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

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Neuro-Oncology; 2015, 17(7): 935-941

ABSTRACT

Background

The efficacy of novel targeted therapies is often tested at the time of tumor recurrence. However, for glioblastoma patients, surgical resections at recurrence are performed only in a minority of patients and 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 only be used 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 GBM samples that were uniformly treated according to the current standard of care, 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 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 that care should be taken in extrapolating *EGFRvIII* expression from the primary tumor, particularly when expressed at first diagnosis.

INTRODUCTION

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 when this occurs, treatment options are limited. In fact, no standard of care exists for recurrent GBM patients. Nitrosoureas, retreatment with (dose-intense) temozolomide, and re-irradiation 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 are 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 tumour 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 re-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 which 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

Glioblastoma samples were collected from two hospitals in the Netherlands (Erasmus MC, Rotterdam and MC Haaglanden, the Hague) from patients operated from 1999-2013, 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. Kros), samples with insufficient tumor content (< 30 %) were omitted from the analysis.

RT-qPCR

DNA and RNA were isolated using the Allprep DNA/RNA FFPE kit (Qiagen, Venlo, the Netherlands) 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 was determined by (RT-) qPCR, using assays from Life Technologies (Bleiswijk, the Netherlands). The assay for *EGFR* DNA (assay number Hs02501405_cn) was designed ~ 1100 bp downstream of exon 1 as 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 – 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, Santa Clara Ca), examples are shown in supplementary figure 1.

EGFRvIII expression was determined using RT-Q-PCR using a custom made primers/probe set designed over the exon 1-8 transition. Control RT-Q-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* wildtype (wt)). In this case, 30 % expression of *EGFRvIII* indicates *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 was observed in five independent samples. Slope of the dilution curve was also similar between the three primers used, which allows direct comparison between primers used. All (RT-) Q-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 patients were identified, of which tissue of 76 patients 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 two samples due to low tissue amounts (n = 7), too low tumor content (n = 1), insufficient DNA quality (n = 10) or the block did not contain tumor tissue (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 40/55 (73 %) samples at first diagnosis. High copy *EGFR* amplification, i.e. those tumors having a ΔCt > 5, (~ 32 fold, 2^5) was observed in 23/55 (41 %) samples. The patient cohort examined in this study therefore has 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 re-treatment) of *EGFR* amplified tumors.

To test whether *EGFR* amplified tumors are more frequently eligible for resurgery, we have tested for such a selective enrichment on GBM samples treated within the 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 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 tumours 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 provides 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 where 20/39, 2/5 and 11/18 patients received resurgery (tumors assigned to IGS-18, IGS-22 and IGS-23 respectively).

We have 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 to the historical cohort (90.1 ± 8.7 v. 81.6 ± 17.0, P < 0.0001, Ttest), and were of younger

Table 1. Patient characteristics and molecular data

			extent of resection					Tumor (%)					EGFR (dCt)		EGFRvIII (%)	
Pat ID	Age (y)	G	pr	rec	Loc	RT	TMZ	pr	rec	PD (days)	OS (days)	ev	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

Table 1. Patient characteristics and molecular data (continued)

Pat ID	Age (y)	G	extent of resection		Loc	RT	TMZ	Tumor (%)		PD (days)	OS (days)	ev	EGFR (dCt)		EGFRvIII (%)	
			pr	rec				pr	rec				Pr	rec	pr	rec
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

Table 1. Patient characteristics and molecular data (continued)

			extent of resection					Tumor (%)					EGFR (dCt)		EGFRvIII (%)	
Pat ID	Age (y)	G	pr	rec	Loc	RT	TMZ	pr	rec	PD (days)	OS (days)	ev	Pr	rec	pr	rec
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
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	

Table 1. Patient characteristics and molecular data (continued)

Pat ID	Age (y)	G	extent of resection		Tumor (%)					PD (days)	OS (days)	ev	EGFR (dCt)		EGFRvIII (%)	
			pr	rec	Loc	RT	TMZ	pr	rec				Pr	rec	pr	rec
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

G: gender, M: Male, F: Female. Pr: primary tumor, rec: recurrent tumor. TMZ: number of cycles. RT dose (Gy). Loc: tumor location: P= Parietal, F= Frontal, O= Occipital, T= Temporal, FP: fossa posterior

age (51.2 ± 12.7 v. 55.7 ± 13.6, P < 0.0001, Ttest). Our cohort also had a significantly lower male/female ration compared to our historic cohort (48/42 v. 175/84, P = 0.006, Fischers' exact test). There were also some differences in tumor location (n = 27, 15, 7 and 36 *v.* n = 40, 33, 12 and 29 for frontal, parietal, occipital and temporal location respectively), though this difference did not reach statistical significance (P = 0.06, 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.

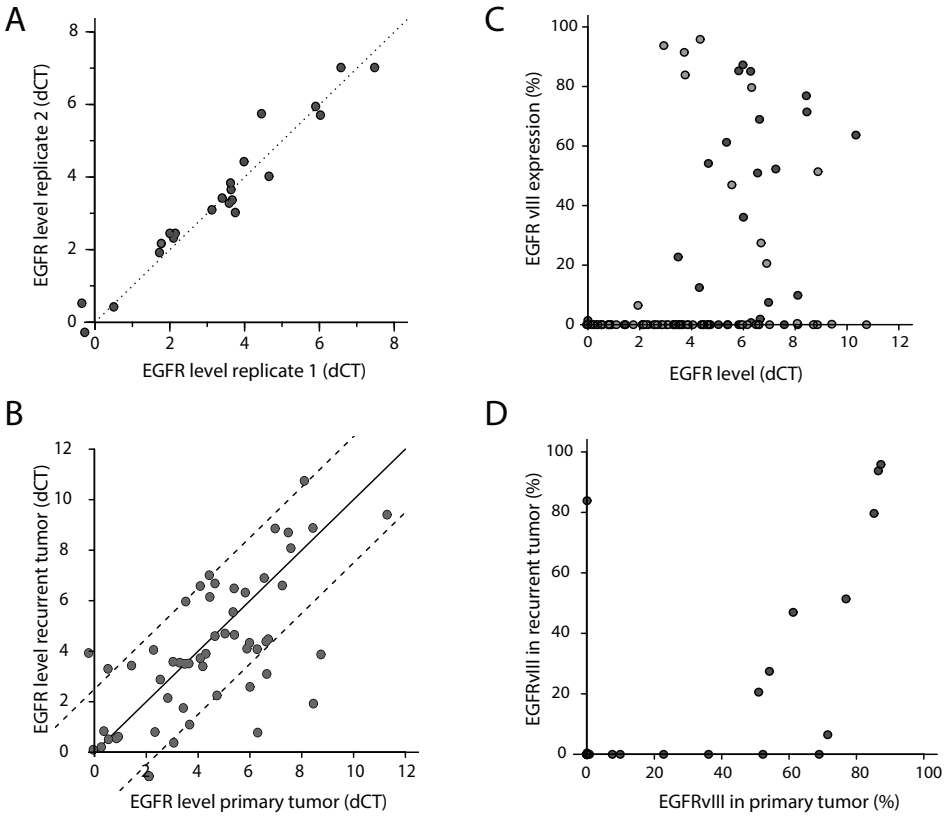

Figure 1. (A) Variability of EGFR amplification within biological replicates. As can be seen, the EGFR status between replicates was relatively constant in our samples. (B) EGFR amplification of primary versus recurrent glioblastomas. Although EGFR amplification varied between the primary and recurrent tumor, the difference was generally within 2.5 DCt 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.

EGFRvIII expression

Of the 76 patients with tissue available from the primary and recurrent tumor, *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 was of insufficient quality in the remaining 27 patients (in most cases, RT-QPCR 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 (figure 1B). For the one sample with *EGFRvIII* expression without *EGFR* amplification (patient CAC) it should be noted that high copy *EGFR* amplification and *EGFRvIII* expression was detected in the primary tumor *but* the recurrent tumor had a much lower tumor content (30%). *EGFRvIII* expression was detected in 17/35 (49%) of primary tumors with *EGFR* amplification ($\Delta Ct > 3$), which is a similar frequency as previously reported[6].

Similar to reported [15], our data show that *EGFR* amplification status was highly variable between tumors: while some tumors show 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 is variable between tumors, within biological replicates the *EGFR* status was relatively constant (n = 22, figure 1A). *EGFRvIII* expression was also highly variable between different tumors and ranged from < 1% up to > 90% of all *EGFR* transcripts being *EGFRvIII*. *EGFR* amplification and *EGFR* gene expression levels were correlated (figure 2).

Most GBMs 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 (figure 1C). The overall concordance correlation coefficient between primary and recurrent tumors was 0.65. Cases where 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 four 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, whilst we do observe a slightly higher tumor content in the primary tumor (71% ± 14% v. 63% ± 17%, $P < 0.001$, paired T-test) this change is unlikely to explain discrepancies in *EGFR* amplification status between the tumor at initial diagnosis and at recurrency:

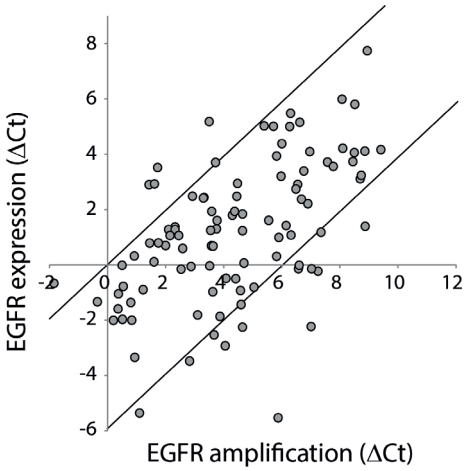

Figure 2. Correlation between *EGFR* amplification status (x-axis) and *EGFR* gene expression levels (y-axis) as determined by (RT-)QPCR on tumor DNA or RNA.

a two-fold decrease in tumor content would result in a maximal decrease in Ct value of one (i.e. one PCR cycle). The *EGFR* amplification status would change in eight/thirteen samples showing a change > 2.5 ΔCt values between primary and recurrent: Five from amplified to non-amplified (of which three from high copy amplification i.e. ΔCt values > 5 to not-amplified) and three from *EGFR* not amplified to amplified (all of which resulting in moderate levels of *EGFR* amplification i.e. ΔCt values > 3 but < 5). Overall, the *EGFR* amplification status (dichotomized to either non-amplified or amplified) remained identical in most tumor pairs (46/55; 84 %, table 2).

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

		***EGFR* in recurrent tumour**		
		Non-amp	Amp	n
***EGFR* in primary tumour**	Non-amp	10	5*	15
	Amp	7*	33	40
	n	17	38	55

		***EGFRvIII* in recurrent tumour**		
		Absent	Present	n
***EGFRvIII* in primary tumour**	Absent	25	2	27
	Present	7	8	15
	n	32	10	42

Cutoff value for *EGFR* amplification is ΔCt>3 between *EGFR* and control probes. * Of the samples that change *EGFR* status from wt > amplified or from amplified > wt, 9 shown a difference in ΔCt value > 2.5 between the primary and recurrent tumor. When considering a change in EGFR amplification status also requires > 2.5 ΔCt values difference between primary and recurrent tumors, 46/55 (84%) tumors retain their EGFR status. Only 5 show a difference in ΔCt value > 3 between the primary and recurrent tumor.

GBMs can lose EGFRvIII expression at tumor recurrence

The relative expression of *EGFRvIII* often was lower in recurrent tumors than that 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 (figure 1D). In fact, in seven out of fifteen *EGFRvIII* positive tumors at first surgery, the *EGFRvIII* variant was lost at the time of progression. 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 recurrency between 0 and 3), eventhough *EGFRvIII* expression decreased (n = 8) or stayed the same (n = 1). In fact only 3/14 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 33/42 (79 %) samples: in 25 samples *EGFRvIII* was absent from the primary and recurrent tumor, in 8 samples it was expressed in both (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 recurrent then represents clonal selection of the tumor. Indeed, gliomas are heterogeneous tumors in which distinct subpopulations of cells, each with different genetic makeup, exist[5,21]. However, recent evidence also suggests the genomic *EGFRvIII* deletion is an early event and that *EGFRvIII* expression is regulated by the tumor[19]. In fact, mice experiments demonstrated that at regrowth, the ratio of *EGFRwt/EGFRvIII* expression is similar to the primary tumor even when sorting for *EGFRvIII* high or low expressing tumor cells[22] (see also [23]). Loss of *EGFRvIII* expression then is a result of epigenetic regulation.

In summary, our data show that, in spite of some quantitative differences, the *EGFR* amplification status remains 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 % retain *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 in extrapolating *EGFRvIII* expression, in trials on recurrent glioblastoma that target EGFRvIII mutations a rebiopsy should be considered.

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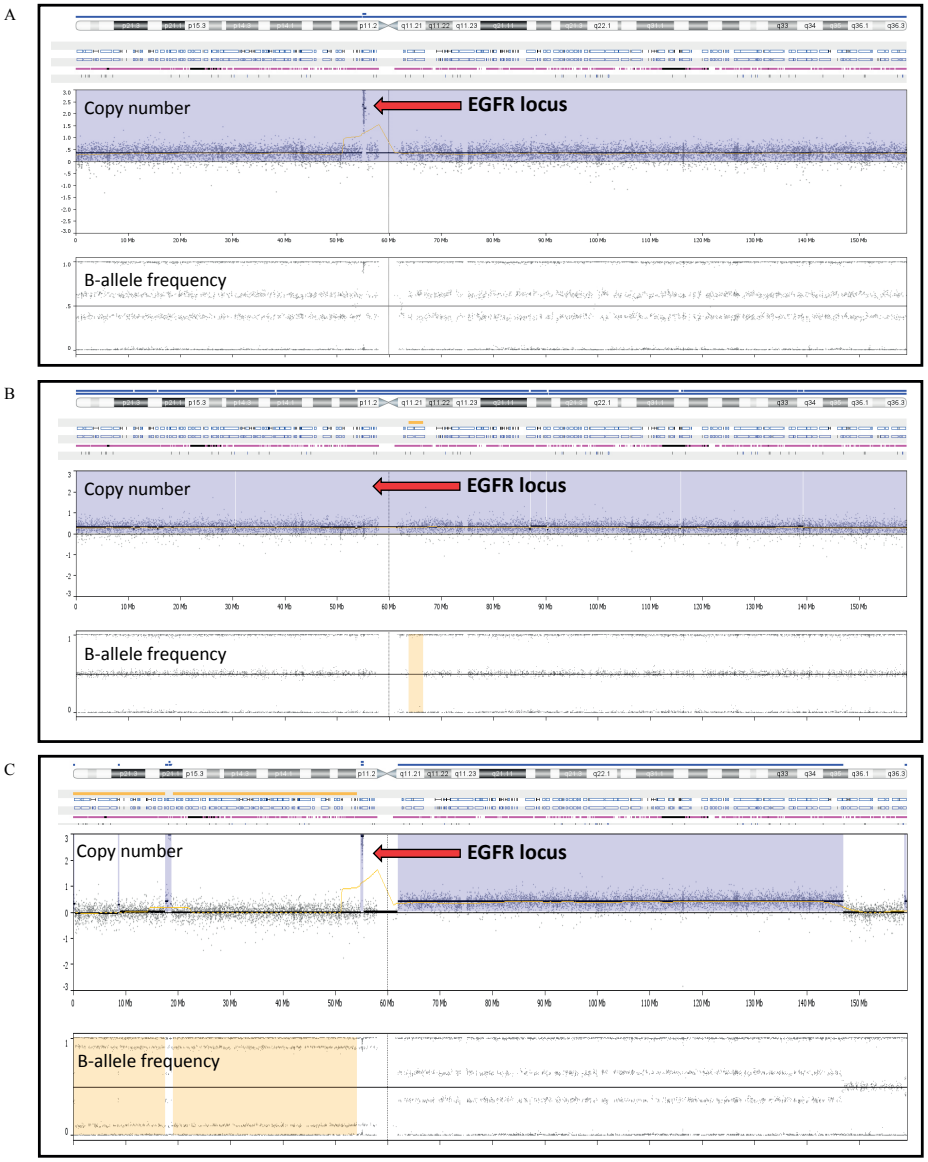

Supplementary Figure 1. Correlation Q-PCR with CNV by Oncoscan DX arrays. Three examples showing Q-PCR results (DCt) and copy number and B-allele frequency of chromosome 7. A: Example 1: EGFR amplified. Patient CCZ, sample at first diagnosis. EGFR amplficiation is visualized by the focal increase in copy number. QPCR DCt results for this sample was 8.4. On top of the high copy EGFR ampification, trisomy of chromosome 7 is seen. B: Example 2: EGFR wt. Patient CBH, sample at first diagnosis. QPCR DCt = 1.0. C: Example 3: EGFR amplified. Patient CCV, sample at first diagnosis. QPCR DCt = 8.1.