Original article

# Epidermal growth factor receptor gene expression in high grade gliomas

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## Abstract

*Background:* *Epidermal growth factor receptor (EGFR) gene amplification and protein expression in malignant gliomas (anaplastic astrocytoma, AA and glioblastoma, GBL) were suggested to be correlated with the degree of malignancy. Large deletions within the EGFR gene occur frequently in glioma patients. The aim of our study was to analyse EGFR gene expression by real-time PCR by three different amplicons located across the gene and relate it to the age of patients and EGFR mutation status.*

*Material and methods:* *We analysed EGFR gene expression in 75 patients, median age 58 years (range 28-75), 52% of glioblastomas, 39% of anaplastic astrocytomas and 9% of low grade gliomas. EGFR expression was measured by real-time PCR, three amplicons located at exons 2-3, 13-14, and 17-18 junctions were analysed, gene expression was normalized by 18S RNA expression. EGFRvIII deletion was detected by RT-PCR.*

*Results:* *EGFR was found to be expressed in 61.8% of brain gliomas, with strongly positive expression in 12.2% of them. We simultaneously analysed by RT-PCR the EGFRvIII status and found the deletion in 21.3% of tumours. In our group EGFRvIII mutation was significantly more frequent in patients older than 50 years of age (48.6%) than in younger patients (23.5%, p < 0.05). When only GBL patients were assessed, none of the patients younger than 50 years of age had EGFRvIII mutation, whereas in the older subgroup they constituted 36.67% of subjects. We observed that younger patients (below 50 yrs) had slightly lower EGFR expression in comparison to older patients, but this difference was not statistically significant.*

*Conclusions:* *As nearly 1/3 of high grade gliomas do not demonstrate abnormal gene expression levels, EGFR status should be taken into account in any targeted therapy attempt. The significance of EGFR axis-related differences between young and old glioma patients and their impact on the prognosis warrant further study.*

*Key words:* *anaplastic astrocytoma, glioblastoma, EGFR, Q-PCR.*

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## Introduction

Anaplastic astrocytoma (AA) and glioblastoma (GBL) are the most frequent primary brain malignancies in adults. In the general population the frequency of these two high grade gliomas ranges from 0.2 to 0.5 per 100 000 children aged below 14 years and from 1.7 to 4.5 per 100 000 adults aged more than 45 years. The most often occurring genetic disturbances in gliomas are mutations in the p53 gene [10,25,50], amplification and rearrangements of the *MDM2* proto-oncogene [36] and amplification of such oncogenes as *N-myc*, *C-myc*, *N-ras*, *K-ras* and *PDGFR-α* [5,7,8]. Several underlying cytogenetic abnormalities are observed, such as loss of heterozygosity (LOH) in chromosomes 17p [10-12], 10 [46,47], 9p [19] and 19q [40,47]. Additionally, in AA several deletions in chromosomes 13q, 17 p and 22p are observed. However, the strongest stimulus for the in vitro-observed tumour growth in malignant gliomas is the gene-level activation of receptors for platelet-derived growth factor (*PDGF*) and epidermal growth factor (*EGF*) [35]. It is suggested that the degree of astrocytoma malignancy is positively correlated with both the concentration of EGFR protein and with *EGFR* gene amplifications [26]. In primary glioblastomas, which typically affect older patients, amplifications of the epidermal growth factor receptor gene (*EGFR*) occur along with deletions/mutations of phosphatase and tensin homologue tumour suppressor gene (*PTEN*), a negative regulator of the phosphatidyl inositol 3 kinase/Akt signalling pathway [43].

Amplifications and overexpression of the EGFR gene are seen in 30-50% of cases of adult high grade gliomas. However, some differences are observed between primary and secondary GBL. Watanabe *et al.* observed EGFR overexpression in 63% of primary GBL, but only in 10% of secondary GBL. In the same study, p53 mutations were found in 11% of primary GBL and 67% of secondary GBL, but in 37% of primary GBL accompanied by the accumulation of p53 protein.

The human EGFR gene is located on chromosome 7. Its high expression is observed in many types of cancer, such as in lung [33,42], breast [37], head and neck [15], bladder carcinoma [31], as well as in gliomas [26]. EGFR overexpression can induce abnormal proliferation of cells, and neoplastic phenotype. Pathway stimulation may be a result of gene amplification, but also ligand-independent activa-

tion can induce protein phosphorylation and binding with adaptor proteins [19,53].

Technically, the analysis of EGFR over-expression should take into account the frequent mutations of this gene. Deletions can appear both in mRNA encoding intracellular domains of the receptor, as well as in sequences of its intracellular part. Loss of the tyrosine kinase domain inhibits signal transduction even in the presence of a ligand [18,39,55]. *EGFRvIII*, the large deletion located in the extracellular domain of EGFR, is the most frequent mutation of the EGF receptor, appearing in over 50% of low and high grade astrocytomas [19,23,34,39,44,45,55].

Some differences in the EGFR-related pathways leading to the formation of de novo primary GBL are suggested between children and adults [6,13,39,55]. Although EGFR protein overexpression is noted in nearly 1/4 of paediatric cases of GBL, corresponding amplification of the EGFR gene in this population is relatively rare. This fact may be of clinical importance, as younger patients with primary GBL exhibit relatively long overall survival compared to older patients [23,34,44].

## Aim of the study

The aim of the study was to assess the expression level of *EGFR* gene in high grade brain gliomas, for both wild type and mutated form. The particular aim was to relate the differences in *EGFR* expression to patients' age.

## Material and methods

EGFR gene expression was analysed in 75 tumour samples obtained from patients treated in the Department of Neurosurgery of the Medical University of Silesia in Katowice. The study was approved by the local Bioethics Committee. Median age was 58 years (28-75 years). 49.3% were women. All patients were operated on for supratentorial intrahaemispheric tumour. A primary tumour was resected in 61.2% of patients, while 38.8% were reoperated due to recurrent tumour. All of them had postoperative radiation therapy. Expression of *EGFR* in 26 samples of breast cancer was also analysed and used as a control group. Detailed histopathological findings are presented in Table I.

Total RNA was isolated from tumour tissue, using the TRIzol method and followed by purification with the RNeasy Mini Kit method (QIAGEN). Quantification of cDNA for the EGFR gene and 18S RNA (internal

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Table I. Histopathological status

| Histopathological diagnosis | Number | Percent [%] |
|---|---|---|
| Glioblastoma with anaplastic astrocytoma component | 9 | 12.0 |
| Glioblastoma | 30 | 40.0 |
| Glioblastomas (total) | 39 | 52.0 |
| Anaplastic oligoastrocytoma | 13 | 17.3 |
| Anaplastic astrocytoma | 12 | 16.0 |
| Anaplastic gemistocytic astrocytoma | 4 | 5.3 |
| Anaplastic astrocytomas (total) | 29 | 38.6 |
| Fibrillary astrocytoma | 3 | 4.0 |
| Oligodendroglioma | 1 | 1.3 |
| Oligoastrocytoma | 3 | 4.0 |
| Low grade gliomas (total) | 7 | 9.3 |
| Total | 75 | 100.0 |

Table II. WHO grading of analysed tumours

| Grade | Number | Percent [%] |
|---|---|---|
| II | 6 | 8.0 |
| II/III | 4 | 5.3 |
| III | 26 | 34.7 |
| III/IV | 1 | 1.3 |
| IV | 38 | 50.7 |
| Total | 75 | 100.0 |

reference gene) was performed using real-time quantitative PCR (ABI PRISM 7700 Sequence Detection System – Taqman; Applied Biosystems).

EGFR expression was studied using three amplicons, complementary to different fragments of the mRNA molecule for EGFR (three exon junctions: 2-3, extracellular domain of EGFR molecule, 13-14, extracellular domain of EGFR molecule, 18-19, intracellular domain of EGFR molecule).

Exons 2 and 3 (in the region of EGFRvIII mutation):
sense starter, F:
5'-GCAGAG GAATTA TGATCT TTCCTT CT-3',
antisense starter, R:
5'-TGTTGA GGGCAA TGAGGA CA-3',

probe:
FAM-5'-AACCAG CCACCT CCTGGA TGGTCT TT-3'-TAMRA.
    Exons 13 and 14:
sense starter, F:
5'-TGCGTC TCTTGCC GGAAT-3',
antisense starter, R:
5'-GGCTCA CCCTCC AGAAGC TT-3',
probe:
FAM-5'-ACGCAT TCCCTG CCTCGGC TG-3'-TAMRA.
    Exons 18 and 19:
sense starter, F:
5'-GCGTTC GGCACG GTGTAT-3',
antisense starter, R:
5'-TTGATA GCGACG GGAATT TTAAC-3',
probe: FAM-5'-TTCTCA CCTTCT GGGATC CAGAGT CCCT-3'-TAMRA.

As the reference gene, 18S rRNA was used:
sense starter, F:
5'-CGGCTA CCACAT CCAAGG AA-3',
antisense starter, R:
5'-GCTGGA ATTACC GCGGCT-3',
probe: FAM-5'-TGCTGG CACCAG ACTTGC CCTC-3'-TAMRA.

A standard curve was made from cDNA serial dilutions of a sample mixture of high grade gliomas. The results after normalization by 18S RNA expression are given in arbitrary units (au, one arbitrary unit is the EGFR gene expression in a reference RNA sample, after normalization with 18S RNA) (Table II).

Immunohistochemical analysis of EGFR expression was performed on paraffin slides with Dako Cytomation anti-EGFR antibody, Clone 11 H, diluted 1 : 200. Labelling was performed by the streptavidin-biotin procedure (LSAB K 0690), staining with DAB Dako 3468 reagent.

## Results

In the first step we performed immunohistochemical (IHC) analysis of EGFR expression in brain tumour samples available for IHC analysis. In 52.8% of high grade gliomas EGFR expression was strongly positive (Table III, Fig. 1).

Gene expression level was measured by quantitative real-time PCR. At the first step the non-normalized data were assessed. We found the highest levels of mean expression in the set containing linking of exons 13 and 14 (mean 0.165 au). Similar results were obtained for the set containing linking of exons 18 and 19 (mean 0.155 au). The results for

**Table III.** Immunohistochemical analysis of brain tumour samples

| Tumour type | Negative | | Positive | | | | | Strongly positive (++ and +++) | |
|---|---|---|---|---|---|---|---|---|---|---|
| | No | % | + | | ++ | | +++ | | No | % |
| GBL | 15 | 28.8 | 18 | 34.6% | 14 | 26.9% | 5 | 9.7% | 19 | 36.6 |
| AA | 13 | 27.7 | 6 | 12.8% | 18 | 38.3% | 10 | 21.3% | 28 | 59.6 |
| Oligodendroglioma and oligoastrocytoma | 13 | 86.7 | 2 | 13.3% | 0 | 0% | 0 | 0% | 0 | 0 |
| Astrocytic low grade glioma | 6 | 66.7 | 3 | 33.3% | 0 | 0% | 0 | 0% | 0 | 0 |
| Total High Grade Gliomas | 28 | 20.2 | 24 | 26.9% | 32 | 35.9% | 15 | 16.9% | 47 | 52.8 |
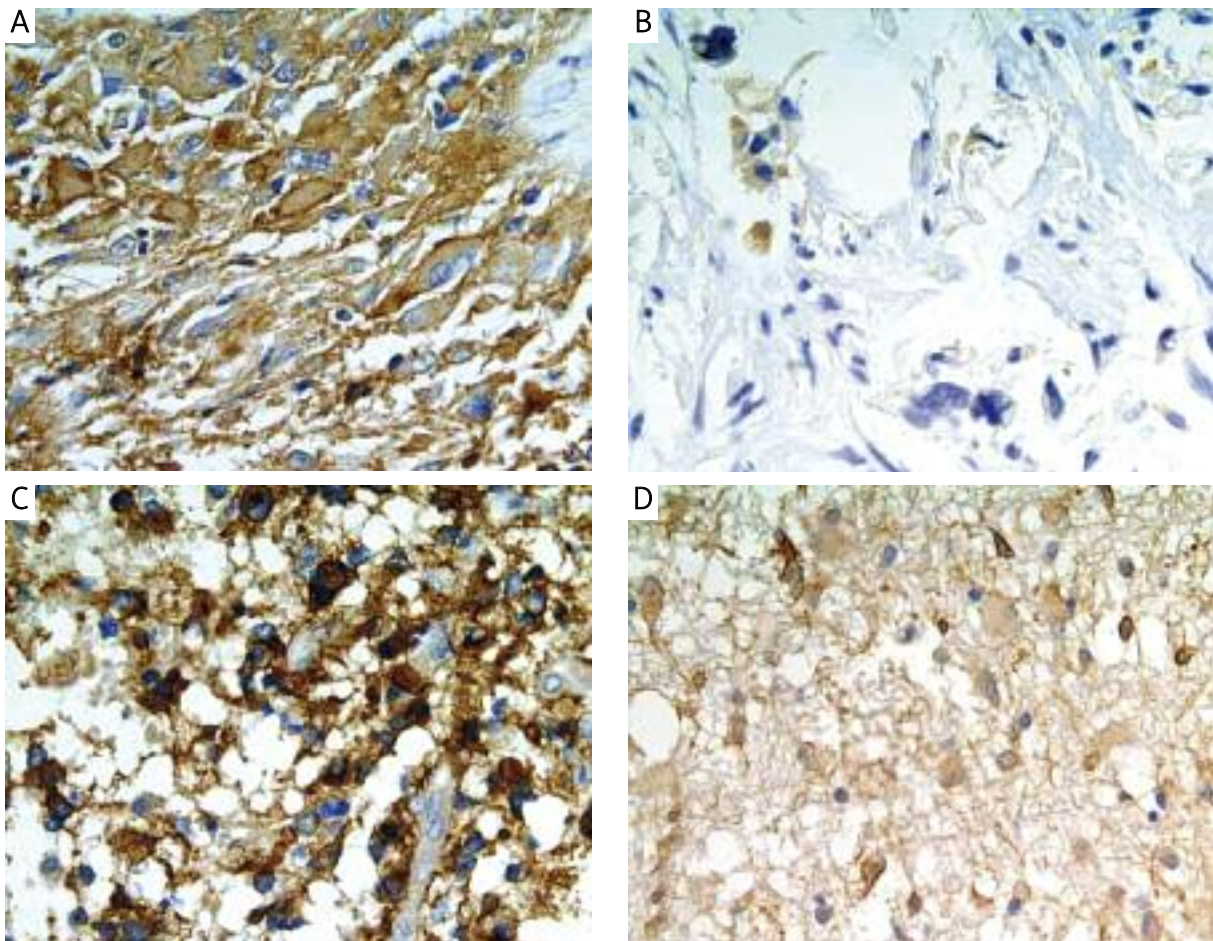


**Fig. 1.** Example of immunohistochemical staining for EGFR. **A)** GBL with strongly positive EGFR, **B)** GBL with negative EGFR, **C)** AA with strongly positive EGFR, **D)** AA with negative EGFR.

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**Table IV.** Levels of expression of particular *EGFR* regions (IQR, interquartile range)

|  | Median expression (au) | IQR | Minimum (au) | Maximum (au) |
|---|---|---|---|---|
| Exons 2-3 | 0.119 | 0.312 | 0.00005 | 5.61 |
| Exons 13-14 | 0.164 | 0.502 | 0.0002 | 16.62 |
| Exons 18-19 | 0.155 | 0.364 | 0.00002 | 6.88 |
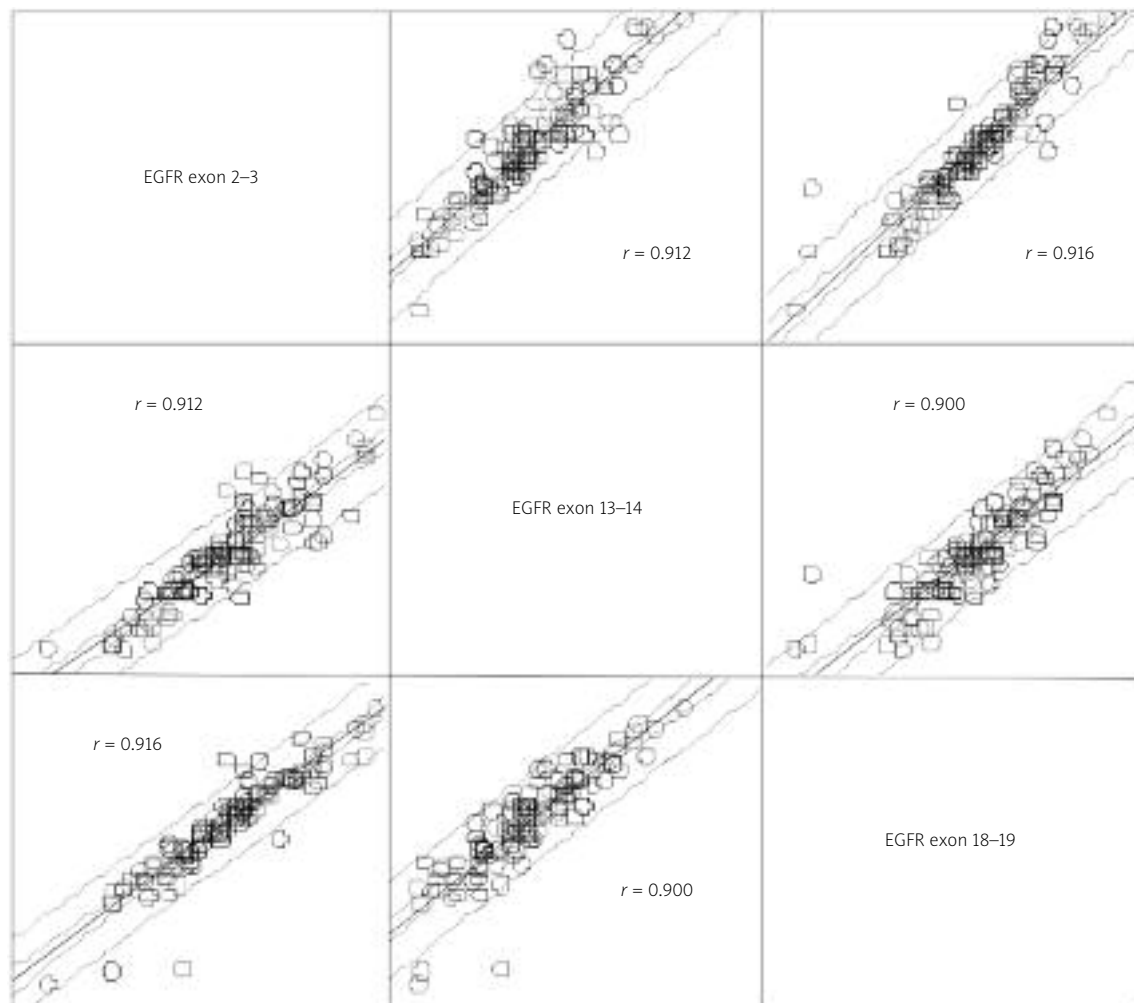


**Fig. 2.** Correlations between three Q-PCR reactions (levels of expression within different exons).

the set containing linking of exons 2 and 3 (extracellular domain) revealed relatively low expression (mean 0.119 au). Detailed results are presented in Table IV. For non-normalized data, we did not find any statistically significant differences in expression levels of *EGFR* between subgroups of primary and secondary GBL.

Further analysis was carried out on EGFR gene expression normalized to 18S RNA. Correlation analysis

of gene expression of the three amplicons studied was performed. It revealed a strong correlation amongst analysed sets. Detailed results are presented in Fig. 2.

The highest values of expression were observed for exons 18-19 of the EGFR gene (median 0.99 au), slightly lower in exons 13-14 (median 0.85 au) and exons 2-3 (median 0.81 au). The difference in the expression levels was statistically significant (Friedman's test, $p = 0.021$).

**Table VA.** *EGFR* expression level normalized to 18S rRNA in the analysed group of patients, according to the amplicon tested (IQR, interquartile range)

|  | Median expression (au) | IQR | Minimum (au) | Maximum (au) |
|---|---|---|---|---|
| exons 2-3 | 0.806 | 1.939 | 0.1 | 24.73 |
| exons 13-14 | 0.844 | 3.237 | 0.3 | 91.33 |
| exons 18-19 | 0.991 | 2.796 | 0.003 | 37.43 |

**Table VB.** *EGFR* expression levels for breast cancer

|  | Median expression level (au) | Minimum (au) | Maximum (au) |
|---|---|---|---|
| Exons 2-3 | 0.343 | 0.04 | 6.34 |
| Exons 13-14 | 0.509 | 0.03 | 2.42 |
| Exons 18-19 | 0.419 | 0.06 | 2.62 |
| Mean | 0.445 | 0.05 | 3.34 |

**Table VC.** Statistical comparison of EGFR gene expression between breast cancer and high grade brain gliomas

|  | EGFR mean | Exons 2-3 | Exons 13-14 | Exons 18-19 |
|---|---|---|---|---|
| Mann-Whitney U | 529.000 | 543.000 | 487.000 | 589.000 |
| *P* | 0.008 | 0.011 | 0.002 | 0.032 |

As a reference, we also analysed the expression of *EGFR* in the set of epithelial cancers (breast cancer) (Table VB). When the *EGFR* expression levels in brain gliomas were compared with breast cancers, significantly higher *EGFR* expression was found in the glioma set (Table VC).

In the next step we tried to find cases with *EGFRvIII* mutation, which is a deletion of the extracellular domain containing exons 2-3. For this purpose mean values of expression of exons 13-14 and exons 18-19 were calculated. In samples with lower expression of exons 2-3 compared to mean expression of the other two domains probably *EGFRvIII* mutation is present. The mutation in selected samples was confirmed in RT-PCR (Fig. 3).
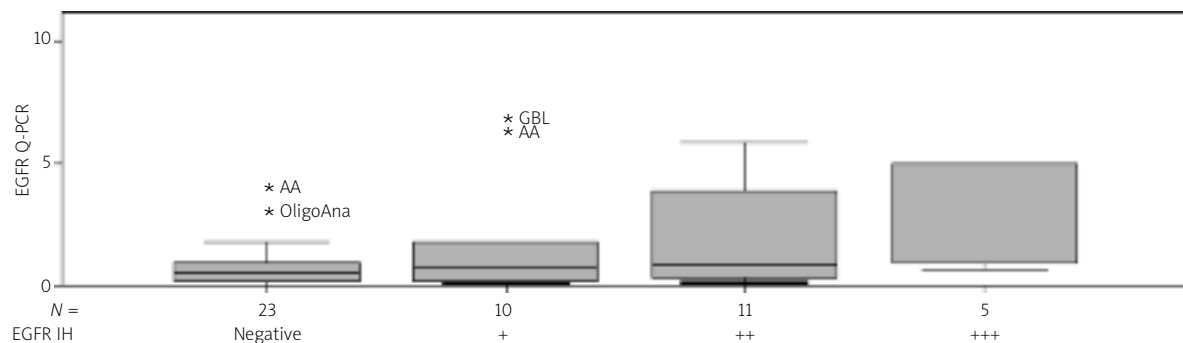
In the whole group of brain tumours *EGFRvIII* mutation was found in 26 samples (34.67% of all tumours). In the GBL group mutation was present in 34.21% of cases and in 40% of the AA group. In the control group of breast cancer *EGFRvIII* mutation was present in 24% of samples.

Comparison of subgroups with present *EGFRvIII* mutation with expression levels of exons 13-14
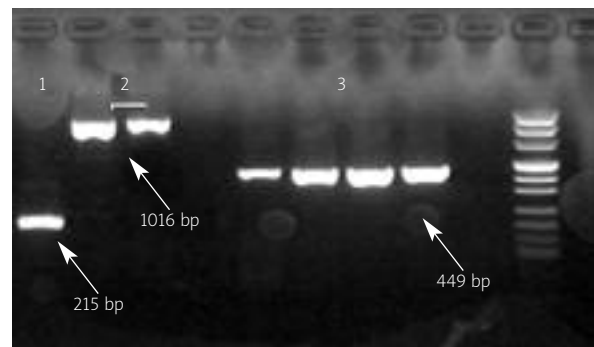


**Fig. 3.** Detection of *EGFR* mutation (RT-PCR). 1 – mutated form with deletion of exons 2-7, 2 – wild type for exons 2-7 deletion, 3 – wild type for exons 14-15 deletion (in our material there was only one case of a mutant gene with deletion of exons 14-15 not shown here).

and 18-19 revealed that tumours without mutation have higher expression levels (Mann-Whitney U test $p < 0.05$). The differences were statistically significant for the whole group of tumours, but we did not obtain significant results within histological subgroups of GBL and AA.

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**Fig. 4.** Comparison of EGFR expression assessed by immunohistochemistry and quantitative PCR ($p = 0.05$, Mann-Whitney U test).
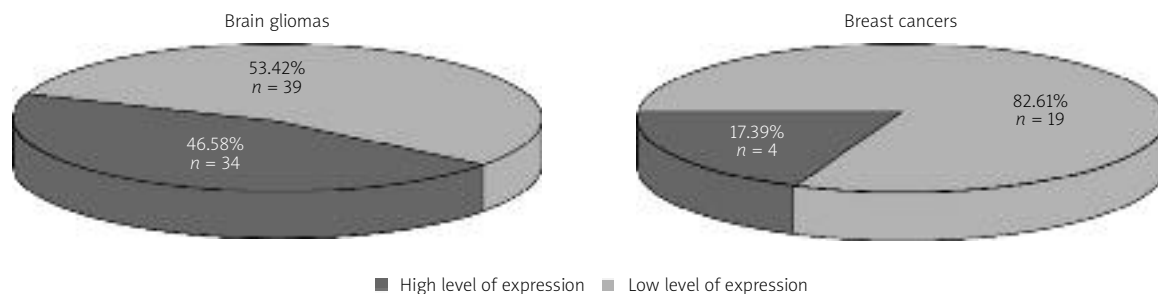


**Fig. 5.** *EGFR* over-expression in brain gliomas and breast cancer group.

When EGFR gene expression, obtained both at protein (IH) and gene (QPCR) level, was compared, we observed significant differences in gene expression between samples with different protein staining levels (Fig. 4), although there were four outlier samples with low protein level, highly expressing EGFR mRNA.

In the next step, we attempted to estimate the frequency of *EGFR* gene over-expression. We approached this by relating gene expression in gliomas to epithelial cancer (breast cancer), and we have taken the median value of gene expression in breast cancer as the non-overexpressed level of gene expression. The median *EGFR* expression in breast cancer is approximately 0.5 au. We found that *EGFR* expression > 0.5 au is significantly more frequent in brain gliomas (47%) than in breast cancers (17%) (Fig. 5).

We also divided the whole group of patients with brain tumours according to age (< 50 and > 50 years). We did not find, however, any statistically significant difference in the expression level of *EGFR* according to age group. Nevertheless, the younger patients (below 50 yrs) exhibited a trend towards lower expression of the *EGFR* gene in both mean expression and in two

intracellular domains. The same results were seen for subgroups with GBL or AA tumours (Table VI).

Analysis of *EGFRvIII* mutation in age subgroups revealed that patients older than 50 years of age more often exhibited a mutated *EGFRvIII* gene (48.6%) than younger patients (23.5%). This difference was also seen for the GBL subgroup, where none of the patients younger than 50 years of age had the *EGFRvIII* mutation, while in the subgroup of older patients 36.67% exhibited this mutation (Fisher exact test, $p < 0.05$). For AA tumours we did not find a statistically significant difference according to age (older than 50 years old 66.67%, younger 50%).

In comparison of *EGFR* gene overexpression frequency in the context of age, we did not find significantly higher *EGFR* expression in the older patient subgroup.

## Discussion

In our study we analysed the expression of wild-type *EGFR*: it was found to be expressed in 61.8% of brain gliomas, with strongly positive expression in 12.2% of them. The results are in the range observed

**Table VI.** Levels of *EGFR* expression according to age of patients

| Group | | Overall EGFR expression | | Exons 13-14, 18-19 | |
|---|---|---|---|---|---|
| | | Mean (au) | Range (au) | Mean (au) | Range (au) |
| All brain tumours | < 50 y.o. | 0.97 | 0.04-4.43 | 0.86 | 0.04-3.01 |
| | > 50 y.o. | 3.82 | 0.01-51.27 | 2.89 | 0.01-31.22 |
| GBL | < 50 y.o. | 0.56 | 0.11-2.13 | 0.39 | 0.07-2.13 |
| | > 50 y.o. | 4.62 | 0.01-51.28 | 3.45 | 0.01-31.22 |
| AA | < 50 y.o. | 0.76 | 0.24-1.75 | 0.67 | 0.24-1.63 |
| | > 50 y.o. | 3.14 | 0.07-7.69 | 2.22 | 0.07-6.05 |

by others: Yoon *et al.* (RT-PCR, 36 patients with GBL) found *EGFR* expression in 36% of cases [54]; Schlegel *et al.* (RT-PCR and Southern blot, 42 GBL) observed increased *EGFR* expression in 64%, but gene amplification in 45% [38]; Arjona *et al.* (86 brain gliomas) observed overexpression in 40% of cases, but no *EGFR* expression in 3.5% of analysed tumours [2]. Aldape and colleagues analysed *EGFR* expression by both RT-PCR and immunohistochemistry; they found gene expression in 66% of cases, and presence of mutated *EGFRvIII* form in 44%. Deletion was observed in parallel to gene over-expression.

In our study, for a detailed analysis of EGFR transcript expression we used three different amplicons located within the EGFR gene: within the exon 2-3 junction, extracellular domain, including the most frequent EGFRvIII deletion site; exon 13-14 junction, anchoring domain of receptor within the cellular membrane (Voldborg *et al.* observed that a deletion in the transmembrane domain makes the protein unable to anchor in the membrane [45]), with relatively low mutation rate in gliomas; and the third amplicon was located at the exon 18-19 junction, corresponding to the intracellular gene domain. Simultaneous analysis of three different amplicons allowed us to obtain a reliable estimate of gene expression. We simultaneously analysed by RT-PCR the EGFRvIII status and found the deletion in 21.3% of tumours, in the range observed by other authors (20 to 67% of cases [16,49]). It was found that gene amplifications can be found in both wild and mutated form of the molecule [27,32,40,51,52], and this seems to be confirmed by our data.

One of the consequences of mutation of tyrosine kinase genes such as EGFR or PDGFR is stimulation of
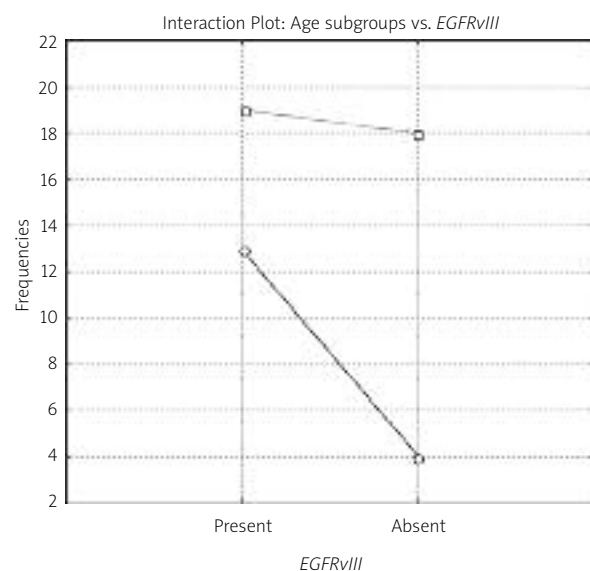


**Fig. 6.** *EGFRvIII* mutation according to age in high grade gliomas.

cell proliferation and their transformation under stimulation of activated Ras proteins [48]. Malignant phenotype of glial tumours may also be a result of high expression of factors promoting invasion and cell migration (such as protein kinase C – PKC [29], or mitogen activated protein kinase – MAPK [9]), as well as autocrine loops activating receptors propagating invasion (e.g. EGFRvIII [17]). Production of proteolytic factors such as MMP is also essential for infiltration of nerve tissue [24].

EGFR has been a target for successful anti-cancer therapeutic strategies in different malignancies, including colon, head and neck and lung cancer. Studies concerning EGFR biology in glioma biology play

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a significant role, especially to understand the inherent differences underlying the growth and therapy resistance of these tumours [15]. *EGFR* activation promotes dedifferentiation of cells, disturbs natural processes of proliferation and simultaneously has anti-apoptotic capabilities. Moreover, stimulation of EGFR also causes an increase of neoplastic angiogenesis and promotes mechanisms of cell migration and invasion of tumours into surrounding nervous tissue. Additionally, frequent mutations of the gene (including *EGFRvIII*), especially in combination with amplification, lead to hyperactivity of the receptor independently of ligands, and are important factors in malignant biology of gliomas [4]. *EGFRvIII* has also been shown to confer resistance to both radiotherapy and chemotherapy [24]. Very interesting in a clinical context is the fact of different *EGFR* expression and its mutation according to age of patients. Malignant gliomas in children seem to be clinically and biologically distinct from their adult counterparts [3,22]. As mentioned above, GBLs in the adult population are classified as primary or secondary based on progression from pre-existing low-grade tumours and on distinct patterns of molecular abnormalities. The primary (*de novo*) GBLs which typically affect older patients are characterized by amplification of *EGFR* along with deletion or mutation of the phosphatase and tensin homologue tumour suppressor gene (*PTEN*), a negative regulator of the phosphatidyl inositol 3 kinase/Akt signalling pathway. Secondary GBLs, which occur in younger individuals, often have mutations of the tumour suppressor gene *TP53* but only infrequently have amplification of *EGFR* or alterations of *PTEN* [3,30,43]. It seems (considering molecular biology) that paediatric cases are similar to secondary adult GBLs [3,30,43]. But on the other hand, these neoplasms rarely originate from pre-existing low-grade lesions. What is important, also other malignant primary brain tumours (e.g. anaplastic ependymomas) show similar differences when comparing paediatric, young adult and older populations [28]. The aforementioned molecular differences concern EGFR expression and its mutation. The EGFR gene is also overexpressed in the most common malignant primary brain tumours in children – medulloblastomas [21]. EGFRvIII mutation was described in 4 of 6 cases of astrocytoma in children, and in 6 of 7 cases of medulloblastomas [45].

In our group *EGFRvIII* mutation was significantly more frequent in patients older than 50 years of age

(48.6%) than in younger patients (23.5%). There was also a statistically significant difference in subgroups of patients with GBL, where no tumours derived from patients younger than 50 years of age had *EGFRvIII* mutation whereas in the older subgroup of patients the frequency of this mutation reached 36.67%. On the other hand, we did not find any statistically significant difference in the frequency of *EGFR* overexpression between age groups. Nevertheless, younger patients (below 50 years of age) had lower expression levels in comparison to older patients in both mean expression of all three analysed domains as well as in mean expression level for two intracellular domains. The same results were obtained for subgroups with GBL or AA tumours.

Certainly, the role of EGFR should be discussed widely in the context of MGMT epigenetic regulation, confirmed as an important prognostic factor in glioma and clearly related to cell sensitivity to genotoxic factors. Similarly, its relation to mutations in the isocitrate dehydrogenase 1 gene (IDH1), possibly correlated with overall survival [34], needs to be discussed, especially due to its potential importance in younger patients. To shed more light on the age-dependent differences in *EGFR* expression we intend to compare the expression of the gene in three age groups, i.e. paediatric, young adults and older patients with high-grade gliomas, in a subsequent study, stratified according to MGMT and IDH1 status.

To conclude, in our analysis we observed *EGFRvIII* mutation to be more frequent in patients older than 50 years, with a non-significant trend toward higher gene expression in older subjects. As nearly 1/3 of high grade gliomas do not demonstrate abnormal gene expression levels, EGFR status should be taken into account in any targeted therapy attempt. The significance of EGFR axis-related differences between young and old glioma patients and their impact on the prognosis warrant further study.

## Acknowledgments

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