Comparison of Biomarker Assays for EGFR: Implications for Precision Medicine in Patients with Glioblastoma

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Abstract

Purpose: Patients with glioblastoma (GBM) have a poor prognosis and are in desperate need of better therapies. As therapeutic decisions are increasingly guided by biomarkers, and EGFR abnormalities are common in GBM, thus representing a potential therapeutic target, we systematically evaluated methods of assessing EGFR amplification by multiple assays. Specifically, we evaluated correlation among fluorescence in situ hybridization (FISH), a standard assay for detecting EGFR amplification, with other methods.

Experimental Design: Formalin-fixed, paraffin-embedded tumor samples were used for all assays. EGFR amplification was detected using FISH (N = 206) and whole-exome sequencing (WES, N = 74). EGFR mRNA expression was measured using reverse transcription-polymerase chain reaction (RT-PCR, N = 206) and transcriptome profiling (RNAseq, N = 64). EGFR protein expression was determined by immunohistochemistry (IHC, N = 34). Significant correlations among various methods were determined using Cohen's kappa (κ = 0.61–0.80 defines substantial agreement) or R2 statistics.

Results: EGFR mRNA expression levels by RNA sequencing (RNAseq) and RT-PCR were highly correlated with EGFR amplification assessed by FISH (κ = 0.702). High concordance was also observed when comparing FISH to WES (κ = 0.739). RNA expression was superior to protein expression in delineating EGFR amplification.

Conclusions: Methods for assessing EGFR mRNA expression (RT-PCR, RNAseq) and copy number (WES), but not protein expression (IHC), can be used as surrogates for EGFR amplification (FISH) in GBM. Collectively, our results provide enhanced understanding of available screening options for patients, which may help guide EGFR-targeted therapeutic approaches.

Introduction

Therapeutic decisions in glioblastoma (GBM), as with many other cancers, are increasingly reliant on biomarker analysis. Alterations such as amplification or mutation of the EGFR gene are a hallmark of disease pathogenesis in GBM (1), with EGFR amplification observed in approximately 50% (1,4). It has been shown that focal high-level amplification of the EGFR gene is associated with activation and overexpression of EGFR mRNA in GBM (2).

There are several methods available to assay for EGFR abnormalities in tumor tissue. Here, we describe correlations among fluorescence in situ hybridization (FISH) to assess gene amplification, real-time reverse transcription-polymerase chain reaction (RT-PCR) to assess mRNA transcription, and immunohistochemistry (IHC) to assess protein translation, as well as whole-exome sequencing (WES) and transcriptome profiling (RNAseq), to assess EGFR status. We further compare assays to determine concordance with FISH, which is often considered the standard in detecting gene amplification. Collectively, these results inform on comparability of various methods to evaluate EGFR in GBM, and potentially other tumor types, and may help guide personalized medicine decisions to better treat patients.

Materials and Methods

Study design and collection of tumor samples

Archival formalin-fixed, paraffin-embedded (FFPE) GBM tissue was analyzed in a designated central laboratory from patients screened for a phase I clinical trial (NCT01800695, also known as M12-356) of the EGFR antibody–drug conjugate deputuxizumab mafodotin (depotux-m, formerly ABT-414) currently under investigation for the treatment of EGFR-amplified GBM, as described previously (6–9). The study was performed in accordance with the 1964 Declaration of Helsinki and its later amendments. All patients or appropriate surrogates provided written informed consent for the trial and use of tissue for research studies prior to enrollment according to national regulation, and the study design was approved by the institutional review board and/or ethics committee of each participating institution. Values/
Translational Relevance

Therapeutic decisions in glioblastoma (GBM) are increasingly reliant on the molecular characterization of a patient’s tumor. EGFR gene amplification occurs in approximately 50% of GBMs, and thus presents an important target for therapeutic intervention and as a potential predictive biomarker. Various methodologies are available to assess EGFR amplification and expression status. A systematic study evaluating different methods to assess EGFR amplification and expression was undertaken to understand comparability and concordance of various assays to evaluate EGFR status in GBM. Using EGFR amplification as detected by FISH as the reference method, we found that amplification detection using whole-exome sequencing and RNA expression by either RT-PCR or RNAseq were well correlated, whereas protein detection by IHC was not. Collectively, these results provide information on comparability of various methods to evaluate biomarkers in GBM, and potentially other tumor types, and may help guide precision medicine–oriented decisions with EGFR-directed therapies.

disposition for all samples across all assays described below can be found in Supplementary Table S1.

FISH

FISH was performed by a central laboratory on 206 GBMs (Fig. 1; Supplementary Fig. S1) using the Vysis EGFR Cdx Assay (Abbott Molecular; not on market) comprising two DNA probes labeled with spectrally distinct fluorophores: orange locus-specific identifier (LSI) EGFR probe that hybridizes to 7p11.2-7p12 region, and green chromosome enumeration probe (CEP) 7 probe that hybridizes to a centromere of chromosome 7. Slides with probe mix were codenatured at 73°C for 5 minutes and then hybridized at 37°C for 14-24 hours on a ThermoBrite (Abbott Molecular). Sample pretreatment and posthybridization washes were performed using the Vysis Universal FFPE Tissue Pretreatment and Wash Kit (Abbott Molecular; not commercially available).Slides were reviewed using fluorescence microscopy with orange, green and DAPI (4’6-diamidino-2-phenylindole) filters. FISH signal counts (copy number) for orange and green were recorded for a total of 50 nuclei in the targeted tumor areas, respectively (Supplementary Fig. S2). A tumor was considered EGFR-amplified when there was focal EGFR gene amplification defined as \( \text{EGFR/CEP}\) ratio greater than or equal to 2 in ≥15% recorded cells. Tumors with polysomy for chromosome 7 (excess copies of the entire chromosome defined as CEP/EGFR < 2 and CEP 7 copy number > 3), but without focal amplification of the EGFR gene ≥ 15% were considered to be EGFR-nonamplified.

RT-PCR

Real-time RT-PCR was used to determine relative EGFR mRNA expression levels in 206 GBMs. Relative EGFR mRNA expression was also determined from 20 non-GBM, normal brain tissue specimens (ProteoGenex). Briefly, one ≥5-μm section containing a minimum of 50 mm² total tissue area from the FFPE block was processed for RNA extraction using the QIAGEN RNeasy FFPE Extraction Kit (QIAGEN Sciences) as per manufacturer's instructions. For non-GBM normal brain tissue specimens, one ≥5-μm section containing a minimum of 50 mm² total tissue area from the FFPE block was processed for RNA extraction using the TargetPrep RNA Pro Kit (Abbott Molecular; not commercially available). FFPE sections were deparaffinized and cells were lysed in the presence of Proteinase K. The nucleic acids were de-crosslinked from formalin and DNAase treated to remove DNA content, captured using microparticles, washed, and eluted. Purified RNA was combined in a 96-well plate with Mastermix containing primers and probes for amplification and detection of total EGFR and β-actin on the Abbott m2000 RealTime System (Abbott Molecular). β-actin served as an endogenous control and to provide relative quantitative values for total EGFR expression in the samples. The difference (ΔCt) between β-actin Ct and total EGFR Ct was calculated and reported.

WES

WES was performed on 74 GBMs to assess EGFR gene amplification. Tumor DNA was obtained by macrodissection of the tumor area (>50% tumor content) from FFPE slides. Tumor genomic DNA was extracted using the QIAGEN AllPrep Kit (QIAGEN Sciences). WES libraries were prepared using the SureSelect Clinical Research Exome kit (Agilent). Sequencing was performed with an Illumina HiSeq 2500 (2 × 100 base pairs; Illumina). Profiling aimed to achieve a 150x mean on-target coverage. ArrayStudio (Omicsoft Corporation) was used for sequence alignment and quality control. Copy number variations (CNV) were estimated from WES data using both Sentieon and GATK4 beta versions following suggested CNV best practice guidance. Briefly, sequencing alignment, deduplication, and realign-recalibration were performed using Sentieon Genomics Tools (Sentieon, Inc.; ref. 10). Realigned bam files of tumor samples were used to calculate library-size normalized mean read depth (coverage) for each WES interval. Further normalization and noise smoothing of the coverage of tumor samples were done by target normalization against a panel of normal samples (PON). CNVs were identified and merged into larger segments using CBS algorithm. A cutoff of >3 copies of the EGFR gene was used to define amplification.

Whole transcriptome sequencing (RNAseq)

RNA sequencing (RNAseq) was performed on 64 GBMs to determine EGFR gene transcription. Library preparation was performed with 1–50 ng of total RNA. Double-stranded complementary DNA (ds-cDNA) was prepared using the SeqPlex RNA Amplification Kit (SeqPlex) as per manufacturer’s protocol.
cDNA was blunt ended, had an A base added to the 3' ends, and then Illumina sequencing adapters ligated to the ends. Ligated fragments were amplified for 12 cycles using primers incorporating unique index tags. Fragments were sequenced on an Illumina HiSeq 2500 or HiSeq 3000 using single reads extending 50 bases. Twenty-five to 30 million reads per library were targeted.

RNAseq reads were aligned to the Ensembl release 76 assembly with STAR version 2.0.4b. Transcript counts were derived from the number of uniquely aligned unambiguous reads by Subread: featureCount version 1.4.5. Transcript counts were produced by Sailfish version 0.6.3. Sequencing performance was assessed for total number of aligned reads, total number of uniquely aligned reads, genes and transcripts detected, ribosomal fraction known, junction saturation, and read distribution over known gene models with RSeQC version 2.3.

**Results**

**Threshold determination for FISH and RT-PCR assays**

FISH was performed on 206 tumor samples. As above, a sample was defined as **EGFR**-amplified if it had an **EGFR/CEP 7** copy number ratio \( \geq 2 \) in \( \geq 15\% \) recorded cells. Most tumors had clear results for **EGFR** amplification, with few ambiguous cases (Fig. 1). For example, 93% of GBMs harbored either a very high \( \geq 80\% \) cells; 69% of samples) or very low \( \leq 5\% \) cells; 24% of samples) number of amplified cells showing **EGFR** amplification, with few \( \leq 6\% \) falling mid-range. This is consistent with historical work, which has also shown a clear dichotomy between "amplified" or "nonamplified." For example, in early studies from the 1980s–1990s describing **EGFR** abnormalities in GBM, amplification was typically unambiguous \( 20x \times (14) \) to \( 50x \times (15) \) increases in gene copy number. Finally, one patient with a partial response to \( \text{depauw-m} \) in our dataset had a tumor harboring **EGFR** amplification in 16% of cells \( (9) \), which contributed to the establishment of a minimum threshold at \( 15\% \) to delineate **EGFR** amplification.

Among 91 samples analyzed, 56 (62%) demonstrated chromosome 7 polysomy. Of those, only 13 (23%) also had concurrent focal **EGFR** amplification. There was not a significant correlation of polysomy with increased **EGFR** mRNA expression (Supplementary Fig. S3).

**Concordance of amplification by FISH and WES**

Of the 74 samples that underwent WES, a 92% concordance rate (68/74) with **EGFR** amplification status was observed when comparing WES to FISH results (Supplementary Table S2) and substantial agreement was observed \( k = 0.739 \) (95% confidence interval \( CI = 0.538–0.939 \)). The majority of the discordant cases were low FISH positive (Fig. 2), and thus not captured by WES, which normalizes copy number across the tissue sample instead of on a cell-by-cell basis as with FISH. **EGFR** expression data, as determined by RT-PCR, was tightly associated to WES copy number determination \( \left( R^2 = 0.619 \right) \) (Fig. 3). Furthermore, all samples screened by WES underwent mutational analysis; 37 unique point mutations were identified, some of which were present in more than one sample (Supplementary Fig. S4; Supplementary Table S3). Three mutations have also been identified as pathogenic in the ClinVar database (https://www.ncbi.nlm.nih.gov/clinvar/). However, we did not identify any mutations that were significantly associated with **EGFR** amplification, likely because of the small sample size.

**Correlation of FISH with mRNA transcription and protein expression**

We further evaluated **EGFR** mRNA and protein expression in the context of focal gene amplification. In 201 samples tested by both FISH and RT-PCR, a 94% concordance rate (188/201) was observed between the assays, and we again observed substantial agreement \( k = 0.702 \) (95% CI \( = 0.473–0.931 \); Supplementary Table S4; Supplementary Fig. S5). RNAseq was comparable with RT-PCR, demonstrating correlation of \( R^2 = 0.790 \) (Fig. 4). A 94% concordance rate \( (60/64) \) was observed in samples that had both...
RNAseq and FISH results with substantial agreement ($\kappa = 0.796$ (95% CI = 0.603–0.990); Supplementary Table S5). As with focal gene amplification testing by FISH, mRNA results were typically unambiguous; for example, total $\text{EGFR}$ mRNA by RT-PCR was approximately 19-fold higher in patient samples with FISH-defined $\text{EGFR}$ amplification versus those without (Fig. 5A).

A less well-defined association between $\text{EGFR}$ amplification as determined by FISH and protein expression as assessed by IHC was observed (Fig. 5B). Protein expression was determined as a continuous variable (H-score) in 33 GBMs. Numerically, there was a trend between higher protein expression in $\text{EGFR}$-amplified versus -nonamplified cases. However, when defining high $\text{EGFR}$ protein expression as an H-score $\geq 200$, there was only 73% concordance (24/33) with FISH, and fair agreement was observed ($\kappa = 0.369$ (95% CI = 0.018–0.721); Supplementary Table S6). Neither lowering the threshold for high $\text{EGFR}$ expression to 150, nor performing similar comparisons with H-score subcomponents (data not shown), increased accuracy of IHC to discriminate between samples that were amplified with high $\text{EGFR}$ expression versus nonamplified with high $\text{EGFR}$ expression as easily as assays measuring $\text{EGFR}$ mRNA; thus, an IHC cutoff threshold could not be determined.

Discussion

Here, we have described different methods used to determine $\text{EGFR}$ excess in GBM (Table 1). When performing FISH, we observed a distinct dichotomy: those with a high proportion of $\text{EGFR}$-amplified cells, and those with very few amplified cells. We found that $\text{EGFR}$ mRNA relative expression had a higher association with $\text{EGFR}$ amplification as determined by FISH than did protein expression as determined by IHC. As the phase I trial
progressed, it became apparent that radiographic responses to depatux-m were observed exclusively in patients with GBMs that harbored EGFR amplification rather than EGFR overexpression by IHC. Therefore, routine performance of IHC was aborted mid-trial.

The lack of specificity of IHC to accurately identify patients responsive to depatux-m was also observed in a phase I/II trial for advanced solid tumors (none of which were GBM; ref. 16). In that study, 21 patients (38%) had a tumor sample with an EGFR H-score ≥ 150, but only 1 patient had a partial response. In contrast, of the 35 samples tested for amplification by FISH, only 6 (17%), including one from the responsive patient, were EGFR-amplified. Moreover, the vast majority of GBMs demonstrate EGFR protein overexpression. For example, Schlegel and colleagues (15) found EGFR gene amplification (using Southern blot) in 49% of GBMs, consistent with our results, but reported EGFR overexpression at the protein level by IHC in 92%, lending further support to our conclusion that EGFR protein overexpression cannot be used effectively as a predictive biomarker as its presence is nearly ubiquitous. These data, combined with previous studies that have shown discrepancies in IHC concordance with other antibodies, tests across multiple sites, and reproducibility (17, 18), raise further concern with the use of IHC as a screening method to identify the appropriate targeted population. Accordingly, fewer samples were tested for EGFR IHC than by other methods, and central testing of amplification by FISH became an eligibility criterion for patients accrued to multiple clinical trials of depatux-m (NCT01800695, NCT02573324, NCT02343406, NCT02590263). To that end, using FISH as the gold standard for amplification, mRNA expression and amplification detection by WES were highly associated, with a major contributing factor likely to be the multi-log dynamic range that encompasses low to high expression of EGFR. IHC had a weaker association, which may be partly attributed to its insufficient analytic dynamic range to measure the large biological dynamic range at the higher end of EGFR expression observed in GBM, demonstrated by 8 of 27 samples with low EGFR expression by H-score still classified as EGFR-amplified by FISH, and 1 of 6 samples with high H-score classified as EGFR-nonamplified (Supplementary Table S5). With an ever-growing list of targeted therapies in GBM as well as other cancers, a firm understanding of concordance of molecular methods measuring biomarkers is critical.

Importantly, our results demonstrate that an array of methods beyond FISH can be used to assay for EGFR gene amplification, including WES and RNAseq (but excluding IHC), all with equivalent validity to identify cases for appropriate therapy, thereby reducing the potential for depleting tissue as a precious resource in performing multiple tests for the same biomarker. Furthermore, comparison of screening results obtained by central FISH assay versus a local FISH (or chromogenic in situ hybridization) assay developed and performed by an independent academic molecular pathology laboratory suggest a high concordance rate of 90% (19). This suggests that local biomarker results may be adequate to identify EGFR amplification, which could help to streamline the process of biomarker testing and conserve tissue.

Of note, data presented here demonstrate that "newer" assays, which look across the exome or transcriptome (i.e., WES, RNAseq), are well associated with mature technology (i.e., FISH) and may offer opportunities to look at multiple biomarkers in the context of one another as opposed to a univariate view (Table 1).

### Table 1. Comparison of EGFR testing methods

<table>
<thead>
<tr>
<th>Method</th>
<th>Cutoff</th>
<th>Relative copy number</th>
<th>Normalized to β-actin</th>
<th>δCt of β-actin – EGFR used and δCt ≥ -5.5</th>
<th>RPMK &gt; 40 categorized as overexpressed</th>
<th>Indeterminate</th>
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<tbody>
<tr>
<td>FISH</td>
<td>15% tumor cells with amplification defined as EGFR/CEP 7 ratio ≥ 2</td>
<td>EGFR exons (excluding 2-7) compared with chromosome 7</td>
<td>EGFR categorized as amplified</td>
<td>EGFR overexpression by FISH</td>
<td>EGFR RT-PCR</td>
<td>EGFR protein overexpression by IHC</td>
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<td>WES</td>
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<td>Indeterminate</td>
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<td>RT/PCR</td>
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<td>RNAseq</td>
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### Correlation with FISH

- Not applicable
- Substantial agreement with amplification by FISH
- Substantial agreement with amplification by FISH
- Highly associated with EGFR RT-PCR
- Low specificity to detect amplification

### Pros

- Widely used methodology
- Fluorescence allows for more multiplexing as compared with similar techniques such as chromogenic in situ hybridization (CISH)
- Highly flexible and can assess many genetic changes in parallel
- Multiple assay options
- Highly flexible and can assess many targets in parallel
- Broadly used, widely available method of protein expression
- Cost effective
- Latest automation minimizes human variable

### Cons

- Fluorescence fades over time
- Complex process and algorithms with more room for variation
- Detectors mRNA expression as a surrogate for amplification
- Not a direct measurement of gene amplification
- Quick turnaround
- Fluorescence technology more expensive than CISH
- Loss of cell and tissue morphology
- More expensive and longer turnaround time than FISH
- More expensive and longer turnaround time than FISH
- Measures protein expression only
- Semi-quantitative
- False positive and false negative cases
The differences tended to be in samples with low amplification, indicating FISH was more sensitive. Although these techniques are complex, they are becoming more common and offer multiple options to assess the genome as a whole. This may refine predictive biomarkers in a patient and allow a patient to be screened for multiple potential therapies at one time. Beyond EGFR, there are molecular markers that are already commonly tested for in GBM (20, 21), and screening to identify other events may become more common as further targeted therapies, and novel combination therapies, emerge in the treatment landscape. Systematic studies cross-comparing various assay approaches can help elucidate the analytic strengths and weaknesses of biomarker methodologies so that trade-offs in terms of sensitivity versus throughput can be optimized. In our ongoing studies, we continue to use FISH for central testing when weighing the pros and cons in comparison with other assays (Table 1).

As mentioned, in the phase I study M12-356 of patients with GBM treated with depatux-m, radiographic responses occurred exclusively among patients with EGFR-amplified disease by FISH (7, 9, 22). Recently reported results from the INTELLENCE-2 study in EGFR-amplified rGBM revealed a survival benefit from the combination of depatux-m and temozolomide in multiple subgroups (23, 24). Thus, the positive correlation of EGFR amplification with clinical benefit further emphasizes that a clinically relevant biomarker for patient selection, proper screening, and a personalized medicine approach is of paramount importance and EGFR amplification was therefore used for eligibility criterion in further clinical trials. These findings may inform future studies in a targeted population, including the ongoing INTELLENCE-1 trial (NCT02753324) in newly diagnosed GBM. Collectively, these results provide a better understanding of screening options for patients, and may help to further guide EGFR-targeted therapy approaches in GBM and potentially other cancers.

Disclosure of Potential Conflicts of Interest

A.B. Lassman reports receiving commercial research grants from Genentech/Roche, NCI, Amgen, AbbVie, Millennium, UCL, Cellnex, Novartis, Pfizer, Artema Zentaris, Northwestern University, Karyopharm, RTGC Foundation, Kadmon, VBI Vaccines, Beigene, and Oncocentrics, other commercial research support from Karyopharm, Northwest Biotherapeutics, Global Coalition for Adaptive Research, New York University, Agios, Celgene, NovoCure, Toragen, Yale University, Kadmon, Radiological Society of North America, US Food & Drug Administration, Genentech/Roche, Sapience, VBI Vaccines, American Academy for Clinical Oncology (ASCO), American Academy of Neurology (AAN), NRG Oncology Foundation, Oncocentrics, and AbbVie, is a consultant/advisory board member for Northwest Biotherapeutics, AbbVie, Agios, ASCO, Bioscientia, Celgene, prME Oncology, Sapience, WebMD, Physicians & Education, Clinical Cancer Research, Bioclinica, Celgene, prME Oncology, Sapience, WebMD, Physicians & Education, Society for Clinical Oncology (ASCO), American Academy of Neurology, and is a consultant/advisory board member for Life Science Pharmaceuticals. No potential conflicts of interest were disclosed by the other authors.

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