

EGFRvIII – A Stable Target for Anti-EGFRvIII Therapy

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Abstract. *Background: Epidermal growth factor receptor (EGFR) gene alterations play important roles in pathogenesis of glioblastoma. Antibodies against EGFRvIII have been recently developed. Their efficacy depends on numerous factors, including the co-existence of EGFRvIII with other genetic alterations, and especially with point mutations of EGFR. Materials and Methods: We examined 91 patients diagnosed with glioblastoma in order to determine the prevalence and mutual relationships between EGFR alterations. Real-time polymerase chain reaction (real-time PCR), fluorescent in situ hybridization (FISH), and sequencing were used to analyze prevalence of the amplification of EGFR gene, polysomy of chromosome 7, EGFRvIII mutation, and point mutations in exons 7-8 and 15 of EGFR. Results: We revealed that all these alterations can occur independently from each other. Nevertheless, the co-existence of EGFRvIII mutation and excessive copies of EGFR gene was observed in most cases (10/14). Similarly, the point mutations in exons 7-8 and 15 co-existed with an excessive number of EGFR copies in nearly all cases. Conclusion: EGFRvIII is a reliable and stable target for anti-EGFRvIII therapy.*

Glioblastoma (GBM) is the most precarious primary tumor of the brain. Alteration of epidermal growth factor receptor (EGFR) gene is the most frequent abnormality detected in GBM (1). The spectrum of the observed alterations of EGFR includes amplification, polysomy of chromosome 7, mutations leading to formation of EGFRvIII, and point mutations in the exons of this gene (2, 3).

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EGFR is involved in signal transduction via numerous pathways important for the cell, such as phosphoinositide 3-kinase (PI3K), mitogen-activated protein kinase (MAPK), and Janus kinase-signal transducer and activator of transcription (JAK-STAT) (4, 5). It plays an important role in the control of proliferation, apoptosis, and growth and differentiation of the cell (6). In this study, we analyzed the prevalence of point mutations in exons 7-8 and 15 of EGFR, and EGFRvIII mutation (7), and examined the occurrence of polysomy of chromosome 7 and amplification of EGFR gene using a new real-time polymerase chain reaction (real-time PCR) method with fluorescent in situ hybridization (FISH)-confirmed method of distinguishing between amplification and polysomy (8). FISH is currently the only method able to establish the true cause for the occurrence of excessive copies of the EGFR gene. The use of classic real-time quantitative PCR did not allow for the precise identification of the reason for an increased number of copies of EGFR gene. Since distinguishing between the phenomena of polysomy and amplification is not possible, only the increase in the quantity of genetic material (9), the interpretation of the findings is imprecise. Numerous authors expressed the need for a rapid and efficient method of identifying the relevant alterations of EGFR gene (10, 11). Consequently, due to the high cost of FISH, the number of published analyses involving large number of samples with co-existing amplification of the EGFR gene and polysomy of chromosome 7 is sparse; moreover, information on co-existing additional alterations of this gene, e.g. EGFRvIII or hot-spot point mutations, is lacking in such cases (12, 13).

Defining the number and type of point mutations present in glioblastoma has become vitally important when searching for monoclonal antibodies and tyrosine kinase inhibitors (14) for targeted anti-EGFR/EGFRvIII therapy. Most literature evidence refers to monoclonal antibodies (15, 16) against EGFRvIII. These antibodies were developed on the basis of epitope created by the joining of exons 1 and 8 (such a combination is characteristic of EGFRvII). However, aside from the joining of exons 1-8, the efficacy of these molecules can also result from the influence of other factors. Additional

mutations can alter the conformation of the epitope and be reflected in a complete lack of therapeutic effect. Therefore, identifying the number and type of point mutations of *EGFR* gene and *EGFRvIII* has profound new implications. These problems were studied by Idhah *et al.* (17) among others. They reported alteration C866T in exon 7 of *EGFR* gene and detected changes in exon 15 (G1793T). Mutations identified by these authors were associated with the extracellular domain of the receptor. Idhah *et al.* (17) found missense mutations associated with the presence of *EGFRvIII* mutation and amplification of this gene. The missense mutations co-existed with the amplification of *EGFR*. Our study also revealed an association between the amplification of *EGFR* gene and *EGFRvIII* mutation. Previous studies by Idhah *et al.* (17) and Frederic *et al.* (18), based on sequencing the exons of *EGFR* gene in glioblastoma, confirmed these findings by revealing that mutation in the extracellular domain is frequently strongly correlated with the amplification of *EGFR*.

Materials and Methods

Tumor samples. The analyzed group consisted of 91 patients, aged from 30 to 75 years, who had undergone neurosurgical resection of glioblastoma at the Norbert Barlicki University Clinical Hospital No. 1 in Lodz, Poland. All samples were collected using protocols approved by the Ethical Committee of the Medical University of Lodz (no. RNN/9/10/KE). The patients were diagnosed at the Department of Pathomorphology, Medical University of Lodz, according to the World Health Organization criteria for brain tumor classification (19). All patients were diagnosed with glioblastoma WHO grade IV by a neuropathologist. None of the patients had an earlier diagnosis of astrocytoma, thus, all cases are regarded as primary glioblastoma. In this study, 91 tumor samples from patients with glioblastoma were analyzed.

DNA/RNA isolation and reverse transcriptase PCR. Total cellular DNA and RNA were isolated from frozen tissue samples (stored at -80°C) using an AllPrep DNA/RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. RNA samples were treated with DNase following isolation. RNA and DNA concentrations were measured spectrophotometrically; 200 ng of total RNA were reverse-transcribed into a single-stranded cDNA using a QuantiTect Reverse Transcription Kit (Qiagen).

Analysis of the prevalence of *EGFRvIII* mutation. The PCR method was used to assess the occurrence of *EGFRvIII* mutation. We employed primers specific to the extracellular domain of *EGFR* overlapping the *EGFRvIII* deletion region: forward: ATGCGACCCTCC GGGACGGC, and reverse: GCACAGGTGG CACACATGGC. Amplicon size was 1860 bp for wild type (wt) *EGFR* and 1059 bp for *EGFRvIII*. The reaction conditions were: initial denaturation at 94°C for 5 min, denaturation at 94°C for 30 s, annealing at 62°C for 30 s, elongation at 72°C for 110 s, and final elongation at 72°C for 5 min, for 35 cycles. The length of PCR products after electrophoresis on 0.9% agarose gel was analyzed on a Gel Doc™ XR+ system (Bio-Rad, Hercules, CA, USA) with UV lamp and the sample was evaluated using Quantity One software.

Simultaneously, real-time qPCR analysis was performed to confirm the results in all samples using *EGFRvIII* forward: 5'-GGCTCTGGAGGAAAAGAAAGGTAAT-3' and reverse: 5'-TCCTCCATCTCATAGCTGTGCG-3' primers, and glucuronidase, beta (*GUSB*) forward: 5'-CTCATTGGAATTTTGCCGATT-3' and reverse: 5'-CCGAGTGAAGATCCCTTTTAA-3' as a reference gene. The reactions were conducted using a Rotor-Gene 3000 instrument (Corbett Research, Mortlake, Australia). A reference sample, namely a mixture of *EGFR*wt sample with *EGFRvIII*, was prepared to obtain a material with low amount of *EGFRvIII*. The results exceeding 0.5 by Pfaffl analysis (20) were considered positive. We were able to unequivocally confirm the occurrence of *EGFRvIII* on the basis of the obtained results being significantly lower or higher than 0.5.

***EGFR* gene dosage analysis: Quantitative real-time PCR at DNA level.** To determine the *EGFR* gene dosage level and the presence of polysomy in original tumor tissue, quantitative real-time PCR was performed using a Rotor-Gene 6000 instrument (Corbett Research, Mortlake, Australia). Analyses were performed according to a previously described protocol (8). The following *EGFR* primers were used for the amplification: forward: AACCATGC CCGCATTAGCTC, reverse: AAAGGAATGCAACTTCCCAA. Each sample was amplified in triplicate in a 10- μl reaction volume containing 10 ng of DNA, a 1x reaction mixture containing Syto9 (Invitrogen) and 35 ng of each of the forward and reverse primers. The cycling conditions for the real-time PCR reactions were as follows: 3 min at 95°C (polymerase activation) followed by 40 cycles of 20 s at 95°C (denaturation), 30 s at 60°C (annealing), and 20 s at 72°C (extension). RNase P (*RPP25*) was used as a reference gene for normalization of the target gene dosage level. Insulin-like growth factor-2 mRNA binding protein 3 (*IGF2BP3*) was used as a reference gene to determine the number of copies of chromosome 7. To confirm the specificity of the amplification signal, the gene dissociation curve was considered in each case. The normalized relative *EGFR* gene dosage level and polysomy of chromosome 7 of the tested samples versus control sample was calculated utilizing the method described previously by Pfaffl *et al.*, based on each sample's average CT value and each gene's average PCR efficiency. As a control sample, DNA derived from non-tumor tissue (leukocytes) was used on the assumption that the gene dosage in normal tissue would be 1.

Sequencing analysis of *EGFR* exons 7-8 and 15. Exons 7-8 and 15 of the *EGFR* gene were amplified by PCR on cDNA template and sequenced using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) for 91 samples following the protocol of the manufacturer. The primers used for the PCR amplification of cDNA sequences were: exons 7-8: forward, CTGCCACAAC CAGTGTGCT and reverse, GCCTGAATCAGCAAAACCC, with annealing temperature of 55°C ; and exon 15: forward, GGCGC CACATCGTTCGGAAG and reverse, CACCCAGCAGTT TGGCCAGC, with an annealing temperature of 57°C . The primers used for cycle sequencing were GGAGGTGCAGTTTGAAGTG (exon 7-8, reverse) and GCACGGTGTATAAGGGACTC (exon 15, forward). Applied Biosystems 3130 Genetic Analyzer system was applied to the separation and analysis of PCR-sequencing products. In order to sequence exons 7-8 in cases with co-existing *EGFRvIII* matrix, the products were separated by means of electrophoresis. The PCR product for wild-type transcript, cut from a 0.9% agarose gel, was used for the sequencing. NucleoSpin kit (Macherey-Nagel, Düren, Germany) was used for isolation of the product from gel.

FISH. The results of real-time PCR were classified into three groups. The first included samples without polysomy or amplification; the second comprised samples with both these alterations; the third group contained samples with only one alteration. After obtaining the results of real-time qPCR analysis, two samples were selected randomly from each group. Material corresponding to these samples was obtained from cell cultures and examined with FISH.

In brief, a commercial probe set (LSI EGFR SpectrumOrange/CEP 7 SpectrumGreen, no. 32-191053; Vysis, Downers Grove, IL, USA) was used for simultaneous determination of the *EGFR* gene and chromosome 7 copy numbers. FISH was performed using the following procedure: the fixed slides were incubated in 2× standard saline citrate (SSC) at 72°C for 5 min. The slides were then placed in 70%, 85%, and 100% ethanol for 1 min each, then air-dried and placed on a 50°C slide warmer for 2 min. The FISH probe set mix (1 µl LSI DNA probe mix, 7 µl LSI Hybridization Buffer, and 2 µl water) was centrifuged and denatured at 73°C for 5 min. The denatured probe was added to each specimen. The slides were then coverslipped and incubated at 37°C overnight in a humidified chamber. Next, the slides were washed with 0.4× SSC/0.3% NP-40 at 73°C for 15 min and rinsed in 2× SSC/0.1% NP-40. Before coverslipping, 10 µl of DAPI II counterstain was added to the slides. To score the samples, a Nikon Eclipse Ci fluorescence microscope equipped with a specially designed filter combination for green and orange spectra was used.

The number of red signals, caused by the binding of the *EGFR*-specific probe, directly reflects the number of *EGFR* copies. The number of green signals, caused by the binding of the CEP 7 probe, directly reflects the number of chromosome 7 copies. FISH evaluation was performed using previously published criteria (21, 22). The *EGFR*/CEP 7 ratio was calculated and nuclei containing three or more signals specific for CEP 7 were defined as having chromosome 7 polysomy. Samples with intrachromosomal amplification ratios of 2 or greater were considered to be amplified for *EGFR* and samples with amplification ratios less than 2 were considered to be non-amplified for *EGFR*. Extrachromosomal amplification of *EGFR* was defined as the presence of at least three- times as many *EGFR* signals as centromere 7 signals per cell (23). A representative FISH result is presented in Figure 1.

Results

The analyses were conducted at the Department of Molecular Pathology and Neuropathology and the Department of Tumor Biology of the Medical University of Lodz.

EGFRvIII. The *EGFRvIII* mutation was detected in 14 (15%) cases, both on agarose gel analysis and by means of real-time qPCR method (Table I).

Analysis of gene amplification and polysomy. Amplification of *EGFR* gene was documented in 20 (22%) and polysomy in 22 (24%) of all examined samples (n=91) (Table I).

EGFR ex 7-8 and 15 sequencing analysis. Upon sequencing exons 7-8 and 15 of *EGFR* gene, three missense point

mutations (3% of cases) were documented in exon 7-8: C866T (A289V), G971T (R324L), and A787C (T263P), along with one mutation (1% of cases), G1736A C579Y, in exon 15 (Figure 2) (Table I).

FISH analysis. Analysis by means of FISH confirmed the results of real-time quantitative PCR analysis in all samples selected from each of the three groups (Figure 1).

Analysis of the co-existence of EGFR gene alterations. Six of the analyzed samples showed both amplification and polysomy. Amongst samples with point mutations in exons 7-8, we found one with amplification of *EGFR* gene, one with both amplification of *EGFR* and the expression of *EGFRvIII*, and one with none of these alterations. The sample with the point mutation in exon 15 had both amplification of *EGFR* gene and the *EGFRvIII* mutation.

Amongst gliomas with *EGFRvIII* mutation, there were seven (50%) cases with amplification, six (43%) with the polysomy, three (21%) with co-existing amplification and polysomy, and four (29%) without polysomy or amplification.

In cases with both amplification and the presence of *EGFRvIII*, we observed the prevalence of the expression of mutated type *EGFR* over the *EGFRwt*.

Discussion

The most frequent genetic alterations associated with GBM include alterations of the *EGFR* gene (1, 24), amplification of *EGFR* (22, 25), and polysomy of chromosome 7 (26). The aim of this study was to determine the frequency of the co-existence of the amplification of *EGFR* gene, polysomy of chromosome 7, *EGFRvIII* form (deletion of exon 2-7), and point mutations in exons 7-8 and 15 (27).

In our study, polysomy and amplification were analyzed as separate alterations; this was possible due to the application of improved real-time qPCR, which was described in another article (8), as well as the FISH technique (28, 29).

The co-existence of *EGFRvIII* was most frequently observed in cases with an excessive number of *EGFR* gene copies, i.e. in cases with amplification, polysomy, or both these alterations. We rarely observed expression of *EGFRvIII* without co-existing polysomy or amplification (30, 31).

These findings are consistent with the results published by Hui K. Gan *et al.* (3) and Liu L. *et al.* (24), who detected *EGFRvIII* in 50-60% of GBM co-existing with amplification or polysomy. However, these authors did not use FISH to confirm their findings. Our study produced highly similar findings as the amplification was documented in seven out of 14 cases with *EGFRvIII*. Shinojima *et al.* (9) also showed that *EGFRvIII* mutation is associated with the amplification of the *EGFR* gene. However, we revealed that the *EGFRvIII* mutation

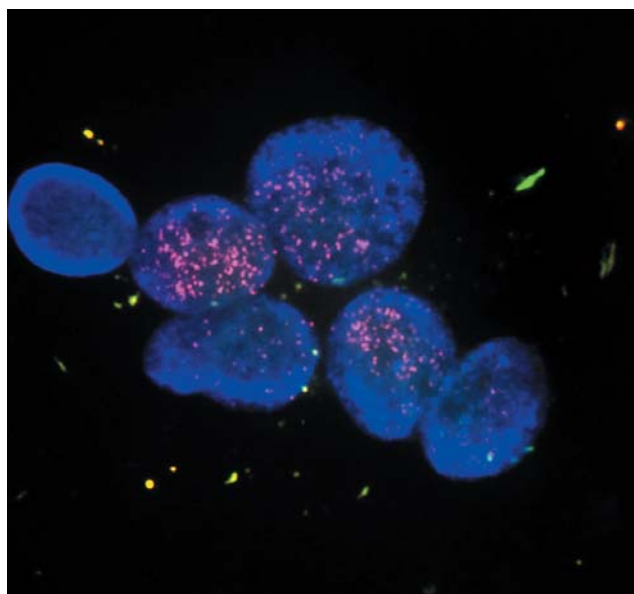


Figure 1. Fluorescence in situ hybridization analysis of the *EGFR* gene dosage: *EGFR* probe (red/pink signals) and CEP 7 control probe (green signals). Tumor cells in cells with the *EGFR* amplification are present.

co-exists not only with the amplification, but also with the polysomy of chromosome 7. Therefore, the presence of *EGFRvIII* was usually associated with the excessive number of *EGFR* copies (10/14 cases, 71%). Only four samples with the *EGFRvIII* mutation did not show amplification or polysomy.

Taking into account the prevalence of polysomy (22 cases), amplification (20 cases) and *EGFRvIII* mutation (14 cases), it can be concluded that although the occurrence of the latter is not determined by the presence of chromosome 7 polysomy or *EGFR* gene amplification, these alterations very often co-exist.

Sequencing of *EGFR* gene, conducted to identify mutations in exons 7-8 and 15 of *EGFR* gene, revealed low prevalence of point mutations on the cDNA template of *EGFR* gene; noticeably, 3 out of 4 identified mutations were associated with the increased number of copies of the *EGFR* gene. The missense mutations in exon 7-8 and 15 were documented in three cases and one case, respectively.

Point mutations were detected in 2 out of 14 samples with *EGFRvIII* mutation; one involved exon 7 (C866T), while the other referred to exon 15 (G1736A). Both these alterations co-existed with the amplification of *EGFR* gene.

The remaining two point mutations detected in our 91 samples were not associated with *EGFRvIII* mutation or with polysomy of chromosome 7. Nevertheless, one of them co-existed with the amplification of *EGFR* gene. In contrast, none of the samples with point mutation showed any polysomy of chromosome 7.

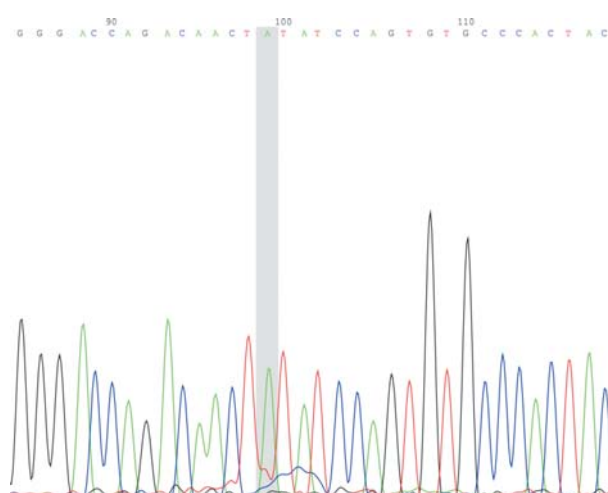


Figure 2. Result of capillary sequencing. A fragment of the sequence of exon 15 of the *EGFR* gene with designated mutation G1736A.

Table I. The combined data on the analyzed alterations of the *EGFR* gene in the studied GBM samples. The samples with identical characteristics were grouped and their number was presented in the column "Number of cases".

Number of cases	<i>EGFRvIII</i>	Amplification	Polysomy	Point mutations	
				Exon 7-8	Exon 15
3	+	+	+	-	-
4	+	-	+	-	-
2	+	+	-	-	-
1	+	+	-	C866T	-
1	+	+	-	-	G1736A
3	+	-	-	-	-
3	-	+	+	-	-
12	-	-	+	-	-
9	-	+	-	-	-
1	-	+	-	G971T	-
51	-	-	-	-	-
1	-	-	-	A787C	-

Detection of *EGFRvIII* mutation in 14% of the cases (2/14) raises some optimism regarding the potential application of monoclonal antibodies or tyrosine kinase inhibitors against *EGFRvIII* receptor. Only in one case, the expression of *EGFRvIII* was associated with point mutation in matrix encoding this mutated receptor. This mutation is located in the close proximity of domain characteristic for *EGFRvIII*. The interaction between altered *EGFRvIII* and specific antibody could be impaired in such a case. However, this situation seems exceptional. Although the

mutation in exon 7 co-existed with EGFRvIII, it could not affect the interaction between EGFRvIII and its specific antibody since the formation of EGFRvIII requires deletion of exons 2-7. On the other hand, our findings show that a specific antibody against EGFRvIII does not necessarily bind to all types of mutated EGFR receptors in GBM cells. Consequences of such events are hardly predictable. The remaining point mutations detected in our material were not associated with the presence of EGFRvIII. Overall, one could expect for some complications in the function of anti-EGFRvIII antibodies in 2 out of 14 cases. Therefore, this problem should not be ignored. Nevertheless, relatively low prevalence of missense mutations co-existing with *EGFRvIII* expression suggests that antibodies which selectively block the EGFRvIII receptor would exert their therapeutic effect in most patients showing this variant of the receptor.

Moreover, we revealed that in cases where the excessive number of copies of *EGFR* gene was associated with the presence of *EGFRvIII*, the *EGFRvIII*-type mRNA predominated over the wild-type mRNA.

In summary, the present study reports the comprehensive analysis of the co-existence of EGFRvIII with the amplification of *EGFR* gene, polysomy of chromosome 7, and hot-spot point mutations of *EGFR* gene.

We revealed that *EGFRvIII* most commonly occurs in cases associated with the excessive number of copies of *EGFR* gene. However, these excessive copies do not necessarily result from the amplification of this gene, but can be also associated with the polysomy of chromosome 7, or a combination of both factors.

Therefore, the co-existence of various alterations in a sample may be characteristic for the injury of a component responsible for the control of cellular processes, thus becoming a therapeutic target.

Our findings confirmed that the sensitivity and specificity of real-time qPCR method (8) in distinguishing between polysomy and amplification is comparable with that of FISH technique, but this earlier method is associated with several-fold lower costs of reagents, equipment, and labor.

Analysis of all these alterations, and especially distinguishing between the multiplication of the *EGFR* gene resulting from the polysomy of chromosome 7 and amplification of *EGFR*, enables better understanding of glioblastoma etiology on the basis of signaling pathways and genetic alterations within the cell. Additionally, it can be helpful during future development of specific therapies for each alteration of the *EGFR* gene. Finally, identification of *EGFRvIII* mutation can play an important role as a therapeutic target (16).

The findings of our study suggest that the presence of EGFRvIII is rarely associated with the occurrence of point mutations that would hinder the effect of specific antibodies against EGFRvIII.

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