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Perspective

Uncovering Therapeutic Targets for Glioblastoma

A Systems Biology Approach

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ABBREVIATIONS

EGFR	epidermal growth factor receptor
RTK	receptor tyrosine kinase
GBM	glioblastoma

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ABSTRACT

Even though glioblastoma, WHO grade IV (GBM) is one of the most devastating adult cancers, current treatment regimens have not led to any improvements in patient life expectancy or quality of life. The constitutively active EGFRvIII receptor is one of the most commonly mutated proteins in GBM and has been linked to radiation and chemotherapeutic resistance. To define the mechanisms by which this protein alters cell physiology, we have recently performed a phosphoproteomic analysis of EGFRvIII signaling networks in GBM cells. The results of this study provided important insights into the biology of this mutated receptor, including oncogene dose effects and differential utilization of signaling pathways. Moreover, clustering of the phosphoproteomic data set revealed a previously undescribed crosstalk between EGFRvIII and the c-Met receptor. Treatment of the cells with a combination employing both EGFR and c-Met kinase inhibitors dramatically decreased cell viability in vitro. In this perspective, we highlight the use of systems biology as a tool to better understand the molecular basis of GBM tumor biology as well as to uncover non-intuitive candidates for therapeutic target validation.

INTRODUCTION

Glioblastoma (GBM) ranks as one of the most aggressive forms of adult cancers, afflicting 12,000 new patients in the United States annually with an average patient life expectancy of less than 15 months. Unlike many of the advances seen in the clinical management of other malignant tumor types such as breast, lung or colon cancer, treatment regimens for GBM have lagged.[1] Current treatments are mostly poorly effective and result in an almost certain recurrence of the tumor after surgical resection, primarily due to the presence of radio- and chemo-resistant lesions that remain in the brain following surgery. The dearth of treatment options suggests that this disease may benefit from new approaches to understand the molecular mechanisms of tumor initiation and progression as well as to guide the selection of new molecular targets for therapeutic development.

We have recently employed a systems biology approach to study cellular signaling networks activated by a truncated form of the epidermal growth factor receptor (EGFR), EGFRvIII.[2] EGFRvIII is found to be expressed in about 40% of GBM tumors expressing the wildtype EGFR receptor. Although EGFRvIII is structurally incapable of binding to the EGFR family of ligands, this mutant receptor is constitutively active and enhances tumorigenic activity both in xenografts and genetically engineered mouse models.[3,4] EGFRvIII has also been shown to confer resistance to both radiation and chemotherapy.[5,6] In addition, a recent study of 49 GBM patients found that expression of EGFRvIII in combination with the loss of the PTEN tumor suppressor gene resulted in resistance to second generation cancer therapeutics targeting EGFR.[7]

While a decade of experiments using classical genetics and molecular biology have provided many insights into the biology of EGFRvIII in the context of GBM, a comprehensive description of the EGFRvIII cellular signaling network is still lacking. New systems biology-based techniques attempt to tackle this challenge by simultaneously examining the relationships of large numbers of species in a particular biological system.[8] Along these lines, to obtain a more comprehensive view of EGFRvIII-mediated signaling, we have employed an unbiased mass spectrometry-based phosphoproteomic approach to quantify tyrosine phosphorylation events that occur in the cell upon expression of titrated levels of EGFRvIII.[2] This analysis highlighted several aspects of EGFRvIII biology, including effects of oncogene dose on receptor activation and employment of downstream biological

signaling networks, differential pathway utilization between wildtype EGFR and EGFRvIII, and the identification of crosstalk between EGFRvIII and other receptor tyrosine kinases.

ONCOGENE DOSE EFFECTS

Cancer is a heterogeneous disease in which oncogene levels vary both within the tumor in individual patients and between tumors in different patients. Although previous studies have estimated that the EGFRvIII receptor may be expressed at several million copies per cell in GBM tumors,[9] this value may vary significantly within the tumor and across patients. Moreover, it has previously been shown in a xenograft model that tumorigenicity is directly proportional to EGFRvIII receptor load.[10] In order to better understand the effect of receptor load on resultant downstream signal transduction pathways, we examined the signaling network of GBM cells expressing titrated receptor levels ranging from 1.5 million to 3 million copies per cell. This study resulted in quantification of 99 phosphorylation sites on 69 proteins, including one serine and seven tyrosine phosphorylation sites on the EGFRvIII receptor.

Intriguingly, all eight phosphorylation sites on EGFRvIII were differentially phosphorylated as a function of titrated receptor levels, suggesting independent regulation of each site, and that each site may perform a different function in the propagation of downstream signal transduction pathways and the resulting tumor phenotype. Clustering of the data revealed that the three phosphorylation sites with the most similar profiles were Y1173, Y1148 and Y1068. Previous site directed mutagenesis studies have indicated that each of these three phosphorylation sites were critical for tumorigenesis in vivo.[11] The biological consequence of the other phosphorylation sites remains unknown; these sites therefore represent obvious targets for future mutagenesis studies.

For the above-mentioned activating phosphorylation sites (Y1173, Y1148 and Y1068) on EGFRvIII, phosphorylation did not increase linearly with receptor expression, indicating the presence of both a threshold and saturation level for receptor activation. At low receptor levels (1.5 million copies per cell), phosphorylation levels remain low and similar to the control cells. However, increasing the expression level to 2 million copies per cell led to a dramatic increase in the phosphorylation of these sites, corresponding to increased activation status of the receptors. Further increase in receptor levels (to 3 million copies per cell) led to saturation of receptor activation and minimal change in phosphorylation of these sites. It is tempting to speculate that regulation of activation at the level of the receptor may be the consequence of receptor dimerization. Unlike wildtype EGFR where ligand binding catalyses the receptor dimerization event, the lack of ligand binding potential in EGFRvIII may mean that a critical receptor level is required before homo-dimerization can occur. Saturation of EGFRvIII activity at higher receptor levels may be due to the presence of downstream negative regulation such as the activation of protein tyrosine phosphatases.

These observations raise clinically important issues for the use of EGFR-targeted drugs in the treatment of EGFRvIII expressing tumors. For instance, it may not be necessary to use high doses of inhibitors to completely shut down the EGFRvIII receptor; instead it may be sufficient to simply decrease activation below the threshold level. Also, treatment with EGFR inhibitors might not affect all phosphorylation sites equally, potentially leading to residual phosphorylation of differentially regulated sites, which may account for the drug resistance observed in patients. Lastly, this data suggests that dimerization inhibitors may be as effective as ATP-analog inhibitors in treating EGFRvIII expressing tumors.

In addition to differential regulation of phosphorylation at the receptor level, we have also demonstrated that there is a modulation of downstream signal transduction networks as receptor levels are increased. For example, the STAT3, MAP kinase (MAPK), and PI3K pathways are activated equivalently in cells expressing 1.5 million copies of the receptor. However, as EGFRvIII levels increase, pathway utilization shifts to favor the PI3K pathway. This observation indicates that oncogene load has dramatic effects on downstream pathways and creates a heterogeneous activation profile which influences the phenotypic outcome, and underscores the need to quantify oncogene levels when performing pair-wise comparisons. In addition, it implies that therapies combining receptor inhibitors with pathway blockade may require temporal monitoring and inhibitor selection as receptor signaling strength changes. A recent study focusing on titrated levels of Ras in a mouse model of breast cancer also reinforces the importance of oncogene levels in the development of tumors.[12] In that study, low Ras expression led to increased proliferation but not transformation while high Ras expression induced cellular senescence in vivo. Thus in order for tumorigenesis to occur in Ras-driven tumors, the senescence checkpoints in the cell must be overcome. Although the signaling mechanisms underlying these contrasting outcomes remain to be elucidated, these studies suggest that quantification of activated oncogene levels in tumors may aid in the design of treatment protocols targeting dominant signaling pathways that are preferentially activated as a function of oncogene dose.

DIFFERENTIAL PATHWAY UTILIZATION

It has been shown previously in a xenograft model that EGFRvIII is more tumorigenic than wildtype EGFR (wtEGFR).[11] Paradoxically, although EGFRvIII activation is sustained, EGFRvIII phosphorylation levels are only 10% that of activated wtEGFR.[11] This apparent contradiction in tumor phenotype and signaling may be explained in part by differential pathway utilization by the two receptors. We have recently performed a temporal phosphoproteomic analysis of human mammary epithelial cells (HMECs) stimulated by EGF.[13] This study showed a dramatic increase in the phosphorylation of both MAPK and STAT3 within one minute of stimulation of wtEGFR. This activation is transient, degrades over time and is consistent with a recent study in which several negative regulators present in the delayed early gene cluster after EGF stimulation of HeLa cells were shown to regulate the immediate early signaling components.[14] These negative regulators include the dual specificity phosphatases (DUSPs) which downregulate phosphorylation of active MAPK.

Our analysis of EGFRvIII signaling demonstrates that the expression of high levels of EGFRvIII favors the activation of the PI3K pathway over the MAPK and STAT3 pathways (Fig. 1). Since transient wtEGFR activation is a balance of immediate early signaling components and subsequent delayed negative regulation,[14] it would be interesting to see if the EGFRvIII gene transcription network represents a hybrid of this model, in which certain negative regulators such as the DUSPs are constantly activated due to the sustained signaling of the EGFRvIII receptor while alternative immediate early

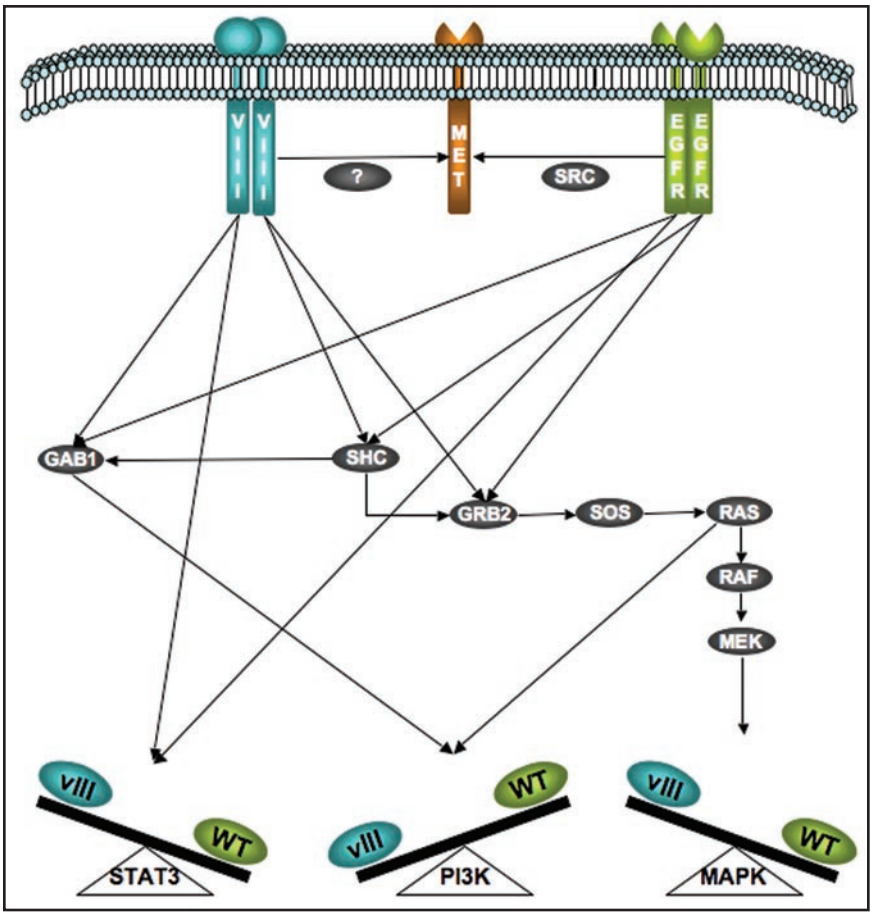

Figure 1. Differential pathway utilization by EGFRvIII and wtEGFR. EGFRvIII favors the utilization of the PI3K pathway while wtEGFR activates the STAT3 and MAPK pathways. Crosstalk with the c-Met receptor has been observed in both wtEGFR and EGFRvIII activation. Activation of c-Met by EGFRvIII may occur via an intermediary molecule yet to be elucidated.

signaling components such as PI3K remain active due to the lack of downstream negative regulation. It should be noted that other constitutively active oncogenic receptor tyrosine kinases such as Tpr-Met and Fig-Ros also favor the utilization of the PI3K pathway over the MAPK pathways.[15,16] It is tempting to speculate that constitutively activated receptors may share a common signaling profile, or at least common network utilization.

The differential pathway activation of EGFRvIII and wtEGFR also suggests that a therapeutic approach toward tumors expressing these two distinct receptors should be fundamentally different. Consistent with our data on PI3K pathway activation by EGFRvIII, there has been some success in the combination of EGFR kinase inhibitors with PI3K/mTOR inhibitors in xenograft models.[17,18] Our data would suggest that therapeutic approaches that combine EGFR kinase inhibitors with MEK inhibitors may be more successful in the treatment of wtEGFR expressing tumors as compared to EGFRvIII expressing tumors. Phosphoproteomic-based systems level signaling network analysis thus provides a means for informed target selection for therapeutic intervention.

RECEPTOR CROSSTALK

Upon cluster analysis of our dataset, we observed that the c-Met receptor was strongly phosphorylated as a function of EGFRvIII receptor levels. This phosphorylation site on c-Met (Y1234) corresponds to one of three phosphorylation sites required for kinase activity. The c-Met receptor has been shown to be oncogenic in metastatic forms of lung, breast and renal cell carcinoma and has been associated with cell scattering and invasion.[19] Our data indicates that EGFRvIII expression results in activation of the c-Met receptor and we have now demonstrated that there is therapeutic value in combining both EGFR and c-Met kinase inhibitors. In this study, inhibiting c-Met kinase activity in EGFRvIII expressing GBM cells sensitized the cells to an EGFR inhibitor in cell viability assays. These results suggest that the tumorigenic properties previously ascribed to the activation of EGFRvIII were due in part to its crosstalk with c-Met. This combination approach may have clinical utility particularly for EGFRvIII expressing PTEN-null tumors which have been shown to be resistant to EGFR kinase inhibition.[7]

A recent study of gefitinib sensitive lung cancer cells that had been cultivated to develop gefitinib resistance found that these cells achieved resistance by undergoing "oncogene switching" through overexpression of the c-Met receptor.[20] Further, the resistance was due to activation of the PI3K pathway via c-Met activation of the ErbB3 receptor that was independent of EGFR phosphorylation status. In the context of GBM cells expressing EGFRvIII, it is unlikely that the same mechanism exists because GBM tumors do not normally express the ErbB3 receptor.[21,22] However, it is possible that EGFRvIII activates the c-Met receptor via an intermediary signaling component. It has previously been shown in a human bladder carcinoma cell line that autocrine stimulation of wtEGFR activates the c-Met receptor via Src (Fig. 1); a similar mechanism may be applicable to EGFRvIII.[23]

In addition to c-Met receptor activation, the Axl receptor tyrosine kinase also follows a similar phosphorylation response as a function of EGFRvIII levels. Axl is expressed at very high levels in GBM and inhibition of Axl signaling led to a decrease in glioma cell proliferation, migration and invasion.[24] The activation of multiple receptors by EGFRvIII represents a paradigm shift in the fundamentals of receptor-mediated signaling. Whereas in the past, activation of a single receptor by a single ligand was thought to activate a specific set of pathways, we have now shown that the activation of a single receptor actually leads to the activation of multiple surface components, each of which has its own set of pathways. Data from systems-wide studies focusing on pair-wise activation of receptors suggests that the co-activation of multiple receptors probably leads to an integrated downstream signal, different from that resulting from the independent activation of each contributing receptor.[25,26] The specificity of multi-receptor crosstalk is also context dependent. In addition to GBM, EGFRvIII is found to be expressed in breast, ovarian, prostate and head and neck cancers. It is likely that crosstalk

mechanisms observed in these tumor types are different from that seen in GBM. For instance, in the context of breast cancer it has been shown that EGFRvIII activates the ErbB2 receptor which is abundantly expressed in a subset of breast cancer cells.[27] Quantitative comparison of EGFRvIII-mediated signaling networks in these various tumors will identify differential aspects of the signaling networks as well as common nodes which may serve as targets for future multi-functional therapeutics.

FUTURE PERSPECTIVES

Global versus targeted phosphoproteomic analysis. While studies on phosphotyrosine-mediated signaling are informative, especially in the context of aberrant RTK signaling in cancer, it represents less than 1% of the global phosphoproteome of the cell.[28] Phosphoserine and phosphothreonine-mediated signaling represent ~93% and ~7% of the phosphorylation events in the cell, respectively, and not unexpectedly have been shown to regulate many critical cellular processes. These cellular processes are not limited to kinase signaling cascades but also impinge on other important cellular functions such as transcription, translation, splicing and protein degradation amongst others.

There exist several strategies to quantitatively measure global phosphorylation levels in cells. One recent study used an unbiased mass spectrometry-based approach to study temporal EGFR signaling in HeLa cells[29] by quantitatively measuring 6,600 phosphorylation sites on more than 2200 proteins, 98% of which consisted of phosphoserine/phosphothreonine sites. As an added dimensionality to the data set, phosphorylation levels were also quantified as a function of subcellular localization. While this heroic effort represents a major advance in the cataloging of cellular phosphorylation sites important in EGF signaling, only a small subset (14%) of all the phosphorylation sites exhibited at least a two-fold change upon EGF stimulation. This limited recovery of EGFR-responsive sites highlights the limitation of unbiased global approaches to quantifying cellular phosphorylation.

An alternative, more targeted approach to studying phosphoserine/ phosphothreonine signaling events is to employ motif-specific antibodies and protein binding domains to enrich for specific subsets of phosphorylated protein substrates. This approach has recently been applied in two studies that focused on the phosphorylation response to DNA damage after irradiating cells with ionizing radiation (IR). In the first study, human embryonic kidney 293T cells were subjected to IR followed by peptide immunoprecipitation with 68 antibodies recognizing pSQ or pTQ containing motifs.[30] These motifs are characteristic of substrates recognized by the ATM and ATR DNA damage checkpoint kinases. The authors used mass spectrometry and quantitatively measured 900 phosphorylation sites on 700 proteins that were upregulated at least four-fold upon DNA damage. These results represent a huge advance over the 25 previously confirmed ATM and ATR substrates. In a second study, the same authors used the BRCT domain of the BRCA1 tumor suppressor protein to enrich for peptides containing the pSXXF motif, again upon irradiation of 293T cells with IR.[31] Using this approach, they identified a novel BRCA1 protein complex that is required for the DNA damage response to IR. These studies are particularly relevant to GBM tumor biology as GBMs are notoriously difficult to treat due to the clinically observed resistance to DNA damaging agents and radiation.[1] Previous studies have demonstrated that EGFRvIII receptor phosphorylation is modulated as a function of cisplatin or radiation treatment.[5,32] It would therefore be important to integrate the above-mentioned approaches with quantitative phosphotyrosine measurements to study the effect of DNA damage on EGFRvIII-driven GBM and in doing so further elucidate the molecular mechanisms of EGFRvIII-mediated resistance.

Intelligent data mining and target candidate generation. Large scale phosphoproteomic studies such as those described above bring about a new set of data mining challenges. There currently exists no informed way for therapeutic target selection in the field of cancer drug discovery. In our EGFRvIII study, we employed clustering algorithms such as self organizing maps to generate potential therapeutic target candidates by "guilt by association", in this case, a co-regulation of increasing EGFRvIII receptor levels and c-Met receptor phosphorylation. A large component of the current drug discovery effort is focused on the development of targeted kinase inhibitors. Many of the kinase inhibitors currently approved for clinical use or in drug discovery pipelines focus on either targeting the initiating kinase such as EGFR or intuitive core process signaling nodes in the network such as PI3K/Akt, the MAPK cascade, or mTOR. While some of these inhibitors have shown promise in preclinical trials, it remains to be seen whether they will perform well in the clinic.

Intelligent data mining offers an alternative by simplifying and assembling large data sets and therefore provides a way to access non-intuitive therapeutic targets. Due to the limited understanding of kinase and phosphatase substrate consensus motifs, one of the challenges of mining phosphoproteomic data sets is the inability to assign kinase and phosphatase substrate specificity to the identified phosphorylation sites. A recently developed algorithm known as NetworKIN aims to overcome this challenge by employing a combination of known kinase substrate consensus motifs together with network context such as interaction databases, gene expression studies and literature mining.[33] When applied to the curated phosphoELM phosphorylation site database, the authors generated a human phosphorylation network of predicted kinase-substrate interactions and experimentally demonstrated that this network correctly predicted two substrates of ATM and CDK1. While a large proportion of known phosphorylation sites are still not accounted for by this improved data mining technique, it allows one to start distilling the ever increasing mass spectrometry generated phosphoproteomic data sets into a manageable reduced set for target candidate discovery and validation.

Beyond in vitro cell signaling. All of the work described above has primarily been limited to in vitro (cultured cells) systems. Tumors are heterogeneous in nature and represent a mixture of multiple cells types with distinct genetic backgrounds. In addition, changes in the tumor microenvironment have been shown to have a critical influence in the initiation and progression of tumors. These important characteristics are ultimately lost when cellular signaling is studied in vitro. Although some of these limitations can be overcome by growing cancer cells in 3D cell culture systems, it still represents an artificial system for studying signaling networks pertinent to cancer progression. It is thus important to extend our phosphoproteomic analysis to the study of tumors, both from murine xenograft models and genetically engineered mouse models of GBM as well as from human clinical GBM samples. These analyses will provide the molecular basis of the pathophysiological hallmarks of GBMs, including

the tremendous proliferative and invasive potential of these tumors.[1] It will also provide an insight into the contribution of the tumor microenvironment to tumor signaling networks, in particular the development of angiogenesis. Unlike gene microarray analysis which only gives a global view of gene expression levels, phosphoproteomics provides a quantitative characterization of the activation states of proteins, the primary targets in the drug development process. It is envisioned that phosphoproteomic analyses of GBM tumors will not only uncover clinically relevant therapeutic targets but will also identify important biomarkers for disease prognosis, thereby predicting response to targeted therapeutics.

CONCLUSION

The results and approaches highlighted in this review demonstrate the utility of phosphoproteomics as a systems biology-based tool for understanding the molecular mechanisms governing GBM tumor biology and generating non-intuitive candidates for target validation. In this specific example, we have identified a previously undescribed crosstalk between EGFRvIII and c-Met which has the potential to be explored as a combination strategy in the clinic. Extending this approach to include global phosphorylation analysis of tumors in vivo will provide a comprehensive picture of GBM tumor signaling networks and reveal critical nodes which may serve as non-intuitive therapeutic targets for the treatment and management of this devastating disease.

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