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EGFR and EGFRvIII interact with PUMA to inhibit mitochondrial translocation of PUMA and PUMA-mediated apoptosis independent of EGFR kinase activity

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Abstract

EGFR and its constitutively activated variant EGFRvIII are linked to glioblastoma resistance to therapy, the mechanisms underlying this association, however, are still unclear. We report that in glioblastoma, EGFR/EGFRvIII paradoxically co-expresses with p53-upregulated modulator of apoptosis (PUMA), a proapoptotic member of the Bcl-2 family of proteins primarily located on the mitochondria. EGFR/EGFRvIII binds to PUMA constitutively and under apoptotic stress, and subsequently sequesters PUMA in the cytoplasm. The EGFR-PUMA interaction is independent of EGFR activation and is sustained under EGFR inhibition. A Bcl-2/Bcl-xL inhibitor that mimics PUMA activity sensitizes EGFR/EGFRvIII-expressing glioblastoma cells to Iressa. Collectively, we uncovered a novel kinase-independent function of EGFR/EGFRvIII that leads to tumor drug resistance.

Keywords

EGFR; EGFRvIII; glioblastoma; apoptosis; PUMA; chemoresistance

1. Introduction

GBM is the most common and deadliest brain malignancy in adults and, approximately, 90% of patients with these tumors survive only 12-14 months after diagnosis [1]. It is, therefore, an urgent task to better understand the biology of these aggressive tumors in order to improve their therapy. The gene encoding EGFR is often amplified and over-expressed in human GBM [2;3;4]. EGFRvIII, the EGFR constitutively active variant, is a product of rearrangement with an in-frame deletion of 801 bp of the coding sequence within the extracellular domain, resulting in a deletion of residues 6 through 273 and a glycine insertion at residue 6 [3;5;6]. Expression of EGFRvIII is common in GBMs. Both EGFR and EGFRvIII are associated with tumor growth

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Conflict of interest

The authors declare that there is no competing financial interest in relation to the work described.

and progression and are, thus, major therapeutic targets for many human cancers, including, GBM [7;8;9;10].

Small molecule EGFR inhibitors and monoclonal anti-EGFR antibodies have been evaluated clinically for the efficacy against GBM patients both as single agents and in combination with chemotherapeutic agents [11]. These therapies have, however, demonstrated only modest effects [12;13;14;15]. The mechanisms underlying these poor results remain unclear. Gain-of-function mutations within the EGFR kinase domain are commonly found in lung cancer and lead to their hyper-sensitivity to EGFR inhibitors [16;17]. However, such somatic mutations have not been found in gliomas [18]. These observations indicate that our understanding of the biology of EGFR that underlies its role in tumor drug resistance and its response to EGFR-targeted therapy in GBM is insufficient.

Most anti-cancer agents induce intrinsic mitochondria-mediated apoptosis rather than extrinsic death receptor-mediated apoptosis [19;20]. The anti-apoptotic members of the Bcl-2 family of proteins, including, Bcl-2, Bcl-xL and Mcl-1, are guardians of mitochondrial integrity [19; 21;22]. Conversely, the proapoptotic members of the Bcl-2 family of proteins function by antagonizing the anti-apoptotic proteins and include the multi-BH3 domain proteins (Bax, Bak, and Bok) and the BH3-only proteins (Bid, Bim, Bmf and PUMA). Although, these proapoptotic proteins appear to be functionally redundant, their expression has been shown to be tumor-specific [23] and one of the main ones, PUMA, has recently been shown to mediate EGFR inhibitor-induced apoptosis in head and neck cancer [24]. Although, a direct regulatory role of EGFR/EGFRvIII in apoptosis has not been reported, it has been recently shown that ErbB4, a member of the EGFR family of receptor tyrosine kinases, interacts with the anti-apoptotic Bcl-2 protein to promotes apoptosis [25].

Therapeutic activation of the apoptotic pathway has emerged as an attractive treatment strategy for a number of cancers, including, GBM [26;27]. In GBMs, however, to date, a systemic analysis of the expression profile of the proapoptotic members of Bcl-2 family of proteins has not been reported and the relationship between these proapoptotic proteins and EGFR/EGFRvIII remains unclear in GBMs. In this study, we investigated the interaction between PUMA and EGFR and its potential role in EGFR-targeted mono and combinational therapies of GBMs. Our results show that, paradoxically, both wild-type EGFR and EGFRvIII co-express with proapoptotic protein PUMA in GBM cells, *in vitro* and *in vivo*. EGFR and EGFRvIII both interact with PUMA constitutively and under apoptotic stress, leading to cytoplasmic sequestration of PUMA in GBM cells. In contrast, PUMA is exclusively localized on the mitochondrial membranes, as expected, in EGFR-negative GBM cells. These results, together with additional data from functional studies provide evidence for a novel inverse functional link between the EGFR/EGFRvIII signaling and the proapoptotic pathway and this can be a mechanism that accounts for EGFR-associated drug resistance in GBM and, potentially, in other cancer types that also co-express EGFR and PUMA.

2. Materials and methods

2.1. Cell lines, cell culture and xenografts

Human GBM and anaplastic astrocytoma (AA) cell lines were established in Dr. Ali-Osman's laboratory from primary specimens [28], with the exception of U87MG, T98G and U373MG that were obtained from American Type Culture Collection, ATCC (Manassas, VA). Human breast carcinoma MDA-MB-468 cells were also from ATCC. All cell lines were maintained in DMEM supplemented with 10% fetal calf serum. U87MG-vector, U87MG-EGFR and U87MG-EGFRvIII stable transfectant lines were previously established from the parental U87MG cells that express very low levels of EGFR [9]. These stable transfectants were cultured in DMEM with 10% fetal calf serum and 1 mg/ml G418. GBM xenografts were established in

the flanks of nude mice in the xenograft facility of Duke University Preston Robert Tisch Brain Tumor Center.

2.2. Reagents and chemicals

All chemicals were purchased from Sigma (St. Louis, MO) unless otherwise stated. Rabbit polyclonal anti-EGFR antibody used in western blotting was purchased from Santa Cruz Biotech. (sc-03; Santa Cruz, CA). The EGFR and EGFRvIII expression vectors were previously generated in our laboratory [9] and both proteins were expressed as Myc-tagged fusion proteins. Anti-Myc mouse monoclonal antibody was purchased from Roche (Indianapolis, IN). Anti-lamin B mouse monoclonal antibody was from Calbiochem (San Diego, CA). β -actin and α -tubulin antibodies were obtained from Sigma. Rabbit polyclonal Cox IV antibody was from Abcam (Cambridge, MA), whereas rabbit polyclonal PUMA, Bad, Bim, Bmf, Bok, pEGFR (Y1068), Bcl-2, Bcl-xL and Mcl-1 antibodies were purchased from Cell Signaling (Danvers, MA). All transfections were performed using lipofectamine LTX (Invitrogen, Carlsbad, CA) and FuGENE HD (Roche). All siRNAs were purchased from Upstate/Dharmacon (Lafayette, CO) and the sequences are 5'-CGGACGACCUCAACGCACA-3' (human PUMA siRNA) and 5'-UGGUUUACAUGUCGACUAA-3' (control siRNA). The Bcl-2/Bcl-xL inhibitor, 2-methoxyantimycin A3 (2-MA A3), was purchased from BIOMOL (Plymouth Meeting, PA).

2.3. Determination of binding of EGFR/EGFRvIII to PUMA via immunoprecipitation and western blotting

To immunoprecipitate EGFR and EGFRvIII, supernatants of whole cell extracts were pre-cleared with 1 μ g mouse IgG and 20 μ l protein G-agarose for 1 hr at 4°C, and incubated with 1 μ g anti-EGFR mouse monoclonal antibody (Ab-13, Neomarkers) or control mouse IgG at 4°C overnight with gentle agitation. Following addition of protein G-agarose and incubation for 30 min at 4°C, protein G-agarose pellets were collected and washed for multiple cycles at 4°C. The washed immunoprecipitates were subjected to SDS-PAGE and western blotting, as described previously [29].

2.4. Immunohistochemistry (IHC) for EGFR/EGFRvIII and PUMA expression in glioma primary specimens

This was performed, as we described previously [9]. For EGFR/EGFRvIII, two tissue arrays (Imgenex; IMT-01240 and IMT-01241) were immunostained. A tissue array (IMT-01255) was previously stained for EGFR/EGFRvIII [9]. The anti-EGFR mouse monoclonal antibody used in IHC recognizes the C-terminus of both EGFR and EGFRvIII. For PUMA, we immunostained all three tissue arrays that are consisted of 12 normal brain tissues and 101 primary gliomas. The tissue sections were deparaffinized, dehydrated, and subjected to antigen retrieval in an EDTA-containing buffer in an oven. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide and the slides incubated with 10% normal goat serum for 30 min and then with anti-EGFR mouse monoclonal antibody (1:50; Novocastra RTU-EGFR-384) and anti-PUMA rabbit polyclonal antibody (1:100; Cell Signaling) at 4°C overnight. Following washes with PBS, the slides were incubated with biotinylated secondary antibodies and then with avidin-biotin-horseradish peroxidase complex. Detection was performed using 0.125% aminoethylcarbazole chromogen. After counterstaining with Mayer's hematoxylin (Sigma), the slides were mounted. Scoring was performed by a pathologist. Histologic scores (H-Scores) were computed from both % positivity (A%, A=1-100) and intensity (B=0-3) using the equation, H Score=A $\times$ B, according to a well-established IHC scoring system [30].

2.5. Determination of apoptosis via the TUNEL assay

This was performed to quantify fragmented DNA, an indicator of apoptosis, using an assay kit (Invitrogen), according to manufacturer's instructions. Briefly, the cells were fixed in 70% ethanol, washed and incubated in labeling solution containing 5-bromodeoxyuridine 5'-triphosphate and deoxynucleotidyl terminal transferase for 1 hr at 37 °C. The incorporated bromouridine was detected by Alexa Fluor® 488 dye-labeled anti-BrdU antibody at room temperature for 30 min. The cells were then counterstained with propidium iodide and observed under a Zeiss LSM 510 upright confocal microscope. Yellow signals indicate nuclear fragmented DNA, the merged products of fragmented DNA (green fluorescence) and nuclei (red fluorescence). A total of 250-300 cells were examined in each experiment and three independent experiments were conducted to derive means and standard deviations. Extent of apoptosis was subsequently computed using the equation, (# of nuclei with fragmented DNA) / (# of total nuclei).

2.6. Determination of the extent of PUMA mitochondrial translocalization

This was performed via mitochondrial fractionation using an assay kit from Pierce (Rockford, IL), according to manufacture's instructions, to yield mitochondrial and non-mitochondrial fractions. Both fractions were subjected to protein extraction using 1% SDS/0.1% NP-40 and sonication followed by centrifugation for 20 min at 15,000 xg at 4°C. In these studies, we subjected 25% of the mitochondrial proteins and 2.5% of the non-mitochondrial proteins to western blotting. Band signals from mitochondrial PUMA and non-mitochondrial PUMA were determined densitometrically using the NIH ImageJ software, as we previously described [31]. Subsequently, the extent of PUMA mitochondrial translocalization, designated as mtPUMA Index, was computed using the equation below.

$$\text{mtPUMA Index} = \frac{\text{mitochondrial PUMA} \div 25\%}{(\text{mitochondrial PUMA} \div 25\%) + (\text{non-mitochondrial PUMA} \div 2.5\%)}$$

2.7. Determination of treatment synergy

This was performed, as we described previously [9;32], using the CellTiter Blue Cell Viability Assay kit (Promega). The assay is a fluorescent method that measures the ability of living cells to convert a redox dye (resazurin) into a fluorescent end product (resorufin). Briefly, tumor cells in exponential growth were seeded in 96-well culture plates and treated with 1% DMSO or with the EGFR inhibitor Iressa (0-100 uM) or 2-MA A3 (0-100 uM) or with a 1:2 molar ratio of Iressa:2-MA A3. After 48 hrs, 25 µl of the CellTiter Blue reagent was added to each well containing 100 µl media, incubated for 4 hrs at 37°C, and the absorbance measured at 560 nm/590 nm using a plate reader (Synergy-HT, BIO-TEK, Winooski, VT). Three independent experiments, each in triplicate, were performed and mean survival fraction was computed for each treatment. Combination index, CI, was computed using the method developed by Chou and Talalay [33] and the computer software CalcuSyn (Biosoft, Cambridge, UK), as we previously described [9;34].

2.8. Statistical analysis

Student *t*-test and regression analysis were performed using STATISTICA (StatSoft Inc., Tulsa, OK) and Microsoft Excel.

3. Results

3.1. EGFR and EGFRvIII paradoxically co-express and complex with PUMA in GBM cell lines and xenografts

We examined a panel of eight GBM and two AA cell lines for expression of EGFR/EGFRvIII and proapoptotic members of the Bcl-2 family of proteins. As shown in Fig. 1a, the majority of these cell lines co-express high levels of EGFR and three proapoptotic proteins, namely, PUMA, Bax, and Bmf. In contrast, these cells express low levels of Bad, Bim and Bok. To determine whether EGFRvIII also co-expresses with proapoptotic proteins similar to EGFR, we examined three EGFRvIII-carrying GBM xenografts since EGFRvIII expression is not maintained *in vitro* [35]. The results (Fig. 1b) showed that these EGFRvIII-expressing GBM xenografts also express PUMA, Bax and Bmf. We also determined PUMA expression levels in GBM xenografts with high and low levels of EGFRvIII and the results (Fig. S1 in Supplemental Data) showed that D-317 MG xenograft co-expressed EGFRvIII and PUMA and that D-320 MG and D-456 MG xenografts with undetectable EGFR/EGFRvIII expression expressed PUMA at low and high levels, respectively.

In light of a previous study [25] showing that ErbB4 interacts with Bcl-2 and the fact that EGFR/EGFRvIII and ErbB4 share structural homology, we examined whether EGFR/EGFRvIII forms a complex with the three proapoptotic proteins that co-express with the receptors. As indicated by the results of the immunoprecipitation (IP)/western blotting using an EGFR antibody for IP (Fig. 1c), EGFRvIII forms a complex with PUMA, but not with Bax and Bmf in GBM xenografts with endogenous EGFRvIII (D-270 MG and D-317 MG) and with stably transfected EGFRvIII (U87MGEGRvIII). Similarly, in three EGFR-expressing GBM cell lines, PUMA was shown to coimmunoprecipitate with EGFR (Fig. 1d). IgG did not yield signals indicating immunoprecipitation specificity. EGFR-PUMA interaction was confirmed via reverse IP using a PUMA antibody (Fig. S2 in Supplemental Data). A previous report [36] showed that EGFR can exist in the cytoplasm in a free non-membrane-bound form. In this context, activated cell-surface EGFR is endocytosed and trafficked to the ER where it associates with Sec61beta, a component of the Sec61 translocon, and is then retrotranslocated from the ER to the cytoplasm as a non-membrane-bound receptor [37]. Our results in Fig. S2 in Supplemental Data show that PUMA primarily interacts with the EGFR protein that is not associated with plasma membranes. Collectively, these results indicate that EGFR and EGFRvIII both paradoxically co-express with proapoptotic proteins and specifically interact with PUMA in human GBM.

3.2. Levels of EGFR/EGFRvIII and PUMA expression and their co-expression correlate with glioma grade

Using a cohort of 101 gliomas and 12 normal brain tissues, we examined the levels of EGFR/EGFRvIII and PUMA via IHC. The results indicate a significant positive correlation between expression levels of EGFR/EGFRvIII and PUMA ($p=0.039$). As shown in Fig. 2a, the extent of EGFR/EGFRvIII-PUMA co-expression is significantly higher in high-grade/malignant gliomas (20% in AAs and 34.1% in GBMs) than in low-grade gliomas (6.3-7.7%) and normal brain tissues (0%). Regression analysis further indicates that EGFR alone, PUMA alone and EGFR/PUMA co-expression correlate significantly with glioma grade (Fig. 2a). Two representative immunostained gliomas are shown in Fig. 2b in which a GBM (top panel) shows positive staining for both EGFR/EGFRvIII and PUMA and in contrast, a grade II glioma (bottom panel) stains negatively for both proteins. The antibody used in the EGFR/EGFRvIII IHC recognizes a C-terminal epitope present in both EGFR and EGFRvIII receptors, and consecutive tumor sections were used in these studies. The high degree of EGFR/EGFRvIII-PUMA co-expression in primary GBM specimens is consistent with the observations in Fig.

1 with the GBM cell lines. In line with the results in Fig. S1, a sub-population of GBMs were found to express PUMA but not substantial levels of EGFR/EGFRvIII.

3.3. Transcriptional down-regulation of PUMA and over-expression of EGFRvIII induce apoptosis in GBM cells

To address whether PUMA is essential for the induction of apoptosis in GBM, PUMA-specific siRNA was used to transcriptionally down-regulate PUMA expression and the response of U87MG GBM cells, which express very low levels of EGFR, to anisomycin, a potent inducer of apoptosis [38], was examined. As shown in Fig. 3a, the TUNEL assay showed that PUMA knockdown renders U87MG cells resistant to apoptosis (2.5%), induced by 48 hr anisomycin treatments. In contrast, U87MG cells transfected with the control siRNA undergo significant anisomycin-induced apoptosis (45.5%). In the assay, the yellow merged signals represent nuclear fragmented DNA. As expected, untreated cells showed no evidence of apoptosis (data not shown). The western blotting (Fig. 3b) indicated that PUMA expression was specifically and efficiently down-regulated by the PUMA-specific siRNA.

Similar to the PUMA down-regulation, over-expression of EGFRvIII in U87MG cells resulted in significant resistance to anisomycin-induced apoptosis (Fig. 3c,d). After treatment with anisomycin, there was only 3.1% apoptosis in U87MG-EGFRvIII cells. In contrast, the isogenic low EGFR expressing U87MG-vector cells underwent massive apoptosis (49.8%), similar to the level observed with PUMA down-regulation (45.5%). The results in Fig. 3, collectively, indicate that GBM apoptosis is positively regulated by PUMA and negatively impacted by EGFR/EGFRvIII, suggesting that PUMA and EGFR/EGFRvIII pathways are functionally but inversely linked to apoptosis in GBM cells.

3.4. EGFR and EGFRvIII both exist in complex with PUMA constitutively and the interactions are sustained under apoptotic stress

The results in Fig. 4 indicate that, in GBM cells, EGFR/EGFRvIII and PUMA form complex constitutively under unstressed conditions and that the complex is sustained even following treatment with the apoptosis-inducer, staurosporin (ST). These observations were found in U87MG-EGFRvIII (top panel) and U87MG-EGFR (mid panel) stable transfectants, as well as, in T98G GBM cells that express high levels of endogenous EGFR (bottom panel). Mouse IgG used as negative controls did not yield any signal, indicating specificity of the assay. It is noticeable that endogenous EGFR levels were slightly reduced by staurosporin in T98G cells (right panel). This is potentially attributed to caspase-mediated cleavage following staurosporin treatment [39].

3.5. PUMA is sequestered in the cytoplasm of EGFR- and EGFRvIII-expressing GBM and breast cancer cells

PUMA is known to localize on the mitochondrial membranes to initiate apoptosis upon appropriate stress [40;41]. Rationalized by our data showing that EGFR and EGFRvIII co-express and interact with PUMA and that these two pathways are inversely linked to apoptosis, we examined whether EGFR/EGFRvIII might regulate PUMA sub-cellular localization. Thus, we analyzed the cytoplasmic/mitochondrial distribution of PUMA in the isogenic pair, U87MG-EGFRvIII and U87MG-vector cells. The mitochondrial fractionation/western blotting (Fig. 5a) showed PUMA to be primarily localized in the non-mitochondrial fractions of U87MG-EGFRvIII cells under unstressed condition and to undergo a modest mitochondrial translocation following exposure to the apoptotic inducers, staurosporin (ST) and anisomycin (AN). As indicated by the low mtPUMA index (0-0.02), the majority of PUMA is sequestered in the non-mitochondrial fractions of the EGFRvIII-expressing U87MG-EGFRvIII cells (Fig. 5a). In contrast, in U87MG-vector cells with very low level of EGFR expression, PUMA was exclusively present in the mitochondrial fractions independent of apoptotic stress

(Fig. 5b). Effectiveness of cell fractionation is indicated by the absence of the cytoplasmic marker, α -tubulin, in the mitochondrial extracts and the absence of the mitochondrial marker, Cox IV, in the nonmitochondrial extracts.

Similar observations were further found in T98G GBM cells that naturally express EGFR (Fig. 5c). The modest detection of mitochondrial PUMA in these cells may potentially be the result of insufficient cytoplasmic EGFR to interact with and sequester all the PUMA molecules in the cytoplasm. Importantly, siRNA-mediated EGFR expression knockdown led to a significant increase (16-fold) of mitochondrial PUMA (Fig. 5c-left), further suggesting the ability of EGFR to modulate PUMA mitochondrial translocation. EGFR-specific siRNA was effective in reducing EGFR expression as shown by the western blots (Fig. 5c-right). In addition to GBM cells, we found PUMA to be localized in the cytoplasm of MDA-MB-468 human breast cancer cells (Fig. 5d) that are known to express significant levels of endogenous EGFR [34]. Collectively, these results demonstrate that PUMA is sequestered in the cytoplasm of EGFR- and EGFRvIII-expressing GBM and breast cancer cells, constitutively and under apoptotic stress.

3.6. EGFR-PUMA interaction is independent of ligand-mediated receptor activation and is sustained under EGFR inhibition

To gain insight into the factors that modulate the interaction of EGFR with PUMA, we examined the requirement of EGFR activation for the EGFR-PUMA interaction. Fig. 6a (left panel) shows that the ability of EGFR to bind to PUMA was similar in U87MG-EGFR cells with and without EGF stimulation following serum starvation. As indicated by the absence of auto-phosphorylated EGFR (p-EGFR, Y1068), serum-starved U87MG-EGFR cells express inactive EGFR (Fig. 6a-right). In contrast, p-EGFR is readily detected in EGF-treated cells, indicating that EGF efficiently activated EGFR in these cells. To further determine whether EGFR kinase activity is required for the EGFR-PUMA interaction, we treated U87MG-EGFR cells with a small molecular weight EGFR kinase inhibitor, Iressa, that is in clinical use to inhibit EGFR activity. As shown by Fig. 6b (left panel), Iressa did not alter EGFR binding to PUMA but, as expected, significantly reduced the level of EGFR auto-phosphorylation/activation.

Furthermore, we found most GBM cell lines analyzed to express high levels of anti-apoptotic members of the Bcl-2 family of proteins, Bcl-2 and Bcl-xL, but not Mcl-1 (Fig. 6c). Rationalized by this observation and the data shown in Fig. 6b indicating that Iressa treatment did not significantly affect the interaction between EGFR/EGFRvIII and PUMA, we hypothesize that EGFR/EGFRvIII-mediated antagonism of PUMA-mediated intrinsic apoptosis contributes to GBM resistance to EGFR-targeted therapy and that restoring the intrinsic apoptosis by inhibiting Bcl-2/Bcl-xL can sensitize these cells to EGFR-targeted therapy. Thus, we treated U87MG-EGFR and U87MG-EGFRvIII cells with a Bcl-2/Bcl-xL inhibitor, 2-MA A3, that has been shown to induce apoptosis [42]. Tumor cells were treated with Iressa alone (0-100 μ M), 2-MA A3 alone (0-100 μ M) and in combination at a molar ratio of 1:2 (Iressa:2-MA A3). Forty eight hrs later, cell survival rates were determined and combination index (CI) computed using Median-effect analysis, as we previously described [9;34]. Treatment synergy is indicated by CI values less than 1.0; additive effect, CI=1.0; antagonistic effect, CI>1.0. As shown in Fig. 6d, the combination of the EGFR inhibitor (Iressa) with the Bcl-2/Bcl-xL inhibitor (2-MA A3) resulted in synergistic cell kill in both U87MG-EGFR and U87MG-EGFRvIII cell lines. These results indicate that simultaneous inhibition of the kinase-independent function of EGFR/EGFRvIII using a Bcl-2/Bcl-xL inhibitor and the kinase-dependent activity by EGFR kinase inhibitors can lead to synergistic therapeutic effects in GBM cells.

4. Discussion

Over-expression of EGFR and EGFRvIII is a major hallmark of GBM. Although, both receptors have been linked to GBM resistance to chemotherapy, the mechanisms underlying this association are still unclear. Our findings in this study provide evidence that both EGFR and EGFRvIII negatively regulate intrinsic mitochondria-mediated apoptosis by binding to PUMA, a proapoptotic protein that is highly expressed in the majority of GBM. Following the interactions of EGFR/EGFRvIII with PUMA, PUMA is sequestered in the cytoplasm, leading to impaired apoptotic response in GBM. Our results also demonstrate that EGFR/EGFRvIII-mediated antagonism of PUMA is independent of EGFR/EGFRvIII kinase activity and thus, may define a novel mechanism of tumor resistance to apoptosis-inducing EGFR inhibitors. Our data also provide a rationale for a novel GBM therapy, in which both the kinase-dependent and -independent activities of EGFR/EGFRvIII are targeted simultaneously in order to improve EGFR-based mono and combinational therapies.

The results in this study showing GBMs, known to be highly resistant to therapy, to express high levels of the proapoptotic protein, PUMA, is paradoxical. To gain insight into this paradox, we investigated EGFR and EGFRvIII, frequently over-expressed in GBM similar to PUMA, and found both pathways to be inversely linked to the apoptotic response of GBM cells. These results allow the speculation that a subset of GBMs (34%) is capable of up-regulating EGFR/EGFRvIII expression in order to negatively regulate PUMA and thereby, escape therapy-induced apoptosis. Our results, however, also indicate that PUMA can be negatively regulated by EGFR/EGFRvIII-independent mechanisms, given the fact (Fig. 2a) that a portion of PUMA-expressing GBMs do not express EGFR/EGFRvIII. Future investigation is thus needed to identify these mechanisms in order to augment the apoptotic effects of anti-GBM therapy.

The functional interaction between EGFR/EGFRvIII and PUMA potentially represents a new class of protein-protein interaction that involves a receptor tyrosine kinase and a proapoptotic protein. It is well known that EGFR can engage in protein-protein interactions with a variety of proteins, including, transcription factors, STAT3 [43], STAT5 [44] and E2F1 [45], DNA-dependent protein kinase [46], and the DNA replication and damage repair protein PCNA [47]. EGFR-STAT3 interactions lead to transcriptional activation of several cancer-related genes, including, inducible nitric oxide synthase [34], TWIST [48] and COX-2 [49]. EGFR has been shown to interact with and stabilize sodium/glucose cotransporter 1, leading to maintenance of intracellular glucose level and prevention of autophagic cell death [50], and to bind to and phosphorylate the human GSTP1 protein [51]. Interestingly, the tumor suppressor, p53, has been shown to translocate onto the mitochondria and bind to anti-apoptotic Bcl-xL, leading to apoptosis [52]. Similarly, ErbB4 undergoes mitochondrial translocalization and subsequently interacts with the anti-apoptotic Bcl-2, leading to apoptosis [25]. Unlike p53 and ErbB4, EGFR and EGFRvIII interact with proapoptotic PUMA to antagonize mitochondrial transport of PUMA, leading to reduced levels of apoptosis and increased cell survival. Together, these findings describe a new class of protein-protein interactions that occurs between Bcl-2 and non-Bcl-2 proteins, and that these interactions regulate intrinsic mitochondria-mediated apoptosis.

Although EGFR and EGFRvIII are best known for their tyrosine kinase function, the results presented in the current study and from previous reports, indicate that both proteins also have kinase-independent functions. For example, EGFR has been shown to undergo nuclear translocalization and to function as transcriptional regulators, through the transactivation domain and interactions with DNA-binding transcription factors [34;45;53]. Nuclear EGFRvIII has been shown to interact with STAT3 to promote malignant transformation of glial cells [54]. EGFR prevents autophagic cell death by maintaining intracellular glucose level through EGFR kinase-independent interaction and stabilization of the sodium/glucose

cotransporter 1 [50]. In line with these observations, PTEN elicits phosphatase-dependent and -independent functions [55]. These findings indicate that both EGFR and EGFRvIII possess kinase-dependent and -independent activities and have a profound potential to interact with other important pathways in cancers.

Our results showed that Iressa, an EGFR-targeted tyrosine kinase inhibitor, fails to disrupt the interaction between EGFR/EGFRvIII and PUMA, suggesting that this kinase-independent anti-apoptotic activity may be an important mechanism underlying the limited clinical efficacy demonstrated by EGFR-targeted therapy. These observations also suggest that a higher therapeutic efficacy may be achieved by targeting both kinase-dependent and -independent functions of EGFR. In support of this premise, our data showed that mimicking PUMA's proapoptotic activity using a Bcl-2/Bcl-xL inhibitor sensitized both EGFR- and EGFRvIII-expressing GBM cells to Iressa and that most GBM cell lines we analyzed expressed high levels of Bcl-2 and Bcl-xL. Collectively, these findings provide strong evidence for a novel mechanism by which EGFR confers GBM resistance to EGFR-targeted therapy and potentially other therapies, as well as, provide a rationale for a novel combinational anti-GBM therapy that target both EGFR and intrinsic apoptotic pathways.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

EGFR and EGFRvIII both paradoxically co-express with proapoptotic proteins and specifically interact with PUMA in GBM. (A) Expression of EGFR and proapoptotic proteins in human malignant glioma cell lines. Total cell extracts isolated from eight GBM and two AA cell lines were subjected to western blotting to determine the levels of EGFR and Bcl-2 family of proapoptotic proteins, including, Bad, PUMA, Bax, Bim, Bmf and Bok. α -tubulin was determined as loading controls. Cell lines: 1, UW14; 2, UW281; 3, MGR1; 4, MGR2; 5, MGR3; 6, T98G; 7, U373MG; 8, UW5; 9, UW15; 10, UW19. All are GBM cells except for MGR1 and UW5 that are AA cells. (B) Co-expression of EGFRvIII and proapoptotic proteins in GBM xenografts. Three EGFRvIII-expressing GBM xenografts, namely, D-270 MG, D-317 MG and U87MG-EGFRvIII, were examined for levels of EGFRvIII, proapoptotic proteins and α -tubulin, by western blotting. (C) EGFRvIII interacts with PUMA, but not Bax and Bmf, in GBM xenografts. Proteins extracted from three EGFRvIII-expressing GBM xenografts, as named in panel b, were subjected to immunoprecipitation/western blotting to determine the extent to which EGFRvIII interacts with co-expressed proapoptotic proteins. An EGFR antibody (Ab) was used to immunoprecipitate EGFRvIII. (D) EGFR interacts with PUMA in GBM cell lines. Using proteins purified from three EGFR-expressing GBM cell lines, immunoprecipitation/western blotting was conducted to investigate the interaction between EGFR/EGFRvIII and PUMA. An EGFR Ab was used to immunoprecipitate EGFR, whereas control IgG was used as negative control for immunoprecipitation. Top panel: immunoprecipitation/ western blotting. Lower panel: western blotting.

A

Tissues	EGFR+/PUMA+*	EGFR+**	PUMA+***
normal brain	0/12 (0%)	0/12 (0%)	3/12 (25%)
grade I glioma	1/13 (7.7%)	1/13 (7.7%)	5/13 (38.5%)
grade II glioma	2/32 (6.3%)	6/32 (18.7%)	14/32 (43.8%)
grade III glioma/AA	3/15 (20.0%)	4/15 (26.7%)	7/15 (46.7%)
grade IV glioma/GBM	14/41 (34.1%)	15/41 (36.6%)	31/41 (75.6%)

normal brain; N=12 gliomas; total N=101

*, **, ***Correlations with glioma grade.

*p=0.018, R=0.94

**p=0.00005, R=1.0

***p=0.021, R=0.93

B **Fig. 2.**

EGFR/EGFRvIII and PUMA co-express in GBM primary specimens and the extent of co-expression significantly correlates with glioma grade. (A) EGFR/EGFRvIII significantly co-expresses with PUMA in primary GBMs. A cohort of 101 gliomas and 12 normal brain tissues were subjected to IHC staining for EGFR/EGFRvIII and PUMA, in which 55 gliomas and 5 normal brain tissues have been previously stained for EGFR/EGFRvIII [9]. The EGFR antibody used in these studies recognized both EGFR and EGFRvIII. Regression analysis showed that expression of EGFR correlates significantly and positively with PUMA ($p=0.039$, $R=0.9$) and that EGFR alone, PUMA alone and their co-expression correlated significantly and positively with glioma grade (*EGFR/PUMA, $p=0.018$, $R=0.94$; **EGFR, $p=0.00005$, $R=1.0$; ***PUMA, $p=0.021$, $R=0.93$). (B) Representative immunostained primary gliomas.

Top panel: a primary GBM stained positively for both EGFR/EGFRvIII and PUMA. Bottom panel: a grade II glioma showing negative staining for both proteins. Consecutive tumor sections were used in these studies.

Fig. 3.

Effects of PUMA expression down-regulation and increased EGFRvIII expression on the induction of apoptosis in GBM cells. Extent of apoptosis is computed as: (# of apoptotic nuclei) / (total # of nuclei). In both panels, 250-300 cells were examined in each experiment and three independent experiments were conducted to derive means and standard deviations. (A) PUMA expression knockdown renders GBM cells resistant to apoptosis, as indicated by the TUNEL assay. U87MG cells were transfected with control siRNA and PUMA-specific siRNA for 24 hrs and exposed to 100 ng/ml anisomycin, a potent apoptosis inducer, for 48 hrs. The cells were then subjected to the TUNEL assay to determine the extent of apoptosis. Red fluorescence: nuclei stained by propidium iodide. Green fluorescence: fragmented DNA. Yellow merged signals: nuclear fragmented DNA. (B) Western blotting indicates specific and efficient PUMA expression knockdown following transfection of PUMA-specific siRNA, but not control non-targeting siRNA. (C) Increased EGFRvIII expression confers resistance of GBM cells to apoptosis. U87MG-vector and U87MG-EGFRvIII stable transfectant cells were exposed to 100 ng/ml anisomycin for 48 hrs and subjected to the TUNEL assay to determine the extent of apoptosis. Generation and characterization of the isogenic cell lines were described previously [9]. (D) Western blotting indicates that U87MG-vector cells express a very low level of endogenous EGFR (higher molecular weight; 175 kD) whereas U87MG-EGFRvIII cells contain significant EGFRvIII (140 kD) expression.

Fig. 4.

EGFR and EGFRvIII both exist in complex with PUMA constitutively and the interactions are sustained under apoptotic stress. U87MG-EGFR and U87MG-EGFRvIII stable transfectants and T98G GBM cells with endogenous EGFR were treated with 1% DMSO and 1 μ M apoptosis-inducer staurosporin (ST), harvested and subjected to immunoprecipitation/western blotting (left panel) and western blotting (right panel), as described earlier.

Fig. 5.

PUMA is sequestered in the cytoplasm of EGFR- and EGFRvIII-expressing GBM and breast cancer cells, independent of apoptotic stress. The extent of PUMA mitochondrial translocation, mtPUMA Index, is computed as described earlier in Materials and Methods. (A) PUMA is primarily localized in the cytoplasm of U87MG-EGFRvIII cells. U87MG-EGFRvIII cells treated with vehicle control, staurosporin (ST, 1 μ M) or anisomycin (AN, 100 ng/ml) were harvested and fractionated into mitochondrial and non-mitochondrial fractions. Protein extracts from both fractions were subjected to western blotting to detect PUMA, α -tubulin (cytoplasmic marker) and Cox IV (mitochondrial protein). Lack of α -tubulin and Cox IV in the mitochondrial and non-mitochondrial fractions, respectively, indicated fractionation effectiveness. Low mtPUMA indices indicate that PUMA is primarily localized in the cytoplasm of U87MG-EGFRvIII cells, under unstressed and stressed conditions. (B) PUMA is exclusively localized in the mitochondrial fractions of U87MG-vector cells. U87MG-vector cells were treated, fractionated and proteins analyzed, as described earlier in panel **a**. The mtPUMA indices in these cells are 1.0, indicating that PUMA is exclusively detected on the mitochondria of these cells, independent of apoptotic stress. (C) EGFR expression knockdown by siRNA leads to increased PUMA mitochondrial translocation. In control siRNA-treated T98G natural GBM cells, PUMA is primarily localized in the cytoplasm. In EGFR-specific siRNA-treated cells, we observed a marked increase of mitochondrial PUMA (left panel). EGFR-specific siRNA was effective in reducing EGFR expression as shown by the western blots (right panel). Lamin B: nuclear protein. ME: mitochondrial extracts. NME: non-mitochondrial extracts. (D) PUMA is sequestered in the cytoplasm of EGFR-expressing human breast cancer cells. MDA-MB-468 cells, known to express high levels of endogenous EGFR [34], were treated with and without staurosporin, fractionated into mitochondrial and non-

mitochondrial fractions and protein extracts analyzed as described earlier. PUMA is primarily localized in the cytoplasm of these cells, under unstressed and stressed conditions, as indicated by the low mtPUMA indices.

Fig. 6.

EGFR-PUMA interaction is independent of ligand-mediated receptor activation and is sustained under EGFR inhibition. (A) EGFR-PUMA interaction occurs independent of ligand-mediated receptor activation. U87MG-EGFR cells were serum-starved for 24 hrs and stimulated with EGF for 20 mins. The cells were harvested and subjected to immunoprecipitation/western blotting (left panel) and western blotting (right panel). An EGFR antibody was used to immunoprecipitate EGFR. Control IgG did not yield signals indicating specificity. Auto-phosphorylated Y1068 residue serves as an indicator for EGF-induced EGFR activation. (B) EGFR kinase activity is not required for their interaction with PUMA. U87MG-EGFR cells treated with and without Iressa (25 μ M) for 2 hrs were subjected to immunoprecipitation/western blotting (left panel) and western blotting (right panel). Control IgG did not yield signals indicating specificity. Iressa treatments effectively inhibited EGFR kinase activity, but did not significantly affect interaction between EGFR/EGFRvIII and PUMA, indicating that the interaction did not require EGFR kinase activity. (C) Anti-apoptotic proteins Bcl-2 and Bcl-xL are highly expressed in most GBM cell lines. Western blotting was conducted to determine expression levels for anti-apoptotic members of the Bcl-2 family of proteins. Noticeably, Bcl-2 and Bcl-xL are highly expressed in the majority of the GBM cell lines analyzed, but not Mcl-1. (D) Restoring intrinsic apoptosis sensitizes EGFR/EGFRvIII-expressing GBM cells to EGFR-targeted therapy. U87MG-EGFR and U87MG-EGFRvIII cells were treated with Iressa alone (0-100 μ M), 2-MA A3 (a Bcl-2/Bcl-xL inhibitor and a potent apoptosis-inducer; 0-100 μ M) alone and in combination with the molar ratio of 1:2 (Iressa:2-MA A3). Forty-eight hrs post treatments, cell survival rates were determined and CI computed using Median-effect analysis, as we previously described [9;34]. CI values: CI<1.0, synergy; CI=1.0, additive effect; CI>1.0, antagonistic effect. The Combination treatment showed synergistic killing effects in both cell lines, as indicated by low CI values.