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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 associated 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. *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.*

Introduction

The *EGFR* gene is the most commonly amplified and overexpressed proto-oncogene and a frequent mutational target in glioblastoma (for reviews, see refs. 1, 2). *EGFR* gene amplification is detectable in approximately 40% of all glioblastomas (3–5) and is particularly common in the classic or receptor tyrosine kinase (RTK) type 2 subgroup of isocitrate dehydrogenase (IDH)-wild-type glioblastoma (6). Approximately 50% of *EGFR*-amplified glioblastomas do not only amplify and overexpress the wild-type *EGFR* gene, but additionally carry a tumor-specific deletion variant (EGFRvIII) that is characterized by an in-frame deletion of exons 2-7 (7, 8). This particular rearrangement results in overexpression of a truncated receptor protein that lacks major parts of the extracellular domain, is unable to bind its ligands and is constitutively active, thus constituting a prototypic oncoprotein (for review, see ref. 9). Furthermore, the EGFRvIII protein carries a unique peptide sequence generated by the fusion of exons 1 and 8 that may serve as a tumor-specific target for anti-EGFRvIII immunotherapy approaches, including antibody-based approaches, genetically modified T cells, as well as peptide-based vaccination strategies (for review, see ref. 10).

Standardization of detection and quantification of *EGFR* amplification and EGFRvIII mutation in routinely processed tumor tissues remain challenging. Few studies suggested that EGFRvIII may occur in the absence of *EGFR* amplification in

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Translational Relevance

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.

minor subsets of anaplastic astrocytomas and glioblastomas (11, 12). However, most studies indicate a close link between *EGFR* amplification and EGFRvIII expression (1, 9, 13), which both are nowadays considered as typical alterations in IDH-wild-type glioblastomas (6, 14, 15).

The prognostic role of *EGFR* amplification and EGFRvIII mutation in glioblastoma patients remains controversial. Individual studies suggested that these alterations are associated with shorter overall survival (OS) among anaplastic astrocytoma patients (12) and glioblastoma patients (11), while other authors found a prognostically favorable role of EGFRvIII positivity in glioblastoma patients (16). Yet other studies, including previous publications of the German Glioma Network (13, 17), as well as a recent meta-analysis of eight publications (18), did not confirm a distinct prognostic role of *EGFR* amplification and EGFRvIII positivity, although a trend toward decreased long-term survival with EGFRvIII-positive glioblastoma has been reported (13, 19, 20).

Because of the ongoing development and clinical evaluation of various targeted treatment strategies directed against wild-type EGFR and/or against EGFRvIII (10), including, for example, peptide-based vaccines such as rindopepimut (21) and monoclonal antibody (mAb)-based immunotoxins such as ABT-414 (22), a better understanding of the biological role and the prognostic significance of *EGFR* amplification and EGFRvIII status in glioblastoma is urgently needed. In particular, the prognostic role of EGFRvIII within the population of patients with *EGFR*-amplified glioblastoma has not been conclusively determined. Moreover, although most novel targeted agents are initially being tested in patients with progressive glioblastoma after failure of standard therapy, consisting of local fractionated radiotherapy with concomitant and maintenance chemotherapy with temozolomide (temozolomide/radiotherapy→ temozolomide), data regarding the expression and role of EGFRvIII in the recurrent setting are sparse and in part conflicting (23, 24). Accordingly, we explored the prognostic role of EGFRvIII expression among newly diagnosed patients with *EGFR*-amplified glioblastomas and determined the stability of *EGFR* amplification and the EGFRvIII status at recurrence following standard-of-care treatment. In a subset of patients, we performed targeted sequencing of the *EGFR* gene to evaluate additional *EGFR* sequence alterations for associations with *EGFR* gene amplification and prognosis, as well as for changes upon tumor recurrence.

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 between primary and 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 frozen tumor tissue samples either by ultracentrifugation (27, 28) or by using the JETQUICK Tissue DNA Spin Kit (Genomed) and the RNeasy Mini Kit (Qiagen). DNA and RNA preparation from formalin-fixed and paraffin-embedded (FFPE) samples was performed with the QIAmp DNA FFPE Tissue Kit (Qiagen) and the RNeasy 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′-cactgcctcatctctcaccatc-3′) and EGFR-R

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(5′-gactcaccgtagctccagac-3′). Primers for the *WI-3306* locus on 2q that served as reference locus were: WI-3306-F (5′-catgactgcgagcc-caagatg-3′) and WI-3306-R (5′-caggtggtgtcatcagaatcag-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 $\Delta\Delta C_t$ 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 8 were used to generate products of 92 bp for the *EGFRvIII* and 893 bp for *EGFR* wild type (wt) mRNA sequences: EGFR-Ex1-F: GAGTCGGGCTCTGGAGGAAA; EGFR-Ex8-R: CCATCTCATAGCTGTCGGGCC. The EGFRwt product of 893 bb, however, was only detected in 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: CAGT-TATTGAACATCCTCTGGAG) generating a product of 111 bp representing *EGFR* wild-type sequences (Fig. 1). PCR was performed with HotStarTaq-Polymerase (Qiagen) for 15 minutes at 95°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 antiserum (lot #6549, Celldex) 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 (J. Felsberg and G. Reifenberger).

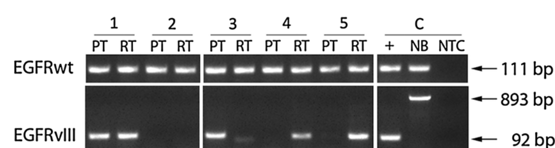

Figure 1.

Agarose gel electrophoresis images of results obtained by RT-PCR for EGFR wild-type (wt) and EGFRvIII mRNA expression in pairs of primary tumors (PT) and recurrent tumors (RT) of five selected patients (1, patient 97; 2, patient 81; 3, patient 99; 4, patient 79; 5, patient 100) with *EGFR*-amplified glioblastomas as demonstrated by real-time PCR (see Supplementary Table S1). Note that EGFRwt transcripts are expressed in all tumors as indicated by a 111-bp PCR product obtained by RT-PCR with primers detecting *EGFR* sequences in exons 1 and 2 (top). In the bottom, RT-PCR was performed with primers specific for *EGFR* exons 1 and 8 that amplify an 893 bp from EGFRwt transcripts and a 92-bp fragment from EGFRvIII transcripts. However, the 893-bp is obtained only when high-molecular weight RNA extracted from frozen tissue is used as template (in case of normal brain, NB), but absent when degraded RNA from FFPE material is used (in case of the tumor samples). Patient 97 expressed EGFRvIII transcripts in both primary and recurrent tumor. Patient 81 lacked EGFRvIII expression in primary and recurrent tumor. In patient 99, EGFRvIII expression in the primary tumor was lost in the recurrent tumor; in contrast, patient 79 had a clear EGFRvIII signal in the recurrent tumor that was absent in the primary tumor, whereas patient 100 showed a very weak, barely detectable EGFRvIII band in the primary tumor and a strong EGFRvIII signal in the recurrent tumor. C, controls: +, positive control with known EGFRvIII expression, NTC, nontemplate control (negative control).

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 (HISS 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 amplicons 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* promotor 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

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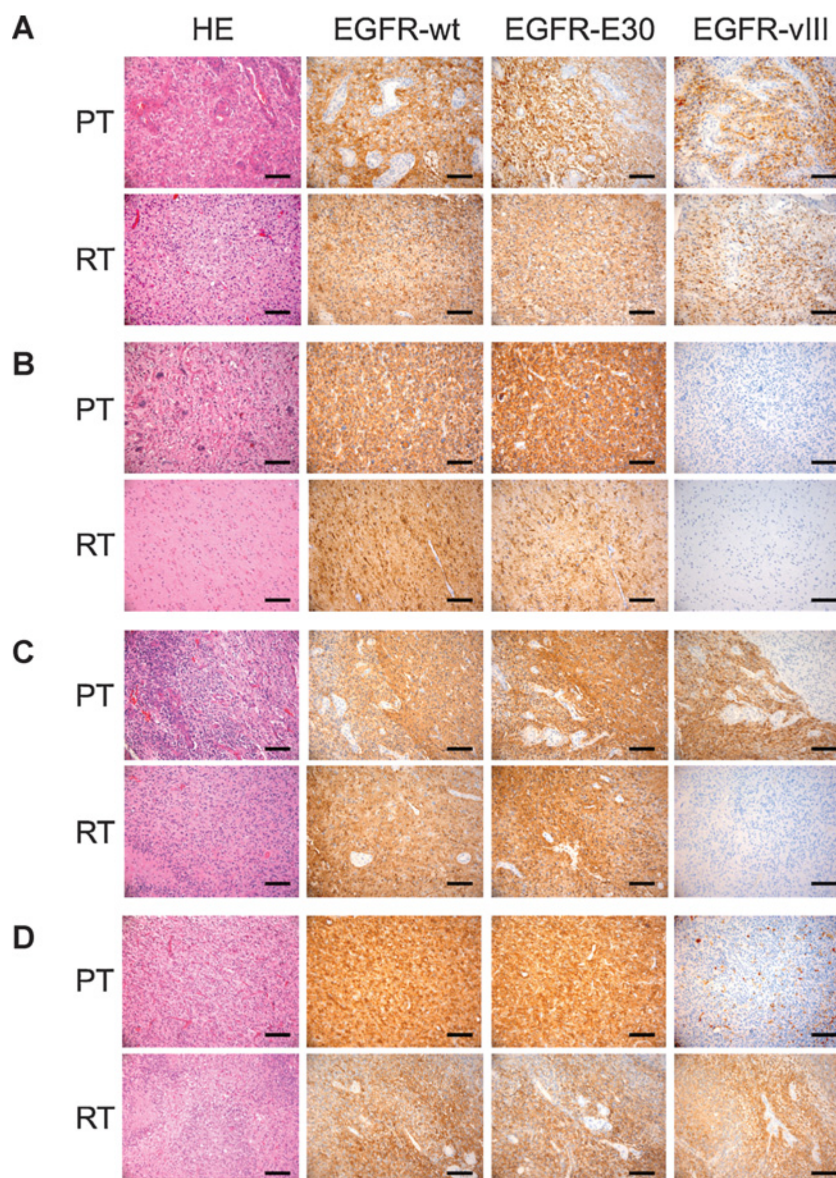

Figure 2.
Representative IHC results obtained in four glioblastoma patients (**A–D**). Shown are histologic features in hematoxylin and eosin (H&E)–stained sections and immunostainings for pairs of primary tumor (PT) and recurrent tumor (RT) from each patient. IHC stainings were done using antibodies detecting wild-type EGFR (EGFRwt), wild type EGFR and EGFRvIII (EGFRwt/vIII, E30), or EGFRvIII. All four depicted tumor pairs showed *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 μm.

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 *EGFR* was classified as "SNV in *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 *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 *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

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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
Range (years)	37–72	29–86	37–72	29–82	39–72	39–86
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						
Frontal	12 (26.1%)	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 (10.3%)	—	2 (6.7%)
No data		1				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 (56.5%)	3 (15.8%)	9 (36.0%)
Partial resection (<50%)	3 (7.0%)	5 (10.4%)	2 (8.3%)	3 (13.0%)	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[a]	34 (73.9%)	43 (71.7%)	15 (60.0%)	19 (65.5%)	19 (90.5%)	24 (77.4%)
TMZ cycles (median)	6 (1–25)	5 (1–24)	5 (2–12)	5 (1–24)	6 (1–25)	5 (1–10)
Patients with information on number of TMZ cycles	28/35	30/43	13/15	13/19	15/20	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 (6.5–9.9)	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 (14.3%)	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.
[a]Includes 6 patients who received radiotherapy and adjuvant temozolomide.

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

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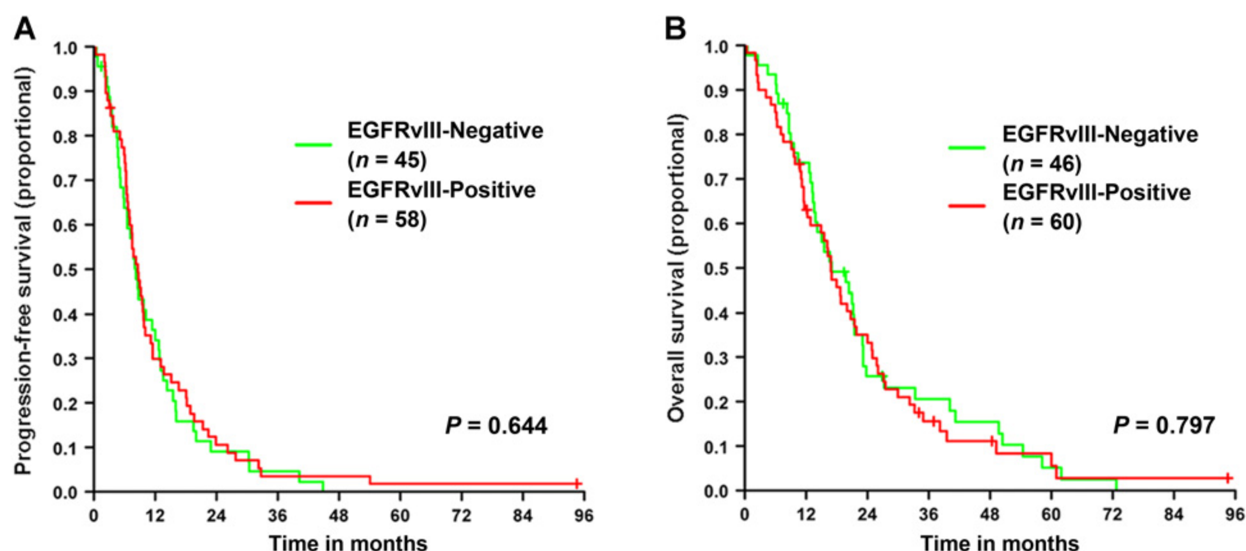

Figure 3.

Survival outcome in 106 patients with *EGFR*-amplified glioblastomas, IDH-wild-type, stratified according to the EGFRvIII status. Progression-free survival (PFS; **A**) and overall survival (OS; **B**) show no difference according to EGFRvIII status.

[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). EGFRvIII 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

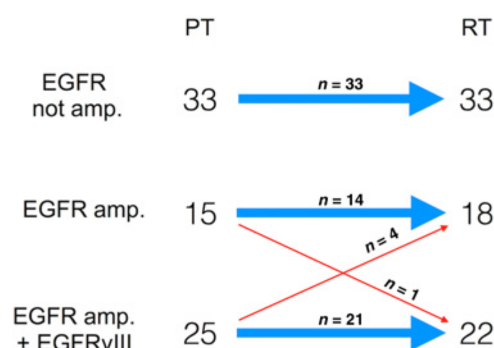

Figure 4.

Schematic representation of changes in EGFRvIII status in pairs of primary tumors (PT) and recurrent tumors (RT) of 73 patients with glioblastomas. Note that in 68 of the 73 tumor pairs, EGFRvIII status remained identical from primary to recurrent tumor. In 5 tumor pairs, a change was observed, including 4 instances with loss of EGFRvIII positivity upon recurrence and a single instance with newly gained EGFRvIII positivity upon recurrence. EGFR amp., *EGFR*-amplified primary tumors; EGFR not amp., *EGFR*-nonamplified primary tumors; EGFR amp. + EGFRvIII, *EGFR*-amplified, and EGFRvIII-positive primary tumors.

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EGFR amplification, we additionally investigated paired primary and recurrent glioblastoma tissues samples from 33 patients with *EGFR*-nonamplified primary glioblastomas. Recurrent tumors in none of these patients demonstrated newly acquired *EGFR* amplification or EGFRvIII positivity (Supplementary Table S2).

Association of *EGFR* SNVs with *EGFR* amplification, tumor recurrence, and OS

To assess a role of *EGFR* sequence alterations other than EGFRvIII in recurrent glioblastoma, we performed NGS of the *EGFR* coding sequence in 27 paired samples of primary and recurrent glioblastoma, including 13 paired samples with *EGFR* amplification. The detected *EGFR* SNVs generally corresponded to missense mutations, most of which are pathogenic (http://cancer.sanger.ac.uk/cosmic) and have been previously reported in other studies as summarized in Supplementary Table S3. NGS analysis also detected EGFRvIII, however, at somewhat lower sensitivity when compared with RT-PCR analysis (8/10 investigated tumors with EGFRvIII positivity), in line with published data (31). *EGFR* SNVs were found in 8 of 13 *EGFR*-amplified primary tumors (including 7 of 11 EGFRvIII-positive tumors and one of two EGFRvIII-negative tumors), but only in one of 14 *EGFR*-nonamplified primary tumors ($P < 0.01$, Fisher exact test). Presence of an *EGFR* missense mutation was not associated with distinct OS in our cohort of 27 patients (Supplementary Fig. S4).

In 6 of 14 GGN patients with one or more *EGFR* SNVs detected by NGS, the individual SNVs identified in the primary tumor were retained in the respective recurrence, albeit with different mutant allele frequencies in some patients. In three patients, *EGFR* SNVs were lost from primary to recurrent tumor, whereas 6 patients showed *EGFR* SNVs in their recurrent tumors that were not detectable in the matched primary tumors (Supplementary Table S3).

Validation studies based on TCGA glioblastoma patients

In line with the findings in the GGN cohort, interrogation of the TCGA database showed no OS difference in a cohort of 150 IDH-wild type glioblastoma patients treated with temozolomide when patients were stratified according to *EGFR* amplification status or according to the presence of at least one EGFR SNV (Supplementary Fig. S5A and S5B; Supplementary Table S4). Presence of *EGFR* SNVs was significantly associated with *EGFR* gene amplification in the TCGA cohort (25/79 *EGFR*-amplified glioblastomas vs. 6/71 *EGFR*-nonamplified tumors, $P < 0.001$). Within the group of 79 patients with *EGFR*-amplified glioblastomas, IDH-wild type, additional presence of *EGFR* SNVs was not associated with OS (Supplementary Fig. S5C). In the 66 cases with available information on EGFRvIII status, there was no association between the presence of an *EGFR* SNV and EGFRvIII positivity (3 *EGFR* SNV-positive among 12 EGFRvIII-positive tumors versus 14 *EGFR* SNV-positive among 54 EGFRvIII-negative tumors, $P = 0.95$). Finally, EGFRvIII positivity was not associated with distinct OS in the subgroup of 32 TCGA patients with *EGFR*-amplified tumors and available information on EGFRvIII status (Supplementary Fig. S5D).

Discussion

Interest in the biological role and the clinical significance of *EGFR* amplification and other EGFR alterations, in particular the constitutively active EGFRvIII deletion variant, in glioblastoma has increased over recent years. Accumulating preclinical evidence has attributed an important function of EGFRvIII-expressing glioblastoma cells in driving tumor heterogeneity and progression by promoting glioma cell proliferation, invasion, angiogenesis, stemness, and therapy resistance in different model systems (36–43). In addition, several therapeutic approaches targeting overexpressed wild type EGFR protein or specifically EGFRvIII have already entered, or are about to enter clinical evaluation, including peptide-based vaccines (44–46), chimeric antigen receptor (CAR) T cells (47, 48), as well as anti-EGFR antibody-based approaches (22, 49, 50).

Previous studies reported on conflicting results concerning the prognostic role of EGFRvIII, with a meta-analysis of eight published studies indicating no obvious association of *EGFR* amplification or EGFRvIII positivity with survival of glioblastoma patients (18). Our current study confirms these data and additionally shows that the presence of EGFRvIII is not prognostic among patients with *EGFR*-amplified glioblastoma treated according to current standard of care (Table 1; Fig. 1).

In our patient cohort, *MGMT* promoter methylation was prognostic in patients treated with radiochemotherapy and was particularly associated with longer OS in the subgroup of patients with EGFRvIII-negative tumors. In contrast, *MGMT* promoter methylation was not prognostic in the subgroup of patients with EGFRvIII-positive glioblastomas. However, we could not confirm the suggestion of a prognostic interaction between EGFRvIII expression and *MGMT* promoter methylation in a published data set of 13 patients with EGFRvIII-positive tumors and available *MGMT* promoter methylation status (16), and in the large ACT IV phase III data set (46).

In line with previous studies, our IHC findings confirm that EGFR wild type protein expression is strong and widespread in *EGFR*-amplified 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). In

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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 EGFRvIII expression is 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. S5C). 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.

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