In situ analysis of mutant EGFRs prevalent in glioblastoma multiforme reveals aberrant dimerization, activation and differential response to anti-EGFR targeted therapy

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Abstract

Aberrations in Epidermal Growth Factor Receptor (EGFR/ErbB1) are the most common oncogenic alterations in glioblastoma multiforme (GBM), the most common primary brain tumour. Interactions between wild type (wt) and mutant EGFRs and their subsequent activation are of biological and potential therapeutic importance in GBMs. We recently demonstrated in situ proximity ligation assay (PLA) allows for quantitative evaluation of EGFR dimerization and activation in intact cells.

Utilizing this in situ platform, we demonstrate the aberrant homo-/hetero-dimeric properties of EGFRvIII and EGFRc958 mutants, the two most common EGFR mutants in GBMs. In addition, dimer phospho-activation status could be detected by PLA with superior SNR (>17-fold) and sensitivity (>16-fold) than IF-based phospho-EGFR measurements. Dimer activation analysis indicated quantitative activation differences of mutant dimers. These aberrant features were not overexpression dependent but appear independent of cellular expression levels, suggesting inherent properties of the mutant receptors. Moreover, we observed in situ detection of wt-vIII heterodimerization in GBM specimens, supporting our cell line observations. Notably, currently used anti-EGFR therapeutics Cetuximab, Matuzumab and Panitumumab could effectively block EGFRwt dimerization and activation, but did not equally impair vIII homodimers, wt-vIII, or vIII-c958 heterodimers. EGFRvIII appears to have intrinsic phospho-activation independent of dimerization since Matuzumab blockade of homodimerization had no effect on receptor phosphorylation levels. This data suggests differences in the dimerization-blocking efficacy of EGFR mAbs since mutant EGFR dimer configurations prevalent in GBMs can evade blockade by anti-EGFR treatments. Further studies are warranted to evaluate whether this evasion contributes to poor therapeutic response or therapeutic resistance.
Introduction

The epidermal growth factor receptor (EGFR) is deregulated in several human malignancies. In GBMs, the most common and malignant adult CNS tumour, EGFR is frequently observed to undergo genomic amplification and is usually overexpressed at the protein level (1, 2). In addition, several mutant forms of the receptor are often detected in cases where the wild-type receptor (EGFRwt) has undergone genomic amplification. Notably, the most frequent mutation is the oncogenic EGFRvIII (delta2-7) seen in about 40% of cases where the EGFRwt is amplified (3). EGFRvIII expression has also been reported in several other human cancers including non–small cell lung, breast, and prostate, ovarian and head and neck cancers, albeit at lower frequency and not at the level of genomic rearrangements (4, 5). The second most common EGFR mutation is the EGFRc958 variant, observed in about 20% of cases where the EGFRwt is amplified and is expressed mostly, if not always in tumours that also express the vIII alteration (6, 7).

Recent reports have suggested that EGFR dimerization has an important role in sustaining a tumourigenic state in GBMs and evading EGFR targeted therapies (8). Inhibitors that selectively impair dimerization of EGFRs are hence of potential interest requiring understanding of the intricacies of EGFR dimerization, specifically the interaction capacity of EGFR mutants that are relevant in the context of GBMs. The possibility of dimerization among EGFRwt and EGFR mutants may be an important signal generator in GBMs, and may confer a mechanism for differential strength and type of signalling, altered downregulation and trafficking, as well as facilitating resistance to EGFR targeted therapies. While previous studies have investigated the ability of GBM-relevant EGFR mutants to dimerize using traditional biochemical
methodologies, such as chemical cross-linking and immunoprecipitations, the interaction status still remains controversial (9).

Recently, we have described our work developing and optimizing a novel in situ proximity ligation assay (PLA) for quantitative EGFR dimerization analysis on intact cells (10). Briefly, antibody based proximity probes conjugated to oligonucleotide extensions, bind to monomers of epitope-tagged EGFR and are brought into requisite hybridization proximity upon receptor dimerization. These proximity probes guide the formation of circular DNA strands that are subsequently amplified to create detection sites for fluorescent probes and act as a reporter for protein dimerization, Figure 1A. Each fluorescently labeled bundle represents the formation of one molecular interaction, facilitating the visualization, quantitation and localization of in situ EGFR dimer formation in individual cells. In the present body of work, we systematically analyzed the in situ homodimerization, heterodimerization and phospho-activation states of EGFRwt, EGFRvIII and EGFRc958 using PLA. Additionally, studies were conducted to assess the ability of clinically approved and investigational antibody-based EGFR therapies to block mutant receptor dimerization, activation and downstream signalling. We also provide, to our knowledge, the first in situ evidence of wt-vIII heterodimerization in GBM specimens, and a potential novel diagnostic or theranostic marker.

Materials and Methods

Cell culture and transfections

Chinese hamster ovary (CHOK1) cells (American Type Culture Collection) were cultured in 1× F-12K nutrient medium containing 10% FCS (Wisent). For imaging and PLA experiments, cells were grown to 70% confluence on 18×18-mm glass coverslips (Fisher Scientific) placed in six-well plates (BD Biosciences) and transfected with indicated expression constructs using Fugene
HD (Roche) according to the manufacturer’s instructions. After 6h, the transfection medium was changed to serum-free medium, incubated another 18h and subsequently fixed in 4% PFA. For TET-inducible expression experiments, CHO-K1 TET-On (Clontech) cells were cultured in 90% high-glucose DMEM (Wisent) containing 10% Tet System Approved FBS. At 70% confluence, cells were transfected with TRE-expression constructs in the presence of Doxycycline (DOX) (Sigma) for 6h, serum-starved in the presence of DOX and incubated another 18h prior to beginning the experiment.

**SDS-PAGE/Immunoblotting**

Cells were lysed in modified PLC lysis buffer and analyzed by SDS-PAGE as described previously (10). Membranes were probed overnight at 4°C with antibodies to pEGFR1068, pAKT, AKT, pERK1/2, ERK and p27 (1:1000, Cell Signaling); EGFR (1:1000, Upstate); FLAG and MYC (1:2000, Sigma-Aldrich); and anti–β-actin antibodies (1:10000, Sigma-Aldrich).

**In situ PLA analyses in cultured cells**

Cells were grown on coverslips and co-transfected with expression constructs as described in the “Cell culture and transfection” section. Transfected cells were stimulated with 100 ng/mL EGF for 3 min, washed, fixed, and permeabilized, and blocked as described previously (10). For EGFR dimerization analysis, mouse anti-MYC (1:1000; Sigma-Aldrich) and rabbit anti-FLAG (1:200; Sigma-Aldrich) primary antibodies were used. EGFR phospho-activation analysis was performed using mouse anti-EGFR (1:400; Upstate) and rabbit anti-pEGFR1068 (1:400; Cell Signalling). In situ proximity ligation was performed following primary antibody incubation as described previously (10, 11). PLA probe dilution/incubation time and rolling circle amplification (RCA) times were all optimized for this specific application. FITC anti-actin (1:100; Abcam) or FITC anti-EGFR (1:50; Upstate), as indicated, were added during the PLA
reaction. Coverslip samples were mounted and examined with a Zeiss Axiovert 200 M epifluorescence microscope under a 40× objective.

**Quantitative PLA image analysis**

Images were collected using AxioVision acquisition software from several fields of view per condition per experiment. Typically, ~30 cells per condition were quantified using Blobfinder 3.1 (Centre for Image Analysis, Uppsala University) semi-automated image analysis. Transfected cells were identified with a FITC anti-EGFR stain. Blobfinder analysis involved identifying cells using the signal from one focal plane of Hoechst nuclear stain (ie. one nucleus/cell), and delineation of cellular boundaries. Point-like PLA signals were analyzed to define true signal as local intensity maximum above a background threshold. Blobfinder assignment of cellular boundary and separation of multi-nucleated structures was manually validated and adjusted for each cell included in the quantitation to ensure accurate approximation of cellular area. The number of PLA signals per cell was counted from three Z-plane images. Statistical analysis was performed using GraphPad Prism 4.0 software. Two-tailed Student's t-test were used for calculating the significance of the differences and significance was accepted when P<0.05. Mean values ± SEM are presented from quantitation of at least three independent experiments.

**pEGFR PLA SNR and sensitivity analysis**

Immunofluorescence was performed as described previously (10) with rabbit anti-pEGFR1068 (Cell Signalling Technologies) at dilutions indicated in text and FITC anti-EGFR (1:50; Upstate). Samples were incubated with Texas red anti-rabbit (Abcam) secondary antibodies, counterstained and imaged as above. Single antibody in situ proximity ligation was performed as described in the section “In situ PLA analyses in cultured cells” using anti-rabbit PLA MINUS
and anti-rabbit PLA PLUS secondary probes. The mean signal intensity of 10 pixels representative of ‘biological’ positive signal and 10 pixels representative of ‘biological’ negative signal was determined per condition for both the immunofluorescent and PLA images, using Adobe Photoshop (Adobe Systems Incorporated). SNR (signal-noise-ratio) was calculated as follows: Signal intensity ‘biological’ positive signal/Signal intensity ‘biological’ negative signal. Mean values of the SNR for 10 pixels pairs ± SEM are presented. PLA sensitivity was calculated as follows: PLA highest antibody dilution where SNR > 1/ IF highest antibody dilution where SNR > 1.

**Targeting of EGFR dimerization with monoclonal antibodies**

Anti-EGFR monoclonal antibodies (mAb) cetuximab (Erbitux; ImClone Systems Incorporated) and panitumumab (Vectibix, Amgen) were purchased, while matuzumab was gifted (EMD 72000, Merck KGaA).

**Quantitative FLOW cytometry**

The number of EGFR receptors/cell was determined by staining samples with AlexaFluor-labelled anti-EGFR (1μg/μL; Upstate) mAb and performing quantitative flow cytometry using Quantum Simply Cellular microbead standards (Bangs Laboratories Inc.) as described previously (12). FACS plots of microbead standards and receptor/cell vs. fluorescent intensity calibration curves can be found in Supplemental Figure 1.

**In situ PLA analyses of formalin-fixed paraffin-embedded (FFPE) GBM specimens**

A panel of GBM operative samples, verified by our neuropathology collaborator (Dr. S. Croul, UHN, Toronto), was characterized for EGFRwt/\(\\text{\text{IV}}\) status by RT-PCR, Supplemental Figure 3. Corresponding FFPE tissue sections were used, in accordance with UHN REB approval. Sections were de-paraffinized, rehydrated, and washed in distilled H\(_2\)O. Antigen retrieval was
performed by Pepsin digestion [Digest All-3 (Invitrogen)]. All subsequent steps were performed as described in the “In situ PLA analyses in cultured cells” section with the exception of secondary probe co-incubation for 90min. Direct EGFRwt-EGFRvIII heterodimerization analysis was performed using rabbit anti-EGFRvIII (1:200; Abcam) and mouse anti-EGFRwt (1:200; Dako).

**Results and Discussion**

**EGFR epitope-tag expression levels in CHOK1 cells are similar to GBM specimens**

EGFR dimerization and activation features in GBMs, and other human cancers, are of considerable biologic and potential therapeutic importance. While much interest and focus has already been devoted to this issue, such studies have yielded conflicting results and also employed experimental models with significant limitations (9, 12-15). Namely, the use of co-immunoprecipitations and chemical-crosslinking followed by Western blotting analysis is restricted in several ways. This analysis may not preserve transient or weak interactions, requires large amounts of cultured cells that may be difficult to obtain in certain cases, and the act of cell lysis does not preserve the subcellular environment in which certain interactions occur.

In the present body of work, we sought to revisit the issue of mutant EGFR dimerization and activation using a previously described in situ dimerization assay combining epitope-tagging with PLA (10). Constitutive mammalian expression vectors were generated to impart mutually exclusive antibody binding epitopes, whereby EGFRwt, EGFRvIII and EGFRc958 proteins were tagged at the carboxy-terminus with either a FLAG or MYC epitope-tag. Transient transfection into a CHOK1 cell line, devoid of endogenous EGFR expression, and immunoblot analysis revealed the appropriate molecular-weight proteins expressed to qualitatively similar levels, **Figure 1B**. As demonstrated previously (10), this epitope-tagging approach did not perturb the
cellular localization or ligand-induced tyrosine phosphorylation of the receptors. To directly quantify the EGFR expression levels on the surface of these cells, quantitative flow cytometry-based measurements were employed. As shown in Figure 1C, all three EGFR expressing cell populations demonstrated EGFR antibody staining to similar levels, in keeping with the immunoblot expression analysis. Quantitative determination confirmed approximately similar surface EGFR levels: EGFRwt-MYC: 9.3x10^5; EGFRvIII-MYC: 7.2x10^5; EGFRc958-MYC: 7.8x10^5 receptors/cell, Supplementary Figure 1. Previous estimations of mutant EGFR expression levels in freshly disaggregated GBM biopsy tissue have suggested that the majority of samples express mutant receptors at about 10^5 receptors/cell (16). The values in our experiments are therefore consistent with ‘disease-relevant’ expression found in human GBMs.

**PLA has higher SNR and sensitivity compared to standard techniques**

To investigate the advantages of using PLA detection of pEGFR over traditional IF detection, we performed a ‘head-to-head’ comparison of IF pEGFR1068 detection against PLA-mediated detection of the same pEGFR1068 antibody. In this case, a ‘single recognition’ (ie. one primary antibody) PLA is employed and a pair of MINUS and PLUS secondary probes raised in the same species are utilized. This approach was chosen to make the comparison conditions more equivalent, since the IF approach is typically carried out using a single primary antibody as well. Immunofluorescent analysis of pEGFR was difficult to interpret, as the pEGFR signals of the EGFR stimulated cells were very similar in appearance to the non-stimulated cells, Figure 1D, left; compare arrows, and non-specific background signal (asterisk). Using pEGFR PLA detection, the non-phosphorylated and phosphorylated forms of EGFR were clearly detected, Figure 1D, right. A striking difference between the IF and PLA images was the reduced PLA ‘noise’ signal in the non-phosphorylated samples. This can be attributed to: absence of spurious
secondary antibody binding; binding specificity of the Texas red-labelled PLA detection oligos; or the amplification step which greatly enhances the local intensity of true signal. Quantitative image analysis of the PLA and IF experiments confirmed that the PLA approach exhibited superior SNR (>17-fold) compared to IF at low antibody dilutions and had >6-fold SNR even at very high antibody dilutions of 1:800, Figure 1E. Consistent with this SNR advantage, pEGFR PLA was more sensitive (>16-fold) at detecting pEGFR even at antibody dilutions of 1:800. In principle, the PLA platform enables users the ability to monitor dimerization characteristics of various RTKs, and more importantly, allows concurrent analysis of the respective activation state of those receptors.

**Mutant EGFRs display aberrant dimerization characteristics**

EGFR genomic amplification is observed in tumours that express mutant EGFRs such as EGFRvIII or EGFRc958. Additionally, the EGFRc958 alteration is expressed mostly, if not always in tumors that also express the EGFRvIII alteration (7). Thus, tumor co-expression suggests at least the potential for dimerization events to occur between EGFRwt and mutant EGFRs. To evaluate the dimerization features of EGFRwt and mutant EGFRs we performed quantitative in situ PLA analysis on cells expressing various combinations of receptors. Homodimer formation was tested in cells co-expressing EGFRwt-MYC + EGFRwt-FLAG, EGFRvIII-MYC + EGFRvIII-FLAG and EGFRc958-MYC + EGFRc958-FLAG. Additionally, heterodimer formation was tested in cells co-expressing EGFR constructs EGFRwt-MYC + EGFRvIII-FLAG, EGFRwt-MYC + EGFRc958-FLAG and EGFRvIII-MYC + EGFRc958-FLAG. Previously, we demonstrated that PLA could detect and quantify EGFR dimerization on intact cells at single cell resolution (10). Consistent with previous data, preformed EGFRwt homodimers were detected even in -EGF conditions, though high levels of EGFRwt dimerization
require +EGF (17-20), Figure 2A,B. However, EGFRvIII homodimers were detected constitutively, and showed no difference under –EGF or +EGF conditions, Figure 2A,B. It is difficult to give an exact percentage of EGFRvIII that exists as homodimers, however it appears from a relative standpoint that a smaller percentage forms dimers compared to EGFRwt. Our data suggests that EGFRvIII homodimers form ~50% fewer dimers than EGF-induced EGFRwt homodimers, even though the number of receptors/cell was approximately equal among samples. EGFRc958 homodimers were also detected primarily in response to EGF stimulation, Figure 2A,B, and were noticeably fewer in comparison to ligand-induced EGFRwt homodimers. Heterodimer quantification revealed reduced but persistent presence of EGFRwt-EGFRvIII heterodimers compared to EGFRwt homodimers. While EGF stimulation showed a modest increase in EGFRwt-EGFRvIII dimer levels, this gain was not significant ($P=0.2868$). However, the elevated levels of EGFRwt-vIII dimers in the unstimulated condition (relative to EGFRwt dimers in the unstimulated condition) indicate that EGFRvIII has a dominant ligand-independent activity driving formation of this dimeric unit. The mutant EGFRvIII-EGFRc958 heterodimer had constitutive dimerization, with a slight but not significant ($P=0.1453$), increase in dimer levels following EGF stimulation. Another novel finding was that EGFRc958 demonstrated a compromised ability to form both homodimers and EGFRwt-EGFRc958 heterodimers. This was not expected since EGFRc958 retains all the extracellular dimerization interfaces found in EGFRwt and also responds in a ligand-induced receptor mediated dimerization mechanism. This impaired dimerization ability is likely due to the absence of recently described dimerization-related sequences which are absent in the EGFRc958 truncated receptor (21). This detection by PLA of the extracellularly-deleted EGFRvIII to dimerize with EGFRwt or EGFRc958 has been of debate and not been resolved with standard techniques to date.
**Phospho-activation status is quantitatively altered in mutant EGFR dimers**

In addition to interaction detection, dual-recognition PLA can allow monitoring of posttranslational modifications (PTMs) on individual molecules. To directly measure the phosphoactivation status of EGFRs in CHOK1 cells, we sought to utilize the PLA approach using antibodies that would enable detection of the phosphorylation status on individual receptors. We hypothesized that the combination of antibodies targeted towards i) the intracellular C-terminal region of EGFR and ii) a unique epitope present only when tyrosine autophosphorylation occurs, would enable detection of pEGFRs when subjected to subsequent dual-recognition PLA analysis. To directly measure the phospho-activation states of the EGFR homodimer and heterodimer configurations, the PLA dual recognition approach was modified using mouse anti-EGFR (C-terminus) and rabbit anti-pEGFR1068 primary antibodies. The pEGFR1068 antibody selectively recognizes the phosphorylated form of Y-1068. Tyrosine 1068 is one of the major autophosphorylation sites critical for tumourigenicity, suggested to be an important site of inter-receptor phospho-feedback connectivity, and is an accepted indicator of pEGFR status (14, 22). To test the possibility of measuring pEGFRs using in situ PLA, CHOK1 cells were transfected with EGFRwt-FLAG and subjected to in situ pEGFR PLA using anti-EGFR and anti-pEGFR1068 primary antibodies. Of note, the Y-1068 site is deleted in EGFRc958 and hence not detected by the pY-1068 antibody used in our experiments. This fact served as an important technical and biological negative control, as demonstrated by EGFRc958 homodimers not generating any observable PLA signal irrespective of EGF stimulation, **Figure 3B**. In contrast, EGF stimulation of EGFRwt transfected CHOK1 cells demonstrated an increase in PLA signal, **Figure 3A-bottom left**, compared to those cells that were transfected but not stimulated with EGF, **Figure 3A-top left**. Autophosphorylation of Y-1068 is dependent on the
competence of the receptor’s kinase domain as cells treated with the tyrosine kinase inhibitor AG1478, **Figure 3A-bottom right**, failed to generate an increase in PLA signal. Taken together, this data demonstrates that the observed signal was dependent on EGFR activation and moreover, the EGFR/pEGFR1068 antibody combination could selectively detect phosphorylated EGFR.

To assess the activation status of the mutant EGFRs, cells co-expressing various combinations of EGFRwt, EGFRvIII, and EGFRc958 were analysed by in situ pEGFR PLA. Preformed EGFRwt homodimers were not active to a large extent, with full activation of receptors dependent on EGF ligand binding, **Figure 3A-bottom left** and **Figure 3B**. In contrast, constitutively formed EGFRvIII homodimers may have functional importance, since reduced but persistent phosphorylated EGFRvIII dimers were detected, **Figure 3B**. The EGFRwt-EGFRvIII heterodimers also demonstrate constitutive phosphorylation, with a statistically significant further activation upon stimulation, **Figure 3B** (*P*=0.0154). The mutant EGFRvIII-EGFRc958 heterodimers were also associated with low-level but constant activation. Levels of mutant dimerization and activation fell below levels for EGF-stimulated EGFRwt. This is consistent with a report demonstrating threshold levels of EGFRvIII and activation may be important for oncogenic signaling and defective downregulation (14). Overall, these data demonstrate mutant EGFR dimerization complexes with quantitatively altered phospho-activation profiles and likely associated aberrant downstream tumour promoting signalling.

**Aberrant dimerization and phosphorylation are independent of receptor expression levels**

Receptor overexpression has been suggested to cause receptor clustering and spontaneous receptor activation (23). Since our aforementioned observations of EGFR dimerization and activation were acquired from cell lines overexpressing EGF receptors (i.e. >10⁵ receptors/cell)
we sought to discern whether receptor overexpression was responsible for spurious formation of
i) ligand-independent wt-homodimers, and ii) aberrant mutant homo- and hetero-dimer complexes. In order to investigate the effects of surface EGFR expression levels on dimerization and activation, an inducible expression system was generated whereby the EGFR epitope-tag construct CMV promoter was replaced with a Doxycycline-responsive TRE promoter. Previous reports suggest that $<10^4$ EGF receptors/cell is critical for preventing overexpression artefacts and furthermore, is a physiologically relevant level of receptor (23, 24). Accordingly, we co-transfected CHOK1 cells with various combinations of TRE-EGFR epitope tag constructs and determined that a Dox concentration of 0.1μg/mL was sufficient to reproducibly generate cells expressing $\sim10^4$ EGFRs/cell, Supplemental Figure 2. Quantitative in situ PLA analysis for EGFR dimerization and EGFR phospho-activation was then conducted on the various receptor combinations under low-level expression conditions.

Receptor overexpression itself was not responsible for preformed wt homodimers, since similar levels ($\sim25\%$) of preformed dimers were observed under low-level expression, Figure 4A. Furthermore, the reduced levels of cellular EGFR did not impair the ability of mutant dimers to form. Indeed, persistent EGFRvIII homodimers, EGFRwt-EGFRvIII heterodimers, and mutant EGFRvIII-EGFRc958 heterodimers could all be observed at similar relative levels. Interestingly, the induction of ligand-dependent EGFRc958 homodimers was far more dramatic under low-level expression compared to overexpression. This data suggests that EGFR overexpression commonly observed in GBMs, and genomic amplification-mediated overexpression, is not strictly required for mutant dimer formation but that the receptors themselves have intrinsic properties that facilitate EGFR interactions.
In addition, and of potential clinical importance, in situ PLA activation analysis indicated low EGFR expression levels do not impair mutant phospho-activation. In fact, EGFRvIII homodimer, EGFRwt-EGFRvIII and mutant EGFRvIII-EGFRe958 activation have higher relative levels, compared to that observed under overexpression conditions, Figure 4B. These observations demonstrate that cellular levels of EGFRwt dimerization and phosphorylation vary proportionally with receptor expression level. However, EGFRvIII-EGFRvIII, EGFRwt-EGFRvIII and EGFRvIII-EGFRe958 have absolute levels of activation that are maintained independent of the total cellular levels of receptor.

**Therapeutic anti-EGFR monoclonals have differential effects on mutant EGFR dimers**

In situ PLA dimerization inhibition studies were conducted to assess the ability of clinically approved and investigational antibody-based EGFR therapies to block receptor dimerization. While several EGFR therapeutics exist for blocking ligand-binding and subsequent dimerization, previous studies have focused on monitoring receptor activation as a measure of therapy efficacy (25, 26). A better measure of efficacy would be to directly examine the dimerization event that these antibodies were designed to impair. Treatment doses for Cetuximab, Panitumumab and Matuzumab were established by treating EGFRwt expressing cells with various concentrations of inhibitor and performing immunoblot analysis for pEGFR levels. As expected, all therapeutics effectively blocked ligand-induced activation, Figure 5A. In situ PLA pEGFR analysis served as an alternative methodology for measuring EGFR mAb inhibition.

In situ PLA pEGFR analysis of EGFRwt expressing cells treated with 10μg/mL of mAbs, concurred with the immunoblot analysis, with a reduction in PLA signal observed, Figure 5B. Quantitative analysis confirmed the ability of the EGFR mAbs to block ligand-induced activation of EGF receptors, Figure 5C. Furthermore, in situ PLA dimerization analysis demonstrated that
Cetuximab, Panitumumab and Matuzumab were able to achieve this effect by actually blocking ligand-induced homodimerization of the receptor, Figure 5D. Moreover, Matuzumab demonstrated significantly superior ability to impair EGFR phosphorylation than Cetuximab ($P=0.0009$) and Panitumumab ($P=0.0052$). This was likely due to enhanced inhibition of homodimerization compared to Cetuximab ($P=0.0040$) and Panitumumab ($P=0.0081$) as shown in Figure 5D. These analyses highlight the potential utility of in situ PLA for identifying and quantitatively evaluating anti-dimerization agents.

All antibody-based EGFR therapeutics to date have established their clinical dose based on their ability to block EGFRwt activity. It remains to be seen what efficacy these “EGFRwt-effective” doses will have on mutant EGFRs commonly expressed in GBMs, such as EGFRvIII and EGFRc958. To address this issue, we performed in situ PLA dimerization and activation analysis on cells expressing various mutants that had been treated with EGFRwt-inhibiting doses of anti-EGFR mAbs Cetuximab, Panitumumab and Matuzumab. Interestingly, we observed that Matuzumab had the greatest ability to significantly block EGFRvIII-EGFRvIII homodimer formation, Figure 6A-black columns. This finding implies that the established steric-hindrance effects of Matuzumab (27) are critical towards interfering with EGFRvIII interaction. In contrast, the indirect blockage of ligand-binding by Cetuximab and Panitumumab is not important to a receptor which is unable to bind ligand to begin with (28). Of interest, this apparent reduction in EGFRvIII homodimer levels had no significant effect on EGFRvIII receptor phosphorylation status, Figure 6A-grey columns. In line with these resistance observations, complementary WB analysis of receptor phosphorylation and downstream signalling confirmed that EGFRvIII-EGFRvIII homodimer containing cells had no apparent alterations to pEGFR1068, pERK, or p27 following mAb treatment, Figure 6C-lanes 6,7,8 compared to 5. Only Matuzumab had a hint of
inhibiting pAKT in EGFRvIII expressing cells. Likewise, Matuzumab, Cetuximab and Panitumumab were also observed to significantly impair EGFRwt-EGFRvIII preformed dimers and prevent further interaction from ligand stimulation, Figure 6B-black columns. However, no mAb demonstrated an ability to fully ablate basal EGFRwt-EGFRvIII heterodimer phosphorylation regardless of their ability to impair heterodimer formation, Figure 6B-grey columns. Interestingly, the greater potency of Matuzumab towards EGFRwt-EGFRvIII heterodimers observed in PLA experiments was also detected in WB signalling analysis. The observed trend was that Matuzumab had modestly enhanced inhibition of pEGFR1068 and pAKT, compared to Panitumumab and Cetuximab, Figure 6C-lane 12. The enhanced signalling inhibition by Matuzumab may be a secondary effect of increased receptor downregulation as it tended to downregulate EGFRvIII and EGFRwt-vIII, better than either Cetuximab or Panitumumab, Figure 6C-EGFR blot. Intriguingly, this increased downregulation of EGFRwt-vIII by Matuzumab also resulted in a paradoxical decrease in p27 levels perhaps indicating a decreased need for cell cycle blockage due to concomitant decrease in oncogenic receptors. The preferential effect of Matuzumab on EGFRwt-EGFRvIII heterodimers may have functional implications as the proliferation of GBM cell line U87-wt/vIII was reduced when treated with Matuzumab, Supplementary Figure 5. In terms of EGFRvIII-EGFRc958 heterodimer formation, none of the therapeutics had a dramatic effect on basal heterodimer levels, but all three were observed to inhibit the ligand-induced dimer fraction. Importantly, no substantial affects were observed on EGFRvIII-EGFRc958 phosphorylation, regardless of the anti-EGFR mAb tested. Supplementary Table 1 summarizes the level of dimerization and phosphorylation inhibition observed for the mAbs tested amongst various homo- and heterodimer configurations. Furthermore, these results hint at a non-conventional mechanism of EGFRvIII homodimeric
association, independent of the canonical domain II dimerization arm that is deleted in EGFRvIII. The extracellular deletion in EGFRvIII may result in a dramatic rearrangement of the remaining extracellular domains yielding a novel dimerization interface for EGFRvIII interactions. Conversely, mutant EGFRvIII may dimerize in a domain IV-dependent manner, similar to that of the extracellular deleted v-ErbB (29). Eventual crystal structures of EGFRvIII may shed light on these outstanding questions. These analyses suggest mutant EGFR dimers can evade anti-EGFR therapy, yet also point to the opportunity to block mutant EGFR activity depending on the specific mAb utilized. A corollary of these observations is that EGFRwt-inhibitory doses may be insufficient to block mutant EGFR function, and the effects of higher-level dosing remains to be seen.

**Mutant EGFRwt-EGFRvIII heterodimers are present in GBM operative specimens**

All published studies to date have evaluated mutant EGFR dimerization using in vitro cell culture models, and are thus of limited relevance (9, 12-15). To address the possibility of mutant EGFRwt-EGFRvIII heterodimerization in GBM specimens, we profiled the EGFR expression status of 47 frozen samples of GBM operative specimens. RT-PCR and agarose gel analysis revealed the co-expression of EGFRvIII (243 bp product) and EGFRwt (1044 bp product) in 5 of the samples tested: GBM 2186, 2548, 2318, 2275, and 2230, Supplemental Figure 3. Using this sub-group of EGFRwt and EGFRvIII co-expressing samples, we performed in situ PLA for EGFRwt-EGFRvIII heterodimer analysis using anti-EGFRwt and anti-EGFRvIII specific antibodies, Supplemental Figure 4. In total 4/5 samples tested demonstrated in situ PLA signal for EGFRwt-EGFRvIII dimers, Figure 7. Overall, tumours which expressed only one partner of the heterodimer (i.e. EGFRwt+/EGFRvIII-) demonstrated the technical specificity of detection in the GBM samples, as these GBMs failed to generate any PLA signal, Figure 7-GBM2539.
Notably, intra-sample heterogeneity was apparent since regional variability in EGFRwt-EGFRvIII dimer signal could be observed within the same sample, Figure 7-bottom middle. It would appear that regional EGFRvIII expression differences, as observed in other reports, are partly responsible for the presence of wt-vIII dimers in some areas and lack of heterodimer signal in other areas of the same tumour (30-32). We would therefore expect that some cells in the tumour samples tested were simply not co-expressing both EGFRwt and EGFRvIII and these cells failed to demonstrate wt-vIII heterodimer signal. Moreover, inter-sample heterogeneity was evident as EGFRwt-EGFRvIII dimer signals were variable among specimens, even though they co-expressed the EGFRwt and EGFRvIII receptors, Figure 7-bottom right. This data using PLA analysis suggests a stoichiometric ratio of receptor expression level that favours heterodimer formation, as hinted by the differential EGFRwt:EGFRvIII expression ratios observed in our RT-PCR analysis, Supplemental Figure 3.

Conclusions

Overall, this study illustrates that EGFR mutants prevalent in malignant gliomas possess aberrant interaction and activation features, as well as varied susceptibilities to anti-EGFR mAb therapies. Of novel clinical pertinence, EGFRwt-EGFRvIII mutant heterodimers were directly detected in GBM specimens. Further studies may determine whether there is a biological or clinical relevance in the quantitative level and regional variability of EGFRwt-EGFRvIII dimers within patient samples. For example, cells and/or regions of cells where mutant heterodimers are observed may be associated with differential downstream signalling (ie. pAKT, pERK, pSTAT3, pPLCγ, etc.) compared to cells lacking this mutant heterodimer thereby creating microenvironments of signaling activity. Furthermore, since EGFR amplification or expression in GBMs have limited utility as prognostic factors for response to anti-EGFR therapeutics, the
current data suggests further investigation of EGFR mutant dimerization as a potential parameter for predicting anti-EGFR therapy response.
References

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Figure Legends

**Figure 1**

A) Dual binding of primary antibodies to epitope-tagged EGFR followed by species-selective PLA probe binding provides a scaffold to allow hybridization of connector oligos (left). Ligation of connector oligos and polymerase-mediated amplification create a concatemeric amplification product that can then be visualized by hybridization of complementary fluorescence-labeled probes to the amplification product (right). B) Immunoblot analysis of EGFR expression in transfected CHOK1 cells with antibodies to EGFR, FLAG, MYC and β-actin. CHOK1: non-transfected CHOK1 cells. C) Analysis of surface EGFR expression levels in cells stained with fluorescently labelled anti-EGFR antibody. Solid horizontal line indicates maximum fluorescence levels (auto-fluorescence) of CHOK1 stained parental cells. Histogram signifies fluorescence due to antibody binding. Representative histograms of duplicate experiments are shown. D) Detection of pEGFR1068 on CHOK1-EGFR-MYC serum-starved (top) and EGF-stimulated (bottom) cells by immunofluorescence microscopy (left) or in situ PLA (right). Inset: Total EGFR stain (green) was included in analysis to identify EGFR positive transfected cells. Images of the condition with the highest SNR are presented. E) Quantitative comparison of SNR and sensitivity of pEGFR1068 detection by immunofluorescence and PLA.

**Figure 2**

A) In situ PLA dimerization analysis was performed on untreated (top) or EGF treated (bottom) CHOK1 cells transfected with EGFRwt-FLAG/MYC (left), EGFRvIII-FLAG/MYC (middle), and EGFRc958-FLAG/MYC (right). Samples co-stained with anti–β-actin (green) allow visualization of cell borders. Cells were counterstained with Hoechst33258 (blue) to visualize nuclei; scale bars = 20 μm. Inset top left: Zoomed view showing reduced but present preformed
EGFRwt homodimers were detected in -EGF conditions. B) In situ PLA quantitation of EGFR dimerization in samples co-expressing various epitope-tagged EGFR receptor combinations.

**Figure 3**

A) In situ PLA pEGFR analysis was performed on both serum-starved (top) and EGF stimulated conditions (bottom) in the absence (left) or 1h incubation with 10μM EGFR TKI AG1478 (right). PLA signal (red) and Hoechst counterstain (blue) are shown. Total EGFR stain (green) was included in analysis to identify EGFR positive transfected cells. B) In situ PLA quantitation of pEGFR1068 in samples co-expressing various epitope-tagged EGFR receptor combinations. Scale bars = 20 μm.

**Figure 4**

A) In situ PLA quantitation of EGFR dimerization and B) In situ PLA quantitation of pEGFR activation was performed on DOX treated samples co-expressing various TRE-epitope-tagged EGFR receptor construct combinations.

**Figure 5**

A) Immunoblot analysis of pEGFR1068 and total EGFR levels in CHOK1 cells transfected with EGFRwt-MYC/FLAG constructs following treatment with various anti-EGFR monoclonal antibodies. UNTFD: non-transfected CHOK1 cells. B) In situ PLA pEGFR analysis was performed following EGF-stimulation and treatment with various anti-EGFR monoclonal antibodies, on samples co-expressing EGFRwt-FLAG/MYC receptor. Total EGFR stain (inset; green) was included in analysis to identify EGFR positive transfected cells. Cells were counterstained with Hoechst33258 (blue) to visualize nuclei; scale bars = 20 μm. C) In situ PLA quantitation of EGFR phosphorylation, and D) in situ PLA quantitation of EGFR dimerization was performed. CET-Cetuximab; MAT-Matuzumab; PAN-Panitumumab.
Figure 6

In situ PLA quantitation of EGFR dimerization (black columns-background series) and in situ PLA quantitation of EGFR phosphorylation (grey columns-foreground series) was performed on cells co-expressing various combinations of epitope-tagged EGFR homodimers (A) and heterodimers (B), as indicated below graph, following 24h treatment with indicated anti-EGFR monoclonal antibodies. Mean PLA signals/cell are shown compared to untreated reference (bold bars) C) Immunoblot analysis of downstream EGFR pathways in CHOK1 cells expressing various homodimer and heterodimer constructs following 24h treatment with various anti-EGFR monoclonal antibodies (10μg/mL) and EGF stimulation. Gel densitometry values are reported below blots as fraction of untreated control in each group. C-Cetuximab; M-Matuzumab; P-Panitumumab.

Figure 7

A) Direct in situ PLA EGFRwt-EGFRvIII detection was performed using anti-EGFRvIII and anti-EGFRwt antibodies on FFPE GBM samples. Note the absence of PLA signal on tissue sample GBM2539 lacking EGFRvIII expression, but positive for EGFRwt expression. Note the clear presence of bright, peripheral EGFRwt-EGFRvIII heterodimer signal on several GBM specimens (red; arrowheads). Cells were counterstained with Hoechst33258 (blue) to visualize nuclei; scale bars = 10 μm.
Figure 2

A

EGFRwt  EGFRvII  EGFRc958

B

Unstimulated  EGF stimulated

*** p=0.0001  NS p=0.334  * p=0.0409  NS p=0.2666  NS p=0.0039  ** p=0.1453
Figure 3

(A) Immunofluorescence images showing the effect of AG1478 on EGF-induced signal transduction. -AG1478 and +AG1478 conditions are compared.

(B) Bar graph showing the PLA signal per cell for different conditions. Unstimulated and EGF-stimulated conditions are compared. Significance levels are indicated with * (p<0.05), ** (p<0.01), and *** (p<0.001).
Figure 4

A

Figure 4A: Bar chart showing PLA signal/cell for different combinations of wtEGFR, vIIIEGFR, and c958EGFR. The chart is divided into two sections: HomoDimers and HeteroDimers.

B

Figure 4B: Bar chart showing PLA signal/cell for different combinations of wtEGFR, vIIIEGFR, and c958EGFR. The chart is divided into two sections: HomoDimers and HeteroDimers.
Figure 5

A

<table>
<thead>
<tr>
<th></th>
<th>Cetuximab</th>
<th>Panitumumab</th>
<th>Matuzumab</th>
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<tr>
<td>mAb (µg/mL)</td>
<td>1</td>
<td>10</td>
<td>100</td>
</tr>
<tr>
<td>EGF (100 ng/mL)</td>
<td>+</td>
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<td>+</td>
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<tr>
<td>UNTFD</td>
<td>-</td>
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</tr>
</tbody>
</table>

B

+EGF

+EGF, +CET

+EGF, +MAT

+EGF, +PAN

C

EGFRwt phosphorylation inhibition

D

EGFRwt dimerization inhibition

Graphs showing the inhibition of EGFR phosphorylation and dimerization with different antibodies and EGF conditions.
Molecular Cancer Research

In situ analysis of mutant EGFRs prevalent in glioblastoma multiforme reveals aberrant dimerization, activation and differential response to anti-EGFR targeted therapy

Aaron S Gajadhar, Elena Bogdanovic, Diana Marcela Munoz, et al.

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