Screening for \textit{EGFR} Amplifications with a Novel Method and Their Significance for the Outcome of Glioblastoma Patients

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Abstract

Glioblastoma is a highly aggressive tumour of the central nervous system, characterised by poor prognosis irrespective of the applied treatment. The aim of our study was to analyse whether the molecular markers of glioblastoma (i.e. \textit{TP53} and \textit{IDH1} mutations, \textit{CDKN2A} deletion, \textit{EGFR} amplification, chromosome 7 polysomy and \textit{EGFRvIII} expression) could be associated with distinct prognosis and/or response to the therapy. Moreover, we describe a method which allows for a reliable, as well as time- and cost-effective, screening for \textit{EGFR} amplification and chromosome 7 polysomy with quantitative Real-Time PCR at DNA level. In the clinical data, only the patient’s age had prognostic significance (continuous: \textit{HR} = 1.04; \( p < 0.01 \)). At the molecular level, \textit{EGFRvIII} expression was associated with a better prognosis (\textit{HR} = 0.37; \( p = 0.04 \)). Intriguingly, \textit{EGFR} amplification was associated with a worse outcome in younger patients (\textit{HR} = 3.75; \( p < 0.01 \)) and in patients treated with radiotherapy (\textit{HR} = 2.71; \( p = 0.03 \)). We did not observe any difference between the patients with the amplification treated with radiotherapy and the patients without such a treatment. Next, \textit{EGFR} amplification was related to a better prognosis in combination with the homozygous \textit{CDKN2A} deletion (\textit{HR} = 0.12; \( p = 0.01 \)), but to a poorer prognosis in combination with chromosome 7 polysomy (\textit{HR} = 14.88; \( p = 0.01 \)). Importantly, the results emphasise the necessity to distinguish both mechanisms of the increased \textit{EGFR} gene copy number (amplification and polysomy). To conclude, although the data presented here require validation in different groups of patients, they strongly advocate the consideration of the patient’s tumour molecular characteristics in the selection of the therapy.

Introduction

Glioblastoma is the most common tumour of the central nervous system in adults with annual occurrence of about 3 per 100,000 population\cite{1}. As a highly aggressive neoplasm, it is characterised by the median survival of untreated patients of about 3 months. Neurosurgery prolongs the survival to about 10 months and the following temozolomide-based radio-chemotherapy (currently the standard treatment) to about 15 months\cite{2,3}. Although numerous novel therapeutic methods are being introduced, only targeting integrins was reported to extend the survival of glioblastoma patients to more than 20 months\cite{4}. Taking into account the high heterogeneity of glioblastoma, it is reasonable to assume that certain molecular subtypes may be characterised by a different response to distinct therapies. To date, this has been shown only for the classical subtype (according to Verhaak’s classification) as well as for the \textit{MGMT} promoter methylation in the temozolomide-based radio-chemotherapy\cite{5,6}. Additionally, the molecular characteristics may be potentially informative with regards to the prognosis; however, no molecular marker has been unanimously validated in glioblastoma for that purpose. The clinical factors of the recognised prognostic value are the age and the general condition of the patient (both included in the Radiation Therapy Oncology Group, RTOG, classification) as well as the extent of neurosurgical resection\cite{5,7}. The aim of this article is to analyse the impact of several molecular alterations characteristic for glioblastoma (i.e. \textit{TP53} and \textit{IDH1} mutations, \textit{CDKN2A} deletion, \textit{EGFR} amplification, chromosome 7 polysomy and \textit{EGFRvIII} expression) in patients treated with a neurosurgical operation and with or without the following therapy (radiotherapy or radio-chemotherapy).
Materials and Methods

1. Analyzed Group/Clinical Data
The analyzed group consists of 83 glioblastoma patients who underwent a neurosurgical resection of the tumor at Norbert Barlicki University Clinical Hospital No. 1 in Łódź and at Maria Skłodowska–Curie Provincial Specialist Hospital in Zgierz. Tumor and blood samples for molecular analyses as well as clinical data were obtained according to protocols approved by the ethical committee of Medical University of Łódź. Written informed consent was obtained from all patients and their data were processed and stored according to the principles expressed in the Declaration of Helsinki. 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 were regarded as primary glioblastoma. Karnofsky Performance Status (KPS and, hence, RTOG classification) data were not available for the most of the patients and were not included in our analysis. The clinical data gathered for this project included: the age of the patient at the time of the diagnosis, sex, location of the tumour, the extent of resection, the following therapy (radiotherapy or radio-chemotherapy) and the overall survival time. The patients were aged from 23 to 84 years (the median age was 60 years), the M:F ratio was 1.18. The survival times were available for 60 patients (51 complete and 9 censored responses) and varied between 1 and 48 months (the median survival was 10 months).

2. DNA/RNA Isolation and Reverse Transcription
Total cellular DNA and RNA were isolated from non-marginal fragments of frozen tumour samples (stored at -80°C) and frozen peripheral blood leukocytes obtained from the patients using AllPrep DNA/RNA Mini Kit (Qiagen, Germany) according to the manufacturer’s protocol. RNA samples were treated with DNase following the isolation. RNA and DNA concentrations were measured spectrophotometrically. The content of tumour cells in each sample was estimated as >70%, according to the STR analysis performed as described earlier [8]. 100 ng of total RNA was reverse transcribed into a single-stranded cDNA in a 20 µl reaction volume using QuantiTect Rev. Transcription Kit (Qiagen, Germany) according to the manufacturer’s protocol.

3. Novel Method of the EGFR Gene Analysis by Quantitative Real-Time PCR at the DNA Level
To determine the EGFR gene dosage level in each sample quantitative Real-Time PCR was performed using Rotor-Gene 6000 system (Qiagen, Germany). Each sample was amplified in triplicate in a 10 µl reaction volume containing 10 ng of DNA, a 1x reaction mixture containing Syto9 (Life Technologies, US) and 35 ng 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). All primer sequences are listed in Table S1. The gene dissociation curve was analysed for each sample to confirm the specificity of the amplification signal. The normalised relative gene dosage level in each sample was estimated as

\[
\text{Gene Dosage Level} = \frac{C_{\text{EGFR}}}{C_{\text{GPER}}} \times \frac{C_{\text{GPER}}}{C_{\text{RNaseP}}} 
\]

4. Standard Analysis of the EGFR Gene Dosage by Quantitative Real-Time PCR at the DNA Level
For the comparative purposes, the standard method of the EGFR gene copy number assessment with quantitative Real-Time PCR has been applied. The reactions for EGFR and RNaseP were performed as explained in point 2.3. and the ratio of EGFR to RNaseP was calculated analogously. The results were interpreted in accordance with the criteria developed by the authors.

![Figure 1](https://example.com/figure1.png)

Figure 1. A diagram depicting the premises upon which the EGFR gene copy number analysis is based. In normal cells both the ratio of EGFR to GPER and the ratio of GPER to RNaseP is equal to 1. In cells with chromosome 7 polysomy the ratio of GPER to RNase increases, while in cells with EGFR amplification the ratio of EGFR to GPER increases. In cells with both the polysomy and the amplification both ratios are increased and the ratio of EGFR to RNase is equal to their product. A. normal cell; B. cell with chromosome 7 polysomy; C. cell with extrachromosomal EGFR amplification.

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the following manner: the ratio of EGFR to \( \text{RNaseP} \) between 1.5 and 5 was considered as resulting from the polony, while the ratio of EGFR to \( \text{RNaseP} \) higher than 5 was considered as resulting from the amplification.

5. **Fluorescence in situ Hybridization (FISH)**

FISH was performed with FISH Pretreatment Reagent Kit (Abbott Molecular, US) according to the manufacturer’s protocol. In brief, a commercial probe set (Vysis LSI EGFR SpectrumOrange/CEP 7 SpectrumGreen; Abbott Molecular, US) was used to simultaneously detect the copy numbers of the EGFR gene and of chromosome 7. FISH was performed using the following procedure: the fixed slides were incubated in 2x standard saline citrate (SSC) at 72°C for 5 min, immersed in protease solution for 10 min at 37°C, washed with PBS for 5 min at room temperature, fixed with 1% formaldehyde for 5 min at room temperature, washed in PBS for 5 min at room temperature, dehydrated 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 mix 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 2 min, rinsed in 2×SSC/0.1% NP-40 for 1 min at room temperature and air dried in darkness. Before coverslipping, 10 µl of DAPI II counterstain was added to the slides. To score the samples, an Olympus BX-41 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 copies of \( \text{EGFR} \). The number of green signals, caused by the binding of the CEP 7 probe, directly reflects the number of copies of chromosome 7. FISH evaluation was performed using previously published criteria [16]. For each sample at least 100 nuclei were analysed. The EGFR/CEP 7 ratio was calculated and samples containing three or more signals specific for CEP 7 per nucleus were defined as having chromosome 7 polysomy. Samples with intrachromosomal amplification ratios of 2 or greater were considered to be amplified for \( \text{EGFR} \). Extrachromosomal amplification of \( \text{EGFR} \) was defined as the presence of at least three times as many \( \text{EGFR} \) signals as centromere 7 signals per cell [17]. An exemplary FISH result is presented in Fig. S1A.

6. **FISH in Formalin-Fixed, Paraffin-Embedded (FFPE) Samples**

FISH in FFPE samples processed for routine histopathology was performed with Paraffin Pretreatment Reagent Kit (Abbott Molecular, US) according to the manufacturer’s protocol. In brief, a commercial probe set (Vysis LSI EGFR SpectrumOrange/CEP 7 SpectrumGreen; Abbott Molecular, US) was used to simultaneously detect the copy numbers of the EGFR gene and of chromosome 7. FISH was performed using the following procedure: the slides were deparaffinised in xylene two times for 20 min at room temperature, dehydrated in a 100%, 80% and 70% ethanol for 1 min each at room temperature and air dried on 50°C slide warmer. Then, the slides were immersed in 0.2N HCl for 20 min at room temperature, washed with purified water and wash buffer for 3 min each at room temperature, incubated in pretreatment solution for 30 min at 80°C and washed in purified water for 1 min at room temperature and in wash buffers two times for 5 min at room temperature. Next, the slides were incubated in protease solution for 30 min at 37°C, washed in wash buffers two times for 5 min at room temperature and air dried on 50°C slide warmer. Then, the slides were fixed in 10% buffered formalin for 10 min at room temperature, washed in wash buffers two times for 5 min at room temperature and air dried on 50°C slide warmer. The FISH probe mix 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 2 min, rinsed in 2×SSC/0.1% NP-40 for 1 min at room temperature and air dried in darkness. Before coverslipping, 10 µl of DAPI II counterstain was added to the slides. The evaluation was performed with confocal laser scanning microscope Olympus FluoView1000 according to the criteria mentioned in point 2.5. Exemplary FISH in FFPE sample results are presented in Fig. S1BC.

7. **TP53 and IDH1 Sequencing Analysis**

Exons 5-8 of the TP53 gene and exon 4, including codon 132, of the IDH1 gene were amplified by PCR on cDNA template and sequenced using the deoxy termination method and SequiTherm Excel DNA Sequencing Kit (Epicentre Technologies) following the manufacturer’s protocol. The primer sequences are listed in Table S1. LICor automatic sequencer system was applied to the separation and analysis of PCR-sequencing products. To verify the results of sequencing the semi quantitative densitometric analysis was performed. The intensity of wild-type and mutated bands was estimated by comparison to the neighbouring bands in the same sequencing lane used as a reference. The results of the TP53 sequencing have been deposited in GenBank (Accession Numbers: KC820708-KC820786). Exemplary sequencing results are presented in Fig. S1D.

8. **Detection of the CDKN2A Deletions at the DNA Level by Quantitative Real-Time PCR**

To determine the CDKN2A exon 1 and/or exon 2 deletions quantitative Real-Time PCR reactions were performed as described above. The primer sequences are listed in Table S1. The reference gene was \( \text{RNaseP} \). DNA derived from non-neoplastic tissue (leukocytes) was used as a control and the gene dosage in normal tissue was assumed to be 1. Each sample was analysed three times. An average value lower than 0.5 was considered to represent the deletion of the tested gene in the general population of cells. CDKN2A exon 1 and/or exon 2 deletion was confirmed by agarose gel electrophoresis using BioRad Quantity One 1-D Analysis Software.

9. **Detection of the EGFRvIII Expression at the cDNA Level by Quantitative Real-Time PCR**

To determine the EGFRvIII expression quantitative Real-Time PCR reactions were performed as described above. The EGFRvIII-specific primers were based on a previous report [18], the primer sequences are listed in Table S1. GUSB was used as a reference gene for the normalization of the target gene expression level. To evaluate the EGFRvIII expression, cDNA derived from tumour tissue positive for EGFRvIII was used as a control. The Real-Time PCR was preceded by a conventional RT-PCR applied to examine the tested tumour samples in terms of EGFRvIII expression.

10. **Statistical Analysis**

Statistical analyses were performed using STATISTICA 10 software (StatSoft, US). In order to assess the significance of given feature a series of analyses was performed. Firstly, Kaplan-Meier diagrams were plotted and the differences between groups were assessed with Gehan’s Wilcoxon test (GW). Next, its association
with the age of incidence was verified by means of box plots and Mann-Whitney U test. Finally, the analysis of Cox’s proportional hazard was performed both as univariate analysis and as multivariate analysis adjusted for age. Spearman’s rank correlation test was applied for the assessment of the correlation between the age and the survival (only complete responses were included in this analysis).

**Results**

1. Comparison of the Results Obtained with Real-Time PCR and FISH

FISH results were obtained for 37 cases (20 with normal EGFR gene copy number, 5 with polysomy, 5 with amplification and 7 with both polysomy and amplification), data in Table S2. The standard method gave true results in 27/37 cases (7/17 true positive and 20/20 true negative). The results obtained with the novel Real-Time PCR method were consistent with FISH results (both false positive and false negative detection rate for the novel method was equal to 0); and, therefore, were used for further analyses.

2. Analysis of the Clinical Aspects

Initially, the clinical data were evaluated in order to select the set of data for which to adjust the multivariate analyses. The significance of patient’s age was firstly assessed with the Spearman’s rank test. Spearman’s rho was -0.41 with \( p = 0.003 \). Next, an univariate analysis of Cox’s proportional hazard was performed both for age counted in years (Hazard Ratio, \( HR = 1.04; \ p = 0.0014 \)) and in decades (\( HR = 1.41; \ p = 0.0056 \); Table 1, Fig. 2A). The analysis of the therapeutic process was performed both in the univariate and multivariate (adjusted for the age) manner. Albeit suggesting a possible effect with GW analysis (\( p = 0.20 \)), the exten of resection appeared insignificant in the multivariate analysis (\( HR = 0.88, \ p = 0.67 \) (Fig. 2B)). Neither did the comparison of gross total resection vs. the lower extents show any significant correlation with the outcome (\( p = 0.54 \)). Next, the analysis of subsequent therapy showed that the patients treated with radiotherapy lived longer (\( p = 0.02 \)), but also were significantly younger (\( p < 0.01 \)). The multivariate analysis showed no significance of the radiotherapy and attributed the differences in survival mostly to the age of the patient (\( p = 0.88 \)) (Fig. 2C). Similarly, radio-chemotherapy was associated with both longer survival (\( p = 0.01 \)) and younger age of the patients (\( p = 0.01 \)), however, in the multivariate analysis a positive, yet insignificant, association was shown (\( HR = 0.57, \ p = 0.24 \)) (data in Table 1, Fig. 2D). For the other analysed clinical data no significant correlation with the outcome was found (Table 1, complete data in Table S3).

3. Analysis of the Molecular Aspects

3.1. Direct analysis of the entire cohort. We observed the analysed alterations with the following frequencies: \( TP53 \) mutation in 22% (17/79); \( EGFR \) amplification in 27% (22/82); chromosome 7 polysomy in 27% (21/79); \( CDKN2A \) deletion in 50% (40/80); \( EGFR:III \) expression in 18% (14/80) and \( IDH1 \) mutation in 3.9% (3/77).

We did not observe any difference in the overall survival between the patients with and without \( TP53 \) mutations in the direct analysis. We found neither \( EGFR \) amplification nor chromosome 7 polysomy to be prognostically significant, \( EGFR:III \) expression appeared as a positive factor and although the GW test gave borderline result (\( p = 0.04 \)), the multivariate analysis was confirmatory (\( HR = 0.34, \ p = 0.04 \) (Fig. 3A). Conversely, the significance of homozygous deletions of the \( CDKN2A \) gene, despite the suggestive Kaplan-Meier diagram (Fig. 3B), was negated both by GW test (\( p = 0.10 \)) and by the multivariate analysis (\( p = 0.30 \)). No statistical analyses were performed for the \( IDH1 \) gene due to the low number of mutations.

3.2. Analysis of age-dependant subgroups. In this part, the aforementioned analysis was performed in separate groups in relation to the age of the patients (with the threshold of 60 years, being the median age in the analysed group). Intriguingly, \( EGFR \) amplification appeared to have an opposite effect on survival in both groups. It seems to be associated with a shorter survival in younger patients and with a longer survival in older patients. Multivariate analysis confirmed the significance of \( EGFR \) amplification only in younger patients (\( HR = 3.75, \ p = 0.01 \) (Table 1, Fig. 4A).

3.3. Analysis of combinations of molecular characteristics. We also analysed combinations of molecular alterations in relation with the survival. As mentioned before, homozygous deletions of \( CDKN2A \) were suspected to be related with a longer survival. Thus, it was intriguing to observe that such deletions were associated with an enhanced outcome in patients with \( EGFR \) amplification (\( HR = 0.12; \ p = 0.01 \) (Fig. 3C)), while no difference was observed in patients without the amplification (\( p = 0.94 \)). Next, the combination of both mechanism of the increased \( EGFR \) gene copy number appeared to be significant. In the group with the amplification the polysomy was associated with a poor outcome (\( HR = 14.88; \ p = 0.01 \); Fig. 3D), while no such association was observed in the group without the amplification (\( p = 0.61 \); Table 1).

3.4. Correlation with the therapy applied. We evaluated the association between the molecular characteristics and the effectiveness of radiotherapy (reflected by the overall survival) and the only divergence was observed for \( EGFR \) amplification. In patients who did not receive radiotherapy the \( EGFR \) amplification did not influence the survival (\( p = 0.50 \)), while in patients who were treated with radiotherapy it was related to an impaired survival (\( HR = 2.71; \ p = 0.03 \) (Fig. 4B)). Intriguingly, we did not observe any differences between the patients with \( EGFR \) amplification treated with radiotherapy and the patients who had not received radiotherapy (irrespective of the \( EGFR \) status) (\( p = 0.20 \) (Fig. 4C)). To investigate the correlation between the amplification and the survival in younger patients and those treated with radiotherapy, two additional analyses were performed – one in a cumulative group of younger patients and those treated with radiotherapy (HR = 3.39; p = 0.01; Fig. 4D) and the other only in patients fulfilling both criteria simultaneously (HR = 2.78; p = 0.06). A similar analysis for the chemotherapy was not conducted due to the small group of patients who underwent such a treatment.

**Discussion**

The presented technique for the \( EGFR \) gene overdosage analysis allows for a clear discrimination between \( EGFR \) amplification and chromosome 7 polysomy. The outspoken need for a simple method serving such purposes was expressed in several publications [19,20,21]. The verification of the results proved the method to be highly reliable (both false negative and false positive detection rate were equal to 0 in this group, however, it cannot be excluded that false results may occur in a more numerous group or as a result of low quality DNA, lower tumour cell content or technical errors, etc.). Until now, such an approach for the detection and discrimination between the \( EGFR \) amplification and chromosome 7 polysomy with Real-Time PCR at the DNA level was not described. The commonly applied methods for \( EGFR \) amplification
gene copy number assessment at the DNA level are based on the ratio of EGFR to the reference, with the threshold for the amplification’s recognition ranged from more than 1 to more than 5, while chromosome 7 polysomy was rarely taken into account [14,15,22,23,24]. The analysed interpretation of the standard Real-Time PCR (the ratio of EGFR to the reference gene between 1.5 and 5 is considered as polysomy, while a ratio higher than 5 is indicative of amplification) allows for the detection and the discrimination between both mechanisms of the increased EGFR gene copy number with a limited precision. Such an approach is effective in some cases, but it can lead to confusing results in 3 situations: the amplification present in a low proportion of cells or a low-magnitude amplification (mistaken for the polysomy), a high-magnitude polysomy (mistaken for the amplification) and the coincidence of amplification and polysomy (polysomy not detected). The proposed technique was efficient in the recognition of each of these situations. Obviously, it is not our suggestion that this method may replace FISH at its position as the golden standard, particularly due to the fact that it allows for the detection of the alterations at a single cell level. Nevertheless, the presented technique allows for the reliable as well as time- and cost-effective analysis of the general population of cells at DNA level, which makes it an attractive possibility for the screening of the EGFR overdosage.

The frequencies of the observed alterations were in general similar to the literature data. In the analysed group, the frequency of IDH1 mutation was in accordance with the analysis by Nobusawa et al. (3.9% vs. 3.7%) [25]. The analysed group was characterised by an overrepresentation of CDKN2A deletions (50% vs. 31%) in comparison to the analysis by Ohgaki et al. [1]. Other markers were underrepresented in the analysed group. The frequency of TP53 mutation was 23% (vs. 28%), of EGFR amplification 27% (vs. 36%) [1]; of EGFRvIII expression 18% (vs. 27% or 31% [26,27]) and of chromosome 7 polysomy was 27% (vs. 39% [28]).

In the clinical data, the age of a patient was a significant prognostic factor in accordance with the published data [7,29,30]. Two other factors (KPS and RTOG classification), described in

Figure 2. Kaplan-Meier diagrams depicting differences in survival times related to the clinical aspects. The attached table presents statistical data for each diagram. Cox’s proportional hazard refers to univariate analysis for diagram A and to multivariate analysis for diagrams B, C, D. The calculated HR values pertain to the second subgroup listed ("total" subgroup for diagram B), while the HR values of the first subgroup (cumulatively of "partial" and "subtotal" subgroups for diagram B) equal to 1. † - complete responses; Δ - censored responses. A. age of the patient, the threshold of 60 years included in the "younger" subgroup; B. extent of resection; C. radiotherapy; D. radio-chemotherapy.

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| Criterion                          | Group size | GW Test | Cox’s Proportional Hazard |
|-----------------------------------|------------|---------|---------------------------|
| **Clinical Data**                 |            |         |                           |
| Age (years)                       | 60         | NA      | HR = 1.042                | p = 0.0014 |
| Age (decades)                     | 60         | NA      | HR = 1.409                | p = 0.0056 |
| Age (>60 y. o.)                   | 60         | p = 0.004 | HR = 2.011                | p = 0.016  |
| Radiotherapy                      | 58         | p = 0.023 | HR = 1.051                | p = 0.882  |
| Radio-chemotherapy                | 57         | p = 0.007 | HR = 0.572                | p = 0.239  |
| Extent of resection               | 31         | p = 0.199 | HR = 0.879                | p = 0.671  |
| Gross Total Resection             | 31         | p = 0.054 | HR = 0.667                | p = 0.535  |
| **Molecular Data: direct analysis of the entire cohort** |            |         |                           |
| TP53                              | 57         | p = 0.279 | HR = 0.949                | p = 0.883  |
| EGFR                              | 59         | p = 0.396 | HR = 1.279                | p = 0.456  |
| Poly 7                            | 57         | p = 0.627 | HR = 1.034                | p = 0.920  |
| EGFRvIII                          | 58         | p = 0.037 | HR = 0.337                | p = 0.040  |
| CDKN2A                            | 58         | p = 0.180 | HR = 0.774                | p = 0.383  |
| **Molecular Data: direct analysis of the age-dependant subgroups** |            |         |                           |
| TP53                              | ≤60 y. o.  | 30      | p = 0.144                 | HR = 0.636 | p = 0.356 |
|                                  | >60 y. o.  | 27      | p = 0.896                 | HR = 1.234 | p = 0.683 |
| EGFR                              | ≤60 y. o.  | 32      | p = 0.006                 | HR = 3.745 | p = 0.007 |
|                                  | >60 y. o.  | 27      | p = 0.300                 | HR = 0.601 | p = 0.351 |
| Poly 7                            | ≤60 y. o.  | 30      | p = 0.178                 | HR = 1.478 | p = 0.388 |
|                                  | >60 y. o.  | 27      | p = 0.544                 | HR = 0.646 | p = 0.405 |
| EGFRvIII                          | ≤60 y. o.  | 31      | p = 0.206                 | HR = 0.339 | p = 0.150 |
|                                  | >60 y. o.  | 27      | p = 0.107                 | HR = 0.198 | p = 0.113 |
| CDKN2A                            | ≤60 y. o.  | 31      | p = 0.441                 | HR = 0.922 | p = 0.850 |
|                                  | >60 y. o.  | 27      | p = 0.478                 | HR = 0.706 | p = 0.479 |
| **Molecular Data: the combinations of molecular characteristics** |            |         |                           |
| EGFR amplified                    | TP53       | 14      | p = 0.937                 | HR = 1.127 | p = 0.908 |
|                                  | Poly 7     | 15      | p = 0.049                 | HR = 14.879| p = 0.013 |
|                                  | EGFRvIII   | 14      | p = 0.078                 | HR = 0.094 | p = 0.115 |
|                                  | CDKN2A     | 15      | p = 0.010                 | HR = 0.119 | p = 0.014 |
| EGFR non-amplified               | TP53       | 42      | p = 0.387                 | HR = 1.121 | p = 0.773 |
|                                  | Poly 7     | 42      | p = 0.823                 | HR = 0.822 | p = 0.614 |
|                                  | EGFRvIII   | 43      | p = 0.199                 | HR = 0.420 | p = 0.239 |
|                                  | CDKN2A     | 42      | p = 0.849                 | HR = 1.026 | p = 0.941 |
| **Molecular Data: correlation with radiotherapy** |            |         |                           |
| Radiotherapy                      | TP53       | 30      | p = 0.306                 | HR = 0.932 | p = 0.879 |
|                                  | EGFR       | 33      | p = 0.022                 | HR = 2.713 | p = 0.033 |
|                                  | Poly 7     | 31      | p = 0.238                 | HR = 1.309 | p = 0.539 |
|                                  | EGFRvIII   | 32      | p = 0.134                 | HR = 0.343 | p = 0.155 |
|                                  | CDKN2A     | 32      | p = 0.230                 | HR = 0.976 | p = 0.955 |
| No Radiotherapy                  | TP53       | 25      | p = 0.670                 | HR = 0.827 | p = 0.734 |
|                                  | EGFR       | 24      | p = 0.903                 | HR = 1.535 | p = 0.499 |
|                                  | Poly 7     | 24      | p = 0.944                 | HR = 0.639 | p = 0.436 |
|                                  | EGFRvIII   | 24      | p = 0.548                 | HR = 0.561 | p = 0.566 |
|                                  | CDKN2A     | 24      | p = 0.627                 | HR = 0.749 | p = 0.526 |
many reports as significantly correlated with the outcome [7,30],
could not be assessed in our analysis. The issue of the significance
of the extent of resection remains discordant in the literature,
either marking it as a factor of great importance [5,31] or
diminishing its role [29,30]; our data concur with the latter. Albeit
expected and suggested in the literature [2,3,32], the therapy given
after surgery did not prove to significantly improve the survival of
patients when the analyses were adjusted for age.

To date, EGFRvIII expression has been associated either with
poorer prognosis of glioblastoma (in combination with the
amplification) [29] or, most commonly, no association has been
observed [26,27,33]. Nonetheless, Liu et al. suggested a correlation
of the EGFRvIII expression and a longer survival in anaplastic

Figure 3. Kaplan-Meier diagrams depicting differences in survival times related to the molecular aspects. The attached table presents statistical data for each diagram. Cox’s proportional hazard refers to multivariate analysis. The calculated HR values pertain to the second subgroup listed, while the HR values of the first subgroup equal to 1. ◆ - complete responses; Δ - censored responses. A. EGFRvIII expression; B. CDKN2A deletion; C. The combination of CDKN2A deletion with EGFR amplification; D. the combination of chromosome 7 polysomy with EGFR amplification.
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Thus far, only the report by Montano et al. [7] has shown an association of the EGFRvIII expression with a more favourable prognosis of glioblastoma, which the results presented here support.

The possible prognostic value of EGFR amplification and overexpression has been intensively analysed, in most cases without any significant correlation with the clinical outcome observed [26,27,31,33,34,35]. However, Simmons et al. observed that EGFR overexpression was differently correlated with survival in separate age groups (with the threshold of 55 years), i.e. the overexpression indicated worse prognosis in younger patients and better prognosis in older patients [30], which concurs with the results of our analysis. On the other hand, the analysis by Shinojima et al. suggested that EGFR amplification was associated with poorer prognosis in the entire cohort with an emphasis on younger patients [29]. The analysis of patients younger than 50 years old by Korshunov et al. indicated a negative influence of amplification on survival [36]. Our data suggest that EGFR amplification may be related to a worse prognosis in younger patients and in patients treated with radiotherapy, while improving the prognosis in older patients. Bearing in mind the strong correlation between the age and treatment with radiotherapy \(p<0.01\), it should be verified which factor is the leading one. The analyses of the cumulative group (younger or treated with radiotherapy) and of only younger patients treated with radiotherapy may imply that EGFR amplification may be a negative prognostic factor for patients whose survival is not limited by their overall clinical condition. If verified, these observations may emphasise the need for a therapy specifically targeting the EGFR pathway in this group of patients. Nonetheless, the analysed group in this study is not large enough for such far-reaching conclusions.

To date, however, the analysis of Ang et al. suggested that the
amplification was related to a better outcome in patients treated with radio-chemotherapy [37].

The prognostic significance of chromosome 7 polysomy has not been extensively analysed in glioblastoma. Our data suggest that the combination of EGFR amplification and polysomy may be correlated with poor prognosis, which requires further verification. Moreover, our data also indicate that both mechanisms of the increased EGFR gene copy number (polysomy and amplification) need to be distinguished and analysed separately.

A prognostic significance of homozygous deletions of CDKN2A has not been validated thus far. Amongst the published reports, every possible correlation between the deletion and the outcome (positive [38], negative [39] or no correlation [31,40]) has been observed. Homozygous deletion of CDKN2A was associated with an improved outcome in patients with the EGFR amplification, but not in patients without this alteration. Nonetheless, our data do not allow to unquestionably verify whether this correlation was random or specific.

The reports analysing TP53 mutations in glioblastoma unanimously recognise its lack of prognostic significance in the general population [5,30,31,33,41]. On the other hand, Ruano et al. proposed that simultaneous TP53 mutation and EGFR amplification may be related to a poorer prognosis [41]. Conversely, in the analysis by Simmons et al. the two cases with the concurrent TP53 mutation and EGFR overexpression were characterised by a relatively long survival [30]. The only patient with the simultaneous alterations in this report had survived 11 months from the diagnosis (which was approximately the median survival time). In the report by Simmons et al. the TP53 mutation was somewhat related to a shorter survival (not significant) in the group of patients without the EGFR amplification [30].

A positive prognostic significance of the IDH1 mutation has been suggested by several authors [25,42] due to its relation to the secondary glioblastoma, which are generally characterised by a more favourable outcome [1], however, we could not perform any reliable analysis due to the low number of such mutations.

Conclusion
To conclude, the presented method was efficient and reliable in detection and distinction between EGFR amplification and chromosome 7 polysomy. EGFR amplification was identified as a factor significantly limiting the effectiveness of radiotherapy and the survival of younger patients. Although the presented data are not sufficient to question the indications for radiotherapy for glioblastoma patients with EGFR amplification and require validation in a larger group of patients, they strongly advocate for the consideration of the patient’s molecular status in the putative selection of the therapy, especially in the light of the numerous novel therapeutic possibilities being introduced.

Supporting Information
Figure S1 Exemplary results of FISH and TP53 sequencing. A. Exemplary FISH result presenting both EGFR amplification and chromosome 7 polysomy; magnification 1000x. EGFR signals are red, CEP7 signals are green, scaling bar marks 10 μm. B. Exemplary FISH in FFPE sample result presenting EGFR amplification; magnification 1000x, EGFR signals are red, CEP7 signals are green, scaling bar marks 10 μm. C. Exemplary FISH in FFPE sample result presenting EGFR amplification; magnification 600x, EGFR signals are red, CEP7 signals are green, scaling bar marks 10 μm. D. Exemplary TP53 sequencing result, an arrow marks the mutated nucleotide in codon 237. (TIF)

Table S1 Primer sequences. a – For the sequencing of IDH1 two sense primers were used. (DOC)

Table S2 Comparison of the results obtained with FISH with those obtained with the standard and novel Real-Time PCR based methods. For the standard method the ratios of EGFR to RNaseP and their interpretations (<1.5 – normal; 1.5–5 – polysomy; >5 – amplification) are presented. For the novel method the ratios of EGFR to RNaseP, EGFR to GPER and GPER to RNaseP and their combined interpretations (EGFR/GPER >1.5 – amplification; GPER/RNaseP>1.5 - polysomy) are presented. (DOC)

Table S3 Complete results of the statistical analyses. Cox’s Proportional Hazard values pertain to the univariate analysis for age and to the multivariate analysis (adjusted for age) for other analyses. HR values refer to the presence of given feature. For example: In the group of patients younger than 60 years old, the risk of death over given time is 3.745 times higher in those with EGFR amplification than in those without the amplification. Abbreviations: TP53 – TP53 mutation; EGFR – EGFR amplification; Poly 7 – chromosome 7 polysomy; EGFRvIII – EGFRvIII expression; CDKN2A – CDKN2A deletion; y. o. – years old a – Invasion of given location by the tumour. (DOC)

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Author Contributions
Conceived and designed the experiments: M. Biekiński PR PPL. Performed the experiments: M. Biekiński SP ESF MS M. Banaszczyk MWB BS. Analyzed the data: M. Biekiński SP ESF PR BS. Contributed reagents/materials/analysis tools: EJL DJJ ARZ DJK WP BS. Wrote the paper: M. Biekiński.

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