Research Paper

Low Incidence along with Low mRNA Levels of *EGFR*^{vIII} in Prostate and Colorectal Cancers Compared to Glioblastoma

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Abstract

Background: The presence as well as the potential role of EGFR^{vIII} in tumors other than glioblastoma still remains a controversial subject with many contradictory data published. Previous analyses, however, did not consider the level of *EGFR*^{vIII} mRNA expression in different tumor types. **Methods:** Appropriately designed protocol for Real-time quantitative reverse-transcription PCR (Real-time qRT-PCR) was applied to analyze *EGFR*^{vIII} and *EGFR*^{WT} mRNA expression in 155 tumor specimens. Additionally, Western Blot (WB) analysis was performed for selected samples. Stable cell lines showing EGFR^{vIII} expression (CAS-1 and DK-MG) were analyzed by means of WB, immunocytochemistry (ICC) and fluorescence *in situ* hybridization (FISH). **Results:** Our analyses revealed *EGFR*^{vIII} expression in 27.59% of glioblastomas (8/29), 8.11% of colorectal cancers (3/37), 6.52% of prostate cancers (3/46) and none of breast cancers (0/43). Despite the average relative expression of *EGFR*^{vIII} varying greatly among tumors of different tissues (approximately 800-fold) or even within the same tissue group (up to 8000-fold for GB), even the marginal expression of *EGFR*^{vIII} mRNA can be detrimental to cancer progression, as determined by the analysis of stable cell lines endogenously expressing the oncogene.

Conclusion: EGFR^{vIII} plays an unquestionable role in glioblastomas with high expression of this oncogene. Our data suggests that EGFR^{vIII} importance should not be underestimated even in tumors with relatively low expression of this oncogene.

Key words: EGFR^{vIII}, prostate cancer, colorectal cancer, breast cancer, glioblastoma, Real-time quantitative reverse-transcription PCR.

Introduction

Type III epidermal growth factor receptor *(EGFR*^{vIII}) is a common mutation of *EGFR* [1]. According to the current state of knowledge, EGFR^{vIII} is tumor-specific, ligand independent and constitutively active receptor. Moreover, it might contribute towards more cancerous phenotype, drug resistance and thus is considered an attractive anticancer therapy target [1, 2]. *EGFR*^{vIII} was confirmed to be expressed in patients with glioblastoma (GB) [3, 4]. On the other hand, there have been many contradictory reports on its presence in other tumor types [2, 5]. Previous research focused only on EGFR^{vIII} detection and did not include any quantitative analysis [6, 7]. Therefore, we decided to analyze not only the occurrence but also the level of *EGFR*^{vIII} expression in glioblastomas, prostate, breast and colorectal tumors as well as unique EGFR^{vIII}-positive glioblastoma cell lines – CAS-1, DK-MG, and DK-MG subline with low EGFR^{vIII} expression (DK-MG^{low}). For the first time the relative

and absolute *EGFR^vIII^* expression level was compared between different tumor types.

Materials and Methods

Tumor samples

Surgical specimens were obtained from 46 patients diagnosed with prostate cancer (Pabianice Medical Center; Mikolaj Pirogow Regional Specialist Hospital in Lodz), 43 with breast cancer (Polish Mother's Health Center Research Institute in Lodz), 37 with colorectal cancer (Antoni Troczewski Local Government Hospital in Kutno, Clinical Hospital Military Memorial Medical Academy – Central Veterans' Hospital in Lodz) and 29 with glioblastoma (The Voivodal Specialistic Hospital in Olsztyn). All samples were collected according to the protocol approved by the Bioethical Committee at the Regional Medical Chamber in Lodz (Approval No. K.B. – 3/12 of February 8, 2012) and by the Bioethical Committee of Medical University of Lodz (Approval No. RNN/27/11/KE). Written informed consent was obtained from all patients and their data were processed and stored according to the principles described in the Declaration of Helsinki. Patients were diagnosed according to the World Health Organization Criteria.

Cell lines

DK-MG cell line (DSMZ, Germany) and its subline (DK-MG^low^) were obtained and cultured as previously described by us [8]. CAS-1 cell line (ICLC, Italy) was cultured in DMEM (PAN-Biotech GmbH, Germany) supplemented with 10% FBS (Biowest, France), 1% Penicillin-Streptomycin (Gibco, France), 0.2% Gentamicin Sulfate (Biowest, France), maintained in 5% CO_2 at 37°C and passaged with trypsin-EDTA (0.05% Trypsin, Gibco, France). Serial dilution in a 96-well plate format was employed to perform clonal selection of CAS-1 cell line.

Real-time qRT-PCR for EGFR^vIII^ and EGFR^WT^

RNA was isolated using AllPrep RNA/DNA Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. Isolation was performed on tissue specimens of 30-40 mg and 4-6 mm in diameter, with approximate RNA yield of 100 ng/µL. For each sample 250 ng of total RNA was reverse-transcribed into single-stranded cDNA using QuantiTect Reverse Transcription Kit (Qiagen, Germany) according to the manufacturer's protocol. To compare *EGFR^vIII^* expression level between different tissue samples, equal amounts of cDNA (20 ng) were analyzed in Real-time qRT-PCR reaction using StepOnePlus Real-Time PCR System (Applied Biosystems). PCR products were synthesized from cDNA samples using SYBR® Select Master Mix. *TBP* and *HPRT1* genes were used as reference to normalize expression level of target genes. Primer sequences for *TBP* gene were 5'-GAGCTGTGATGTGAAGTTTCC-3', 5'-TCTGGGTTTGATCATTCTGTAG-3' while 5'-TGAGGATTTGGAAAGGGTGT-3', 5'-GAGCACA CAGAGGGCTACAA-3' were used to amplify *HPRT1* gene. The following specific primers were used for amplification of target genes: 5'-TAGCAGTCTTATCT AACTATGAT-3', 5'-CACTGCTGACTATGTCCCGC-3' for *EGFR^WT^* and 5' GGCTCTGGAGGAAAAGAAA GGTAATTATGT-3', 5' ACCAATACCTATTCCGTTA CACACT-3' for *EGFR^vIII^*.

Normalized relative *EGFR^WT^* or *EGFR^vIII^* expression level in tested samples *versus* control sample was calculated utilizing the method described by Pfaffl *et al.* [9, 10], based on each sample's average Ct value and each gene's average PCR efficiency. cDNA from CAS-1 and DK-MG cell lines was used as a positive control of *EGFR^vIII^* and *EGFR^WT^* expression. BJ human neonatal foreskin fibroblasts (ATCC, USA) provided a negative control for *EGFR^vIII^* detection.

To generate cDNA pool from EGFR^vIII^-positive samples, mRNA isolated from 15 tumor samples expressing the mutated receptor was pooled and diluted 50 times. Specimens were classified as EGFR^vIII^-positive or -negative depending on generation of reaction product at cycle threshold (Ct) ≤ 37. The expression of *EGFR^vIII^* was normalized using pool cDNA, while *EGFR^WT^* was normalized using cDNA from fibroblasts. A fold change of more than 2 was considered as overexpression, while between 1 and 2 as expression within normal range [11].

The absolute quantification of *EGFR^vIII^* expression level within different tissues was performed according to standard curve method [12]. The number of *EGFR^vIII^* transcripts in tested samples was extrapolated from a standard curve based on Ct values obtained for dilution series of plasmid encoding *EGFR^vIII^*.

DK-MG and CAS-1 cell lines analyses

Immunocytochemistry (ICC) and Western Blotting (WB) for EGFR protein expression as well as FISH for *EGFR* copy number detection were performed as previously described by us [8, 13]. Additionally, WB analyses were performed for selected tumor specimens (with initial homogenization step – mechanical disruption of liquid nitrogen frozen sample). Phospho-WB for DK-MG cell line was performed as described previously with the use of rabbit anti-phospho-EGFR (Tyr1068) antibody (Cell Signaling Technology, Inc., Cat. No. 2234; 1 : 500) [8].

Results

EGFRvIII presence and EGFRvIII and EGFRWT expression level in glioblastomas as well as prostate, breast and colorectal tumors

Real-time qRT-PCR analysis of 155 specimens revealed *EGFRvIII* expression in 14 tumor samples (28.39%), which constituted 9.03% of tested group. All specimens considered EGFRvIII-positive generated reaction product at Ct ≤ 37. Glioblastomas expressed *EGFRvIII* in 27.59% (8 out of 29 samples), while this variant was detected in three out of 37 colorectal tumors (8.11%) and three out of 46 prostate tumors (6.52%). All of 43 analyzed breast cancer samples were EGFRvIII-negative. A maximal thousandfold difference in relative *EGFRvIII* expression level was detected between samples (Table 1). The absolute number of *EGFRvIII* mRNA molecules varied remarkably between different specimens, still the general trend between relative and absolute values was followed (Table 1, Figure 1B).

Intriguingly, glioblastomas were divided into two groups with high and low *EGFRvIII* expression (100-fold difference on average) (Figure 1). These groups varied also in terms of *EGFRWT* expression, which was inversely proportional to the levels of *EGFRvIII*. Interestingly, expression of the oncogenic variant in non-CNS EGFRvIII-positive samples (prostate cancer and colon cancer) and glioblastoma specimens with low *EGFRvIII* expression was comparable (Figure 1A).

Table 1. Comparison of relative *EGFRvIII* and *EGFRWT* mRNA expression levels and absolute copy number of EGFRvIII cDNA in EGFRvIII-positive samples by means of Real-time quantitative PCR.

Sample name	Relative gene expression ± SD		Absolute number of EGFRvIII molecules
	EGFRWT	EGFRvIII	
PC25	1.823 ± 0.062	0.053 ± 0.007	77.706
PC33	2.283 ± 0.063	0.015 ± 0.014	20.382
PC46	2.689 ± 0.079	0.844 ± 0.047	2935.646
CC12	1.239 ± 0.255	0.019 ± 0.008	14.835
CC13	1.257 ± 0.006	0.034 ± 0.015	8.708
CC30	0.690 ± 0.078	0.024 ± 0.006	2.473
GB16	11.435 ± 0.770	0.233 ± 0.033	28.609
GB20	3.769 ± 0.062 ***	21.441 ± 1.898 ***	1730.176
GB28	42.482 ± 7.466	38.299 ± 7.538 ***	660833.331
GB31	3.137 ± 0.436 ***	67.149 ± 9.368 ***	672855.128
GB34	21.895 ± 0.666 ***	0.020 ± 0.004	11.694
GB45	105.487 ± 14.168 ***	0.024 ± 0.019	18.747
GB46	35.256 ± 0.886	1.820 ± 0.086	6887.642
GB49	3.851 ± 0.625	0.008 ± 0.002	6.035
DK-MG	1.931 ± 0.272	16.288 ± 1.816 ***	6392.209
DK-MGlow	2.877 ± 0.196	0.806 ± 0.030	265.193
CAS-1	0.937 ± 0.011	0.786 ± 0.023	1467.265
human fibroblasts	1 ± 0.005	0	0

Note: Statistical significance calculated by one-way ANOVA with Tukey's post-comparison test; ***, p < 0.001; PC – prostate cancer; CC – colorectal cancer; GB – glioblastoma. Absolute number of molecules given *per* 20 ng cDNA.
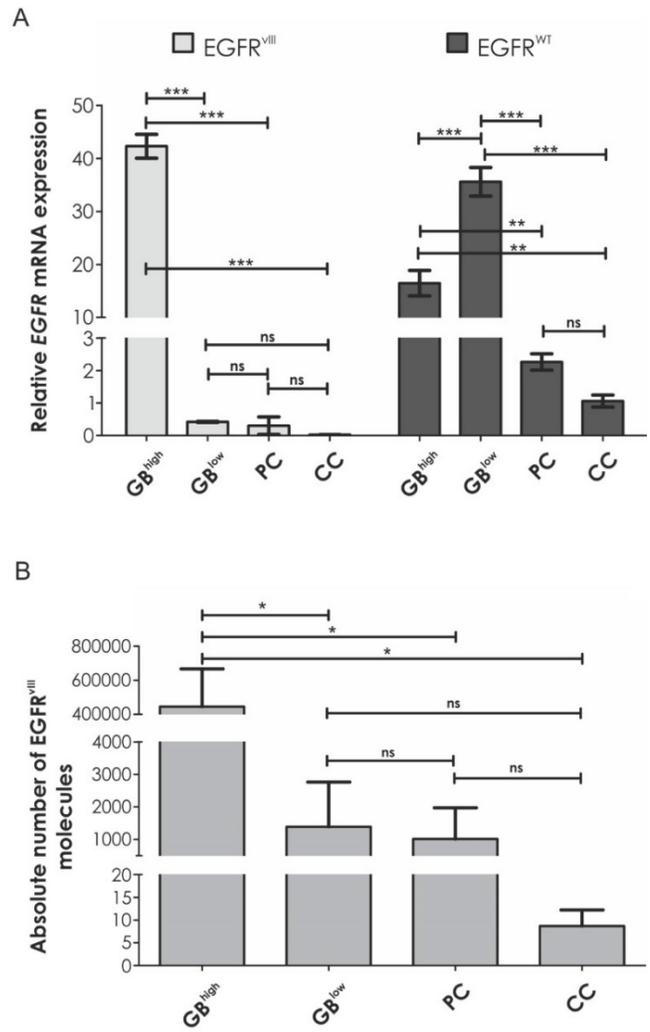
Figure 1. Comparison of **A.** the average relative *EGFRvIII* and *EGFRWT* mRNA expression levels and **B.** the average number of EGFRvIII molecules *per* 20 ng of cDNA between two glioblastoma groups (with high and low *EGFRvIII* expression), prostate cancer and colon cancer. Error bars indicate SEM. Statistical significance calculated by one-way ANOVA with Tukey's post-comparison test; ***, p < 0.001, ns, not significant.

Considering EGFRvIII-positive samples, Real-time qRT-PCR analysis demonstrated on average 20 times higher *EGFRWT* expression level in comparison to tumors other than GB (p < 0.001) and at least three times higher in GB specimens when compared to fibroblasts (8/8, ranging from 3.137 to 105.487).

It is worth mentioning that EGFRvIII was undetectable at protein level in two out of three tumors with comparably low level of this oncogene mRNA expression. In our analysis, the threshold of EGFRvIII detection was 0.8 (Figure 2A, Table 1).

Correlation between EGFRvIII mRNA level and protein expression in CAS-1, DK-MG and DK-MGlow cells

We established DK-MG subline with a minimum content of EGFRvIII-positive cells at 5% (DK-MGlow)

[8]. FISH analysis of CAS-1 cell line revealed numerous extrachromosomal amplicons, in a similar staining pattern to the one observed for DK-MG line, in approximately 1% of cells (Figure 2B). Furthermore, double ICC staining with antibodies against total as well as wild-type EGFR confirmed presence of the truncated receptor and indicated the fraction of EGFRvIII-positive cells to be around 1% (Figure 2C). In case of CAS-1, an attempt to derive a population with smaller fraction of EGFRvIII-expressing cells by means of clonal selection was unsuccessful.

The fraction of EGFRvIII-positive cells in the DK-MG population was further confirmed when population of cells was stimulated with EGF, inducing degradation of the EGFRWT, observed as
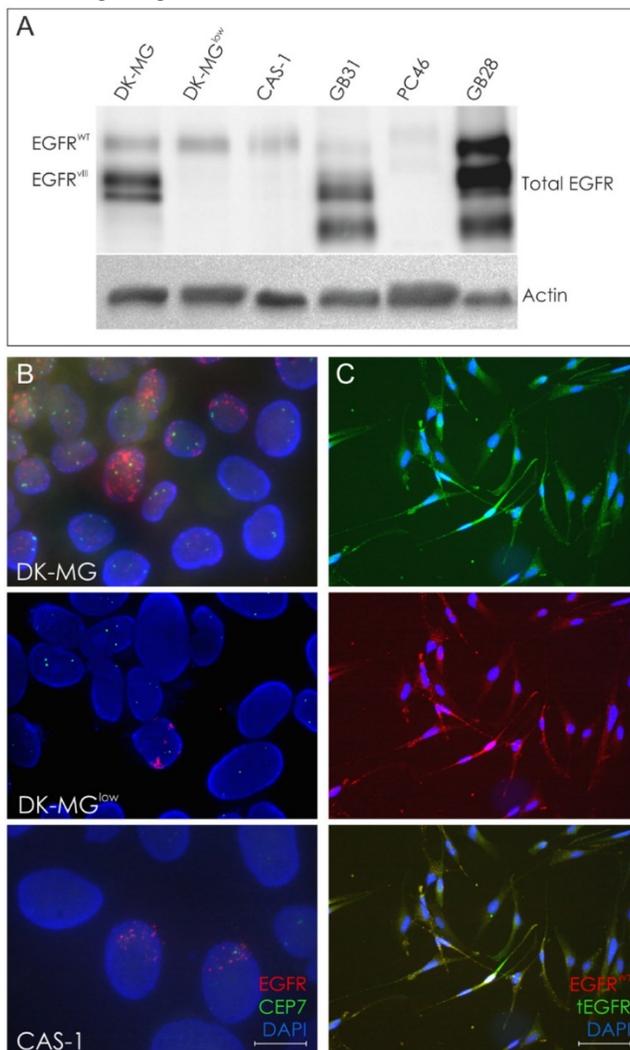
Figure 2. Molecular analyses in glioblastoma cell lines and selected tumor specimens. **A.** Western Blot analysis of DK-MG, DK-MGlow, CAS-1 cell lines and selected EGFRvIII-positive tumor specimens. **B.** FISH analysis of DK-MG, DK-MGlow and CAS-1 cell lines. The number of cells positive for EGFRvIII amplicons varies among these lines; magnification 600x. **C**. Representative images of EGF-mediated degradation of EGFRWT following 1h EGF treatment in CAS-1 cell line, demonstrated as intracellular dots. Cells presenting high cell membrane expression of total EGFR under these conditions are likely to be EGFRvIII-positive.

intracellular dots representing endocytosed protein (Figure 3A). The ligand-mediated degradation of the wild-type receptor, but not EGFRvIII, was confirmed by Western Blotting (Figure 3B).

It is important to note that results of ICC and FISH were consistent for CAS-1, DK-MG and DK-MGlow cells, in each case demonstrating similar percentage of cells with EGFRvIII expression and amplicons, respectively (Figure 2B, 2C and 3A).

Our data suggests, however, that in case of EGFRvIII there is no simple correlation between mRNA and protein level. In spite of the fact that both CAS-1 and DK-MGlow showed similar *EGFRvIII* mRNA expression, the percentage of EGFRvIII-positive cells detected by means of Immunocytochemistry differed between the lines and was undetectable with Western Blotting (Figure 2C), even on overexposed blots (data not shown). Hence, we suggest that Western Blotting may be insufficiently sensitive to detect the low protein level. Moreover, WB analysis of EGFRvIII in frozen tumor sections may give misleading results, due to possible detection of EGFRWT degradation products, which might appear similar in molecular weight to EGFRvIII (Figure 2C).

Discussion

The presence of EGFRvIII in glioblastoma is unquestionable [3, 4], while its occurrence in other tumor types still remains very controversial [6, 7, 14, 15, 16, 17]. There are no reports analyzing *EGFRvIII* mRNA level in a wider range of tumors, since previously it was considered satisfactory to classify tumor samples as EGFRvIII-positive or -negative.

Our work showed for the first time the relative levels of *EGFRvIII* mRNA in specimens of glioblastomas, as well as prostate, colorectal and breast cancer. Interestingly, those levels varied substantially between analyzed tumor types – several thousandfold differences were detected between specimens with the highest and lowest expression, even within glioblastoma cases. It has to be noted that in contrary to other tumor types, glioblastoma is resected with a minimal non-malignant tissue margin. Consequently, the substantial diversity in *EGFRvIII* expression levels and absolute number of molecules observed by us within the group of glioblastoma specimens (Table 1) cannot be explained solely by the variation in extension of surgical resection (resulting from differences in content of normal cells in analyzed specimens). Interestingly, all of the glioblastoma samples investigated by us can be subdivided into two subgroups, depending on the level of *EGFRvIII* mRNA expression (Figure 1A). On the other hand, prostate and colorectal cancers showed relatively low

expression of *EGFRvIII* mRNA and low frequency of this alteration when compared to glioblastoma.

Considering other analyzed tumor types, no breast cancer specimen expressing *EGFRvIII* was identified, which is in accordance with Rae *et al.* [16] and contradicts the data published by Del Vecchio *et al.* and Silva *et al.* [18, 19]. Therefore, it may be suggested that previous analyses overestimated the number of cases showing EGFRvIII expression in tumors other than glioblastoma [6, 15, 16].

Our choice of Real-time qRT-PCR as a method to investigate *EGFRvIII* expression in tumors of different origin has been dictated predominantly by reports on relatively low specificity of anti-EGFRvIII antibodies, low sensitivity and semi-quantitative nature of Western blot or limited use of DNA-based methods in assessment of high copy number of genes encoded by amplicons [15, 20-22]. Still, we are aware that percentage of normal cells in tumor specimens, percentage of tumor cells showing EGFRvIII expression, number of *EGFRvIII* amplicons *per* cell, as well as number of *EGFRvIII* mRNA molecules *per* cell remain variables that may hinder the interpretation of Real-time qRT-PCR results.

Despite the variables described above, the strong discrepancies in mRNA expression levels, even within the same tumor type, suggest different roles or modes of action the oncogene can employ to induce tumor progression. EGFRvIII-positive cells have been described to constitute only a small fraction of the overall cancer cell population, however, they remain dispersed throughout the tumor tissue rather than forming cohesive lumps. Taking into account the reports on the EGFRvIII being implicated in inducing secretion of cytokines and growth factors associated with tumor progression [23-25], or the oncogene being secreted in the form of extracellular vesicles that merge with surrounding cells, it ensures that even

very limited expression of EGFRvIII can contribute towards cancer progression [26]. It remains to be seen, whether different levels of mRNA correlate with any particular oncogenic action.

The important role of EGFRvIII in cancer cell progression is further supported by the study on stable cancer cell lines that endogenously express the oncogenic receptor. In case of DK-MG line we were able to reduce the number of EGFRvIII-positive cells below the 5% threshold of the population, but never to zero. Additionally, we were unable to decrease the number of EGFRvIII-positive cells in CAS-1 cell line. Taken together, our results suggest that the aforementioned models are fairly accurate representation of the tumor tissue and correspond with Nishikawa *et al.* who demonstrated that low percentage of EGFRvIII-positive cells is sufficient to maintain proper environment for glioblastoma progression [27]. Additionally, models characterized by low incidence of a particular mutation may be suitable especially for analysis of cancer stem cells hypothesis. In fact, EGFRvIII-positive cells have already been reported to demonstrate characteristic features of cancer stem cells [28].

With the use of Real-time qRT-PCR we demonstrated that prostate and colorectal cancer resections were positive for *EGFRvIII* expression at 6.52% and 8.11% incidence rate, respectively, albeit the relative expression level was low. In contrast, glioblastoma specimens (with incidence rate at 27.59%) varied greatly in respect to the relative and absolute number of mutant receptor's mRNA.

The correlation between varying levels of *EGFRvIII* mRNA and the reported elsewhere pro-oncogenic function of EGFRvIII remain to be elucidated. However, it is clear that even the lowest levels of the oncogenic receptor's expression might be sufficient for cancer progression.
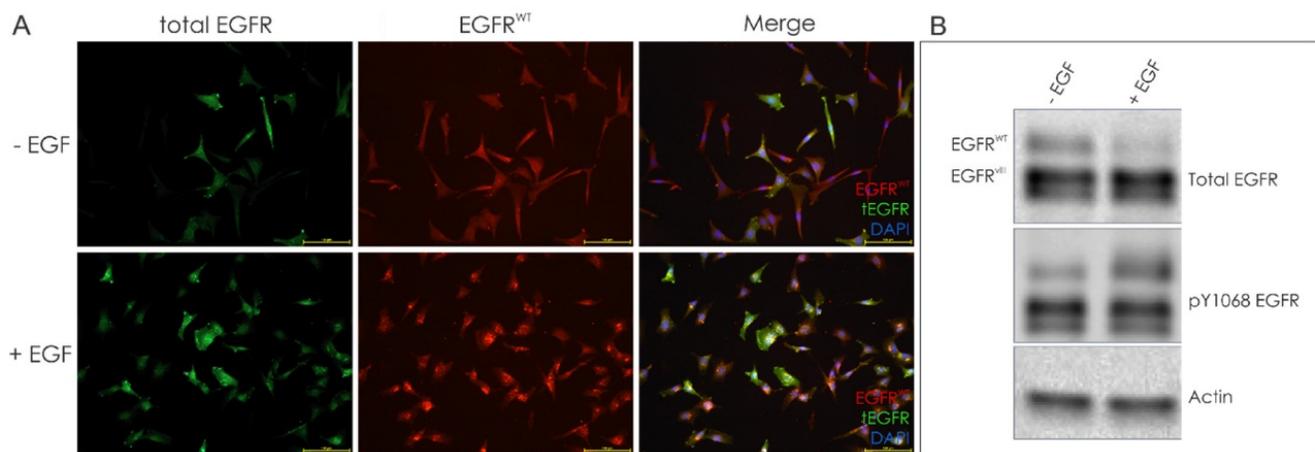
Figure 3. EGF-mediated degradation of the wild-type EGFR allows for identification of EGFRvIII-positive cells. **A.** Representative images of DK-MG cells treated for 1h with EGF, as indicated. Intracellular dots represent EGFRWT undergoing endocytosis. Cells strongly positive for total EGFR signal are likely to be EGFRvIII-positive. **B.** Western blot analysis confirms degradation of the EGFRWT following ligand stimulation.

Abbreviations

CNS: Central Nervous System; EGFRvIII: Epidermal Growth Factor Receptor variant III; EGFRWT: Epidermal Growth Factor Receptor wild-type; GB: Glioblastoma; Real-time qRT-PCR: real-time quantitative reverse-transcription PCR

Competing Interests

The authors have declared that no competing interest exists.

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