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EGFR wild type antagonizes EGFRvIII-mediated activation of Met in glioblastoma

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Abstract

Epidermal growth factor receptor (EGFR)vIII is the most common EGFR mutant found in glioblastoma (GBM). EGFRvIII does not bind ligand, is highly oncogenic and is usually coexpressed with EGFR wild type (EGFRwt). EGFRvIII activates Met, and Met contributes to EGFRvIII-mediated oncogenicity and resistance to treatment. Here, we report that addition of EGF results in a rapid loss of EGFRvIII-driven Met phosphorylation in glioma cells. Met is associated with EGFRvIII in a physical complex. Addition of EGF results in a dissociation of the EGFRvIII–Met complex with a concomitant loss of Met phosphorylation. Consistent with the abrogation of Met activation, addition of EGF results in the inhibition of EGFRvIII-mediated resistance to chemotherapy. Thus, our study suggests that ligand in the milieu of EGFRvIII-expressing GBM cells is likely to influence the EGFRvIII–Met interaction and resistance to

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

treatment, and highlights a novel antagonistic interaction between EGFRwt and EGFRvIII in glioma cells.

Keywords

EGFRvIII; glioblastoma; EGFR wild type; Met; antagonistic interaction; temozolomide

INTRODUCTION

Increased expression of wild type and mutant forms of the epidermal growth factor receptor (EGFR) is widespread in cancer. EGFR gene amplification and mutation are common and striking abnormalities in glioblastoma (GBM) occurring in the classical subtype of the disease and detected in ~40–50% of GBMs.^{1–3} A specific EGFR mutant (EGFR Type III, EGFRvIII, de2–7, EGFR) can be detected in up to one-third of GBMs^{1,4} and other types of cancer.^{5,6} EGFRvIII is the most common EGFR mutant found in GBM and is usually coexpressed with the EGFR wild type (EGFRwt). EGFRvIII is missing exons 2–7 of the EGFR and is unable to bind ligand and signals constitutively. Increased EGFRvIII expression may influence multiple aspects of tumor biology including proliferation of cells, motility and invasiveness, and resistance to treatment.^{1,7}

Multiple receptor tyrosine kinases (RTKs) are expressed in GBM.⁸ c-Met is a RTK that is overexpressed in GBM and also other cancers.⁹ A number of studies have reported a pro-oncogenic role of Met in glioma and that inhibition of Met is effective in inhibiting glioma growth in preclinical models.^{10–12} Transactivation of Met by EGFR signaling has been reported for EGFRwt as well as EGFRvIII in GBMs and other cancers.^{13,14} Phosphorylation of Y1234 is required for Met activity and is particularly responsive to the presence of EGFRvIII.^{15,16} Thus, a number of studies have identified Met as a target of EGFRvIII and documented that EGFRvIII activates Met. Direct inhibition of the EGFR by using tyrosine kinase inhibitors is effective in preclinical models but has not proven effective in GBM.¹⁷ A plausible mechanism of resistance to EGFR inhibition is the activation of multiple RTKs in the same tumor.^{15,18,19} Met is known to be active in glioma and to be activated by EGFRvIII. A synergistic effect of EGFRvIII and Met was suggested by the finding that combined inhibition of EGFRvIII and Met seems to be more effective than inhibition of either alone in glioma models.^{15,18} It should also be noted that a number of EGFRwt ligands are expressed in GBM, and the availability of ligands in the vicinity of tumor cells is likely to influence EGFRwt activation and secondarily affect EGFRvIII signaling.^{19–22}

Previous studies have concluded that, whereas in some tumors EGFRvIII distribution is more restricted or focal compared with EGFRwt (more generalized), EGFRvIII is usually expressed in cells where EGFRwt is overexpressed.^{23,24} Our recent data derived from limiting dilution of clonal cell populations from primary GBM cultures also support previous work, suggesting that EGFRwt and EGFRvIII are expressed in the same tumor cells.²⁵

In this study, we report a novel antagonistic interaction between EGFRwt and EGFRvIII involving Met activation. Consistent with previous studies, we find that EGFRvIII activates

Met in multiple glioma cell lines. However, surprisingly, addition of EGF to EGFRvIII-expressing cells led to a rapid dephosphorylation of Met. There is no dephosphorylation of EGFRvIII in response to EGF excluding a global inhibition of EGFRvIII. Inhibition of EGFRvIII-induced Met activation by activation of EGFRwt is accompanied by an increased sensitivity to temozolomide, the first-line chemotherapeutic drug used in GBM. We find that EGFRvIII becomes associated in a physical complex with Met. Addition of EGF with the resultant activation of EGFRwt results in a disruption of the physical complex between EGFRvIII and Met. We propose that the EGF-mediated loss of EGFRvIII–Met association leads to a loss of Met activation. Thus, our study identifies an antagonistic interaction between EGFRwt and EGFRvIII involving EGFRvIII-induced Met activation and suggests that the availability of ligand in the vicinity of EGFRvIII and EGFRwt-expressing cells may be a critical determinant of simultaneous EGFRvIII and Met activation in GBMs, influencing the malignant phenotype and response to treatment.

RESULTS AND DISCUSSION

Multiple RTKs are expressed in cancer and have an important role in the malignant phenotype. There is substantial evidence for coexpression and synergistic interactions between RTKs from different families in cancer. For example, a number of studies have reported transactivation of Met by EGFR in cancer.^{13,14,26} Met is linked to aberrant EGFR signaling in GBM. Furthermore, combined inhibition of Met and EGFRvIII is more effective in inhibiting the growth of glioma cells compared with the inhibition of either RTK alone in preclinical models.

We investigated whether activation of coexpressed EGFRwt using ligand would synergize with EGFRvIII to increase Met activation. To investigate the role of EGFRwt in EGFRvIII signaling, we used tetracycline-inducible as well as constitutive expression of EGFRvIII in established glioma cell lines. As EGFRvIII is unable to bind ligand, an inducible system of expression is particularly useful in studying EGFRvIII signal transduction. U87MG cells express endogenous EGFRwt and were engineered to express EGFRvIII conditionally in response to tetracycline (U87vIII-Ind, Figure 1a), as we have described.²⁷ We examined the phosphorylation of Tyr 1234/1235, a critical step for activation of Met tyrosine kinase and known to be highly responsive to EGFRvIII expression. Consistent with previous studies, we find that tetracycline-inducible EGFRvIII expression leads to activation of Met (Figure 1a) in U87vIII-Ind cells. Surprisingly, addition of EGF results in a rapid decrease in Met phosphorylation in these cells (Figure 1a). The loss of Met phosphorylation in response to EGF can also be detected in U251vIII-Ind cells expressing EGFRvIII in response to tetracycline as described previously.²² Expression of EGFRvIII in these cells results in the activation of Met, an activation that is abolished when EGF is added to cells (Figure 1b). Similarly, LN229EGFRvIII cells express EGFRvIII constitutively and show activation of Met. When exposed to EGF, Met phosphorylation is abolished consistent with our results in other cell lines (Figure 1c). In addition, as EGFRvIII is commonly expressed in EGFR-amplified GBMs with increased expression of EGFRwt, we examined a cell line that overexpresses both EGFRwt (constitutively) and EGFRvIII conditionally in response to tetracycline (U251vIII-Ind + wt cells). Addition of EGF also results in a loss of Met phosphorylation in these cells (Figure 1d). Next, we screened a panel of primary GBM

cultures grown as neurospheres in stem cell medium for expression of EGFRwt and EGFRvIII (Figure 1e). EGFRvIII is truncated and migrates faster than EGFRwt in electrophoresis gels. We selected neurospheres derived from tumor 987 with significant expression of EGFRvIII and a lower level of EGFRwt (987-NS). Addition of EGF to 987 neurospheres also resulted in dephosphorylation of Met as seen in established GBM cell lines (Figure 1f). Notably, however, the addition of EGF does not influence tyrosine phosphorylation of EGFRvIII in any cell line (Figure 1). Thus, the dephosphorylation of Met cannot be explained by a global inhibition of EGFRvIII activity. As expected, addition of EGF increased tyrosine phosphorylation of EGFRwt and resulted in increased ERK activation (Figure 1).

Thus, there is an antagonistic interaction between EGFRvIII and EGFRwt that involves Met. Synergistic interactions between EGFRwt have been reported previously and involve autocrine or paracrine loops between EGFRvIII and EGFRwt involving EGFR ligands such as HB-EGF or cytokines such as IL-6 or heterodimerization between EGFRwt and EGFRvIII.^{22,28,29} We have recently shown that EGFRwt has a key role in the activation of EGFRvIII and oncogenicity.¹⁹ In the case of Met activation, however, ligand-mediated activation of EGFRwt antagonizes EGFRvIII-induced Met activation. Antagonistic interactions between RTKs of the ErbB family have, to our knowledge, not been reported previously. We have recently reported one other instance of EGFRwt–EGFRvIII antagonism and shown that EGFRwt activated by ligand abolishes EGFRvIII-mediated NF-kappaB activation.²⁵ Moreover, it is important to note that EGFRwt and EGFRvIII levels in the cell lines used in this study are similar to actual GBM tumors.²⁵

EGF does not influence internalization of Met

To investigate the mechanism used by ligand-activated EGFRwt to inhibit EGFRvIII-driven phosphorylation of Met, we examined whether EGFRwt activation resulted in increased internalization of Met. Internalization of Met could expose it to the activity of phosphatases resulting in dephosphorylation. Internalization of EGFR and Met was examined by labeling extracellular proteins with biotin, stimulating the cells with EGF, cleaving the cell surface-exposed biotin and recovering proteins with Streptavidin followed by western blot, using a method described previously.³⁰ As expected, EGFR is internalized when EGF is added to cells; however, we did not detect internalization of Met in response to EGF (Supplementary Figure 1a). We also examined the distribution of Met in cytoplasmic and nuclear lysates after EGF treatment. We do not detect a significant change in the cytoplasmic Met level after EGF treatment, although there may be some decrease in nuclear Met with EGF treatment (Supplementary Figure 1b). These data suggest that the membrane localization of Met is not significantly altered by EGF treatment.

EGFRvIII forms a physical complex with Met that is disrupted by EGF treatment

Next, we examined the possibility that EGFRvIII forms a complex with Met in U87vIII-Ind cells. EGFRvIII is conditionally expressed in response to tetracycline in these cells. We examined whether EGFRvIII and Met form a complex by immunoprecipitating with a Met antibody and conducting western blot with an EGFR antibody. As can be seen in Figure 2a, Met co-immunoprecipitates with EGFRvIII and this association is rapidly lost when

EGFRwt is activated with EGF. As noted, EGFRvIII does not bind EGF or any other ligand. A similar result is noted in U251vIII-Ind cells (Figure 2b) and in EGFRvIII-expressing glioma cells that have constitutive overexpression of EGFRwt (Figure 2c). These data suggest that EGFRvIII association with Met promotes activation of Met. Addition of ligand with resultant EGFRwt activation results in a loss of Met association with EGFRwt with concomitant loss of Met phosphorylation. It should be noted that EGFRwt and EGFRvIII have been reported to form a complex.²⁹ We have also recently confirmed constitutive co-immunoprecipitation of EGFRwt and EGFRvIII without any change with ligand.²⁵

These data suggest that the EGFRvIII–Met physical complex is likely to have an important role in Met activation. Addition of EGF with EGFRwt activation results in a rapid loss of the EGFRvIII–Met association, with a concomitant loss of Met phosphorylation. We hypothesize that activation of EGFRwt results in a conformational change in the EGFRvIII that is complexed with EGFRwt, leading to a disruption of the EGFRvIII–Met association and loss of Met phosphorylation.

Differential sensitivity to temozolomide with Met dephosphorylation

Temozolomide is the first-line chemotherapy drug used in the treatment of GBM.³¹ As noted, 40–50% of GBMs have EGFR gene amplification, and EGFRvIII is expressed in up to one-third of GBMs. Furthermore, EGFRvIII induces resistance to chemotherapy with temozolomide that is induced, at least in part, by EGFRvIII-induced Met activation.^{15,18} Thus, we examined EGFRvIII-mediated resistance to temozolomide in our experimental system under conditions of Met activation or Met dephosphorylation, by examining resistance to temozolomide-induced toxicity. Firstly, we compared the effect of temozolomide on cell viability using an MTT conversion assay. As can be seen in Figures 3a and b, expression of EGFRvIII confers resistance to temozolomide in both U87- and U251-derived cells. Furthermore, the toxicity of temozolomide is increased by exposure of cells to EGF for 6 h prior to exposing cells to temozolomide. In the absence of EGFRvIII expression, EGF does not influence temozolomide toxicity. We also compared the response to temozolomide in both U87- and U251-derived EGFRvIII-expressing cells using an Annexin-fluorescence-activated cell sorting assay (Figures 3c and d). Similar to the results with the cell viability assay, we find that the toxicity of temozolomide is significantly increased by exposure to EGF for 6 h prior to addition of temozolomide in both lines. As noted, exposure to EGF results in a loss of Met phosphorylation in EGFRvIII-expressing cells. The cell death induced by temozolomide is represented by an increase in Annexin and propidium iodide-positive cells (Figures 3c and d). Thus, the EGFRwt-induced loss of EGFRvIII-driven Met phosphorylation may render cells more sensitive to chemotherapy.

Next, we investigated the combined effect of Met inhibition with EGFRwt activation with EGF in the same cells. The experiment was conducted in the presence of tetracycline, resulting in EGFRvIII expression and Met activation. We used a Met kinase inhibitor, SU11274 (1 μ M). Figures 4a and b show that inhibition of Met in these cells results in decreased viability of glioma cells. Exposure of cells to EGF prior to SU11274 exposure results in a statistically significant increased effect on cell viability compared with SU11274 alone, suggesting a complementary effect. The combination of EGF (with resultant

inhibition of EGFRvIII-induced Met phosphorylation) and low-dose SU11274 may result in a more complete inhibition of Met activity, and thus account for the enhanced toxicity. The same experiment was performed using an Annexin-fluorescence-activated cell sorting assay (Figures 4c and d). Similar to the results with the cell viability assay, we find that the toxicity of SU11274 is significantly increased by prior exposure to EGF for 6 h. It is important to note that EGF alone for 6 h had no effect on the viability of these cells (Figures 4c and d).

Concluding comments

The extent and biological consequences of RTK antagonism in cancer are unknown but potentially quite intriguing and relevant to pathobiology and treatment. The current study provides an insight, suggesting that EGFRwt-mediated inhibition of EGFRvIII-driven Met activation results in a state of increased sensitivity to chemotherapy with temozolomide. In this context, it was recently shown that ligand induced activation of Met primes cells to subsequent Met inhibition.³² In our experiments, when both EGFRvIII and EGFRwt are present, a short exposure to EGF primes cells to treatment with temozolomide, presumably by inhibition of Met. Future studies in animal models will help to determine whether this is a viable strategy for treatment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

EGF abolishes EGFRvIII-induced Met phosphorylation in glioma cells. U251MG and U87MG were used to generate cell lines conditionally expressing EGFRvIII using the T-Rex Tet-on system from Invitrogen (Carlsbad, CA, USA) as described previously.^{22,27} U87vIII-Ind cells and U251vIII-Ind cells conditionally express EGFRvIII in response to tetracycline. EGFRwt in PcDNA 3.1 (Neo) or empty vector were stably transfected into U251EGFRvIII-Ind (inducible) and U87EGFRvIII-Ind-expressing cells to generate U251EGFRvIII-Ind + wt or U87EGFRvIII-Ind + wt cells. LN229 cells have been described previously.³³ Primary GBM cultures were generated directly from resected human GBM tumor specimens according to the IRB-approved protocols and cultured in Neurobasal medium supplemented with B27 without Vitamin A, and with EGF (10 ng/ml) and basic fibroblast growth factor (bFGF) (10 ng/ml). (a) Expression of EGFRvIII results in Met phosphorylation in U87vIII-Ind cells. A western blot showing that exposure to EGF (50 ng/ml) for 15 min results in Met dephosphorylation. There is no change in phosphorylation of EGFRvIII upon exposure to EGF. As expected, EGFRwt becomes phosphorylated when EGF is added. In contradistinction to Met, ERK is phosphorylated upon addition of EGF. (b) The same result in U251vIII-Ind cells. (c) The same result in LN229 cell constitutively expressing EGFRvIII. (d) The same result in U87vIII-Ind + wt cells constitutively overexpressing EGFRwt and conditionally expressing EGFRvIII in response to tetracycline. Cells are exposed to tetracycline in all three lanes. (e) A panel of primary GBM cultures grown as neurospheres was screened using western blot for expression of EGFRwt and EGFRvIII. (f) Neurospheres derived from GBM 987 (987-NS) were cultured in EGF-free medium for 24 h followed by exposure to EGF and western blot for phosphorylation of Met and quantitation

of signal by densitometry. EGFR (06–847) antibody recognizes both EGFRwt and EGFRvIII (Millipore, Burlington, MA, USA), β -actin (sc-47778) (Santa Cruz Biotechnology, Dallas, TX, USA). Phospho-EGFR antibodies (Tyr-1068, no. 2236) pERK (no. 9101), ERK (no. 4695), pMet (Tyr 1234/1235, no. 3077) and Met (no. 8198) antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). Three independent experiments were performed unless specified otherwise.

Figure 2.

EGF disrupts a physical complex between EGFRvIII and Met. **(a)** U87vIII-Ind cells were exposed to tetracycline for 24 h as indicated, followed by addition of EGF (50 ng/ml) for 15 min, followed by immunoprecipitation with Met antibodies, followed by western blot with EGFR antibodies. **(b, c)** The same experiment was conducted in U251vIII-Ind cells and in U87vIII-Ind + wt cells. In U251vIII-Ind cells, there is an initial decrease in Met EGFRvIII association at 15 min followed by a more complete dissociation at 60 min.

Immunoprecipitation was conducted according to the standard protocols using antibodies described in legend to Figure 1.

Figure 3.

EGF enhances sensitivity to temozolomide. **(a)** An MTT conversion assay was performed in U87vIII-Ind + wt cells using a Roche Applied Bioscience (Indianapolis, IN, USA) kit according to the manufacturer's protocol. In the absence of tetracycline+(no EGFRvIII expression), exposure of cells to EGF (50 ng/ml for 6 h prior to adding temozolomide for 24 h) does not influence sensitivity to temozolomide. Addition of tetracycline with EGFRvIII expression increases resistance to temozolomide toxicity compared with cells without EGFRvIII expression ($P < 0.0004$, one-way analysis of variance (ANOVA)). In cells expressing EGFRvIII, addition of EGF results in increased sensitivity to temozolomide ($P < 0.0004$, one-way ANOVA). **(b)** The same experiment was conducted in U251vIII-Ind + wt cells with similar results. **(c)** Annexin-FACS (fluorescence-activated cell sorting) experiment in U251vIII-Ind + wt cells showing that EGF enhances sensitivity to temozolomide (50 μ g/ml) ($P < 0.0001$, one-way ANOVA). Cells were exposed to EGF for +6h prior to adding temozolomide for 24 h. Annexin and PI staining represent apoptotic and necrotic cells, respectively. **(d)** The same experiment in U87vIII-Ind + wt cells. EGFRvIII-expressing cells are resistant to temozolomide but become sensitive once EGF is added ($P < 0.0001$, one-way ANOVA). Cells (1×10^6) were plated in six-well plates serum-starved for 24 h followed by EGF treatment for 6 h (50 ng/ml), followed by treatment with temozolomide (50 μ g/ml). Annexin- and PI-positive cells were detected with flow cytometry using an Annexin-V-FLUOS Staining kit (Roche applied Science) according to the manufacturer's protocol. * indicates degree of statistical significance. ** $P < 0.001$, *** $P < 0.0001$.

Figure 4.

Combined effect of EGFR wild type activation and Met inhibition. **(a)** An MTT conversion assay was performed in U87vIII-Ind + wt cells. Cells were exposed to tetracycline to induce EGFRvIII expression followed by treatment with a chemical inhibitor of Met kinase SU11274 alone (plus control vehicle phosphate-buffered saline) or in combination with EGF. Treatment of these cells with SU11274 results in a decreased viability of cells. Combined treatment with EGF plus SU11274 results in a significantly increased cell death compared with SU11274 alone. **(b)** A similar result was obtained in U251vIII-Ind + wt cells. **(c)** Annexin-FACS experiment in U87vIII-Ind + wt cells showing that EGF enhances sensitivity to SU11274 (one-way ANOVA). Cells were exposed to EGF for 6 h prior to adding SU11274 for 24+h. **(d)** The same experiment in U251vIII-Ind + wt cells ($P < 0.0001$, one-way ANOVA). The concentration of SU11274 was used in this experiment $1 \mu\text{M}$. **(d)** The same experiment was conducted in U251EGFRvIII cells with similar results. * indicates degree of statistical significance. ** $P < 0.001$, *** $P < 0.0001$.