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Changes in the *EGFR* amplification and *EGFRvIII* expression between paired primary and recurrent glioblastomas

Martin J. van den Bent, Ya Gao, Melissa Kerkhof, Johan M. Kros, Thierry Gorlia, Kitty van Zwieten, Jory Prince, Sjoerd van Duinen, Peter A. Sillevis Smitt, Martin Taphoorn, and Pim J. French

Department of Neurology, Erasmus MC, Rotterdam, Netherlands (M.J.v.d.B., Y.G., K.v.Z., J.P., P.A.S.S., P.J.F.); Department of Pathology, Erasmus MC, Rotterdam, Netherlands (J.M.K.); Department of Neurology, Haaglanden MC, The Hague, Netherlands (M.K., M.T.); EORTC Headquarters, Brussels, Belgium (T.G.); Pathology Department, Leiden University Medical Center, Leiden, Netherlands (S.v.D.)

Corresponding Author: Pim French, PhD, Department of Neurology, Room Be430A, Erasmus MC, PO Box 2040, 3000 CA Rotterdam, Netherlands (p.french@erasmusmc.nl).

See the editorial by Niclou, on pages 907–909.

Background. The efficacy of novel targeted therapies is often tested at the time of tumor recurrence. However, for glioblastoma (GBM) patients, surgical resections at recurrence are performed only in a minority of patients; therefore, molecular data are predominantly derived from the initial tumor. Molecular data of the initial tumor for patient selection into personalized medicine trials can therefore be used only when the specific genetic change is retained in the recurrent tumor.

Methods. In this study we determined whether *EGFR* amplification and expression of the most common mutation in GBMs (*EGFRvIII*) is retained at tumor recurrence. Because retention of genetic changes may be dependent on the initial treatment, we only used a cohort of GBM samples that were uniformly treated according to the current standard of care (ie, chemo-irradiation with temozolomide).

Results. Our data show that, in spite of some quantitative differences, the *EGFR* amplification status remains stable in the majority (84%) of tumors evaluated. *EGFRvIII* expression remained similar in 79% of GBMs. However, within the tumors expressing *EGFRvIII* at initial diagnosis, approximately one-half lose their *EGFRvIII* expression at tumor recurrence.

Conclusions. The relative stability of *EGFR* amplification indicates that molecular data obtained in the primary tumor can be used to predict the *EGFR* status of the recurrent tumor, but care should be taken in extrapolating *EGFRvIII* expression from the primary tumor, particularly when expressed at first diagnosis.

Keywords: *EGFR, EGFRvIII*, glioblastoma, recurrent tumors.

Gliomas are the most common type of primary brain tumor, of which 60%–70% are diagnosed as glioblastoma multiforme (GBM), the most aggressive variant.[1] The current standard of care for GBM patients includes surgical resection followed by chemo-irradiation.[2] However, tumors invariably relapse, and treatment options are limited when this occurs. In fact, no standard of care exists for recurrent GBM patients. Nitrosoureas, retreatment with (dose-intense) temozolomide and reirradiation are often employed but have limited activity. Progression-free survival of recurrent GBM is 2–4 months, and post-progression survival is 6–8 months with conventional chemotherapy.[3]

Current efforts to improve treatment of GBMs are often based on a personalized medicine approach. In this approach, the efficacy of novel agents is tested on those tumors that harbor specific mutations. Personalized medicine trials will generally be performed after the standard of care treatment at the time of tumor recurrence. However, surgical resections at recurrence are performed on a minority of glioma patients. Since marker testing based on circulating tumor DNA is not feasible (<10% detection rate) for glioma patients,[4] molecular data can only be derived from analysis of the tumor itself. Therefore, using molecular data of the initial tumor for inclusion into personalized medicine trials requires the specific genetic change to be retained in the recurrent tumor. A recent study on a limited set of low-grade gliomas indicated that only ~50% of all mutations present in the primary tumor are also present in the recurrent tumor.[5] Although this percentage was higher for the known causal cancer genes, this demonstrates the need to obtain more insight into the correlation between molecular

changes of the primary and recurrent tumor, especially if this molecular change is the target for treatment at progression. A substantial difference between newly diagnosed and recurrent tumors will indicate that patients require repeat surgery for inclusion into a personalized medicine trial.

The epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase that is frequently amplified and mutated in GBMs.[6,7] The most common mutation found in GBM patients, the *EGFRvIII* mutation, is an in-frame deletion of exons 2–7 that results in the receptor being constitutively active. Because *EGFR* amplification and *EGFRvIII* expression contribute to tumor formation, EGFR is a potential target for treatment in GBM patients.[8–12] In this study we therefore screened for differences in *EGFR* status and *EGFRvIII* expression between tumors at initial diagnosis and at recurrence.

Methods

Samples

GBM samples were collected from 2 hospitals in the Netherlands (Erasmus MC in Rotterdam and MC Haaglanden in The Hague) from patients operated between 1999 and 2013, who had resurgery at first recurrence. Use of patient material was approved by the Institutional Review Board of the respective hospitals. Patients were uniformly treated with chemoradiation with temozolomide.[2] All samples were evaluated for tumor content by a central review pathologist (J.M.K.), and samples with insufficient tumor content (<30%) were omitted from the analysis.

Quantitative Reverse Transcriptase Polymerase Chain Reaction

DNA and RNA were isolated using the Allprep DNA/RNA FFPE kit (Qiagen) according to the manufacturers' instructions except for an extension of the prot K incubation step from 15′ to overnight. *EGFR* amplification status and *EGFRvIII* expression were determined by quantitative reverse transcriptase (qRT-)PCR using assays from Life Technologies. The assay for *EGFR* DNA (assay number Hs02501405_cn) was designed ~1100 bp downstream of exon 1 because few genomic changes occur in this region; genomic breakpoints giving rise to *EGFRvIII* occur further downstream in this intron.[13] Control probes for DNA were *RNase P* (TaqMan copy number reference assay) and *BRAF* (HS04949885). *EGFR* status was determined as the average of the Ct values of control probes minus the average *EGFR* Ct values. The qPCR assay used correlated with *EGFR* amplification status as determined by copy number arrays ($n = 5$, Oncoscan DX, Affymetrix); examples are shown in Supplementary material, Figure S1.

EGFRvIII expression was determined with qRT-PCR using a custom-made primers/probe set designed over the exon 1–8 transition. Control qRT-PCR primers were targeted against *EGFR* wt (HS01076078_m1), RPL30 (Hs00265497_m1), and POP4 (Hs00198357_m1). Samples in which *EGFRvIII* expression >35 Ct values were scored as negative. *EGFRvIII* expression was scored as percentage of all *EGFR* transcripts (*EGFRvIII* + *EGFR* wild-type [wt]). In this case, 30% expression of *EGFRvIII*

indicates that *EGFRvIII* expression is 1 Ct value lower than that of *EGFR* wt.

All primers showed linear amplification over a wide range of Ct values (DNA content or RNA expression). This finding was observed in 5 independent samples. Slope of the dilution curve was also similar between the 3 primers used, which allows direct comparison between primers used. All qRT-PCR experiments were run in duplicate. The concordance correlation coefficient (Lin, equivalent to intraclass correlation coefficient ICC), was used to assess similarities between *EGFR* measurements.[14]

Results and Discussion

EGFR Amplification

A total of 89 cases were identified, of which tissue from 76 cases was available from both resections. *EGFR* amplification status could be determined in 55 primary-recurrent tumor pairs (Table 1); in remaining patients, we were unable to determine *EGFR* status in at least one of the 2 samples due to low tissue amounts ($n = 7$), too low tumor content ($n = 1$), insufficient DNA quality ($n = 10$), or no tumor tissue in the block ($n = 3$). Of these, *EGFR* amplification, as defined by a ΔCt >3 between controls and *EGFR* (which corresponds to an approximately 8-fold (2^3) increase) was present in 40 of 55 (73%) samples at first diagnosis. High-copy *EGFR* amplification (ie, those tumors having a ΔCt >5, [~32-fold, 2^5]) was observed in 23 of 55 (41%) samples. The patient cohort examined in this study therefore had a higher percentage of tumors with *EGFR* amplification than reported in other studies.[6,15] This higher percentage of *EGFR*-amplified tumors may reflect sample bias or may be caused by differences in sensitivity of the different techniques used. Alternatively, a higher percentage of *EGFR*-amplified tumors may also be a result of selective enrichment for second surgeries (and retreatment) of *EGFR*-amplified tumors.

To test whether *EGFR*-amplified tumors are more frequently eligible for resurgery, we tested for such a selective enrichment on GBM samples treated within Erasmus MC (between 1989 and 2005) as reported by us.[16] For this analysis, we used molecular subtyping based on gene expression data as a surrogate marker for *EGFR* amplification: *EGFR* amplification occurs predominantly in one molecular subtype (IGS-18, a subtype similar to "classical" GBMs as defined by The Cancer Genome Atlas [TCGA]).[16,17] Of the tumors diagnosed as GBM at initial presentation, 32 were assigned to IGS-18, of which 7 (22%) patients received resurgery. This frequency is ~2–3-fold lower in tumors assigned to other subtypes (where *EGFR* amplification is infrequent) including IGS-22 (1/12, 8.3%) or IGS-23 (6/47 12.8%; this subtype shows overlap with the TCGA mesenchymal GBMs). Although this difference is not statistically significant, it does provide some support for the bias towards resurgery of *EGFR*-amplified tumors found in current. Of note, this potential bias was not observed in the TCGA dataset in which 20 of 39, 2 of 5, and 11 of 18 patients received resurgery (tumors assigned to IGS-18, IGS-22, and IGS-23, respectively).

We also compared clinical data from this study with data from GBMs in a historical cohort ($n = 259$) to screen for potential sample bias.[16] As may be expected, patients in the recurrent GBM cohort had a better performance score compared

Table 1. Patient characteristics and molecular data

Pat ID	Age (y)	Sex	Extent of Resection		Loc	RT	TMZ	Tumor (%)		PD (days)	OS (days)	ev	EGFR (dCt)		EGFRvIII (%)	
			Pr	Rec				Pr	Rec				Pr	Rec	Pr	Rec
AAA	54.2	M	PR	CR	P	60			40%	222	396	1		0.43		
AAB	68.3	M	PR	PR	P	60		80%	80%	434	584	1		2.93	86.5	93.7
AAC	68.3	F	PR	PR	T	60		90%	60%	67	304	1	6.28	4.08	85.1	
AAD	64.3	M	PR	PR	T	60				250	451	1	5.82		0.0	
AAF	43.6	F	PR	PR	F	60		70%	50%	139	590	1	3.43	1.75	0.0	0.0
AAG	43.6	F	PR	PR	T	60	2	70%	70%	108	379	1	0.85	0.55	0.0	0.0
AAI	57.7	M	PR	PR	T	5		70%	60%	26	445	1	3.64	3.51	0.0	
AAJ	60.9	F	PR	PR	T	60	2	70%	50%	143	282	1	0.92	0.61	0.0	
AAK	58.0	F	PR	PR	P	60	4	70%	60%	182	605	1	3.29	3.55	0.0	0.0
AAL	60.1	M	PR	PR	O	60	3	70%	60%	187	373	1	2.54	2.88	0.0	0.0
AAM	63.0	F	PR	PR	T	60	6	70%	60%	271	410	1	5.82	6.32	85.2	79.7
AAN	50.3	F	PR	PR	P	60		70%	70%	455	527	1	6.00	2.59	36.1	0.0
AAS	37.3	M	PR	PR	F	60	6	80%	70%	264	508	1		4.41	0.0	0.0
AAT	62.5	F	PR	PR	F	60	6	80%	50%	833	1277	1	6.62	4.38	68.9	0.0
AAU	52.5	F	PR	PR	FP	60	6	70%	80%	647	1279	1		3.84	0.0	0.0
AAV	60.9	M	PR	PR	T	60	12	70%	60%	532	1412	1	5.39	6.49	0.0	0.0
AAW	40.7	F	PR	CR	F	60	1	70%	60%	104	448	1	0.54	0.51	0.0	0.0
AAX	43.0	F	PR	PR	O	60	1			61	754	1	11.29	9.41		0.2
AAY	69.6	F	PR	PR	F	60		80%	80%	257	315	1	0.36	0.84	0.0	0.0
ABA	52.9	F	PR	PR	T	60	6	70%	80%	241	470	1	3.52	5.97	0.0	0.0
ACA	65.3	M	PR	PR	F	60	6	90%	70%	147	247	1	8.74	3.87	0.0	0.0
ADA	55.7	M	PR	PR	F	60	6			363	602	1	6.71	4.48		0.0
AFA		M	PR	PR	F	60	6	80%	80%	496	850	1	0.26	0.20	0.0	0.0
AGA	50.5	M	CR	PR	T	70	6	90%	70%	305	535	1	5.04	4.70	0.0	0.0
AHA	50.8	M	PR	PR	F	60	4	70%	80%	195	332	1	4.43	7.01		0.0
AIA	65.2	M	PR	PR	T	60	6			280	437	1	2.28	4.05		0.0
ALA	50.5	M	CR	PR	T	60	6	90%	70%	274		0	6.97	8.86	7.5	0.0
AMA	61.9	M	PR	CR	P	60	6	70%	70%	1707	1740	1	4.08	3.72		91.5
AOA	64.5	F	CR	CR	P	60	12	80%	80%	434		0	6.30	0.78	0.7	0.0
AQA	75.1	F	CR	PR	T	40	9	60%	70%	352		0	3.67	1.10	0.0	0.0
ARA	68.9	M	PR	CR	F	60	6			258		0	3.06	0.38		0.0
CAB	55.8	M	PR	PR	O	60	4	50%	60%	214	479	1	−0.23	3.93		
CAC	44.6	M	PR	CR	T	60	5	70%	30%	270	576	1	8.45	1.93	71.5	6.5
CAD	51.6	M	PR	PR	T	60	5	60%	70%	252	348	1	−0.30	3.33		0.0
CAF	28.4	M	PR	CR	T	60	6	70%	70%	276	694	1	4.65	6.68	54.1	27.4
CAK	45.7	F	PR	PR	P	60	2	40%	30%	229	395	1	1.43	3.43	0.0	
CAM	47.2	M	B	PR	T	60		60%	60%	388	520	1	7.48	8.70		0.0
CAN	66.0	M	PR	B	T	60	6	80%	50%	270	494	1	4.08	6.58		0.0
CAO	50.4	M	PR	PR	T	60	6	70%	70%	605	940	1	8.10	10.75	9.9	0.0
CAS	53.8	M	CR	B	F	60		80%	30%	198	560	1	6.65	3.10	1.9	
CAV	31.4	F	PR	PR	T	60	6	70%	70%	451	673	1		3.10	0.0	0.0
CAX	39.8	M	PR	PR	P	60	2	70%	20%	162	1079	1	4.18	3.40		0.0
CAZ	43.0	F	PR	B	T	60	6	80%	50%	905	1240	1	2.83	2.15	0.0	0.0
CBA	56.6	M	PR	PR	F	60		60%	40%	109	190	1	2.33	0.80	0.0	0.0
CBE	53.9	F	PR	PR	T	59	6	80%	70%	297	523	1	4.45	6.15	0.0	0.0
CBF	59.7	F	PR	PR	F	60		80%	70%	232	513	1	5.98	4.33	87.3	95.9
CBG	31.7	M	PR	PR	F	648		90%	80%	389	702	1	1.40		0.0	
CBH	72.8	M	PR	PR	O	40		70%	60%	120	333	1	1.03			0.0
CBI	41.6	F	PR	PR	O	60	6	80%	90%	290	633	1	5.35	5.55	61.2	47.0
CBM	55.3	M	PR	PR		60	6	60%	60%	271	353	1	3.03	3.58	0.0	0.0

Continued

Table 1. *Continued*

Pat ID	Age (y)	Sex	Extent of Resection		Loc	RT	TMZ	Tumor (%)		PD (days)	OS (days)	ev	EGFR (dCt)		EGFRvIII (%)	
			Pr	Rec				Pr	Rec				Pr	Rec	Pr	Rec
CBP	61.0	F	PR	PR	P	60	2	70%	80%	181	546	1	−0.05	0.10	0.0	0.0
CBQ	61.3	M	PR	PR	P	60	6	80%	70%	698	1283	1	5.40	4.65	0.0	0.0
CBR	60.1	M	PR		T	60	2	70%	90%	1291	343	1	1.45		0.0	0.0
CBS	52.7	F	PR	PR	T	60	2	70%	70%	170	260	1	4.73	2.25	0.0	0.0
CBT	52.5	M	PR	CR	F	60	6	60%	70%	289	681	1	6.55	6.90	51.0	20.6
CBV	50.0	M	PR	PR	F	60	6	30%		308	1383	1	3.75			
CBW	49.3	M	PR	PR	T	60	8	70%	60%	1261	1903	1	0.52	3.30	0.0	0.0
CCA	45.4	F	CR	PR	P	60	6	70%	70%	885	1488	1	7.58	8.08	0.0	0.5
CCB	52.1	M	PR	B	T	60	5	60%	60%	202	511	1	4.30	3.90	12.4	
CCD	52.5	F	PR	PR		60		80%	70%	283	327	1	4.65	4.60	0.0	0.0
CCP	43.2	F	PR	PR	F	59				203	279	1	2.10	−0.95	0.0	
CCV	49.2	M	PR		T	60	3	80%		411	413	1	8.10		0.0	
CCW	48.0	F	PR	PR	T	60		70%	30%	191	529	1	7.25	6.60	52.3	0.0
CCX	49.9	M	PR	PR	P	65		30%	70%	2069	2743	1	3.48	3.50	22.7	0.0
CCZ	51.2	F	PR	PR	O	60	3	80%	70%	247	277	1	8.43	8.88	76.9	51.4
CDA	65.6	M	PR	PR	T	60	6	80%	50%	628	890	1	5.88	4.10	0.0	
CDB	36.5	M	PR	PR	T	60		80%	30%	109	223	1		3.75	0.0	83.9
CAY	48.8	F	PR	PR	O	60	6			282	336	1		8.50		7.0
CBO	63.6	M	PR	B	T	60	5			262	512	1	2.20		0.0	
AEA	45.0	F	CR	CR	T	60	6			281	402	1		4.08		0.0
AAE	53.0	F	PR	PR	F	60	6			1026	1357	1		4.60		0.0
AAH	46.8	M	PR	PR	P	60				335	545	1	8.52		1.4	
ANA	65.6	M	CR	PR	T	60	6			430		0		8.95		0.0
AAP	47.9	F	PR	PR	T	60	4			534	1802	1	10.34		63.7	
AAR	52.8	M	CR	PR	P	60	3			186	393	1				2.6
AKA	61.4	F	CR	PR	F	60	3	90%	70%	211	364	1		0.94		0.0

Abbreviations: B, biopsy; CR, complete resection; F, female; M; male; Pr, primary tumor; PR, partial resection; Rec, recurrent tumor; TMZ, number of cycles; RT, radiation therapy dose (Gy); Loc, tumor location (F, frontal; O, occipital; P, parietal; T, temporal; FP, posterior fossa).

with the historical cohort (90.1 ± 8.7 vs 81.6 ± 17.0; $P < .0001$, *t* test) and were younger in age (51.2 ± 12.7 years vs 55.7 ± 13.6 years; $P < .0001$, *t* test). Our cohort also had a significantly lower male-to-female ratio compared with our historic cohort (48:42 vs 175:84; $P = .006$, Fisher exact test). There were also some differences in tumor location ($n = 27$, 15, 7, and 36 vs $n = 40$, 33, 12, and 29 for frontal, parietal, occipital and temporal locations, respectively), although this difference did not reach statistical significance ($P = .06$, chi-square test). However, re-resection of GBMs will only be performed on tumors that are relatively accessible for surgery, which inevitably results in a location bias.

EGFRvIII Expression

Of the 76 cases with tissue available from the primary and recurrent tumors, *EGFRvIII* expression could be evaluated in 111 samples from 69 patients (Table 1). Data from both primary and recurrent samples was generated for 42 patients; data from either the primary or recurrent tumor were of insufficient quality in the remaining 27 patients (in most cases, qRT-PCR could detect transcripts, but the Ct values were too high to reliably allow quantification of *EGFRvIII* expression). *EGFRvIII* expression was detected in 34 samples and, apart from one recurrent sample, only occurred in samples with a genomic amplification of the *EGFR* locus (Fig. 1B). For the single sample with *EGFRvIII* expression without *EGFR* amplification (patient CAC) it should be noted that high copy *EGFR* amplification and *EGFRvIII* expression were detected in the primary tumor, but the recurrent tumor had a much lower tumor content (30%). *EGFRvIII* expression was detected in 17 of 35 (49%) primary tumors with *EGFR* amplification ($\Delta Ct > 3$), which is a similar frequency as previously reported.[6]

Similar to a report by Hobbs et al,[15] our data show that *EGFR* amplification status was highly variable between tumors. While some tumors showed only modest amplification levels (3–4 ΔCt values), other tumors showed a much stronger amplification (up to 10 ΔCt value difference between *EGFR* and controls). Although the *EGFR* amplification status was variable between tumors, the *EGFR* status was relatively constant within biological replicates ($n = 22$, Fig. 1A). *EGFRvIII* expression was also highly variable between different tumors and ranged from
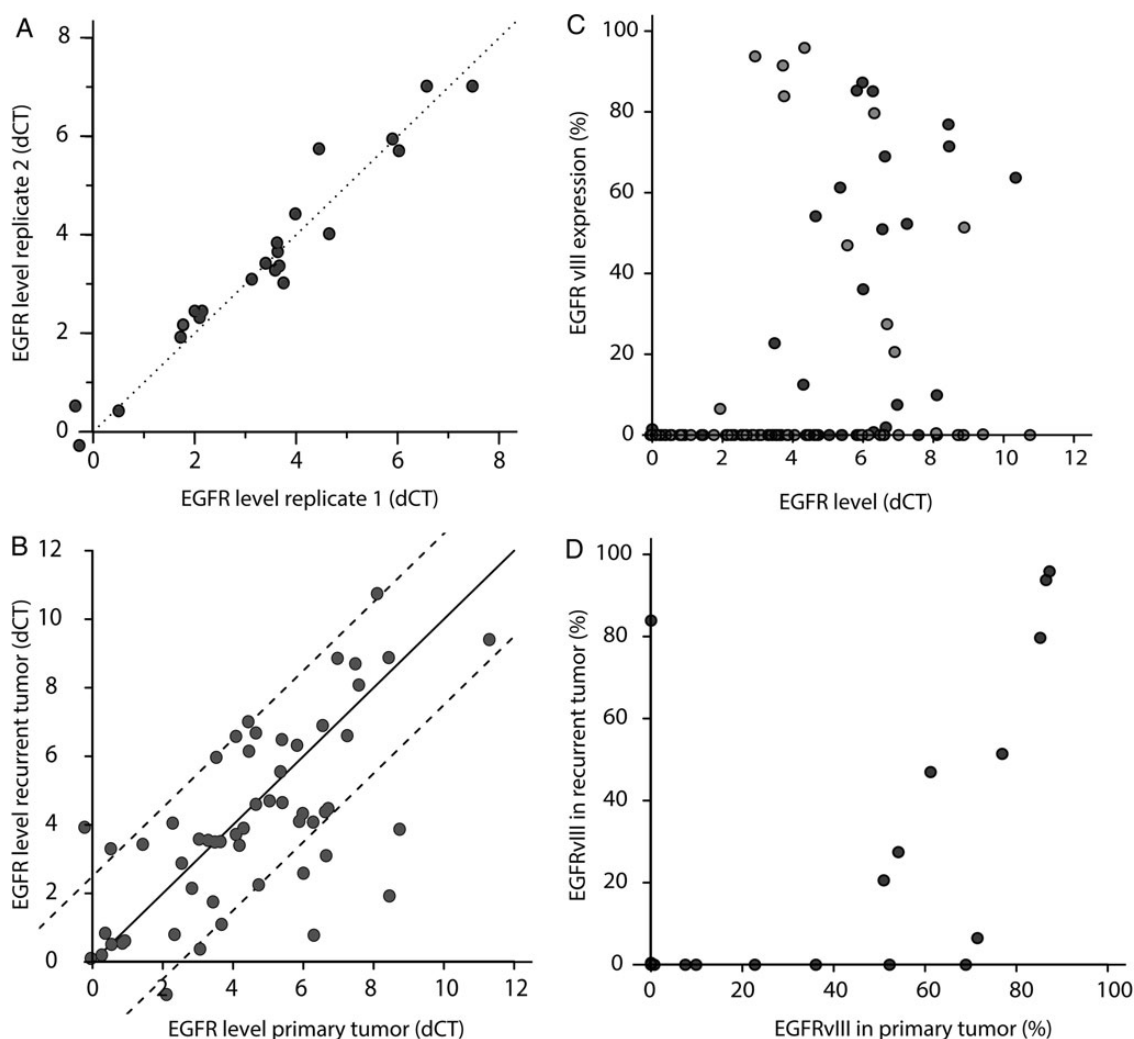
Fig. 1. (A) Variability of *EGFR* amplification within biological replicates. As can be seen, the *EGFR* status between replicates was relatively constant in our samples. (B) *EGFR* amplification of primary versus recurrent glioblastomas. Although *EGFR* amplification varied between the primary and recurrent tumor, the difference was generally within 2.5 ΔCt values (dotted lines) of each other. (C) *EGFRvIII* expression, plotted as a percentage of all *EGFR* transcripts, is predominantly observed in samples with *EGFR* amplification (ie, those with dCt >3). Points in dark grey are from initial diagnoses, and light grey is from the recurrent tumor. (D) *EGFRvIII* expression in primary versus recurrent tumors. As can be seen, the relative expression of *EGFRvIII* was often lower in recurrent tumors than in primary tumors, with 7 samples showing *EGFRvIII* expression only in the primary tumor.

<1% up to >90% of all *EGFR* transcripts being *EGFRvIII*. *EGFR* amplification and *EGFR* gene expression levels were correlated (Fig. 2).

Most Glioblastomas Retain Their EGFR Amplification Status at Tumor Recurrence

EGFR amplification of the recurrent tumor did differ from the primary tumor, but the difference was generally within 2–2.5 ΔCt values of each other (Fig. 1C). The overall concordance correlation coefficient between primary and recurrent tumors was 0.65. Cases in which the difference between primary and recurrent tumors was <2.5 ΔCt values (*n* = 42 tumor pairs) were considered to have retained their *EGFR* amplification status.

In 13 tumors, the difference in *EGFR* amplification between primary and recurrent tumors was >2.5 ΔCt values; only 4 tumors showed a marked (≥4 ΔCt values) difference between the initial tumor at recurrence. More detailed analysis failed to detect any specific characteristics for these tumors with respect to extent of resection, use of steroids, *MGMT* promoter methylation, and tumor location. Also, while we did observe a slightly higher tumor content in the primary tumor (71% ± 14% vs 63% ± 17%; *P* < .001, paired *t* test), this change is unlikely to explain discrepancies in *EGFR* amplification status between the tumor at initial diagnosis and at recurrence: a 2-fold decrease in tumor content would result in a maximal decrease in Ct value of one (ie, one PCR cycle). The *EGFR* amplification status would change in 8 of 13 samples showing a change >2.5 ΔCt values
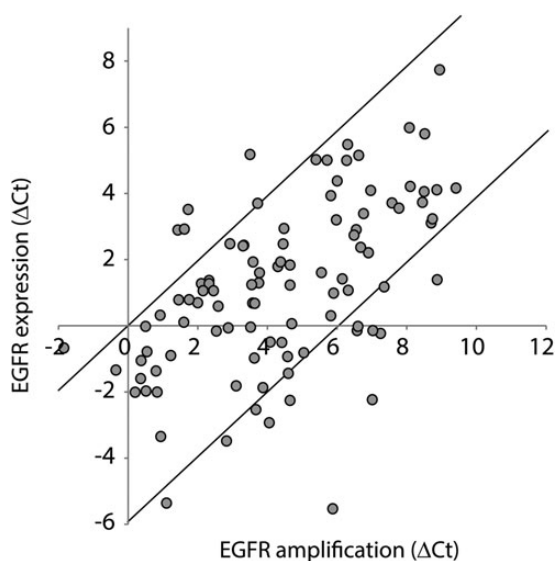
Fig. 2. Correlation between *EGFR* amplification status (x-axis) and *EGFR* gene expression levels (y-axis) as determined by quantitative reverse-transcriptase PCR on tumor DNA or RNA.

Table 2. Summary of *EGFR* and *EGFRvIII* data

		EGFR in Recurrent Tumor		
		Nonamp	Amp	n
EGFR in primary tumor	Nonamp	10	5[a]	15
	Amp	7[a]	33	40
	n	17	38	55
		EGFRvIII in Recurrent Tumor		
		Absent	Present	n
EGFRvIII in primary tumor	Absent	25	2	27
	Present	7	8	15
	n	32	10	42

Abbreviations: Amp, amplified; Nonamp, nonamplified.

Cutoff value for *EGFR* amplification is $\Delta Ct > 3$ between *EGFR* and control probes.

[a]Of the samples that changed *EGFR* status from nonamplified to amplified or from amplified to nonamplified, 9 showed a difference in ΔCt value >2.5 between the primary and recurrent tumor. When considering that a change in EGFR amplification status also requires >2.5 ΔCt values difference between primary and recurrent tumors, 46 of 55 (84%) tumors retained their EGFR status. Only 5 showed a difference in ΔCt value >3 between the primary and recurrent tumor.

between primary and recurrent: 5 from amplified to nonamplified (with 3 from high copy amplification, ie, ΔCt values >5 to nonamplified) and 3 from *EGFR* not amplified to amplified, all of which resulted in moderate levels of *EGFR* amplification (ie, ΔCt values >3 but <5). Overall, the *EGFR* amplification status (dichotomized to either nonamplified or amplified) remained identical in most tumor pairs (46/55; 84%, Table 2).

Glioblastomas Can Lose EGFRvIII Expression at Tumor Recurrence

The relative expression of *EGFRvIII* was often lower in recurrent tumors than in the primary tumor. Of the 15 tumors with detectable *EGFRvIII* expression in the primary tumor, 8 showed a>20% decrease in relative abundance of *EGFRvIII* transcripts (Fig. 1D). In fact, the *EGFRvIII* variant was lost at the time of progression in 7 of 15 *EGFRvIII*-positive tumors at first surgery. These data are in line with data reported in a different study using an unselected patient cohort,[18] although intratumoral heterogeneity may also explain part of this variability.[19,20]

Of the 15 tumors with *EGFRvIII* expression, corresponding *EGFR* amplification status was available for 14. The majority of these (9/14) showed a relative increase in *EGFR* amplification (ΔCt between the tumor at initial diagnosis and at recurrence between 0 and 3), even though *EGFRvIII* expression decreased ($n = 8$) or stayed the same ($n = 1$). In fact only 3 of 14 showed concordant decrease in *EGFR* amplification status (> 2.0 ΔCt values between initial recurrent tumors) and decrease in *EGFRvIII* expression.

Qualitatively *EGFRvIII* status (present or absent) remained similar between the primary and recurrent tumor in 33of 42 (79%) samples: *EGFRvIII* was absent from the primary and recurrent tumor in 25 samples and expressed in both primary and recurrent tumor in 8 samples (Table 2). The loss of *EGFRvIII* expression may be explained by the hypothesis that *EGFRvIII* deletions occur after *EGFR* amplification and that individual cells harbor varying levels of *EGFRvIII*.[5] Loss of *EGFRvIII* expression at tumor recurrence then represents clonal selection of the tumor. Indeed, gliomas are heterogeneous tumors in which distinct subpopulations of cells exist, each with different genetic makeup.[5,21] However, recent evidence also suggests that genomic *EGFRvIII* deletion is an early event and that *EGFRvIII* expression is regulated by the tumor.[19] In fact, mice experiments demonstrated that the ratio of *EGFRwt*-to-*EGFRvIII* expression was similar to the primary tumor at regrowth, even when sorting for *EGFRvIII* high- or low-expressing tumor cells[22] (see also[23]). Therefore, loss of *EGFRvIII* expression is a result of epigenetic regulation.

In summary, our data show that, in spite of some quantitative differences, the *EGFR* amplification status remained stable in the majority (~84%) of tumors evaluated. *EGFRvIII* status also remained similar in 79% of GBMs; however, when focusing on *EGFRvIII*-expressing tumors, only 50% retained *EGFRvIII* expression at recurrence. The relative stability of *EGFR* amplification expression therefore indicates that molecular data obtained in the primary tumor can be used to predict the *EGFR* status of the recurrent tumor. Care should be taken when extrapolating *EGFRvIII* expression; a repeat biopsy should be considered in trials on recurrent glioblastoma targeting EGFRvIII mutations.

Supplementary Material

Supplementary material is available online at *Neuro-Oncology* (http://neuro-oncology.oxfordjournals.org/).

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