Research Paper

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

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Abstract

Background: The presence as well as the potential role of $\text{EGFR}^{\text{vIII}}$ in tumors other than glioblastoma still remains a controversial subject with many contradictory data published. Previous analyses, however, did not consider the level of $\text{EGFR}^{\text{vIII}}$ 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 $\text{EGFR}^{\text{vIII}}$ and $\text{EGFR}^{\text{WT}}$ mRNA expression in 155 tumor specimens. Additionally, Western Blot (WB) analysis was performed for selected samples. Stable cell lines showing $\text{EGFR}^{\text{vIII}}$ expression (CAS-1 and DK-MG) were analyzed by means of WB, immunocytochemistry (ICC) and fluorescence in situ hybridization (FISH). Results: Our analyses revealed $\text{EGFR}^{\text{vIII}}$ 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 $\text{EGFR}^{\text{vIII}}$ 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 $\text{EGFR}^{\text{vIII}}$ mRNA can be detrimental to cancer progression, as determined by the analysis of stable cell lines endogenously expressing the oncogene. Conclusion: $\text{EGFR}^{\text{vIII}}$ plays an unquestionable role in glioblastomas with high expression of this oncogene. Our data suggests that $\text{EGFR}^{\text{vIII}}$ importance should not be underestimated even in tumors with relatively low expression of this oncogene.

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

Introduction

Type III epidermal growth factor receptor ($\text{EGFR}^{\text{vIII}}$) is a common mutation of $\text{EGFR}$ [1]. According to the current state of knowledge, $\text{EGFR}^{\text{vIII}}$ 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]. $\text{EGFR}^{\text{vIII}}$ 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 $\text{EGFR}^{\text{vIII}}$ detection and did not include any quantitative analysis [6, 7]. Therefore, we decided to analyze not only the occurrence but also the level of $\text{EGFR}^{\text{vIII}}$ expression in glioblastomas, prostate, breast and colorectal tumors as well as unique $\text{EGFR}^{\text{vIII}}$-positive glioblastoma cell lines – CAS-1, DK-MG, and DK-MG subline with low $\text{EGFR}^{\text{vIII}}$ expression (DK-MGlow). For the first time the relative
and absolute \( \text{EGFR}^{\text{vIII}} \) 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 Specialist Clinic 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\textsubscript{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\textsubscript{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 \( \text{EGFR}^{\text{vIII}} \) and \( \text{EGFR}^{\text{WT}} \)**

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 \( \text{EGFR}^{\text{vIII}} \) 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. \( \text{TBP} \) and \( \text{HPRT1} \) genes were used as reference to normalize expression level of target genes. Primer sequences for \( \text{TBP} \) gene were 5’-GAGCTGTGATGGAAGGTTCCT-3’, 5’-TCTGGTTTATCATTGCTG-3’ while 5’-TGAGGATTGGAAGGGTG-3’, 5’-GACCA GAGAGGCTACAA-3’ were used to amplify \( \text{HPRT1} \) gene. The following specific primers were used for amplification of target genes: 5’-TACAGTCTTATCTAACATGAT-3’, 5’-CATGCTGACTATGTCCCGC-3’ for \( \text{EGFR}^{\text{WT}} \) and 5’ GGCCTGTGAGAAAGAAA GTAAATTATGT-3’, 5’ ACCAATACCTATTCCGT TACACT-3’ for \( \text{EGFR}^{\text{vIII}} \).

Normalized relative \( \text{EGFR}^{\text{WT}} \) or \( \text{EGFR}^{\text{vIII}} \) 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 \( \text{EGFR}^{\text{vIII}} \) and \( \text{EGFR}^{\text{WT}} \) expression. BJ human neonatal foreskin fibroblasts (ATCC, USA) provided a negative control for \( \text{EGFR}^{\text{vIII}} \) detection.

To generate cDNA pool from \( \text{EGFR}^{\text{vIII}} \)-positive samples, mRNA isolated from 15 tumor samples expressing the mutated receptor was pooled and diluted 50 times. Specimens were classified as \( \text{EGFR}^{\text{vIII}} \)-positive or -negative depending on generation of reaction product at cycle threshold (Ct) \( \leq 37 \). The expression of \( \text{EGFR}^{\text{vIII}} \) was normalized using pool cDNA, while \( \text{EGFR}^{\text{WT}} \) 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 \( \text{EGFR}^{\text{vIII}} \) expression level within different tissues was performed according to standard curve method [12]. The number of \( \text{EGFR}^{\text{vIII}} \) transcripts in tested samples was extrapolated from a standard curve based on Ct values obtained for dilution series of plasmid encoding \( \text{EGFR}^{\text{vIII}} \).

**DK-MG and CAS-1 cell lines analyses**

Immunocytochemistry (ICC) and Western Blotting (WB) for \( \text{EGFR} \) protein expression as well as FISH for \( \text{EGFR} \) 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-\( \text{EGFR} \) (Tyr1068) antibody (Cell Signaling Technology, Inc., Cat. No. 2234; 1 : 500) [8].

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**Results**

**EGFR<sup>iii</sup> presence and EGFR<sup>iii</sup> and EGFR<sup>WT</sup> expression level in glioblastomas as well as prostate, breast and colorectal tumors**

Real-time qRT-PCR analysis of 155 specimens revealed EGFR<sup>iii</sup> expression in 14 tumor samples (28.39%), which constituted 9.03% of tested group. All specimens considered EGFR<sup>iii</sup>-positive generated reaction product at Ct ≤ 37. Glioblastomas expressed EGFR<sup>iii</sup> 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 EGFR<sup>iii</sup>-negative. A maximal thousandfold difference in relative EGFR<sup>iii</sup> expression level was detected between samples (Table 1). The absolute number of EGFR<sup>iii</sup> mRNA molecules varied remarkably between different specimens, still the general trend between relative and absolute values was followed (Table 1, Figure 1).

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

**Table 1.** Comparison of relative EGFR<sup>iii</sup> and EGFR<sup>WT</sup> mRNA expression levels and absolute copy number of EGFR<sup>iii</sup>cDNA in EGFR<sup>iii</sup>-positive samples by means of Real-time quantitative PCR.

| Sample name | Relative gene expression ± SD | Absolute number of EGFR<sup>iii</sup> molecules |
|-------------|-------------------------------|-----------------------------------------------|
| PC25        | 1.823 ± 0.062                 | 0.053 ± 0.007                                 |
| PC33        | 2.283 ± 0.063                 | 0.015 ± 0.014                                 |
| PC46        | 2.689 ± 0.079                 | 0.844 ± 0.047                                 |
| CC12        | 1.239 ± 0.255                 | 0.019 ± 0.008                                 |
| CC13        | 1.257 ± 0.006                 | 0.034 ± 0.015                                 |
| CC30        | 0.690 ± 0.078                 | 0.024 ± 0.006                                 |
| GB16        | 11.435 ± 0.770                | 0.233 ± 0.033                                 |
| GB20        | 3.769 ± 0.062 ***            | 21.441 ± 1.988 ***                             |
| GB28        | 42.482 ± 7.466                | 38.299 ± 7.538 ***                             |
| GB31        | 3.137 ± 0.436 ***            | 67.149 ± 9.368 ***                             |
| GB33        | 21.895 ± 0.666 ***           | 0.020 ± 0.004                                 |
| GB45        | 105.487 ± 14.166 ***         | 0.024 ± 0.019                                 |
| GB46        | 35.256 ± 0.886               | 1.820 ± 0.086                                 |
| GB49        | 3.853 ± 0.625                | 0.008 ± 0.002                                 |
| DK-MG       | 1.931 ± 0.272                | 16.288 ± 1.816 ***                            |
| DK-MG<sub>low</sub> | 2.877 ± 0.196          | 0.806 ± 0.030                                 |
| CAS-1       | 0.937 ± 0.011                | 0.786 ± 0.023                                 |
| human fibroblasts | 1 ± 0.005        | 0                                              |

Note: Statistical significance calculated by one-way ANOVA with Tukey’s post-comparison test; ***, p < 0.001; ns, not significant.

Considering EGFR<sup>iii</sup>-positive samples, Real-time qRT-PCR analysis demonstrated on average 20 times higher EGFR<sup>WT</sup> 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 EGFR<sup>iii</sup> 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 EGFR<sup>iii</sup> detection was 0.8 (Figure 2A, Table 1).

**Correlation between EGFR<sup>iii</sup> mRNA level and protein expression in CAS-1, DK-MG and DK-MG<sub>low</sub> cells**

We established DK-MG subline with a minimum content of EGFR<sup>iii</sup>-positive cells at 5% (DK-MG<sub>low</sub>)
[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 EGFR\textsuperscript{vIII}-positive cells to be around 1% (Figure 2C). In case of CAS-1, an attempt to derive a population with smaller fraction of EGFR\textsuperscript{vIII}-expressing cells by means of clonal selection was unsuccessful.

The fraction of EGFR\textsuperscript{vIII}-positive cells in the DK-MG population was further confirmed when population of cells was stimulated with EGF, inducing degradation of the EGFR\textsuperscript{WT}, observed as intracellular dots representing endocytosed protein (Figure 3A). The ligand-mediated degradation of the wild-type receptor, but not EGFR\textsuperscript{vIII}, 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-MG\textsuperscript{low} cells, in each case demonstrating similar percentage of cells with EGFR\textsuperscript{vIII} expression and amplicons, respectively (Figure 2B, 2C and 3A).

Our data suggests, however, that in case of EGFR\textsuperscript{vIII} there is no simple correlation between mRNA and protein level. In spite of the fact that both CAS-1 and DK-MG\textsuperscript{low} showed similar EGFR\textsuperscript{vIII} mRNA expression, the percentage of EGFR\textsuperscript{vIII}-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 EGFR\textsuperscript{vIII} in frozen tumor sections may give misleading results, due to possible detection of EGFR\textsuperscript{WT} degradation products, which might appear similar in molecular weight to EGFR\textsuperscript{vIII} (Figure 2C).

**Discussion**

The presence of EGFR\textsuperscript{vIII} 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 EGFR\textsuperscript{vIII} mRNA level in a wider range of tumors, since previously it was considered satisfactory to classify tumor samples as EGFR\textsuperscript{vIII}-positive or -negative.

Our work showed for the first time the relative levels of EGFR\textsuperscript{vIII} 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 EGFR\textsuperscript{vIII} 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 EGFR\textsuperscript{vIII} mRNA expression (Figure 1A). On the other hand, prostate and colorectal cancers showed relatively low
expression of EGFR<sup>vIII</sup> mRNA and low frequency of this alteration when compared to glioblastoma.

Considering other analyzed tumor types, no breast cancer specimen expressing EGFR<sup>vIII</sup> 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 EGFR<sup>vIII</sup> expression in tumors other than glioblastoma [6, 15, 16].

Our choice of Real-time qRT-PCR as a method to investigate EGFR<sup>vIII</sup> expression in tumors of different origin has been dictated predominantly by reports on relatively low specificity of anti-EGFR<sup>vIII</sup> 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 EGFR<sup>vIII</sup> expression, number of EGFR<sup>vIII</sup> amplicons per cell, as well as number of EGFR<sup>vIII</sup> 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. EGFR<sup>vIII</sup>-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 EGFR<sup>vIII</sup> 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 EGFR<sup>vIII</sup> 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 EGFR<sup>vIII</sup> 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 EGFR<sup>vIII</sup>-positive cells below the 5% threshold of the population, but never to zero. Additionally, we were unable to decrease the number of EGFR<sup>vIII</sup>-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 EGFR<sup>vIII</sup>-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, EGFR<sup>vIII</sup>-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 EGFR<sup>vIII</sup> 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 EGFR<sup>cell</sup> mRNA and the reported elsewhere pro-oncogenic function of EGFR<sup>vIII</sup> 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](http://www.jcancer.org)

**Figure 3.** EGF-mediated degradation of the wild-type EGFR allows for identification of EGFR<sup>vIII</sup>-positive cells. **A.** Representative images of DK-MG cells treated for 1h with EGF, as indicated. Intracellular dots represent EGFR<sup>WT</sup> undergoing endocytosis. Cells strongly positive for total EGFR signal are likely to be EGFR<sup>vIII</sup>-positive. **B.** Western blot analysis confirms degradation of the EGFR<sup>WT</sup> 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

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Competing Interests

The authors have declared that no competing interest exists.

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