | |
| Radiotherapy alone | 9 (19.6%) | 13 (21.7%) | 9 (36.0%) | 7 (24.1%) | — | 6 (19.4%) |
| TMZ/radiotherapy — TMZ* | 34 (73.9%) | 43 (71.7%) | 15 (60.0%) | 19 (65.5%) | 19 (90.5%) | 24 (77.4%) |
| Patients with information on number of TMZ cycles | 28/35 | 30/43 | 15/25 | 14/29 | 14/29 | 17/24 |
| Chemotherapy alone | 1 (2.2%) | — | — | 1 (4.8%) | — | — |
| No therapy | 2 (4.3%) | 4 (6.7%) | 1 (4.0%) | 3 (10.3%) | 1 (4.8%) | 1 (3.2%) |
| PD (events) | | | | | | |
| Salvage chemotherapy | 21 (91.3%) | 19 (76.0%) | 9 (100.0%) | 5 (83.3%) | 12 (85.7%) | 14 (73.7%) |
| Salvage radiotherapy | 1 (4.3%) | 2 (8.0%) | — | 1 (16.7%) | 1 (7.1%) | 1 (5.3%) |
| Salvage radio-/chemotherapy | 1 (4.3%) | 1 (4.0%) | — | 1 (7.1%) | — | 1 (5.3%) |
| Other | — | 3 (12.0%) | — | — | — | 3 (15.8%) |
| Survival | | | | | | |
| Median PFS (months, 95% CI) | 8.2 (6.9–10.5) | 8.7 (6.9–10.5) | 8.2 (6.4–10.0) | 7.4 (5.9–8.9) | 7.7 (3.0–12.4) | 9.6 (8.1–11.1) |
| Median OS (months, 95% CI) | 17.0 (10.0–23.9) | 16.8 (13.6–20.1) | 13.3 (11.5–15.0) | 9.9 (4.0–15.7) | 21.3 (17.6–25.0) | 24.0 (15.6–32.5) |
| Follow-up range (months) | 7.5–26.9 | 10.7–94.6 | — | 94.6 | 7.5–26.9 | 10.7–48.4 |
| Alive at last follow-up | 3 (6.5%) | 6 (10.0%) | — | 1 (3.5%) | 3 (14.5%) | 5 (16.1%) |

NOTE: Patient characteristics are stratified according to EGFRVIII status at first surgery and according to treatment by second surgery. Abbreviations: CI, confidence interval; KPS, Karnofsky Performance Score; PD, progressive disease; TMZ, temozolomide; TMZ/radiotherapy — — TMZ, radiotherapy with concomitant and maintenance temozolomide.

*Includes 6 patients who received radiotherapy and adjuvant temozolomide.
HR, 0.91; 95% confidence interval (CI), 0.61–1.36; P = 0.644; HR, 1.05; 95% CI, 0.70–1.58; P = 0.798). HRs were observed in the same order after adjustment for MGMT promoter methylation and first-line therapy (HR, 0.88; 95% CI, 0.59–1.32; P = 0.539 for PFS and HR, 1.03; 95% CI, 0.69–1.55; P = 0.878 for OS). MGMT status was not associated with MGMT promoter methylation status (P = 0.265). MGMT promoter methylation, but not EGFRvIII expression, was associated with longer OS in the 77 patients treated with radiotherapy and temozolomide chemotherapy (Supplementary Fig. S2A and S2B). Further stratification of these patients according to EGFRvIII status and MGMT promoter methylation revealed that MGMT promoter methylation was associated with longer OS in patients with EGFRvIII-negative tumors but was not prognostic in patients with EGFRvIII-positive glioblastomas (Supplementary Fig. S2C–S2D).

Changes in EGFRvIII expression between paired primary and recurrent glioblastomas

We investigated 40 glioblastoma patients with EGFR-amplified primary tumors who were treated by second surgery at progression, and from whom representative tissue sections with viable tumor tissue were available from both primary and recurrent tumors. Of these, 25 patients had EGFRvIII-positive primary tumors as detected by RT-PCR, IHC, or both methods (Supplementary Table S1). Important patient characteristics are summarized in Table 1. Compared with patients who did not receive second surgery, patients with second surgery had more often received a gross total resection (28/44 patients vs. 19/47 patients, P = 0.027) and radiotherapy with concomitant and maintenance temozolomide chemotherapy (temozolomide/radiotherapy—temozolomide) as first-line treatment (43/52 patients vs. 34/54 patients, P = 0.023), but clinical characteristics were otherwise similar. One of the initially 15 EGFRvIII-negative tumors (by both IHC and RT-PCR) became EGFRvIII-positive at recurrence by RT-PCR but not by IHC (Fig. 1, patient 79). Among the recurrent tumors of the 25 initially EGFRvIII-positive patients undergoing second surgery, 21 patients (84%) retained EGFRvIII expression as detected by IHC (15 patients, 71%), RT-PCR (18 patients, 86%), or both methods (12 patients, 57%; Figs. 1 and 2; Supplementary Table S1). In four patients, recurrent glioblastomas lost EGFRvIII positivity as determined by both methods for three patients and by IHC for one patient (Figs. 1, 2, and 4; Supplementary Table S1). There were overall no major differences in clinical characteristics as well as PFS, OS, and postrecurrence survival between patients who did or did not receive a second operation when each group was stratified according to EGFRvIII status (Supplementary Fig. S3).

To evaluate whether EGFRvIII-negative and EGFR-nonamplified glioblastomas may newly acquire EGFRvIII positivity and/or...
SNVs were found in 8 of 13 when compared with RT-PCR analysis (8/10 investigated tumors also detected EGFRvIII, however, at somewhat lower sensitivity studies as summarized in Supplementary Table S3. NGS analysis by NGS, the individual SNVs identified in our cohort of 27 patients (Supplementary Fig. S4).

EGFR missense mutation was not associated with distinct OS in glioblastomas. Recurrent tumors in 7 of 15 initially EGFRvIII-positive tumors. In contrast, other authors detected no loss of EGFRvIII positivity after tumor recurrence following standard radiochemotherapy in 15 of 15 patients with EGFRvIII-positive glioblastomas, while 16 of 16 patients treated with anti-EGFRvIII vaccination demonstrated no more EGFRvIII expression upon tumor recurrence (24). In line with the findings in the GGN cohort, interrogation of the TCGA database showed no OS difference in a cohort of 150 IDH-negative glioblastomas vs. 6/71 EGFRvIII-nonamplified glioblastomas (11, 17, 51). We did not assess regional heterogeneity of EGFR gene amplification. However, previous studies reported that EGFR amplification may be restricted to subpopulations of tumor cells, as determined in cases of glioblastomas with amplification of EGFR and PDGFRA (52, 53). With respect to EGFRvIII expression, our data demonstrate that EGFRvIII immunopositivity shows marked regional heterogeneity and is often restricted to subpopulations of tumor cells in glioblastomas, thus confirming previous findings in the GGN patient cohort (17) and in several independent studies (39, 42, 43, 51, 54).

We also addressed the clinically relevant question whether EGFR amplification and EGFRvIII expression may change from primary to recurrent glioblastomas following standard therapy. Montano and colleagues (55) reported on a trend toward lower expression of EGFRvIII in recurrent as opposed to corresponding primary glioblastomas based on the analysis of 13 patients. Van den Bent and colleagues (23) investigated matched pairs of primary and recurrent glioblastomas from 55 patients, including 23 patients with tumors demonstrating high-copy EGFR amplification, and found that the EGFR amplification status remained stable in 46 of 55 patients (84%). EGFRvIII mRNA expression as determined by RT-PCR was found to be lost from primary to recurrent tumors in 7 of 15 initially EGFRvIII-positive tumors. In contrast, other authors detected no loss of EGFRvIII positivity upon tumor recurrence following standard radiochemotherapy in 15 of 15 patients with EGFRvIII-positive glioblastomas, while 16 of 16 patients treated with anti-EGFRvIII vaccination demonstrated no more EGFRvIII expression upon tumor recurrence (24).
our study, we evaluated EGFR amplification and expression at the DNA and protein levels, as well as EGFRvIII expression at the mRNA and protein levels. Thereby, we clearly demonstrated that EGFR amplification and the associated overexpression of EGFR protein in IDH-wild type glioblastomas generally remain stable upon recurrence following standard therapy. In addition, investigation of 33 patients with EGFR nonamplified primary glioblastomas, IDH-wild type, did not reveal a single patient whose tumor newly acquired EGFR amplification upon recurrence. EGFRvIII positivity persisted from primary to recurrent glioblastomas in 21 of 25 patients (84%) with initially EGFRvIII-positive tumors. However, glioblastomas in four patients had lost their initial EGFRvIII positivity upon recurrence, whereas a single patient with an EGFR-amplified glioblastoma showed EGFRvIII positivity only in the recurrent tumor (Fig. 4). The reason for the lower rate of tumors that lost EGFRvIII positivity upon recurrence in our cohort, as compared with the study of van den Bent and colleagues (23), are unclear. We carefully checked by histologic review that all recurrent tumor specimens included in our series indeed contained vital cellular tumor tissue and not just reactive changes due to cytotoxic therapy, in particular radiotherapy. Thereby, we excluded false-negative findings due to insufficient tumor cell content and radiation necrosis. Thus, available data (ref. 23; current study) suggest that EGFRvIII expression may be lost following standard radiochemotherapy in a subset of patients, challenging the significance of previous observations reporting on loss of EGFRvIII positivity specifically in recurrent glioblastomas after peptide-based vaccination against EGFRvIII but not after radiochemotherapy (24). Moreover, the finding that EGFR expression may become more commonly reduced or lost than increased or newly gained upon glioblastoma recurrence suggests a limited role of EGFRvIII in driving radiochemotherapy resistance and disease progression in glioblastoma patients, as suggested by studies in preclinical glioma models (36, 56).

Several studies have reported on various other EGFR sequence alterations than EGFRvIII in glioblastomas, including SNVs as well as larger rearrangements/deletions affecting the extracellular or intracellular domains (8, 14, 57–63). We therefore additionally investigated 27 pairs of primary and recurrent glioblastomas for other EGFR gene alterations using targeted next-generation sequencing of tumor DNA. Thereby, we identified various EGFR SNVs leading to missense mutations, especially in tumors with EGFR gene amplification, thus corroborating data from other groups reporting on a frequent coincidence of EGFR amplification with EGFR SNVs (14, 57–61). Neither the results in our GGN cohort nor data from the TCGA cohort analyzed here revealed evidence for an independent prognostic role of EGFR SNVs in glioblastoma patients treated according to the current standard of care. This finding reflects that EGFR SNVs are closely linked to EGFR amplification, which lacks prognostic significance in IDH-wild type glioblastoma patients (13, 17, 18). Moreover, TCGA data do not support a prognostic role of EGFR SNVs among patients with EGFR-amplified glioblastomas (Supplementary Fig. S3C). We did not evaluate the prognostic role of less common EGFR deletion variants like EGFRvII (deletion of exon 14–15) and EGFRvV (C-terminal deletions; refs. 8, 60, 62, 63); however, a previous study based on TCGA data did not observe a different outcome in glioblastoma patients whose tumors carried either of these variants (61).

We also investigated whether EGFR SNVs may change from primary to recurrent glioblastomas in individual patients. In six of 14 patients, EGFR SNVs detected in primary glioblastomas remained stable at recurrence. However, in three patients point mutations detected in primary tumors were lost upon recurrence while novel EGFR mutations turned up in recurrent glioblastomas of six patients. These findings would be in line with a branched tumor evolution model, suggesting that recurrent glioblastomas following therapy may develop from minor subclones of the respective primary tumor (64, 65). In addition, it is possible that EGFR point mutations detected exclusively in recurrent tumors are induced by therapy, in particular in case of C-G to T-A transitions that are known to be related to DNA-alkylating treatment with temozolomide (66). These issues require further analyses by more comprehensive molecular investigations of longitudinal biopsies in a larger cohort of glioblastoma patients.

In summary, our study shows that presence of EGFRvIII and/or EGFR SNVs is not prognostic in EGFR-amplified glioblastoma patients. Upon tumor recurrence, the EGFR amplification status of the primary tumor is generally retained and the majority of EGFRvIII-positive glioblastomas maintain EGFRvIII positivity at recurrence. However, EGFRvIII expression may change in a subset of patients at recurrence. Thus, in patients with recurrent glioblastoma who are evaluated for EGFRvIII-directed therapy approaches, either on compassionate use or within clinical trials, reassessment of the EGFRvIII status should be performed using recurrent glioblastoma tissue specimens to assure that the therapeutic target is still expressed on the tumor cells.

Disclosure of Potential Conflicts of Interest
G. Reifenberger reports receiving commercial research grants from Merck and Roche and speakers bureau honoraria from Amgen, and is a consultant/advisory board member for Celldex. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
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