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

Andrew B. Lassman[1], **Lisa Roberts-Rapp**[2], **Irina Sokolova**[3], **Minghao Song**[3], **Ekaterina Pestova**[3], **Rupinder Kular**[3], **Carolyn Mullen**[3], **Zheng Zha**[2], **Xin Lu**[2], **Erica Gomez**[2], **Anahita Bhathena**[2], **David Maag**[2], **Priya Kumthekar**[4], **Hui K. Gan**[5], **Andrew M. Scott**[5], **Maria Guseva**[2], **Kyle D. Holen**[2], **Peter J. Ansell**[2], **Martin J. van den Bent**[6]

[1]Department of Neurology & Herbert Irving Comprehensive Cancer Center, Columbia University Irving Medical Center, New York, NY, USA [2]AbbVie, Inc., North Chicago, IL, USA [3]Abbott Molecular, Inc., Des Plaines, IL, USA [4]Department of Neurology, Northwestern University Feinberg School of Medicine, Chicago, IL, USA [5]Austin Health, Olivia Newton-John Cancer Research Institute, and La Trobe University, Melbourne, VIC, Australia [6]Erasmus MC Cancer Center, Rotterdam, the Netherlands

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 between 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 between various methods were determined using Cohen's kappa (κ = 0.61 – 0.80 defines substantial agreement) or R^2 statistics.

Results: *EGFR* mRNA expression levels by 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*

Corresponding Author: Andrew B. Lassman, MD, John Harris Associate Professor and Chief, Neuro-Oncology Division, Department of Neurology & Herbert Irving Comprehensive Cancer Center, Columbia University Irving Medical Center, 710 West 168th Street, New York, NY 10032, Phone: 212-342-0871, Fax: 212-342-1246, ABL7@cumc.columbia.edu.

amplification (FISH) in GBM. Collectively, our results provide enhanced understanding of available screening options for patients, which may help guide EGFR-targeted therapy approaches.

Keywords

EGFR; depatuxizumab mafodotin; depatux-m; GBM; biomarkers

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 *Epidermal Growth Factor Receptor* (*EGFR*) gene are a hallmark of disease pathogenesis in GBM (1), with *EGFR* amplification observed in ~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 (5).

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.

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 1 clinical trial (NCT01800695, also known as M12-356) of the EGFR antibody-drug conjugate depatuxizumab mafodotin (depatux-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/disposition for all samples across all assays described below can be found in Supplementary Table S1.

Fluorescence in situ hybridization (FISH)

FISH was performed by a central laboratory on 206 GBMs (Figure 1, Supplementary Figure S1) using the Vysis EGFR CDx Assay (Abbott Molecular, Des Plaines, IL, USA; 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 co-denatured at 73°C for 5 minutes and then hybridized at 37°C for 14-24 hours on a ThermoBrite (Abbott Molecular, Abbott Park, IL, USA). Sample pretreatment and post-hybridization washes were performed using the Vysis Universal FFPE Tissue Pretreatment and Wash Kit (Abbott Molecular, Abbott Park, IL, USA; not commercially available).

Slides were reviewed using fluorescence microscopy with orange, green and DAPI (4',6-diamindino-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 Figure S2). A tumor was considered *EGFR*-amplified when there was focal *EGFR* gene amplification defined as *EGFR/CEP 7* ratio was greater than or equal to 2 in ≥ 15% recorded cells. Tumors with polysomy for chromosome 7 (excess copies of the entire chromosome defined as *CEP7/EGFR* < 2 and CEP7 copy number > 3) but without focal amplification of the *EGFR* gene ≥ 15% were considered to be *EGFR*-nonamplified.

Reverse transcription-polymerase chain reaction (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, Inglewood, CA, USA). Briefly, one ≥ 5μM section containing a minimum of 50 mm^2 total tissue area from the FFPE block was processed for RNA extraction using the QIAGEN RNeasy FFPE Extraction Kit (QIAGEN Sciences, Germantown, MD, USA) per manufacturer's instructions. For non-GBM normal brain tissue specimens, one ≥ 5 μM section containing a minimum of 50 mm^2 total tissue area from the FFPE block was processed for RNA extraction using the TargetPrep RNA Pro Kit (Abbott Molecular, Des Plaines, IL, USA; 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 *m*2000 RealTi*m*e System (Abbott Molecular, Des Plaines, IL, USA). *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.

Whole exome sequencing (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, Germantown, MD, USA). Whole exome sequencing libraries were prepared using the SureSelect Clinical Research Exome kit (Agilent, Cedar Creek, TX, USA). Sequencing was performed with an Illumina HiSeq 2500 (2 × 100 base pairs) (Illumina, Hayward, CA, USA). Profiling aimed to achieve a 150× mean on-target coverage. ArrayStudio (Omicsoft Corporation, Cary, NC, USA) 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., Mountain View, CA, USA) (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 tangent normalization against a panel of normal samples (PON). CNV were identified and merged into larger segments using CBS algorithm. A cut-off of > 3 copies of the *EGFR* gene was used to define amplification.

Whole transcriptome sequencing (RNAseq)

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 (Sigma-Aldrich, St. Louis, MO, USA) 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.

RNA sequencing reads were aligned to the Ensembl release 76 assembly with STAR version 2.0.4b. Gene 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.

Immunohistochemistry (IHC)

IHC was performed on 34 GBMs to assess EGFR protein expression using the EGFR pharmDx Kit for Dako Autostainer (Agilent, Santa Clara, CA, USA). H-score was calculated as described previously (11) as a continuous variable. In brief, the range of H-score is 0 – 300 and is a quantitative measure of protein expression. A score of 200 – 300 was considered as high EGFR expression. The DAKO antibody clone 2-18C9 recognizes both wild-type and EGFRvIII forms of EGFR, and therefore represents total EGFR protein expression.

Statistical analysis

Cohen's kappa statistic (12) was used to compare categorical agreement between amplification detection by FISH with amplification detection by WES and with mRNA expression by RT-PCR. Briefly, $k < 0$ indicates poor agreement, 0.0 – 0.20 slight agreement, 0.21 – 0.40 fair agreement, 0.41 – 0.60 moderate agreement, 0.61 – 0.80 substantial agreement, 0.81 – 1.0 almost perfect agreement (13). R^2 statistic was calculated by linear regression and was used to correlate mRNA expression determined by RNAseq vs RT-PCR, and association between mRNA expression by RT-PCR and WES copy number.

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 ≥ 2 in ≥ 15% recorded cells. Most tumors had clear results for *EGFR* amplification, with few ambiguous cases (Figure 1). For example, 93% of GBMs harbored either a very high (≥ 80% cells; 69% of samples) or very low (≤ 5% cells; 24% of samples) number of amplified cells showing *EGFR* amplification, with few (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 (20× (14) to 50× (15) increases in gene copy number). Finally, one patient with a partial response to depatux-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 analylzed, 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 Figure S3).

For RT-PCR, the cut-off was determined to be $\Delta Ct \geq -5.50$, and was informed by *EGFR* mRNA expression levels as observed in 20 normal brain samples, and association with *EGFR* amplification status in 94 tumor samples (46%). The samples demonstrating ΔCt of ≥ -5.50 were considered positive for total *EGFR* mRNA expression. The other 112 samples (54%) were tested after the cut-off was set. Using this cut-off, 90% of tumor samples positive for *EGFR* mRNA expression demonstrated *EGFR* amplification.

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 ($\kappa = 0.739$ [95% CI = 0.538, 0.939]). The majority of the discordant cases were low FISH positive (Figure 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 ($R^2 = 0.619$, Figure 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 Figure 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.

Concordance 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 (κ = 0.702 [95% CI = 0.473, 0.931]) (Supplementary Table S4, Supplementary Figure S5). RNAseq was comparable to RT-PCR, demonstrating correlation of R^2 = 0.790 (Figure 4). A 94% concordance rate (60/64) was observed in samples that had both RNAseq and FISH results with substantial agreement (κ = 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 *EGFR* mRNA by RT-PCR was approximately 19-fold higher in patient samples with FISH-defined *EGFR* amplification vs those without (Figure 5A).

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

Discussion

Here, we have described different methods used to determine EGFR excess in GBM (Table 1). When performing FISH, we observed a distinct dichotomy: those with a high proportion of *EGFR*-amplified cells, and those with very few amplified cells. We found that *EGFR* mRNA relative expression had a higher association with *EGFR* amplification as determined by FISH than did protein expression as determined by IHC. As the Phase 1 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 to conserve tissue.

The lack of specificity of IHC to accurately identify patients responsive to depatux-m was also observed in a Phase 1/2 trial for advanced solid tumors (none of which were GBM) (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. By 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 et al (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 analytical dynamic range to measure the large biological dynamic range at the higher end of *EGFR* expression observed in GBM, demonstrated by 8/27 samples with low EGFR expression by H-score still classified as *EGFR*-amplified by FISH, and 1/6 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 vs 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). 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 analytical strengths and weaknesses of biomarker methodologies so that trade-offs in terms of sensitivity vs throughput can be optimized. In our ongoing studies, we continue to use FISH for central testing when weighing the pros and cons in comparison to other assays (Table 1).

As mentioned, in the Phase 1 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 INTELLANCE-2 study in *EGFR*-amplified rGBM revealed a survival benefit from the combination of depatux-m and TMZ 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 INTELLANCE-1 trial (NCT02573324) 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Conflict of Interest Disclosures

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Statement of Translational Relevance

Therapeutic decisions in glioblastoma (GBM) are increasingly reliant on the molecular characterization of a patient's tumor. *EGFR* gene amplification occurs in ~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 fluorescence *in situ* hybridization (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 immunohistochemistry 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.

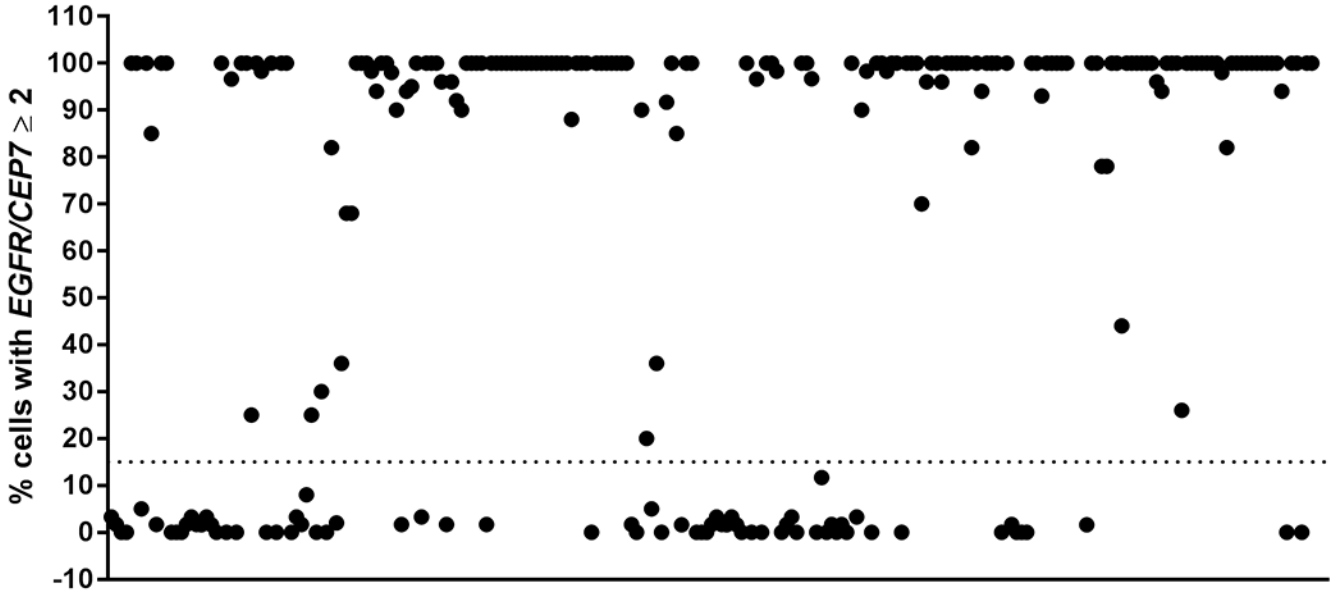

Figure 1. FISH amplification cut-off in tumor samples.

Tumors were deemed positive for *EGFR* amplification if ≥ 15% (dotted line) of cells demonstrated amplification (defined as *EGFR/CEP 7* ratio was ≥ 2). FISH performed on 206 samples; 3 are excluded here (FISH failure).

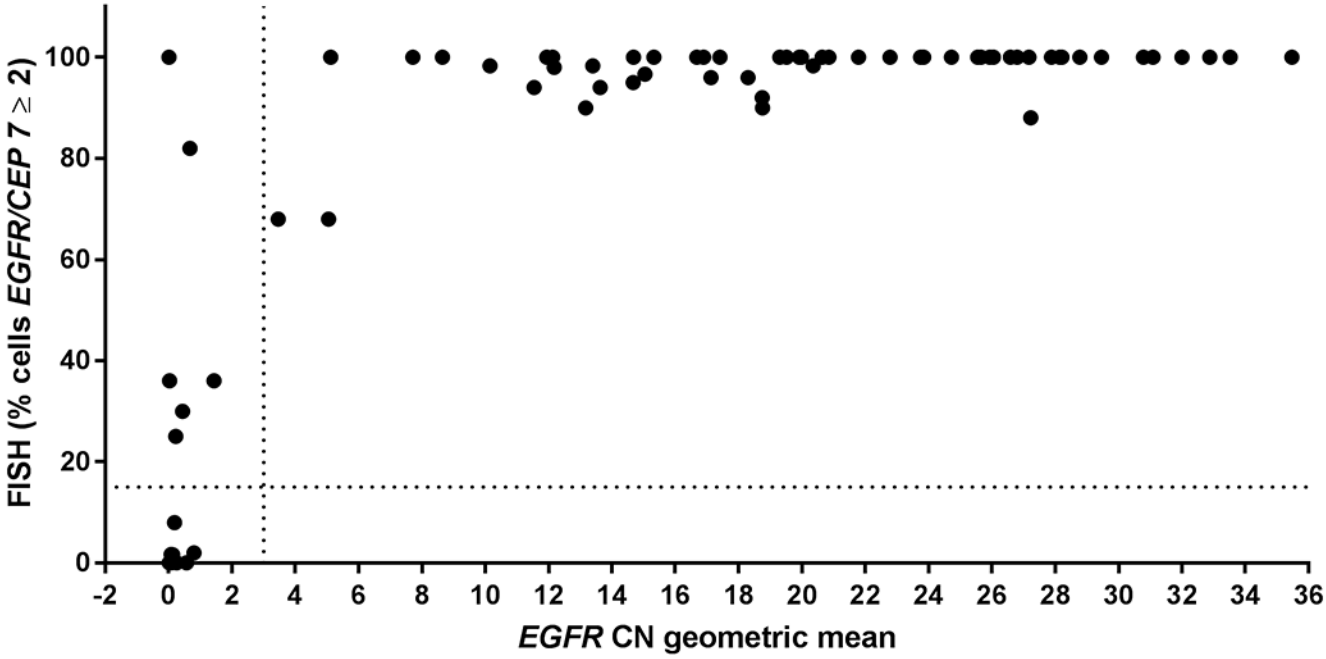

Figure 2. Correlation of *EGFR* amplification by FISH with copy number (CN) determined by WES.

X-axis, geometric mean of *EGFR* copy number (all exons except exons 2–7); linear scale. Vertical dotted line at 3 delineates *EGFR*-amplified (to the right) vs –nonamplified samples (to the left) by CN. Y-axis, percentage *EGFR* amplification by FISH. Cut-off for amplification (≥ 15%) indicated by dotted horizontal line. N = 74 samples.

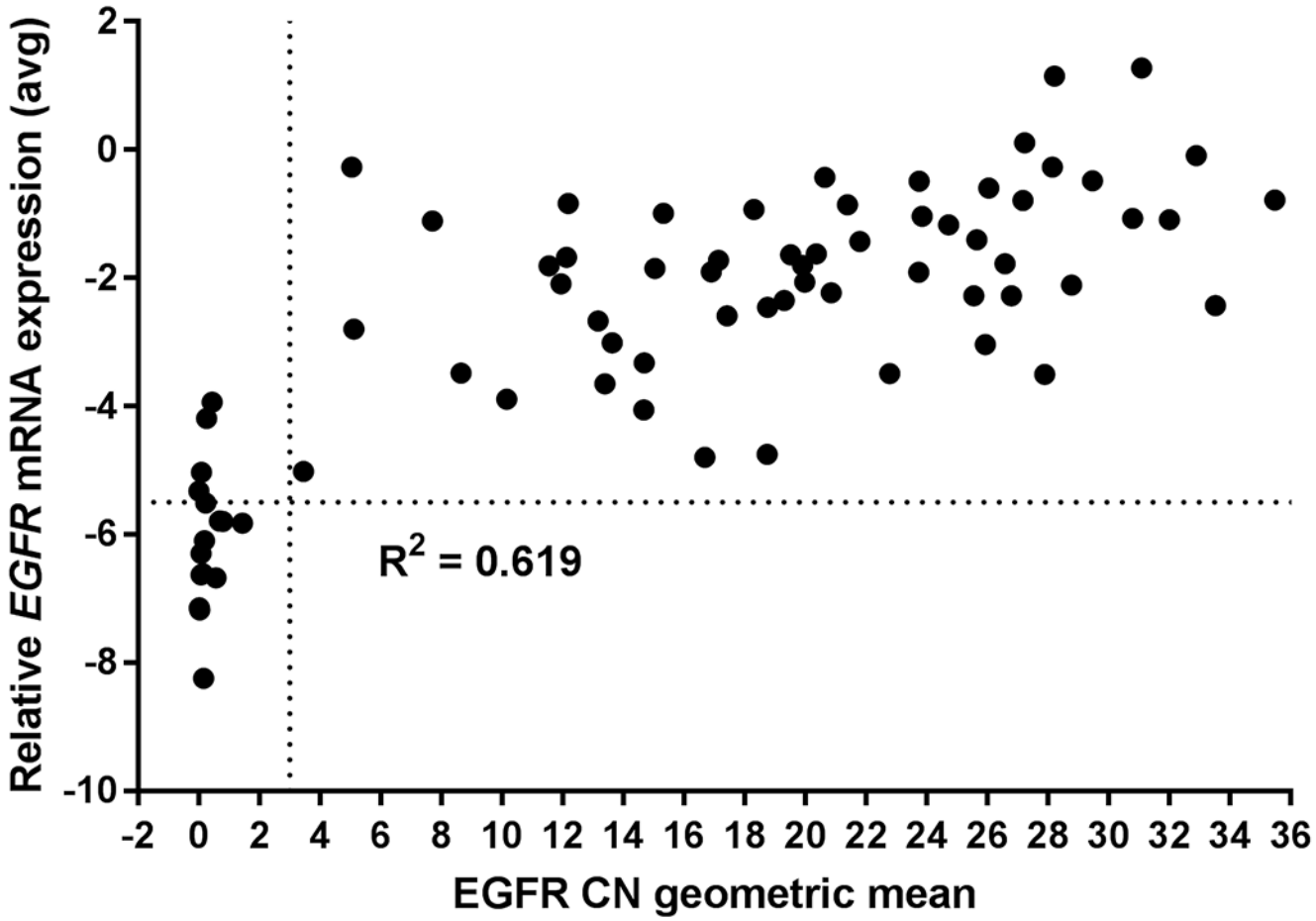

Figure 3. *EGFR* mRNA expression is highly associated with copy number (CN) determined by WES.

X-axis, geometric mean of *EGFR* copy number (all exons except exons 2-7); linear scale. Vertical dotted line at 3 delineates *EGFR*-amplified (to the right) vs –nonamplified samples (to the left) by CN. Y-axis, *EGFR* mRNA expression measured by RT-PCR (ΔCt); linear scale. Horizontal dotted line at −5.50 delineates cut-off between *EGFR*-positive (above line) and -negative (below line) samples. N = 74 samples.

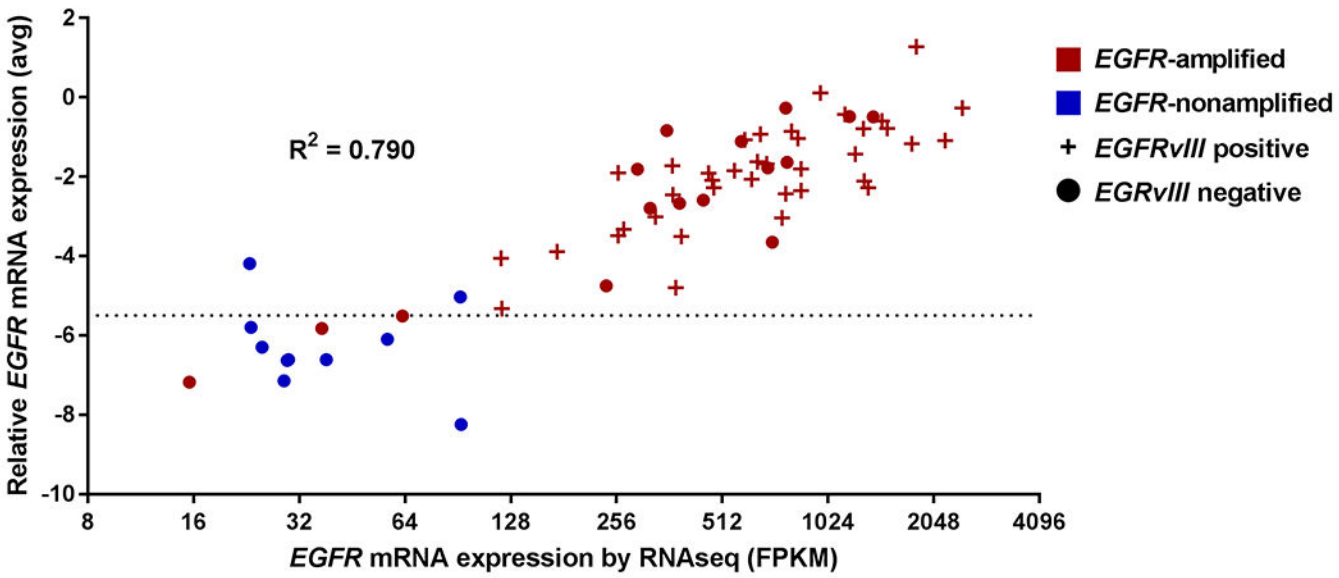

Figure 4. *EGFR* expression by RNAseq and RT-PCR are comparable.

Correlation between RNAseq (x-axis, log2 scale; FPKM, fragments per kilobase million.) and RT-PCR (y-axis, ΔCt, linear scale) results in 64 tumor samples. Horizontal dotted line at −5.50 delineates cut-off between *EGFR*-positive (above line) and -negative (below line) samples. Colors indicate *EGFR* amplification as determined by FISH, symbol indicates *EGFRvIII* mutation (present +, absent •), with mutation detected exclusively among *EGFR*-amplified tumors.

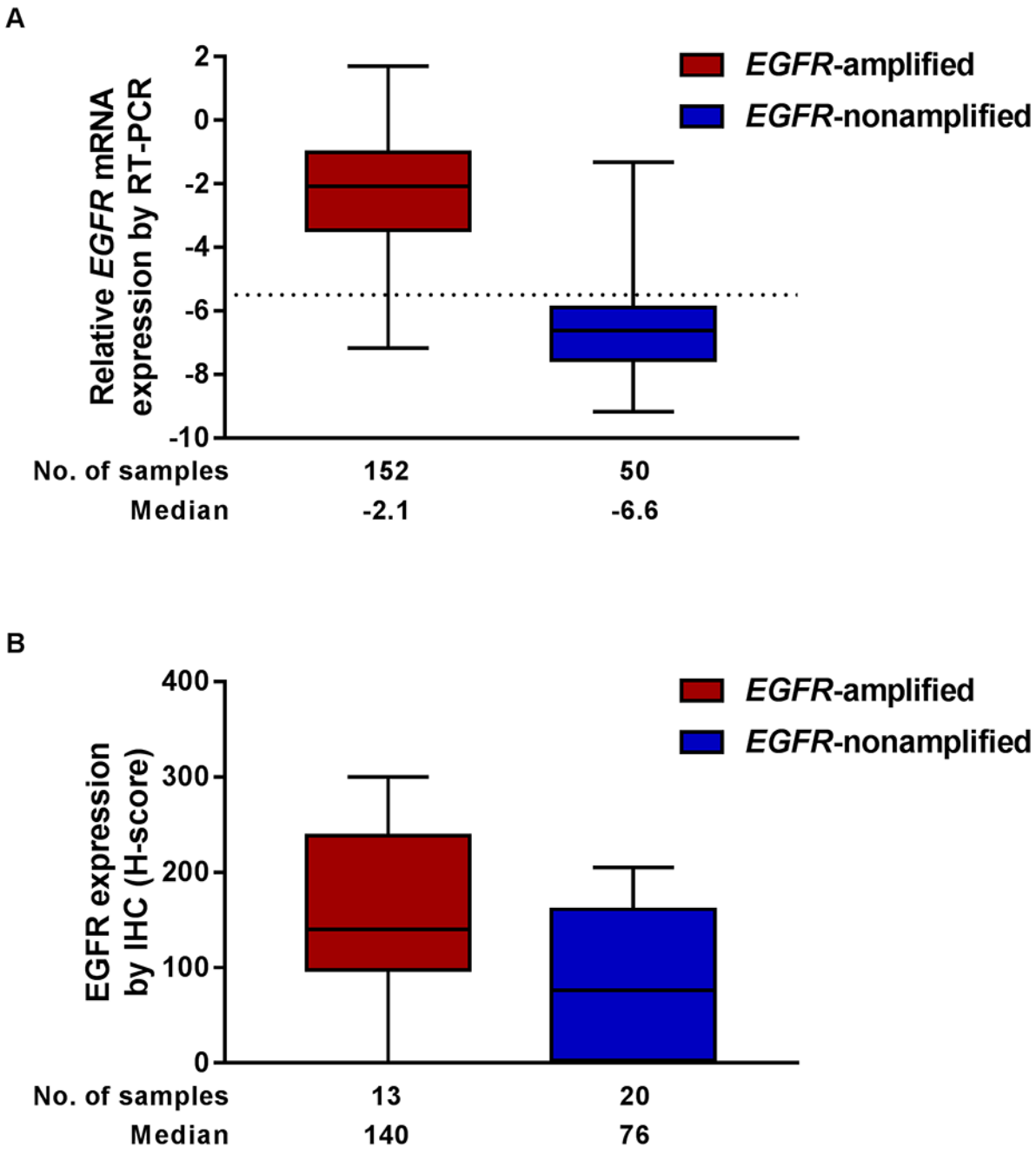

Figure 5. Correlation of *EGFR* amplification with mRNA and protein expression.

A, *EGFR* mRNA expression measured by RT-PCR (ΔCt, linear scale). FISH and RT-PCR assays performed on 202 samples; 4 are excluded here (2 FISH failure, 1 FISH result unreadable, 1 RT-PCR failure). **B**, H-score for EGFR protein expression determined by IHC. Colors indicate *EGFR* amplification as determined by FISH. FISH and IHC assays performed on 34 samples; 1 sample with an H-score of 0 is excluded due to FISH failure. Error bars indicate range.

Table 1.

Comparison of EGFR testing methods

	FISH	WES	RT/PCR	RNAseq	IHC
Cut-off	15% tumor cells with amplification defined as *EGFR/CEP7* ratio ≥ 2	Relative copy number *EGFR* exons (excluding 2-7) compared with chromosome 7 1.3 log increase of *EGFR* categorized as amplified	Normalized to *β-actin* ΔCt of *β-actin* – *EGFR* used and ΔCt ≥ −5.5 categorized as overexpressed	RPKM > 40 categorized as overexpressed	Indeterminate
Correlation with FISH	NA	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 Quick turnaround
Cons	Fluorescence fades over time Fluorescence technology more expensive than CISH	Complex process and algorithms with more room for variation Loss of cell and tissue morphology More expensive and longer turnaround time than FISH	Detects mRNA expression as a surrogate for amplification	Detects mRNA expression as a surrogate for amplification More expensive and longer turnaround time than FISH	Not a direct measurement of gene amplification Measures protein expression only Semi-quantitative False positive and false negative cases