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## Contribution Of Ectodomain Mutations In Epidermal Growth Factor Receptor To Signaling In Glioblastoma Multiforme

Marta L. Rojas

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**CONTRIBUTION OF ECTODOMAIN MUTATIONS IN EPIDERMAL GROWTH  
FACTOR RECEPTOR TO SIGNALING IN GLIOBLASTOMA MULTIFORME**

A

**DISSERTATION**

Presented to the Faculty of

The University of Texas

Health Science Center at Houston

and

The University of Texas

M. D. Anderson Cancer Center

Graduate School of Biomedical Sciences

In partial fulfillment

Of the requirements

For the degree of

**DOCTOR OF PHILOSOPHY**

by

**Marta L Rojas, M.S.**

Houston, Texas

December, 2011

## **DEDICATION**

I would like to dedicate this work and dissertation especially to my parents: my mother, Ana Maldonado de Rojas, my father, Jose Eliecer Rojas for their unconditional love, support and encouragement through all my life and also to my brother Jose Mauricio Rojas and his family for all the advices and support throughout this time.

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# CONTRIBUTION OF ECTODOMAIN MUTATIONS IN EPIDERMAL GROWTH FACTOR RECEPTOR TO SIGNALING IN GLIOBLASTOMA MULTIFORME

Publication No. \_\_\_\_\_

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The Cancer Genome Atlas (TCGA) has conducted a comprehensive analysis of a large tumor cohort and has cataloged genetic alterations involving primary sequence variations and copy number aberrations of genes involved in key signaling pathways in glioblastoma (GBM). This dataset revealed missense ectodomain point mutations in epidermal growth factor receptor (EGFR), but the biological and clinical significance of these mutations is not well defined in the context of gliomas.

In our study, we focused on understanding and defining the molecular mechanisms underlying the functions of EGFR ectodomain mutants. Using proteomic approaches to broadly analyze cell signaling, including antibody array and mass spectrometry-based methods, we found a differential spectrum of tyrosine phosphorylation across the EGFR ectodomain mutations that enabled us to stratify them into three main groups that correlate with either wild type EGFR (EGFR) or the long-studied mutant, EGFRvIII. Interestingly, one mutant shared characteristics of both groups suggesting a continuum of behaviors along which different mutants fall. Surprisingly, no substantial differences were seen in activation of classical downstream signaling pathways such as Akt and S6 pathways between these classes of mutants. Importantly, we demonstrated that ectodomain mutations lead to differential tumor growth capabilities in both *in vitro* (anchorage independent colony

formation) and *in vivo* conditions (xenografts). Our data from the biological characterization allowed us to categorize the mutants into three main groups: the first group typified by EGFRvIII are mutations with a more aggressive phenotype including R108K and A289T; a second group characterized by a less aggressive phenotype exemplified by EGFR and the T263P mutation; and a third group which shared characteristics from both groups and is exemplified by the mutation A289D. In addition, we treated cells overexpressing the mutants with various agents employed in the clinic including temozolomide, cisplatin and tarceva. We found that cells overexpressing the mutants in general displayed resistance to the treatments. Our findings yield insights that help with the molecular characterization of these mutants. In addition, our results from the drug studies might be valuable in explaining differential responses to specific treatments in GBM patients.

## TABLE OF CONTENTS

TITLE PAGE.....	ii
DEDICATION.....	iii
ACKNOWLEDGEMENTS.....	iv
ABSTRACT.....	v
TABLE OF CONTENTS.....	vii
LIST OF FIGURES.....	xii
LIST OF TABLES.....	xv
CHAPTER 1 INTRODUCTION.....	1
1.1 Genetics of Glioblastoma .....	2
1.2 Core pathways in GBM.....	5
1.3 Therapeutics in GBM.....	9
1.4 Dysregulation of EGFR in GBM.....	11
1.5 EGFR inhibitors effectiveness in treatment strategies and resistance in GBM.....	26

1.6 Hypothesis, rationale and significance.....	29
<b>CHAPTER 2 MATERIALS AND METHODS.....</b>	<b>32</b>
2.1 Generation of WT-EGFR and TOPO-Cloning.....	33
2.2 Identification of ectodomain mutations.....	33
2.3 Mutagenesis and cloning.....	33
2.4 Generation of stable cell lines.....	35
2.4.1 Production of Bi-cistronic VSV-G pseudotyped retrovirus stocks.....	35
2.4.2 Transduction of Glioblastoma and CHO cell lines.....	36
2.4.3 Zeocin selection to obtain stable cell lines.....	37
2.5 Protein expression of stable cell lines.....	37
2.5.1 Obtain protein lysates.....	37
2.5.2 Protein quantitation.....	38

<b>2.5.3 Western blot analysis.....</b>	<b>38</b>
<b>2.6 Ray Bio EGFR phosphorylation antibody array analysis using U87 stable cell lines.....</b>	<b>39</b>
<b>2.7 Cell signaling analysis after starved and EGF stimulated conditions.....</b>	<b>40</b>
<b>2.8 Phospho-proteomics assays.....</b>	<b>41</b>
<b>2.8.1 Sample preparation, peptide immunoprecipitation and mass spectrometry analysis.....</b>	<b>41</b>
<b>2-8-2 Database analysis and quantification.....</b>	<b>42</b>
<b>2.9 Cellular distribution of EGFR ectodomain mutations in transient transfected CHO cell lines.....</b>	<b>43</b>
<b>2.9.1 Cellular distribution of EGFR ectodomain mutations in transient transfected U87 cells.....</b>	<b>43</b>
<b>2.9.2 Nuclear and cytoplasmic expression of EGFR ectodomain mutations.....</b>	<b>44</b>
<b>2.10 Cell viability in EGFR ectodomain mutations.....</b>	<b>45</b>
<b>2.11 WST-1 assay of glioma cells overexpressing EGFR ectodomain mutations.....</b>	<b>45</b>

2.12 Anchorage independent colony formation.....	45
2.13 Generation of xenograft models.....	46
2.14 Inhibitor studies.....	47
2.14.1 Erlotinib treatment.....	47
2.14.2 Cisplatin treatment.....	48
2.14.3 Temozolomide treatment.....	48
2.15 Statistical Analysis.....	49
<b>CHAPTER 3 RESULTS.....</b>	<b>50</b>
3.1 Identification of EGFR ectodomain mutations.....	51
3.2 Stratification of ectodomain mutations according to molecular profiles.....	54
3.3 Impact of EGFR ectodomain mutations on cell viability and proliferation.....	69
3.4 Expression of ectodomain mutations enhances anchorage independent colony formation in different glioma cells.....	73
3.5 Expression of EGFR ectodomain mutations decreased tumor latency period in xenograft mice.....	78

<b>3.6 Phosphoscan phospho-tyrosine analysis.....</b>	<b>81</b>
<b>3-7 Cellular distribution of ectodomain mutations.....</b>	<b>84</b>
<b>3-8 EGFR ectodomain mutants determine response</b>	
<b>    to standard therapy.....</b>	<b>88</b>
<b>3.8.1 Cisplatin treatment.....</b>	<b>89</b>
<b>3.8.2 Tarceva treatment.....</b>	<b>91</b>
<b>3.8.3 Temozolomide treatment.....</b>	<b>93</b>
<b>CHAPTER 4 DISCUSSION, SUMMARY AND FUTURE DIRECTIONS.....</b>	<b>98</b>
<b>Discussion.....</b>	<b>99</b>
<b>Summary.....</b>	<b>108</b>
<b>Future Directions.....</b>	<b>109</b>
<b>BIBLIOGRAPHY.....</b>	<b>111</b>

## LIST OF FIGURES

Figure:

1. Probability of survival in GBM patients expressing somatic mutations by TCGA .....	53
2. EGFR point ectodomain mutants overexpressed in stable cell lines.....	55
3. EGFR phosphorylation antibody microarray analysis.....	56
4. Differential phosphorylation profiles for tyr-1173 across EGFR ectodomain Mutants.....	58
5. Differential phosphorylation profiles for Tyr-1068 phosphorylation across EGFR ectodomain mutants.....	59
6. Differential profiles of phosphorylation for Tyr-1148 across EGFR ectodomain Mutants.....	61
7. Differential phosphorylation profiles for Tyr-845 across EGFR ectodomain mutants.....	62
8. Western blots validation for some tyrosine residues of the antibody microarray.....	64
9. Downstream signaling of ectodomain mutants overexpressing U87 cells....	65
10. Downstream signaling of EGFR ectodomain mutants overexpressing	

LNZ308 cells.....	66
<b>11. Basal and downstream signaling activation of EGFR ectodomain mutants overexpressed in LN428 cells.....</b>	<b>68</b>
<b>12. Effect of EGFR ectodomain mutations overexpressed in U87 cells on cell viability.....</b>	<b>70</b>
<b>13. Cellular proliferation effect of EGFR ectodomain mutants overexpressed in different glioma cell lines.....</b>	<b>71</b>
<b>14. Contribution of U87 cells overexpressing ectodomain mutants in anchorage independent colony growth.....</b>	<b>74</b>
<b>15. Contribution of LNZ308 cells overexpressing ectodomain mutants in anchorage independent colony growth.....</b>	<b>75</b>
<b>16. Contribution of LN428 overexpressing ectodomain mutants in anchorage independent colony growth.....</b>	<b>76</b>
<b>17. Kaplan Meier survival analysis in xenograft groups implanted with EGFR ectodomain mutants.....</b>	<b>78</b>
<b>18. Histological analysis of xenograft brain tumors.....</b>	<b>81</b>
<b>19. Phosphoscan analysis of EGFR ectodomain mutants.....</b>	<b>82</b>
<b>20. Cellular distributions of GFP-tagged EGFR ectodomain mutants in CHO</b>	

cells.....	85
<b>21. Profile of distribution of GFP-tagged EGFR ectodomain mutants in</b>	
<b>U87 cells.....</b>	<b>86</b>
<b>22. Nuclear and cytoplasmic expression of EGFR ectodomain mutants in U87</b>	
<b>stable cells.....</b>	<b>87</b>
<b>23. Response of ectodomain mutants overexpressed in U87 cells</b>	
<b>to cisplatin.....</b>	<b>89</b>
<b>24. Effect of tarceva treatment on ectodomain mutants overexpressed</b>	
<b>in U87 cells.....</b>	<b>91</b>
<b>25. Effect of temozolomide treatment on ectodomain mutants overexpressed</b>	
<b>in U87 cells.....</b>	<b>94</b>
<b>26. Dose response effect of temozolomide on ectodomain mutants</b>	
<b>overexpressed in LN428 cells.....</b>	<b>96</b>

## LIST OF TABLES

Table:

1. Primers for site-directed mutagenesis.....	34
2. Confirmation primers.....	35
3. EGFR ectodomain mutants identified by TCGA.....	52
4. Significant differences in survival curves of xenograft groups.....	80
5. Cisplatin IC <sub>50</sub> values for ectodomain mutants overexpressed in U87 cells.....	90
6. Tarceva IC <sub>50</sub> values for ectodomain mutants overexpressed in U87 cells.....	92
7. Temozolomide IC <sub>50</sub> across EGFR ectodomain mutants overexpressed in U87 cells.....	95
8. Temozolomide IC <sub>50</sub> for ectodomain mutants overexpressed in LN428 cells.....	97
9. Stratification of EGFR ectodomain mutants according to the biologic characteristics.....	104
10. Categorization of EGFR ectodomain mutants according	

to tyrosine phosphorylation profiles.....105

## **CHAPTER 1 INTRODUCTION**

## 1.1 Genetics of Glioblastoma

Glioblastoma multiforme (GBM) is the most common and lethal type of primary brain tumor in adults accounting for about 52% of all the glioma cases. About 3 in 100,000 people are newly diagnosed with GBM each year (1), with the mean age of primary GBM being about 62 years(2). GBM occurs more frequently in males (3) Depending on genetic alterations and histological hallmarks, the degree of malignancy in gliomas is ranked on a scale of I to IV, where grade IV tumors exhibit the more aggressive features of the malignancy involving necrosis and vascular proliferation (4). Also, these tumors are more refractory to chemo- and radiation therapy and so confer shorter survival time. Based on clinical presentation, GBMs can be additionally classified into primary or secondary subtypes (5). Although these two categories are histologically indistinguishable, they display different patterns of genetic alterations. Primary or *de novo* GBM category comprises about 90% of the cases where most of them develop very rapidly without indication of a less malignant preceding lesion and have shorter survival times (9-12 months) (6). In contrast, secondary GBMs originates from transformation of lower grade astrocytomas into grade III/IV GBMs. Secondary GBMs are fairly rare and involve about 10% of the GBM cases and is common in patients below the age of 45 years (7). Primary GBMs characteristically show overexpression or amplification of CD13 and MDM2/4, and loss of heterozygosity of chromosome 10 (8). About 40% of these tumors show PTEN mutations, and typically EGFR is amplified in about 45% of the cases. In these EGFR amplified cases, about 14% also show mutations in the receptor (9, 10). The most common genetic alterations in secondary GBMs include p53 mutations, loss of heterozygosity in the

long arm of the chromosome 10, deregulation of RB pathway through mutations as well as PTEN mutations in about 10% of the cases (11-14). The National Cancer Institute (NCI) and The National Human Genome Research Institute (NHGRI) have joined efforts to accelerate the understanding of the molecular basis of GBM and other types of cancers through The Cancer Genome Atlas (TCGA) initiative. The central goal of TCGA is to provide valuable information to the scientific community allowing a better diagnosis, treatment and prevention of multiple types of malignancies including GBM. This group systematically explores the full spectrum of genomic changes in GBMs through the application of genome analysis technologies. TCGA initially started with sequencing of about 600 target genes in 500 GBM cases. They then expanded the target gene list to about 6000 which is available for analysis currently and are currently doing whole exome sequencing for the GBM samples. In addition, whole genome sequencing has been made available for 24 GBM cases along with their matched controls (<http://tcga-data.nci.nih.gov/tcga/>). The marked molecular diversity in GBM has been delineated by this group; they have catalogued key genomic alterations found in this type of cancer based on the results obtained from 201 tumor samples. Analysis from TCGA has allowed the finding of recurrent and important focal alterations not detected previously in GBMs such as amplifications in AKT3, homozygous deletions in PARK2 and NF1 (10).

Additionally, a new algorithm was used to search for copy numbers aberrations (CNAs) that resulted in the uncovering of less frequent focal events including amplification of FGFR2 kinase and IRS2 kinase adaptor genes and deletion of the tumor suppressor NF2 (10, 15). This analysis also indicated loss of heterozygosity mainly at chromosome 17q which encompass the p53 gene, and less frequently at 7q

and 9q, pointing to potential new tumor suppressor genes as candidates for further study (16). In summary, the integrated expression data set highlighted that 76% of the genes with CNAs displayed a relationship between expression pattern and copy number (10).

Sequencing analysis in a cohort of 91 GBM samples, revealed 453 validated non-silenced somatic mutations in 233 unique genes, 79 of which contained two or more variations. In general, mutational background differed substantially between untreated and treated GBMs in a proportion of 1.4 versus 5.8 somatic silence mutations for each sample (10). As a consequence, a reduction in DNA repair ability is inferred in some treated patients having mutations in one or more mismatch repair genes (MMR) including MSH6, MLH1 and MSH2 and mutations in these genes are thought to be causally associated with temozolomide resistance (17) Additionally, TCGA has reported an incidence of about 23% inactivating somatic mutations in NF1 gene. However, the functional implication of these mutants has not been established yet (18, 19). Furthermore, TCGA assessed the promoter methylation status of MGMT, a DNA repair enzyme associated with glioblastoma sensitivity to alkylating agents (20, 21), and found that 19 of 91 tumors exhibited MGMT promoter methylation. Further, these findings showed an association between the hyper-mutated phenotype and the pattern of MGMT methylation characterized by a significant change in the nucleotide substitution spectrum of treated glioblastomas. In the TCGA dataset, the most frequently mutated and amplified gene was EGFR, which reconfirmed data from early studies demonstrating pivotal role played by EGFR in GBMs (12, 22, 23).

## **1.2 Core pathways in GBM**

Decades of work identifying individual genetic events in human glioblastomas, were confirmed in a comprehensive analysis of a substantial set of tumors by TCGA (5). Thereby, the TCGA has provided an integrated view of an interconnected network of aberrations involving primary sequence alterations and significant copy number changes for components in major pathways including p53 and RB tumor suppressor pathways (10), and activation of RTKs. While these studies highlight the high degree of heterogeneity in GBMs in terms of the specific mutations encountered in a given tumor, they also show that a common set of core pathways are invariably activated or inactivated, albeit by different specific events. This suggests that the pattern of mutations may govern therapeutic decisions in the near future (24). The dominant pathways implicated in glioma biology are:

### **(1) Signaling alteration due to mutations and amplifications in receptor tyrosine kinases (RTK) and their downstream effector genes**

Aberrations in RTK signaling arising either from deregulated RTKs and/or components of their downstream signaling events has been identified as one of the primary alteration in GBMs, Among the RTKs that are altered: Epidermal Growth Factor Receptor (EGFR) is dysregulated in a large population of cases, and will be reviewed in detail in section 1. In addition to EGFR, platelet derived growth factor receptors (PDGFR) represent another RTK with a critical role in gliomagenesis, where PDGFR $\alpha$  and its ligands PDGF-A and PDGF-B are expressed at high levels in high

grade gliomas. Robust expression of PDGFR $\beta$  has also been demonstrated in proliferating endothelial cells of GBM tumors (25-28).

In addition to RTKs, signaling hyperactivation of PI3K and MAPK pathways is often a feature of GBM (29). The class IA PI3Ks are heterodimers that are recruited to activated RTKs and adaptor proteins via their regulatory subunit, and there are five isoforms encoded by: p85 $\alpha$ , p55 $\alpha$ , and p50 $\alpha$  (PIK3R1); p85 $\beta$  (PIKR2); and p55 $\gamma$  (PIKR3) (30). The action of class I PI3K enzymes is directly antagonized by the tumor suppressor PTEN, however, this gene is inactivated in about 50% of the GBMs by deletions mutations or epigenetic mechanisms (2, 31). In addition, activation of phosphoinositide 3'-kinase (PIK3) through point mutations has been reported in about 15% of GBM tumors samples. These mutations mostly occur in the adaptor binding domain (ABD) and the C2 helical and kinase domains of the catalytic subunit (PIK3CA) (32-34). More recently, crystallographic studies have suggested that these mutations disrupt interactions diminishing the inhibitory effect of the p85 $\alpha$  on p110 $\alpha$  (35). In contrast, mutations in the regulatory subunit of PIK3 (PIK3R1) are uncommon in cancer. However, sequencing analysis from TCGA indicated the presence of 9 PIK3R1 somatic mutations in a cohort of 91 GBMs samples. Crystallographic studies have suggested that these mutants might diminish the inhibitory effect of p85 $\alpha$  on p110 $\alpha$  (36). Consequently, uncontrolled PI3K activation leading to AKT activation is observed in about 85% of the GBM samples (37).

AKT phosphorylates a broad number of proteins associated with regulation of cell growth, proliferation, metabolism, and apoptosis. Other mechanisms by which AKT activation could be altered in GBM include: elevation of expression of PIKE-A, a small GTPase, which is highly expressed in GBMs and glioma cell lines, and which binds to

phosphorylated Akt and enhances its anti-apoptotic role (38, 39); suppression of expression of PH domain leucine-rich repeat protein phosphatase (PHLPP) which dephosphorylates S473 residue as well as the C terminal modulator protein (CTMP), and which binds and inhibit the phosphorylation of AKT (40-42). A further study suggests that AKT represents an important requirement for cell proliferation and susceptibility to oncogenesis in a p53 independent manner but mTORC1 dependent (43).

## **(2) Alterations in genes involved in cell cycle**

### **A. The p16-CDK4-RB Pathway**

In order to control the fidelity of cell cycle, progression through cell cycle is regulated by cyclin-dependent kinases (CDKs) which are in turn regulated by cyclins (44). In addition, the activity of the different cyclin/CDK complexes is further influenced by binding to cyclin-dependent kinase inhibitors (CKIs). Two CKI families have been identified : the CIP/KIP family, comprising p21<sup>CIP1</sup>, p27<sup>KIP1</sup>, and p57<sup>KIP2</sup> and the INK4 family (inhibitors of CDK4), which include p15<sup>INK4B</sup>, p16<sup>INK4A</sup>, p18<sup>INK4C</sup>, and p19<sup>INK4D</sup> (45). Members of the INK4-family bind exclusively to CDK4 and CDK6, thus preventing them from binding to cyclins or blocking the kinase activity of pre-assembled complexes (46). The retinoblastoma tumor suppressor protein (pRB) is one of the most important substrates regulated by cyclin/CDK activity in mammalian cells (47). pRB is inactivated by phosphorylation and in its hypophosphorylated state binds to transcription factors such as the E2F family members (48). Upon phosphorylation, pRB dissociates from E2Fs allowing E2F target gene expression and cell cycle progression (49).

GBMs contain alterations by genetic and epigenetic events in genes that code for components of the pRB/E2F pathway. The identified abnormalities include homozygous deletion of *RB1* (located at chromosome 13q14) or hemizygous deletion with mutations in the retained allele which results in a nonfunctional RB1 protein (50). Mutations in *RB1* are observed in 30% of GBMs and hypermethylation of the *RB1* promoter, resulting in transcriptional silencing of the gene has also been documented (51). While CDK4 amplification is found in about 15% of GBM (10), mutations in p16<sup>INK4A</sup> gene occur more frequently (52). In the comprehensive TCGA analysis (10), it was shown that the p16<sup>INK4A</sup> gene was altered in about 52% of the GBMs that were evaluated. In addition to loss of heterozygosity (LOH) of the p16<sup>INK4A</sup> locus, alterations in expression of the p16<sup>INK4A</sup> protein have been described (53). These are a result of either decreased mRNA or protein stability or decreased mRNA production due to promoter methylation (54). Homozygous deletion of *CDKN2B* (coding for p15), occur in 47% of glioblastomas (10). Other members of the pathway that are altered in a lower number of patients are *CDKN2C* (deleted- 2%) and *CCND1* and *CDK6* which are amplified in 1% and 2% of the patient population respectively. Disruption in the various pathway members collectively were found to occur in about 80% of the tumors (10)

### **B. The ARF-MDM2-p53 Pathway**

A second pathway involved in control of cell cycle progression is the one involving the p53 protein. p53 is a transcription factor that is induced in response to cellular stress and external insults which brings about either cell cycle arrest or promotes apoptosis (55). One of the main regulators of p53 levels—and thus of the biological response—is Mdm2 (murine double mutant 2), an E3 ligase which binds to p53, and

targets it for degradation. Mdm2 in turn is inactivated by its binding to p14<sup>ARF</sup> consequently blocking the ubiquitination and degradation of p53 (56).

The p53 pathway is inactivated in about 90% of gliomas, either by mutations in the p53 gene, by amplification of *MDM2* or *MDM4*, or by loss of expression of ARF (10). The most common event causing p53 inactivation (at about 35% frequency) is LOH of the *p53* gene accompanied by missense mutations in the remaining allele (10, 16). Amplification and overexpression of MDM2 occurs in 14% of GBMs while that of MDM4 is detected in 7% of GBMs (10). Both *MDM2* and *CDK4* are often co-amplified as they are both located on chromosome 12q13-14 (57), and thus affect both the pRB and the p53 pathways. Similarly, mutations in the p19<sup>ARF</sup> protein which is generated by an alternative reading frame (ARF) of the *INK4A* locus that also encodes for the CKI p16<sup>INK4A</sup> (58) affects the regulation of both the pRB and p53 pathway. *CDKN2A* mutations occur essentially via homozygous gene deletion at a frequency of about 50% of GBMs (10, 59)

### **1.3 Therapeutics in GBMs**

The accumulation of multiple genetic variations is thought to confer GBMs with a notorious plasticity which, in spite of therapeutic interventions, allows recurrence. Despite recent achievements in standard multimodal therapies including resection followed by chemotherapy and radiotherapy, treatments remain ineffective with very poor survival (1-2 years) (60, 61). Thus, the development of novel therapeutics and improved strategies focusing on abnormal genetic events and signaling pathways, tumor stem cell identification and characterization as well as categorization of patients for customization of treatment regimens is of extreme importance (62). Standard treatment in GBMs involves the use of temozolomide (TMZ). This agent is an oral

alkylating agent approved for anaplastic astrocytoma and GBMs. TMZ is an analog of mitozolomide, one of the antitumor imidazotetrazines synthesized by Stevens *et al.* in the 1980s (63), but in contrast to mitozolomide, TMZ showed less toxicity and a wide spectrum of activity on mouse tumors (64). This lower toxicity allows for better tolerance to TMZ treatment in conjunction with radiation in patients. Clinical trials phase I showed better tolerance and response in patients with melanomas and malignant gliomas (65). TMZ has become one of the standard modalities that offer a modest clinical efficacy (no more than 20%) in malignant gliomas (66). The strongest predictive signature for favorable outcome to TMZ treatment is the silencing through promoter methylation of the DNA repair enzyme O (6)-methylguanine-DNA-methyltransferase gene (MGMT), which is detected in about 45% of the GBMs cases (21). However, patients with TMZ-sensitive glioblastoma also relapse eventually (67). Events involved in the innate resistant of GBM patients to TMZ include: a) high activity of MGMT whose main function is the removal of alkyl groups from O6-methylguanine in double-stranded DNA (68), b) loss of PTEN where therapeutic studies have demonstrated that induction of PTEN expression along with an inhibitor of Akt phosphorylation –neflavir- enhances sensitivity to temozolomide in glioblastoma cells (69, 70) and c) strong base excision repair (BER), where a significant proportion of DNA damage induced by TMZ is repaired by the BER pathway (70, 71). In addition, early studies have demonstrated that glioma cell lines with low MGMT expression can also exhibit significant resistance to TMZ, suggesting the existence of alternative mechanisms of resistance (72, 73). Deficiency of the mismatch repair genes is one of the proposed mechanisms for resistance to TMZ (72, 73). Further, nucleotide excision repair mechanism may also be implicated in TMZ resistance as well as the expression

of genes involved in the nucleotide excision such as repair protein, poly (ADP-ribose) polymerase-1 (PARP) (74). In addition, more recent studies have indicated that the expression of HOX gene signatures such as prominin-1 (CD133) predict poor prognosis and postulates CD133 as a stem cell marker in the subpopulation of glioma stem cells that are resistant to TMZ treatment (75).

Although cisplatin and its analogues are most commonly used in head and neck squamous cell carcinoma therapies (76-78), platinum compounds have been also considered in the treatment of gliomas (79). Previous reports have shown response to platinum compounds in ~15% of the patients (80). In vitro studies have demonstrated that cisplatin reduces MGMT activity (81). Thus, combination of TMZ and cisplatin has been the object of clinical trials, where this regimen appears active and with acceptable levels of toxicity in patients with recurrent GBM (82). In pre-clinical models, cisplatin has been demonstrated to induce apoptosis and has become the gold standard for induction of apoptosis in an experimental setup (83).

#### **1.4 Dysregulation of EGFR in GBMs**

EGFR, also known as Human Epidermal Growth Factor Receptor (HER1/ErbB1) is cataloged among the type I tyrosine kinase receptors group. Other members of the HER family include ErbB2/ Her2, ErbB3/ Her3 and ErbB4/ Her4.

EGFR is a 170 KDa glycosylated plasma membrane protein with three main domains: an extracellular domain, a transmembrane domain and an intracellular domain containing the tyrosine kinase activity (TK) (84, 85). The extracellular domain consists of four subdomains, where the ligand binding pocket arises from domains I, II and III, which interact with the cognate ligands (86). EGFR can be activated by

multiple ligands including epidermal growth factor (EGF), transforming growth factor-alpha (TGF- $\alpha$ ), betacellulin (BTC) and amphiregulin (AR) (87). These factors activate the receptor through dimerization that consequently leads to the autophosphorylation of various preferential residues in the cytoplasmic domain of the receptor (88, 89). Tyrosine autophosphorylation plays an essential role in determining the selectivity of downstream signaling cascade triggered by EGFR and facilitates binding of adapters or other signaling proteins (29). This enables trans-phosphorylation of the interacting partners and thus triggers important downstream pathways such as RAS-RAF-MEK-ERK cascade, PI3K-AKT pathways and finally alterations in activation of transcription factors that lead to changes in gene transcription (90-92). Thus, the final biological consequence includes cellular growth and proliferation in tumorigenesis (1, 93, 94).

From the physiological perspective, the function of EGFR during development is not well established. Recapitulation of EGFR overexpression in mice has been unsuccessful, probably due to lethality during mouse development. It has been found that EGFR overexpression leads to premature death between midterm of gestation and post natal day 20 depending of the genetic milieu, with the strain the 129/sv being more susceptible than the C57BL/6 strain These mice showed abnormalities in multiple organs including skin, hair, eyes, lungs, bones, heart and neurodegeneration (95-98). Therefore, the majority of our insight regarding embryonic role of EGFR comes from loss of function studies in mouse models and show that epithelial and glial cells in general are the most affected during development (99, 100).

To approach the function of EGFR in the developing brain, different groups have successfully targeted EGFR knock-out in mice (95, 96, 101). The results of these

studies again differ according to the genetic background of the mice. A homozygous null mutation developed on CF-1 or 129Sv backgrounds caused embryonic lethality (101), while mice with similar genetic manipulation developed on CD-1, C57BL/6 or 129Sv/J Swiss Black backgrounds survived postnatally (97). Early analysis of mice developed on a CD-1 background (96) indicated abnormalities in piriform cortex and cerebellum, which took place postnatally and also thalamus degeneration between postnatal days 5 and 8. Additional findings showed a focal but massive degeneration of olfactory bulb and neocortex with this particular phenotype occurring due in part to apoptosis (100). However, some of the neuronal cell populations that degenerate usually do not express EGFR, suggesting an indirect mechanism of neuronal death (100, 102).

Stimulation of EGFR through multiple ligands generates numerous effects in the central nervous system (CNS). This receptor and its ligands are expressed in both developing and adult brain. Studies with in situ hybridization in mouse brain tissue sections showed TGF- $\alpha$  as the predominant EGFR ligand in neuronal cell populations (102). Early studies indicated different regional distribution of mRNA levels for EGF and TGF- $\alpha$  ligands in adult mouse regions, where the levels of EGF mRNA were 15-170 times lower than TGF- $\alpha$  ligand, with the highest regional concentration of mRNA EGF observed in olfactory bulbs, cerebellum and basal hypothalamus (103). TGF- $\alpha$ 's presence was observed at low level in the dorsal, medial, and lateral extents of the anterior olfactory nuclei, dentate gyrus, accessory olfactory bulb, and the tuberculum olfactorium and in numerous periglomerular and mitral cells of the olfactory bulb, the latter cells forming a laminar cell group adjacent to the granular cell layer (103). Further examination of mRNA levels for EGF and TGF- $\alpha$  ligands showed detectable

levels as early as embryonic day 14 (103-107). Investigations have shown a role for EGFR expression across all the stages of mammary gland development. Studies in mice having mutations in the kinase domain region of the receptor have shown a weak development of the mammary gland (108, 109).

In human cancers of epithelial or glial origin, EGFR is significantly deregulated and shown to drive cellular differentiation, proliferation, motility and survival. The dysregulation of the receptor is implicated in many human malignancies and is present in about 50% of the GBMs (110, 111). EGFR can be deregulated by multiple mechanisms in cancer. These are:

#### **1.4.1 Aberrant enhancement of ligand production**

Autocrine loops, in which both the receptor and its cognate ligand are expressed in the same cells have long been recognized as an important contributor to growth autonomy of cancer cells (112). Various studies have demonstrated that tumor cells coexpress both EGFR and its ligands that lead to its aberrant activation in a variety of neoplasms including GBMs (113, 114). EGF and TGF- $\alpha$  are the two most frequently studied ligands, which particularly bind and activate EGFR. After the screening of multiple cell lines derived from GBMs and normal human brain tissues, an abnormal increase in the expression of genes encoding TGF- $\alpha$  and EGF were observed (115). TGF- $\alpha$  mRNA is overexpressed in significant percentage of malignant astrocytomas and found at very low levels in normal cerebral cortex. After screening of multiple gliomas for levels of transcripts coding for the pre-pro forms of EGF and TGF- $\alpha$ , expression of mRNA levels for one or both of the pre-pro forms of the ligands were detected in every tumor studied (116). In glioblastoma cell lines, coexpression of

EGFR and TGF- $\alpha$  has been described in EGFR amplification-positive glioma , indicating an autocrine growth stimulatory loop which may be involved in the anchorage independent proliferation of these cells (114). TGF- $\alpha$  overexpression appears predominantly in malignant gliomas (glioblastoma and anaplastic gliomas) (117), supporting the role of TGF- $\alpha$  as an oncoprotein marker in brain neoplasms and suggesting a strong positive correlation between tumor grade and extent of TGF- $\alpha$  amounts (118-120). Gene amplification and expression of TGF- $\alpha$  and EGFR in human gliomas has been shown to be highest in recurrent tumors where lower grade tumors had progressed to high grade malignant tumors(121). In addition, it was recently demonstrated that EGFRvIII overexpressing glioma cells produced increased amounts of TGF- $\alpha$  and HB-EGF, thus influencing the growth of EGFR expressing cells (122, 123). In a more recent study, it was shown that autocrine TGF-stimulation leads to enhancement of tumor growth *in vivo*, an effect that was mediated through EGFR activation (124). This TGF- $\alpha$ /EGFR autocrine loop could be downregulated by an EGFR specific tyrosine kinase inhibitor, which resulted in tumor growth inhibition (124), suggesting that interruption of the autocrine loop may be key node for therapeutic intervention.

Lately, substantial attention has been focused on heparin-binding EGF-like growth factor (HB-EGF) as an important ligand for EGFR activation. Early studies have showed that coexpression of EGFR and HB-EGF is found in 44 % of GBMs (125). Importantly, in the same study, the authors showed that neutralizing anti-HB-EGF antibody suppressed the proliferation of glioma cell lines that expressed this ligand (125), indicating that HB-EGF may participate in the autocrine growth of glioma cells. Additionally, it has been shown that EGFR activation by G-protein coupled receptors

(GPCR) is facilitated via metalloproteinase dependent cleavage of pro-HB-EGF in the tumors where the HB-EGF then activates EGFR in either an autocrine or paracrine manner (126).

#### **1.4.2 Increased EGFR protein level (via gene amplification and abnormal gene expression)**

One of the most prevalent characteristics in GBMs is the overexpression and amplification of EGFR. It occurs in 40% - 50% of primary GBMs and is not seen in low grade astrocytomas (2, 127). In GBMs the distribution of cells with EGFR amplification can vary in the same tumor from 10% to 60% (128, 129). Typically amplification of the receptor appears as small fragments of extrachromosomal DNA and it is always associated with increased intensity of protein expression (130) resulting in tumor growth, disease progression, poor prognosis and reduced sensitivity to chemotherapy (131). Recently, a different type of EGFR amplification has been identified, in which extra copies of EGFR inserted in different loci of chromosome 7, and it is present in 28% of the cases (132). A small fraction of GBM tumors can show overexpression of EGFR without gene amplification (14). Interestingly, a similar pattern is seen with ErbB2 receptor in breast cancer patients, where overexpression without amplification is associated with clinical outcome comparable to patients who do not express ErbB2. However, it is unknown if this pattern also seen for EGFR in GBMs patients, largely because the prognosis for patients with GBM is uniformly poor (133).

In the recently advanced subgrouping according to differential gene expression profiles, GBMs can be categorized into three different types: One group where EGFR is overexpressed, a second group characterized by upregulation of genes on the

chromosome 12q13-15 and a third group of GBM which lacks any of these changes (134). Upregulation of EGFR is associated with the group that shows characteristic transcriptional profile such as expressing genes that promote cell growth, cell survival and angiogenesis, which are critical for EGFR-mediated pathogenesis and also can provide different therapeutic targets (134, 135).

### **1.4.3 Malfunction in downregulation**

Ligand induced endocytosis of EGFR is a key deactivation pathway, which leads to receptor downregulation and subsequent degradation. Activation of EGFR leads to recruitment of the c-Cbl E3 ubiquitin-protein ligase directly or indirectly for degradation via the proteosomal pathway (136, 137). c-Cbl is an adaptor protein catalogued as a complex scaffolding protein due to its ability to interact with multiple proteins. Direct binding of Cbl with EGFR is mediated through the phosphorylated tyrosine residue 1045 on EGFR (138), while indirect binding is mediated through the adaptor protein Grb2, which binds to phosphorylated tyrosine sites, 1086 and 1068 on the receptor (138, 139). This binding facilitates efficient internalization via clathrin-coated pits that invaginate to form coated vesicles and then subsequent receptor degradation in the lysosomes (136).

Previous studies involving mutagenesis of c-Cbl and EGFR revealed specific requirements for productive ubiquitination and sorting of the receptor to degradation such as intact SH2 and RING finger domains on c-Cbl as well as EGFR Tyr-1045 phosphorylation, which creates a major docking site for c-Cbl or indirect interaction between c-Cbl and EGFR through adaptor protein Grb2 (138). Malfunction in EGFR downregulation involves the presence of truncated forms of c-Cbl with oncogenic

function where v-cbl form was generated by a truncation in which 60% of the C-terminus has been removed (140). The studies defined the region within Cbl, where the change occurs between non-tumorigenic and tumorigenic forms (140). Further, mutational studies have also evidenced deregulation in downregulation and degradation of the receptor, where mutation of Tyr-1045 reduced ligand-induced down-regulation in living cells and also decreased receptor ubiquitylation (138, 141) thus, enhancing the mitogenic response to EGF (138, 142).

#### **1.4.4 Crosstalk with others receptors**

Lateral activation of EGFR by various membrane bound molecules including other RTKs, cell adhesion molecules, cytokine receptors, ion channels, and G-protein coupled receptors (GPCR) has been demonstrated in tumor cells (143). Cross-talk between EGFR and other RTKs such as c-MET has been extensively described for tumor types where EGFR is a major player in their biology (144-146). This is also true in the case of GBMs where crosstalk between EGFR and c-MET has been described (88). EGFR and c-MET physically interact with other and can induce each other's activity (147). Two large scale phosphoproteomic studies have shown that c-MET and EGFR coactivate in glioma cell lines. The first report was by Huang et al (88) where they showed that cMET phosphorylation levels in glioma cells overexpressing EGFRvIII mutant was dependent on the kinase activity of this mutant. The second study showed that cMET phosphorylation levels was responsive not only to the EGFRvIII mutant but also to ligand stimulated EGFR in gliomas (148). This cross-talk between the receptors could be targeted with specific inhibitors to both, resulting in enhanced cytotoxicity of EGFRvIII-expressing cells compared with either compound

alone, suggesting a potential combination strategy to be explored in the clinic (88, 89, 89, 148).

Cross-talk between EGFR and a number of cell adhesion molecules including members of the integrin family has also been found. Activation of specific integrin molecules by extracellular matrix proteins (ECM) has been demonstrated to trigger tyrosine phosphorylation of EGFR (149, 150). Studies in fibroblast and epithelial cells have provided evidence on the ability of integrins to stimulate EGFR tyrosine phosphorylation in a ligand- independent manner leading to subsequent activation of MAPK and PI3K pathways (151). Similarly integrins is also involved in PLC $\gamma$  activation, which is particularly dependent on EGFR in epithelial cells (149, 151).

A further well defined cross-talk mechanism of EGFR activation involves G-protein coupled receptors (GPCR), which participate in EGFR transactivation. Activation of EGFR in response to a broad number of diverse GPCR agonist has been demonstrated in several different human cancers (152-155). However, the mechanisms by which GPCRs transactivate EGFR have not been well established and seem to differ in distinct cell types. In general, as mentioned earlier, GPCRs activated MMPs which cleave HB-EGF from the cell surface to facilitate activation of EGFR (143, 156). Studies using glioblastoma cells simultaneously expressing EGFR and a formylpeptide receptor (FPR), a member of the GPCR family, have demonstrated transactivation of EGFR, and synergistic cooperation to exacerbate the malignant phenotype in GBMs through cellular growth and tumor formation in athymic mice (157). Other GPCRs have also been shown to transactivate EGFR, including the receptors for lysophosphatidic acid (LPA) (158, 159), thrombin (160, 161), endothelin-

1(162, 163), carbachol (164, 165), angiotensin (166, 167), bombesin (168), and the chemokine SDF1 (169).

#### **1.4.5 Mutations in EGFR**

One of the mechanisms driving functional alteration of EGFR in tumor cells is mutation giving rise to constitutively active variants. Mutations in EGFR comprise three categories: extracellular, intracellular and tyrosine kinase mutations. Extracellular domain EGFR mutations are common in GBM, and characteristically, the majority of these mutations give rise to truncated forms of the receptor. A typical example of this type of mutation is the EGFRvIII (also call  $\Delta$ EGFR). This variant is characterized by an in-frame deletion of exons 2 to 7 encompassing part of the ligand binding domain of EGFR (170). EGFRvIII is the most commonly occurring and comprehensively studied member of this class of mutants.

EGFRvIII variant is present in about 30%-40% of the GBMs where EGFR is amplified (171-173). EGFRvIII exerts a prominent enhancement of tumorigenicity, and this characteristic has been attributed to suppression of apoptosis which is associated with the constitutively active signal observed in EGFRvIII (116, 173, 174). EGFRvIII is also related with a shorter interval to relapse and decrease in survival rates in GBMs (175, 176). EGFRvIII differs from EGFR in the following characteristics:

- 1) It is insensitive to the ligand stimulus, due to rearrangements of the ectodomain region of the receptor that lead to the absence of domain I and II which are important requirements for the ligand binding pocket conformation (172, 177, 178). Therefore, EGFRvIII is unable to bind EGFR-binding ligands and it is constitutively active and

capable of triggering downstream signaling events. Typically EGFR is characterized by absent or low levels of tyrosine phosphorylation in the absence of external ligand. Post EGF ligand stimulation, the levels of activation increase substantially (179-181). From a molecular perspective, similar pathways are activated by both EGFR and EGFRvIII, including MAPK, PI3K and STAT3 pathways (89, 182, 183) but with different levels of intensity. Interestingly, recent studies have indicated that even as wild type EGFR signals through classical pathways, EGFRvIII may not activate the same canonical pathways (184, 185). This variant seems to preferentially utilize the PI3K and STAT3/5 pathways to bring about its downstream biological effects ((89, 186). The dominant pathway that is activated by EGFRvIII in this regard is the PI3K pathway (187, 188). EGFRvIII may interact directly with the PI3K regulatory subunit p85 or indirectly through interactions with Gab1 adaptor (189). In addition, in the context of the loss of the tumor suppressor PTEN, EGFRvIII is strongly associated with the activation of PI3K-Akt pathway and the activation of mTOR and S6 pathways (89, 174, 182, 183, 190, 191).

2) EGFRvIII's low level constitutively active signal is augmented by its capacity to signal continuously as a result of the reduced endocytosis and degradation of the receptor (179). This is associated with restricted c-Cbl binding and inefficient ubiquitinylation (192-194). In addition, the small fraction of receptor that does internalize is recycled rather than delivered to lysosomes (192).

3) EGFRvIII appears to signal strongly to inhibit apoptosis via upregulation of Bcl-xl expression (195). The role of canonical Bcl2 family members (BID, BAX, BCL-XL, MCL-1 BAK, BAD) has been widely studied in gliomagenesis (5, 196). Typically these set of proteins regulate apoptotic process via preservation of mitochondrial membrane

and release of cytochrome c (197). During the transitional process from initial to recurrent GBM there is a change in the balance of anti-apoptotic function (198), where Bcl-x<sub>L</sub> is upregulated by overexpression of EGFRvIII in glioma cells. This has been attributed to be the basis for the enhancement in tumorigenesis and resistance to cisplatin-induced apoptosis observed in EGFRvIII overexpressing cells (174, 195). Additional functions of Bcl2 family members involve enhancement of migration and invasion (199-201).

Patients with EGFRvIII-expressing tumors have a shorter interval to clinical relapse and poorer survival than patients with EGFRvIII-negative tumors (202). For GBM patients who survive 1 year or longer after diagnosis, the expression of EGFRvIII is also an independent negative prognostic indicator of survival (203-205). Current molecular characterization of resistance to targeted therapy has shown that this variant grants distinct properties as compared to EGFR (206). Early studies have reported better response to EGFR tyrosine kinase inhibitors in patients whose tumors express EGFRvIII (207) demonstrating that this variant could sensitize gliomas to EGFR tyrosine kinase inhibitors. However, better response is observed in just 50% of the patients indicating that other aberrations affect the outcome of the treatment. Further, studies have shown evidence that clinical response to targeted therapy could be dictated by other molecular pathways (10, 174, 208). Loss of PTEN is one of the most frequent molecular signatures in GBM patients who exhibit poor response to tyrosine kinase inhibitor treatments. Studies where a cohort of more than 600 patients with newly diagnosed GBMs were examined have suggested that tumors negative for EGFRvIII were less aggressive (209), and complementary clinical trial studies suggested that tumors coexpressing EGFRvIII and PTEN are more prone to respond

to EGFR-tyrosine-kinase inhibitors (207, 210-212). Thus, it has been hypothesized that expression of EGFRvIII and PTEN might be useful prognostic indicators in patients that exhibit a better response to EGFR tyrosine kinase inhibitor therapies (207, 208, 210). In addition, novel strategies involving immunotherapy have been considered and could represent a promising approach in GBM treatment. Preclinical studies have shown that EGFRvIII specific peptides can be used in association with specialized antigen presenting cells, dendritic cells, to induce a long-lasting immunological response that significantly increased median survival times in mice (213, 214). Phase 1 clinical trial was conducted where the dendritic cell presenting the unique peptide of EGFRvIII was administered intradermally to patients. The results from the trial have indicated longer survival in GBM patients (18.7 months) after EGFRvIII-based vaccination (215). More recently, a phase II clinical trial reported an increase in overall survival (26 months) in group of patients with newly diagnosed GBM expressing EGFRvIII who received EGFRvIII targeted vaccine and at the present a randomizing phase III study is in planning stage (216).

Additional less common deletion mutations have been identified in GBMs and include intracellular deletions which lack of three exons 25–27 named EGFRvIVa; or two exons 25 and 26 termed EGFRvIVb) (172, 217) but their oncogenic potential remained uncharacterized until recently. Stable expression of these mutants in mouse fibroblast NIH3T3 cells showed that overexpression of these mutants result in an increase in cellular proliferation (218). Further, subcutaneous implantation of these cells in nude mice demonstrated that these mutants were tumorigenic to a greater extent than EGFR (218). In addition, both mutants exhibited activation in the absence of ligand. This study also suggested that signal transduction from these mutants

shows differences as well as similarities with signaling from EGFRvIII in the same context (218). For instance, both activate AKT and show dependency on chaperone HSP90 for activity and stabilization. In addition, mass spectrometry analysis showed a decrease in the basal levels of phosphorylation of a negative regulator, EPHA2 in these mutants (218). EPHA2 has been reported to be overexpressed in the unphosphorylated state in GBM cells and tumors (218, 219). Other deletion mutations that have been also found specifically in GBM (220), include EGFRvI and EGFRvII, which are believed has a significant role in cell proliferation and invasiveness (220, 221). In addition, the deletion EGFRvV is found in about 15% of the GBMs and has thus far not been identified in any other tumor type (217). This particular mutant lacks the c-Cbl binding site at Tyr-1045 suggesting that there are defects in the receptor internalization (222), but the ability of this mutant to confer tumorigenesis and the signaling mechanism activated have yet to be studied (217).

Recently, a novel class of glioblastoma-associated EGFR mutants has been identified. These are missense mutations in the extracellular domain (9). I have focused my work on this new class, and specifically those that occur between exons 2-7 of the receptor, in order to test the hypothesis that they resemble EGFRvIII in terms of biology and mechanism. It has been shown that ectodomain missense mutants can have constitutive, ligand-independent kinase activity like EGFRvIII. However, they can also still be activated further by the addition of ligand such as EGF, unlike EGFRvIII (9). Many of these missense mutations have been identified in the TCGA dataset.

#### **1.4.5.1 Role of EGFR ectodomain mutations in gliomagenesis:**

Lee et al. sequenced the EGFR coding regions in a cohort of a 151 glioma tumor and cell lines, and identified novel mutations, including a substantial number of

ectodomain missense mutations (9). Approximately 14% (19/132) of glioblastomas and 13% (1/8) of glioblastoma cell lines displayed this type of mutation (9). In addition, they found that these mutants exhibited a stronger transforming phenotype when compared to wild type EGFR as evidenced by the anchorage independent growth assay, where transformation by EGFR overexpression in mouse fibroblast cells was completely dependent on ligand stimulation for colony formation (9). On the other hand, fibroblasts overexpressing ectodomain mutants formed colonies even in the absence of ligand suggesting the ligand-independent oncogenic nature of these mutants. Also, they found that fibroblasts overexpressing these mutants were able to generate tumors after implantation in nude but a similar phenotype was not observed in the group of mice implanted either with fibroblast expressing empty vectors or EGFR (9). In addition, these mutants showed tyrosine kinase activation in serum starved condition as seen for EGFRvIII in BaF3 cells which have no endogenous EGFR (9). Unlike EGFRvIII however, there was more robust tyrosine kinase activation observed after EGF stimulation in cells harboring EGFR with the ectodomain mutations (9). This indicates that these mutants respond to EGF acutely similar to EGFR. The main conclusion of this study is that EGFR missense ectodomain mutations could signify a novel mechanism for activation of EGFR in tumors (9). Their transforming and oncogenic capacity suggests a potentially important role in gliomagenesis but further studies are essential to validate it. Also a complete analysis of downstream signaling pathways of the mutants is critical to establish if they are active through the same or different mechanism when compared to EGFR and EGFRvIII. In particular, as new ectodomain mutants are being identified by the sequencing efforts of TCGA, it is important to determine whether they can be classified into categories, which may have mechanistic and ultimately clinical significance. TCGA

initially analyzed a cohort of 206 GBMs patient samples. Now the panel of has been increased to 500 patient samples. Information contained in the data base of TCGA allowed us to identify EGFR ectodomain mutations situated in the deleted area of the EGFRvIII. The multidimensional data from TCGA gives us further information such as gene copy number, frequency and also patient clinical data, which permits us to associate expression of the ectodomain mutants to the above mentioned parameters. Additionally, high-resolution genomic and exon-specific transcriptomic profiling readily detected the EGFR vIII as well as carboxy-terminal deletions of EGFR in GBMs (10). Also, this analysis detected point mutations associated with focal amplifications of the EGFR. In an analysis from a cohort of 91 GBM samples, 3 different statuses were found: one that exhibited focal amplification without mutations (total 22 cases), one that displayed focal amplification and point mutations (16 cases) and one that showed point mutations without focal amplification (3 cases) (10).

### **1.5 EGFR inhibitors effectiveness in treatment strategies and resistance in GBM**

Overexpression of EGFR has been detected in numerous epithelial tumors during the 1980s and these findings support the theory that deregulated EGFR expression and signaling is an important event in the origin of human cancers (223, 224). This fact has led to the development of multiple drugs targeting EGFR, which include anti-EGFR monoclonal antibodies such as mABC225 and mAB528 (225, 226). These antibodies compete with EGF for EGFR binding and also induce receptor downregulation through internalization and degradation (227). This results in inhibition of cellular proliferation in human fibroblast (228). Initially the mABC225 (Cetuximab) had demonstrated efficient antitumor effect in cell cultures and xenograft models, leading to the development of this therapeutic agent (229). Cetuximab is an

immunoglobulin G1 chimeric mouse–human monoclonal antibody with a mean half-life of approximately 112 h in humans (229). This agent was approved for the use in metastatic colorectal cancer (CRC), and for treatment of squamous cell carcinoma of the head and neck (HNSCC). Both of the tumor types are of epithelial origin and typically express EGFR (230, 231). Cetuximab has also been under active clinical evaluation in progressive non small cell lung cancer (NSCLC) (232-235). More recent studies suggest a new role for this antibody in breast cancer cells, where it has been shown to affect integrins independent of EGFR (236). Cetuximab treatment leads to activation of RhoA and inhibition of breast cancer cell invasion independent of the level of EGFR in the cells, thus providing a basis for using this antibody in metastatic breast cancer independent of the levels of EGFR (236). Preclinical studies in cell cultures and mouse models indicate that cetuximab exerts antitumor and radiosensitizing effects in GBM (237). Preclinical data also suggests that cetuximab binds to and internalizes EGFRvIII leading to significant reduction of active forms of the variant (238). Although very little clinical data of cetuximab in patients with GBM is available, anecdotal cases where patients with recurrent, strongly pretreated, EGFR-expressing GBM responded to treatment with the single agent cetuximab have been reported (239). While in other types of cancers such as CRC and HNSCC, cetuximab has showed promising antitumor activity in clinical trials as monotherapy, it has been efficacious also in combination with chemotherapy and/or radiation (230, 231, 240, 241). Therefore, in more recent clinical trials, the efficacy and safety of cetuximab in combination with other agents such as irinotecan and bevacizumab was investigated in patients with recurrent primary GBM (242). These phase II trials demonstrated that cetuximab in combination with irinotecan and bevacizumab had a satisfactory safety profile and stimulated a considerable number of clinically significant, long-term responses in a

cohort of 43 patients (26%) (241). Another approach to inhibiting receptor tyrosine kinases is with tyrosine kinase inhibitors (TKIs), which are synthetic molecules with low molecular weight that likely allow them to penetrate the blood-brain-barrier, and which inhibit the tyrosine kinase activity by acting at the ATP binding site. TKI's are typically administered orally to patients. The most advanced TKIs in clinical development are erlotinib (Tarceva®, Genentech Inc., San Francisco, CA, USA; OSI Pharmaceuticals Inc., Melville, NY, USA; and F. Hoffmann-La Roche Ltd., Basel, Switzerland) and gefitinib (Iressa®, AstraZeneca, Wilmington, DE, USA), two EGFR-specific, reversible TKIs. Numerous preclinical studies highlight the efficacy of TKIs in GBMs. While erlotinib is able to repress anchorage-independent growth of GBM cells, cells were able to overcome this inhibition by upregulation of EGFR mRNA levels (243). In contrast, erlotinib induced higher levels of apoptosis in cell lines from secondary GBMs, probably because of their inability to increase EGFR mRNA levels when exposed to erlotinib (243). A phase I clinical trial has established a safety profile and indicated a degree of efficacy of erlotinib in combination with TMZ in patients with malignant glioma, who received escalating doses of erlotinib starting with 100 mg/day to 500 mg/day (244). Another study has shown that the combination of erlotinib and radiotherapy in GBM patients has acceptable levels of tolerance in patients receiving doses from 150 to 200 mg/day (245). Various combinations of EGFR-TKIs with other compounds are under investigation including mTOR inhibitors such as sirolimus used in combination with erlotinib in recurrent malignant gliomas, a regimen that was reasonably tolerated and under which 19% of the patients showed a partial response, while 50% had stable disease (246). In gliomas, association between gefitinib sensitivity and EGFR amplification is not apparent (247, 248). Furthermore, this agent appears more effective when mutations are present in the intracellular domain of

EGFR exon 18 to 21 as was shown in NSCLC (249, 250), but these mutations are not present in GBMs (251). GBM tumors expressing EGFRvIII are significantly affected by erlotinib (252, 253). In contrast, these tumors do not display any response to gefitinib treatment (254). More recently, preliminary studies from Lee et al indicate that erlotinib did induce dose-dependent cell death in Ba/F3 cells expressing the EGFR ectodomain mutations including EGFRvIII (9), suggesting that this drug may have uses in a subset of GBM patients. Thus, a series of clinical trials with particularly selected group of patients exhibiting EGFR mutations, who are most likely to benefit from first-line treatment with EGFR TKI therapy needs to be conducted (255-257).

## **1.6 Hypothesis, rationale and significance**

### **Rationale**

The importance of EGFR in the biology of glioma, both in terms of the prevalence of mutations in the gene and in its central position in key signaling pathways, provides a strong case for targeting it with drugs. The last decade of research on targeting of RTKs with antibodies and TKIs has made it clear that the details of the receptor biology of the many mutations in EGFR needs to be considered in detail, as they will profoundly affect the effectiveness of these agents. More recently, large scale sequencing efforts have revealed new mutants in EGFR in glioma, particularly in the extracellular domains where deletion mutations were previously identified. Early studies had identified missense ectodomain mutations in EGFR after screening of a panel of 132 glioblastoma samples and 8 glioblastoma cell lines (9). In their study, Lee et al. have demonstrated anchorage independent colony formation in soft agar of NIH3T3 mouse fibroblast expressing novel EGFR missense mutations as well as tumorigenic capacity in athymic nude mice after subcutaneous implantation of stable

mouse fibroblast expressing these mutants (9), but the mechanisms of receptor activation followed by these mutants remains unclear. In order to realize the promise of EGFR-directed therapy in this disease, it is imperative to study EGFR ectodomain mutations in detail by defining the downstream signaling events specifically activated by these mutations, by determining whether they have essential function in GBMs and by identifying possible implications in novel therapeutic approaches. Furthermore, because many of these mutations occur in the domains deleted in the most common and thoroughly studied EGFR mutant, EGFRvIII, it is important to determine whether point mutations in this region share characteristics with this ligand-independent, oncogene which exhibits persistent but low levels of activity. Therefore, the goal of my work is to evaluate the molecular mechanism of receptor activation and also the oncogenic capacity of EGFR ectodomain mutations located in the region deleted in EGFRvIII.

### **Hypothesis**

1. Signaling from EGFR ectodomain mutants shares characteristics of both EGFR and EGFRvIII.
2. EGFR ectodomain mutants are oncogenic and confer a more aggressive phenotype on GBM cells.
3. The overexpression of EGFR ectodomain mutants in glioma cell lines leads to changes in the pattern of response to chemotherapeutic agents.

### **Significance**

One of the most common genetic abnormalities in GBMs is the activation of RTKs,

and overexpression, amplification and mutation of EGFR is the most prevalent, occurring in about half of GBMs (10, 258). Multimodal treatment involving surgical resection followed by chemoradiation represents the current standard treatment of GBMs, but offers median survival of less than two years (259). Thus, inhibition of EGFR remains an important goal, and with the advent of personalized medicine the possibility of customization of therapeutic schemes to the molecular abnormalities in an individual's tumor provides a compelling rationale for studying EGFR mutations in depth. The finding that EGFR missense ectodomain mutations grant increased receptor sensitivity to small molecule inhibitors such as erlotinib is particularly encouraging in this context (9). It is also clear that the broader molecular context, such as the status of tumor suppressor genes like PTEN, will be important when selecting the appropriate treatment for groups of patients with a particular molecular abnormality (210). Thorough analysis of the recently identified ectodomain mutations is therefore highly significant, as it could ultimately contribute to the stratification of individual GBM patients according to their molecular signatures for more effective treatment.

## **CHAPTER 2 MATERIALS AND METHODS**

## 2.1 Generation of EGFR and TOPO-Cloning

Blunt end PCR products for TOPO-cloning were generated by PCR reaction containing 100 ng of genomic DNA (in house EGFR construct) as template, 10X high fidelity PCR buffer, 10mM dNTP mixture, 50mM MgSO<sub>4</sub>, 2 Units Vent<sub>R</sub>® DNA polymerase from Biolabs (Catalog #MO254S), 20µM forward and reverse primers purchased from Sigma Aldrich: EGFR 5' fwd CACCATGCGACCCTCCGGGACGGCC and EGFR 3'rev TGCTCCAATAAATTCAGTCT (no stop codon) in a 150µl reaction volume. PCR cycling parameters were: One cycle 95°C for 5 min; 32 cycles of 95°C for 15 s, 55°C for 30 s, and 72°C for 6 min; followed by one cycle of 72°C for 7 min. Then PCR products were transformed using One Shot®TOP10 chemically competent E.coli cells from Invitrogen (Catalalog # C4040-10) and then purified using the Promega Wizard®Plus Midipreps DNA purification system (catalog #A-7640). DNA purified products were quantified and then TOPO-cloned in pENTR™/TEV/D-TOPO vector using the pENTR directional topocloning kit from invitrogen ( Catalog #K2525-20).

## 2.2 Identification of ectodomain mutations

EGFR ectodomain mutations R108K, T263P, A289D, A289T were identified using TCGA information at <https://cgwb.nci.nih.gov>.

## 2.3 Mutagenesis and Cloning

TOPO-cloned blunt end PCR products were used to generate EGFR ectodomain mutations using specific forward and reverse primers for every mutation previously identified from the TCGA dataset. The primers were designed using the Primer X software program (<http://www.bioinformatics.org/primerx/>) and are listed below:

<b>Primer Name</b>	<b>Sequence</b>
<b>R108K-F</b>	5'-CCTGCAGATCATCAAGGGAAATATGTACTAC-3'
<b>R108K-R</b>	5'-GTAGTACATATTTCCCTTGATGATCTGCAGG-3'
<b>T263P-F</b>	5'-CACGTGCAAGGACCCATGCCCCCACTC-3'
<b>T263P-R</b>	5'-GAGTGGGGGGCATGGGTCCTTGACAGTG-3'
<b>A289D-F</b>	5'-CAAATACAGCTTTGGTGACACCTGCGTGAAGAAG-3'
<b>A289D-R</b>	5'-CTTCTTCACGCAGGTGTCACCAAAGCTGTATTTG-3'
<b>A289T-F</b>	5'-CAAATACAGCTTTGGTACCACCTGCGTGAAGAAG-3'
<b>A289T-R</b>	5'-CTTCTTCACGCAGGTGGTACCAAAGCTGTATTTG-3'

**Table 1 Primers for site-directed mutagenesis**

Table lists the set of primers used to generate the different EGFR ectodomain mutations along with their sequences.

Then QuikChange II ®Site-Directed Mutagenesis protocol from Stratagene (Catalog # 200523) was followed. DNA products were transformed and purified as described above in section 5-1, and EGFR mutations were confirmed by sequencing analysis using primers:

Primer Name	Sequence
T7-0	TAA TAC GAC TCA CTA TAG GG
EGFR3622-F	AGTGGGCAACCCCGAGTATCT

**Table 2 Confirmation primers**

Sequences for EGFR ectodomain mutations were confirmed in the MD Anderson Cancer Center sequencing core using specific primers contained in this table.

Next, recombination reaction was performed using pENTR EGFR ectodomain mutations and DNA products were subcloned into expression vectors using the Gateway®LR Clonase™ II Enzyme Mix kit from Invitrogen (Catalog # 11791-020). The destination vectors used were pcDNA-DEST 47 from invitrogen (Catalog #12281-010) and the Bi-cistronic retroviruses system 1726-zeo (260).

**2.4 Generation of stable cell lines**

**2.4.1 Production of Bi-cistronic VSV-G pseudotyped retrovirus stocks**

We use the BD Retro-X™ universal packaging cell line system from Clontech (Catalog # 631530) stably expressing gag/pol genes were cultivated at 37°C in 5% CO<sub>2</sub> in 100 mm diameter plates in DMEM media purchased from Cellgro (Catalog #10-017-CV ) supplemented with 10% of FBS and 5% sodium pyruvate. Once the GP2 cells reached ~70% confluence the cells transfected following the modified calcium phosphate protocol and using 20µg of DNA: 10µg VSV-G DNA plasmid acquired from Clontech (Catalog# 631530) and 10µg of the retroviral construct. In brief, the plasmids

were added to a total volume of 437  $\mu$ l of TE [10mM Tris pH 7.9 and 0.1 mM EDTA] to which 63 $\mu$ l of 2 M  $\text{CaCl}_2$  was added. Using a 1 ml culture pipette in a small sterile polystyrene snap cap tube was used to establish a steady flow of air bubbles through 500  $\mu$ l of 2x hepes buffer saline (HBS) [0.05 M HEPES free acid, 0.28 M NaCl and 1.5 mM  $\text{Na}_2\text{HPO}_4$  pH 7.12] and the DNA/ $\text{CaCl}_2$  mixture was added drop wise over the course of about 30 seconds. After incubation at room temperature for about 20 minutes the mixture was added drops wise to the GP2 cells, and then incubated for 4 hours. Subsequently, the media was changed to add fresh media using half the volume (5 ml) and the plates were incubated for 48 hours. There after supernatants were collected and filtered through 0.4  $\mu$ m filter, aliquoted and stored at  $-80^\circ\text{C}$  until further use.

#### **2.4.2 Transduction of Glioblastoma and CHO cell lines**

Glioblastoma cell lines U87 (ATCC®# HTB-14™), LNZ308 and LN428 (courtesy of Dr. Alfred Yung's lab. Neuro-Oncology Department MD Anderson Cancer Center) were cultured in T25 flasks in DMEM media purchased from Cellgro (Catalog #10-017-CV ) supplemented with 10% of FBS and 5% of L-Glutamine and penicillin-streptomycin and incubated at  $37^\circ\text{C}$  in 7%  $\text{CO}_2$  , also CHO cell lines (ATCC®#CCL-61) were cultured in IMDM procured from Hyclone (Catalog # SH 30228.01) supplemented with 10% of FBS, 5% of L-Glutamine and penicillin-streptomycin and 5% of sodium pyruvate and then incubated in standard conditions as above. Thereafter when plated target cells reached 50% confluence, the media was removed, and 1 ml of stock virus with 8 mg/ml of Polybrene [Hexadimethrine bromide, Sigma H-9268, made up in water at 8 mg/ml and sterilized by filtration] were applied onto the cells to

infect them and then were left for 2 hours in the incubator at standard conditions, the media was then removed and replaced with normal growth media and incubated for 48 hours before initiation of drug selection using Zeocin.

### **2.4.3 Zeocin selection to obtain stable cell lines**

Cells were subjected to treatment using the antibiotic zeocin purchased from Invitrogen (Catalog # R250-01) at the lowest lethal dose as determined by titration curve on naive cells as follow:

CHO cell ..... 600 µg /ml

U87 cell..... 100 µg /ml

LNZ 308..... 50µg /ml

LN428..... 50µg /ml

Cells were fed every two days until selection of stable cell lines was achieved (we run in parallel a non-infected culture control cells). Selection of stable cell lines took about 15 days.

## **2.5 Protein expression of stable cell lines**

Protein expression in stable cell lines was confirmed using western blotting analysis according to standard protocols (148).

### **2.5.1 Obtain protein lysates**

Cultured stable cells were washed with pre-cold PBS and after scrapping cells were lysed in RIPA buffer containing 50 mM Tris-HCL buffer (pH 7.4), 150 mM NaCl,

1% NP40, 0.25% Na-deoxycholate, 1mM EDTA, 1mM PMSF, 1µg/ml of aprotinin, 1µg/ml of leupeptin, 1mM Na<sub>3</sub>VO<sub>4</sub>, 1mM NaF 1mM. Thereafter, samples were individually passed through a 25G needle, and then incubated with rotation at 4°C for 45min to 1 Hour, followed by centrifugation at 4°C for 30 min at 14,000 revolutions/min. The supernatants were separated and used for further experiments.

### **2.5.2 Protein quantitation**

Protein concentration was determined using the protocol from BCA™ Protein Assay Kit from Pierce (Catalog # 23227). Then protein concentration of the lysates was adjusted between 10-15 µg/µl.

### **2.5.3 Western blot analysis**

Lysates were boiled in 5X SDS at 95°C for 5-10 min to perform protein separation on 10% SDS polyacrylamide gel in 1X running buffer for 1 Hour at 120 volts. Then membranes were transferred to nitrocellulose membranes for 1 hour at 100 volts at 4°C (Bio-Rad, Hercules California Catalog# 162-0097). Membranes were washed in 1X TBST buffer [0.1% Tween 20, 20 mM Tris base (pH 7.6), 136 mM NaCl and 0.38 mM HCL] and blocked for 1 h at room temperature in either 5% nonfat milk or 5% BSA from Sigma (Catalog # A3059) in 1X TBST buffer and probed with (1000X) anti EGFR rabbit polyclonal antibody (Cell Signaling Technology Catalog # 2232) in 5% of blocking buffer (nonfat milk), and then incubated overnight at 4°C. Next day, blots were washed 3 times using 1X TBST buffer and followed by incubation with (10,000X) horseradish peroxidase linked to the secondary antibody anti-rabbit (EGFR) from Thermo Scientific (Catalog # 31460) for 45 min then washed 3 times in 1X TBST buffer, and followed by enhanced chemiluminescence detection using

SuperSignal®West Dura ThermoScientific (Catalog #34076) according to the manufacturer's instruction. Membranes were stripped at 42°C for 30 min, and then washed overnight and re-probed using monoclonal anti-B-Actin peroxidase from Sigma (Catalog #A3854) or monoclonal antibody vinculin from Sigma (Catalog # V9131) for 1 hour, followed by chemiluminescence detection. We use the ChemiDoc™ XRS+ imaging system from Bio-Rad for the acquisition of all the images.

## **2.6 Ray®Bio EGFR phosphorylation antibody array analysis using U87 stable cell lines**

In this analysis, stable U87 cell lines overexpressing EGFR ectodomain mutations were seeded in 150 mm plates (2 plates per mutation) and cultivated under standard conditions. Once the plates reached ~70% confluence, the plates were separated into two sets. One set was serum starved for 24 hours and the other set was ligand stimulated after starvation using recombinant human EGF from Invitrogen (Catalog # PHG 0311) for 5 minutes using 15 nanograms/ml. Subsequently, cells were harvest by scraping and pellets were collected by centrifugation at 1,000 revolutions/ minute per 5 minutes, then pellets were treated using Ray-Bio kit protocol to obtain protein lysates. The protein concentration was estimated using BCA™ Protein Assay Kit from Pierce (Catalog # 23227) protocol. Thereafter, a preliminary western blot analysis using specific antibodies for Tyr-845, Tyr-1068 and Tyr-1173 sites as well as total EGFR was performed to determine relative signal intensities. Subsequently we assessed for relative levels of autophosphorylation for EGFR Tyr-845, Tyr-1068, Tyr-1148 and Tyr-1173 using the protocol from Ray®Bio human EGFR phosphorylation antibody array kit from RayBiotech, Inc. (Cat# AAH-PER-1-8) followed by chemiluminescence

detection . Images from the membranes were captured using the ChemiDoc™ XRS+ imaging system from Bio-Rad and quantitative analysis of signal densities were carried out using the Quantity One software program from Bio-Rad. Following normalization with the total EGFR fraction and biotinylated controls and after background subtractions, relative levels of autophosphorylation were estimated for the multiple tyrosine sites across all the mutations including EGFRvIII and also intact EGFR in both conditions. Results were graphed using the graphpad prism software program and then subjected to analysis.

Validation of the different autophosphorylation profiles for several tyrosines residues obtained from the Ray-Bio analysis was performed using antibodies for Tyr-845, Tyr-1068 and Tyr-1173 by western blot technique.

## **2.7 Cell signaling analysis after starved and EGF stimulated conditions**

U87 cell lines stably expressing EGFR ectodomain mutations were seeded in 100 mm plates (two plates per mutation) and cultivated in standard conditions. At 60% confluence, one set of plates was serum starved from 18-24 hours and the other set of plates was ligand stimulated after 24 hours of starvation using EGF (15 ng/ml of media for 5 minutes). Next cell cultures were washed with pre-chilled PBS and lysed in RIPA buffer. Protein lysates were obtained and quantitated as described earlier in section 5-1. The membranes were probed using antibodies procured from Cell Signaling: EGFR (Catalog #2232), EGFR-Tyr 1068 (Catalog #2234), EGFR-Tyr-1173 (Catalog # 4407), AKT (Catalog #9272), p-AKT/S473(Catalog #4060), MAP-K p44/42 (Catalog #9102), p-MAP-K (Thr 202/ Tyr 204) (Catalog #9101), S6 (Catalog # 2217) and p-S6 Ribosomal protein (Ser 235/ 236) (Catalog #4858) from Cell Signaling Technology. The HRP-linked anti-rabbit (EGFR) secondary antibody was obtained from

ThermoScientific (Catalog #31460), anti B-Actin peroxidase antibody from Sigma (Catalog#A3854), Monoclonal antibody 4G10 platinum-anti-phospho-tyrosine from Millipore (Catalog # 05-1050) and secondary antibody ECL- anti mouse IgG HRP-linked from Amershan (Catalog #NA931V). After chemiluminescence signaling detection, quantitation was carried out as performed in section 2.6. Following to normalization with EGFR total fraction and actin levels, relative levels of kinase activation for Akt and ribosomal S6 pathways were graphed using GraphPad Prism Program and then subjected to analysis.

## **2.8 Phospho-proteomics assays**

Stable U87 cells overexpressing ectodomain mutations R108K, T263P, EGFRvIII and also EGFR were seeded in 150 mm plates until they reached 70 to 80% of confluence. Thereafter cells were washed twice with PBS and serum starved using serum free media and incubated in standard conditions for 24 hours. After incubation, cells were lysated and trypsinized according to phospho-proteomics protocol. The phospho-proteomic analysis was performed by Dr, Chumbalkar, a collaborator from Dr. Bögler's laboratory.

### **2.8.1 Sample preparation, peptide immunoprecipitation and mass spectrometry analysis**

Samples for mass spectrometry analysis were prepared and analyzed as described earlier. In brief, we used two biological replicates and for each, we extracted protein in urea lysis buffer and later trypsinized it after reduction and alkylation. Thus generated peptides generated were desalted with Sep-Pak C18 column (Waters Corp) and freeze dried. Peptide immunoprecipitation was out carried out using the P-Tyr-100

phosphotyrosine mouse mAb (Cell Signaling Technology), and enriched peptides were further purified with C18 zip-tips (Millipore Corp). LC-MS/MS analysis was done in duplicate with Agilent's 6340 Ion trap system with electron transfer dissociation (ETD) where fragmentation was set to alternate between Collision-Induce Dissociation (CID) and ETD. The CID process consists of fragmentation of isolated peptides by repeated collisions with the helium cooling gas, thus adding internal energy to the peptides and inducing random fragmentation (261). We alternated CID with the ETD process which uses low levels of energy and allows us a complementary fragmentation strategy providing better confidence in peptide identification (262, 263).

### **2-8-2 Database analysis and quantification**

MS/MS spectra were extracted using Bruker Compassxport to mzxml files which were converted to ".mgf" (mascot generic format) for database searches using trans-proteomic pipeline (Seattle Proteome Center). Mascot search engine version 2.3.02 was used for searching human subset of Swissprot database to identify peptides and modifications. Phosphorylation site assignment was confirmed manually looking at spectra. We used Ideal-Q<sup>2</sup> software to align all the runs with each other based on retention time. After this we used Ideal-Q to calculate peak areas manually for all the identified phosphopeptides (264). We normalized these values by total ion chromatogram (TIC) of whole run. At the end we calculated mean of peak areas all the phosphopeptides and calculated statistical significance by unpaired t test. The preliminary cohort of phosphopeptides obtained was filtered based on common phosphopeptides to R108K, T263P and EGFRvIII mutants, where the values of relative phosphorylation for these phosphopeptides were considered as zero (logarithm of one) for EGFR. Then potential targets were selected base on the criteria

of phosphopeptides with high relative levels of phosphorylation common to R108K and EGFRvIII but different to the levels seen for the mutation T263P.

## **2.9 Cellular distribution of EGFR ectodomain mutations in transiently transfected CHO cell lines**

CHO cells were seeded in 6 well plates containing sterile cover slips at low density (30% confluence) and cultured in IMDM media and incubated under standard conditions. Thereafter, cells were transiently transfected using 2 $\mu$ g of DNA (GFP-Tagged EGFR ectodomain mutation plasmids) per well. Protocol for the use of Fugene-HD purchased from Roche (Catalog # 04709713001) was followed for transient transfection. Cells were grown until they reached 60% confluence in standard media conditions (10% serum). After that, plates were rinsed with PBS (5 minutes x 2) and fixed in 4% paraformaldehyde at room temperature for 20 minutes. Following wash (5 minutes x 2) using PBS they were covered with blocking buffer containing 5% normal goat serum (NGS) and 0.1% Triton X-100 using 900 $\mu$ l/well for 1 hour, then rinsed for 5 minutes x 3 with PBS. Then cover slips were lifted out using a fine gauge needle with the tip bent at an angle and placed the top part of the cover slip inside on top of a slide containing a drop of mounting media (80% of glycerol in PBS). The slides were sealed using nail polish and kept at 4°C for confocal microscopic analysis.

### **2.9.1 Cellular distribution of EGFR ectodomain mutations in transiently transfected U87 cells**

Parental U87 or cell lines were cultured at 50-60% of confluence in 6 well plates containing sterile cover slips previously pretreated with poly-L-Lysine, and then cells were transiently transfected using 2 $\mu$ g of DNA (GFP-Tagged EGFR ectodomain mutations plasmids) per well as explained earlier in section (5.9). After 24 hours, the

cells were serum starved for 24 hours and one set of plates were used as such while the other set of plates were stimulated with EGF ligand (15 ng/ml for 30 minutes) and subjected to immunostaining for confocal microscopic evaluation. The staining was done as described earlier in section (2.9) with the following modifications: the plates were counterstained using Topro3 (blue) for 20 minutes.

### **2.9.2 Nuclear and cytoplasmic expression of EGFR ectodomain mutations**

To obtain nuclear and cytoplasmic cellular fractions, we used 100 mm plates of stable U87 cells overexpressing EGFR ectodomain mutations including EGFRvIII and also wt-EGFR. After the plates reached about 80% confluence, cells were serum starved for 24 hours. One set of cells was processed in serum starved condition, while the second set was stimulated using EGF ligand (15 ng/ml for 30 minutes) and then cellular fractionation protocol was followed in order to obtain nuclear and cytoplasmic cellular fractions. Cells were washed twice with ice-cold PBS, collected by scraping and lysed in lysis buffer (20mM HEPES, pH 7.0, 10 mM KCL, 2 mM MgCl<sub>2</sub>, 0.5% Nonidet P-40, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 10 mM NaF, 1 mM phenylmethanesulfonyl fluoride, 2 µg/ml aprotinin and protease and phosphatase inhibitors cocktail). After incubation on ice for 10 minutes, the cells were homogenized by 20 strokes in a tightly fitting dounce homogenizer. The homogenate was centrifuged at 1,500 x g for 5 minute to sediment the nuclei the supernatant was then centrifuged at a maximum speed 14,000 rpm for 20 minutes, and the resulting supernatant formed the non-nuclear fraction. The nuclear pellets were washed three times with lysis buffer to remove any contamination from cytoplasmic fraction. To extract nuclear proteins, the isolated nuclei were resuspended in nuclear extraction buffer (20mM HEPES, pH7.9, 400 mM NaCl, 1mM EDTA, pH 8, 1mM EGTA, pH 7, protease inhibitor cocktail (from Sigma) and

phosphatase inhibitor cocktail) and incubated on ice for 30 minutes with intermittent vortexing. The nuclei were centrifuged for 30-60 minutes at 14,000-16,000 rpm. The supernatant comprised the nuclear extract. Thereafter protein amounts were quantitated and western blot analysis was performed to confirm the expression of EGFR mutations in both nuclear and cytoplasmic cell compartments.

### **2.10 Cell viability in EGFR ectodomain mutations**

U87 cell lines stably overexpressing EGFR ectodomain mutations were seeded (15,000-20,000 cells/well) in 6 well plates and incubated at standard conditions. After 72 hours of incubation, total number of viable cells was counted in the Vi-Cell Analyzer from Beckman, which determines percentage of cell viability via standard trypan blue assay. Data was graph and analyzed using GraphPad Prism program.

### **2.11 WST-1 assay of glioma cells overexpressing EGFR ectodomain mutations**

Complementary to the previous analysis in section 2.10, we seeded U87 and LNZ308 cells overexpressing ectodomain mutants in 96 well plates (1,000 cells per well) and the WST-1 protocol from Roche (Catalog # 1644807) was followed to determine changes in cell viability. After 72 hours of incubation the plates were subject to scanning in the plate reader SPECTRAMAX and then values were graphed and analyzed using GraphPad Prism program.

### **2.12 Anchorage independent colony formation**

U87, LNZ308, LN428 MG cell lines over-expressing EGFR ectodomain mutants were grew under exponential growth phase in standard conditions. Then plates were washed and cells were trypsinized. Thereafter, cell densities were determined in the Vi-Cell analyzer instrument from Beckman in order to seed 2,000 cells/well in 12 well

plates. These plates were previously covered with 1 ml of base agarose solution consisting of 4% of low melt agarose (10 ml) in pre-warmed media (42.5 ml) and FBS (7.5 ml), then adding 1 ml of top agarose solution consisting of 3% of low melt agarose (10 ml) in pre-warmed media (42.5 ml) and FBS (7.5ml) and followed by the addition of 1ml of pre-warmed media after the top matrix has solidified, plates were incubated at standard conditions. After 7 days of culture, plates were scanned for tumor colony formation in the Gel-Count<sup>MT</sup> instrument from Oxford-Optronix and daily readings were recorded until day 12 for stable U87 cells and day 15 for LNZ308 and LN428 stable cells lines. Three independent biological replicates were performed for every set of mutants in the different glioblastoma cell lines. After calculation of colony number and biomass for all the replicates, data was graphed and analyzed using GraphPad Prism software program.

### **2.13 Generation of xenograft models**

The Guide Screw System was followed (265) for xenograft studies and performed by the Brain Tumor Center animal core from MD Anderson Cancer Center. Single cell suspensions of U87 cells were prepared in Serum-free medium at a final concentration of 400,000 cells /10 $\mu$ l which were injected intracranially in sterile nude mice. Groups of 10 mice per mutant were used and mice status was monitored for a period of 2-4 weeks. Tumors were collected and survival time was recorded. Half of the tumors were processed for H&E (paraffin embedded) and the other half for protein preparation (flash frozen in liquid nitrogen). To estimate survival time across all the groups of mice, Kaplan Meier survival estimator was used. In addition, to determine statistical significant differences in survival among the groups we used Gehan-Breslow-Wilcoxon Test. In general,  $P < 0.05$  is considered as statistical significance.

## **2.14 Inhibitor studies**

To evaluate the effect in anchorage independent growth of various agents on EGFR ectodomain mutations overexpressed in U87 cell lines we established IC<sub>50</sub> values using 4 different drug concentrations of cisplatin, tarceva and TMZ. Three independent biological replicates were performed and the protocol for anchorage independent growth as is described in section 2.12 was followed. Data from the relative biomass indexes resulting from the different concentration treatments after days five, seven and nine for cisplatin and tarceva was used to calculate IC<sub>50</sub> values and for TMZ we use data from days seven and nine. The graphpad prism software program was used, where X= logarithm dose and Y are relative biomass indexes. Biomass indexes were calculated by multiplication of volume and colony count values and the relative levels of biomass were obtained after normalization using the biomass indexes values from the mock-treated (DMSO). In addition, we also evaluate the effect of TMZ on EGFR ectodomain mutants overexpressed in LN428 cells.

### **2.14.1 Erlotinib treatment**

Erlotinib Cat-N°E-4007 was purchased from LC Laboratories. Stock solution of (10 mM) was made and then different concentrations of erlotinib were prepared in DMSO: 0.5 µM, 5 µM, 10µM and 20µ M. After 7 days of incubation cell culture plates were scanned in the Gel- Count instrument and readings were recorded. Thereafter the different sets of cells were treated with individual concentrations of erlotinib (0.5, 5.0, 10 or 20 µM) and control cells were treated with equal amounts of vehicle. To assess the inhibitory effect of erlotinib in tumor colony growth, the plates were subjected to multiple readings in the Gel-Count instrument starting from the day one of treatment and then every other day until day nine. Readings obtained from the Gel-

Count instrument were used to calculate colony count and biomass, after that collected data was graphed and analyzed using GraphPad Prism software program as previous described in section 2.14.

### **2.14.2 Cisplatin Treatment**

*cis*-Platinum(II) diammine dichloride Cat N°P-4394 from Sigma-Aldrich was purchased. Stock solution (50 mM) was prepared in DMSO, then different concentrations were prepared as follow: 0.33  $\mu$ M, 3.3  $\mu$ M, 16.5  $\mu$ M and 33 $\mu$ M. After six days of culture the cells were treated with specific doses of cisplatin (0.33, 3.3, 16.5 or 33 $\mu$ M), control cells were treated with equal amounts of DMSO. Then the protocol used for erlotinib was followed to evaluate the effect of cisplatin.

### **2.14.3 Temozolomide Treatment**

Temozolomide compound (Catalog # 1849) was purchased from LKT laboratories, Inc. An initial stock solution of 200 $\mu$ M was prepared and then different drug concentrations were prepared: 1 $\mu$ M, 10 $\mu$ M, 50 $\mu$ M, 100  $\mu$ M and 200  $\mu$ M. Cells were seeded at 500 cells/well in 24 well plates previously covered with agarose as described earlier. Plates were incubated under standard conditions. After 5 days of incubation, TMZ treatment was initiated at the following doses 1.0, 10, 50 and 100 $\mu$ M for LN428 cells and 0, 10, 50, 100 and 200 $\mu$ M for U87. The plates were scanned every other day for tumor colony formation in the Gel-Count<sup>TM</sup> instrument and readings were recorded for both cell lines. After calculation of colony number and biomass, data was graphed and analyzed using GraphPad Prism software program as described in section 2.14.

## **2.15 Statistical Analysis**

The results of xenograft studies were analyzed for survival using Kaplan Meier estimator and for their significance using Gehan-Breslow-Wilcoxon Test. In general, a  $P < 0.05$  was considered as statistically significant. For inhibitor studies we established  $IC_{50}$  using the GradPad Prism software program. For cell viability, WST1 assay and anchorage independent studies statistically differences were determined using the one-way ANOVA test. We considered  $P < 0.05$  or  $P < 0.0001$  as statistically significant.

## **CHAPTER 3 RESULTS**

### **3.1 Identification of EGFR Ectodomain mutations**

EGFRvIII represents the most common mutation in GBMs with an incidence of about 50% in GBM patients whose tumors show amplification of EGFR (94, 266-269). The in frame deletion between the exons 2 and 7 of this variant leads to important changes in the functional characteristics of the receptor, which are recognized as essential to its oncogenic effects (177-179, 182, 270).

In our study we focused our attention on newly described EGFR ectodomain point mutations that are located in the region deleted in EGFRvIII. We used TCGA information to identify these mutations, using the database found at their website (<http://tcga-data.nci.nih.gov/>). We selected the following mutations: R108K, T263P, A289D and A289T for in depth examination because they represent both some of the most common mutants (at T263 and at A289), and mutants predicted to have the strongest structural impact (at R108 – see below). Additional information obtained from TCGA data base is found in Table 3. It includes the analysis of a cohort of 536 tumor samples where the mutation T263P showed the highest frequency of occurrence (5%) followed by the mutation A289T (4.4%) and mutation R108K exhibited the lowest incidence (0.56%). Similar to the occurrence of EGFRvIII with EGFR amplification, the ectodomain mutations that we identified also coexisted with EGFR amplification where the highest level of amplification was observed for the mutation A289T (CNA 10.3) and the lowest for the mutation T263P (CNA 2.9).

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MUTATION	FREQUENCY	CNA / NUMBER OF SAMPLES
R108K	0.56%	3.2 / 24
T263P	5.00%	2.9 / 24
A289D	3.89%	7.2 / 24
A289T	4.44%	10.3 / 24

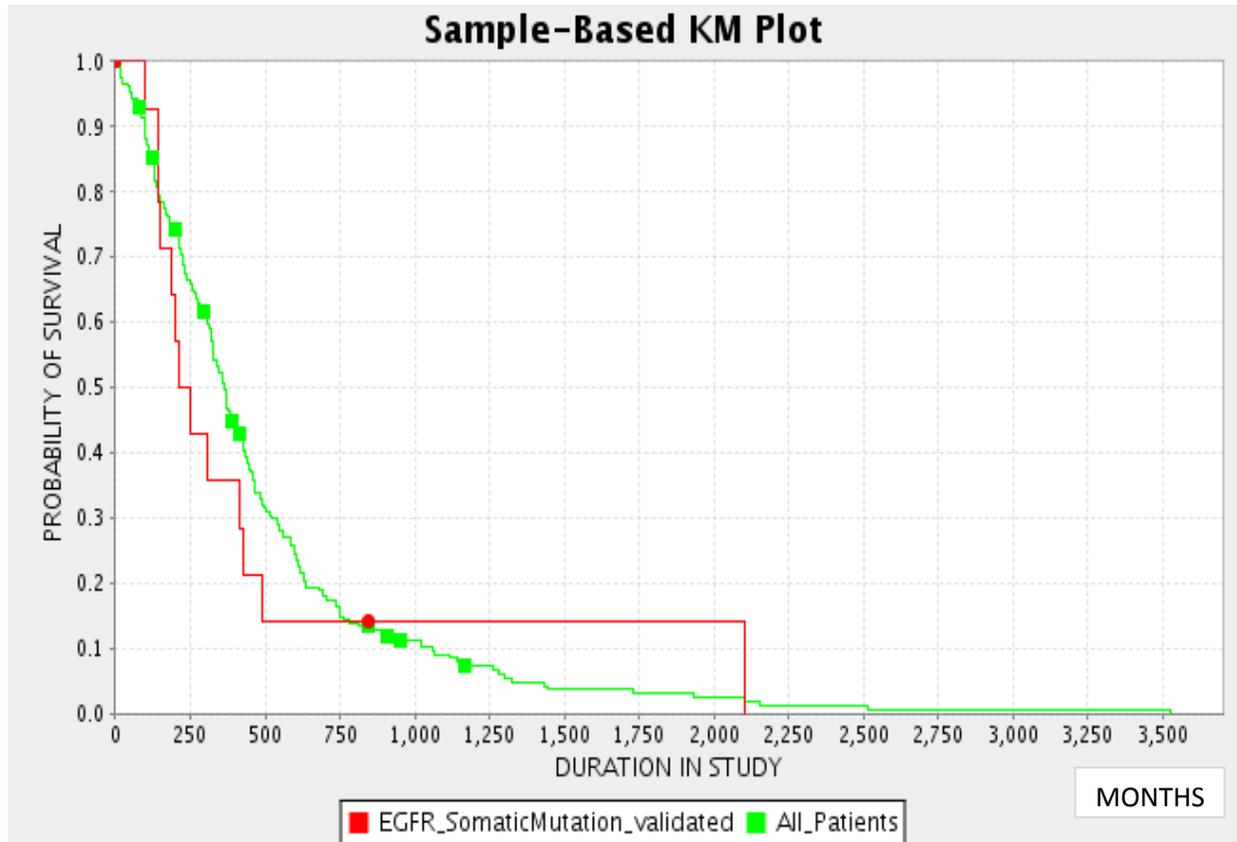
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**Table 3 EGFR ectodomain mutants identified by TCGA**

Ectodomain point mutants occurring between exons 2 and 7 of EGFR recognized after validated sequencing and additional data obtained from the TCGA (Updated 3/31/11). CNA were obtained from TCGA information as single values.

The TCGA web portal (<https://cma.nci.nih.gov/cma-tcga/>) provides survival analysis information, assessing survival differences between patient population that have EGFR somatic mutations or lack it. We found that the group of patients exhibiting EGFR somatic mutations showed shorter survival versus longer survival seen in the

group of patients without EGFR mutations (p-value 0.64). However, there the difference between the two groups is not statistically significant as shown by the log-rank p-value of 0.64 (**Figure 1**).



**Figure 1 Probability of survival in GBM by TCGA**

Graph showing shorter overall survival in GBM patients with EGFR somatic mutations versus all GBM patients. According to TCGA information differences between both groups are not statistically significant after calculate the log rank p-value using the Mantel-Haenszel method (p-value 0.64). Data from TCGA at <https://cma.nci.nih.gov/cma-tcga/>.

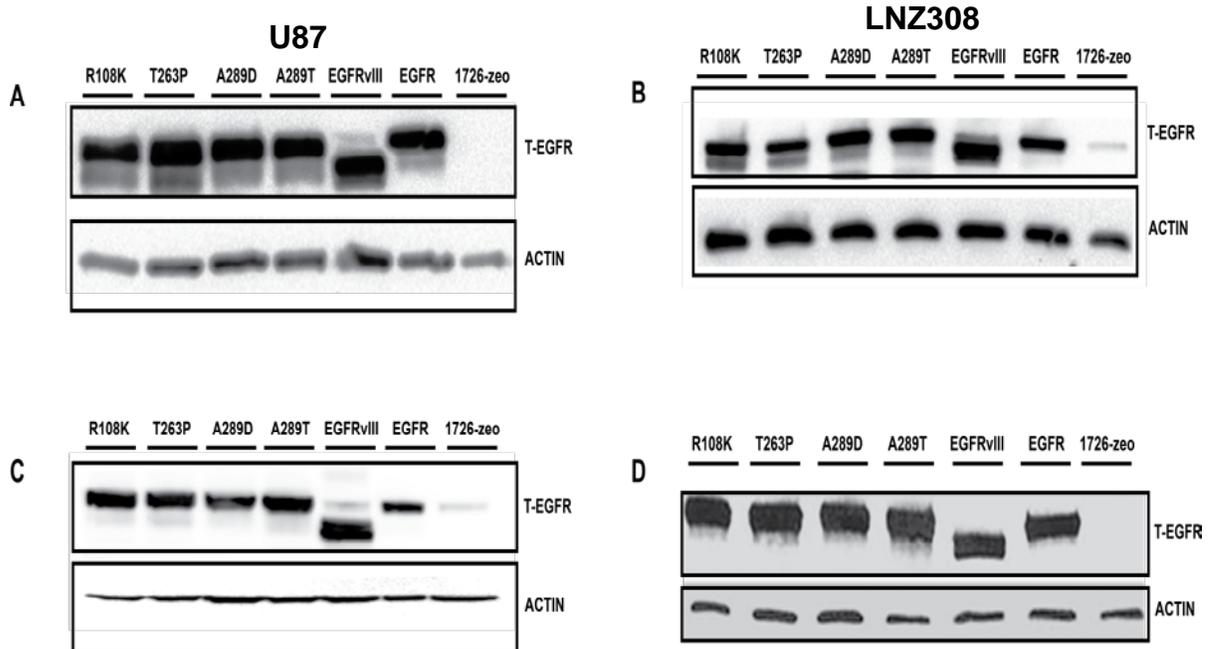
Further, the MSKCC tools allowed us to predict the functional impact of mutations empirically (271), using an algorithm based on the assessment of the evolutionary

conservation of amino acid residues in the protein family. This allows for determination of relevant or irrelevant structural alterations in the parent molecule due to the different genetic variations (272). This analysis has facilitated the stratification of the mutations in 3 groups, where the T263P mutation is categorized as the mutant that demonstrate low functional impact while R108K mutation falls in the group where there is high functional impact and the mutants A289T and A289D were included in the group with medium impact.

### **3.2 Stratification of ectodomain mutations according to molecular profiles**

Mutational studies have demonstrated that the increased tumorigenicity arising from the EGFRvIII mutation is dependent on the autophosphorylation sites situated in the regulatory carboxyl terminus. Tyr-1173 has been shown to be the main phosphorylation site for this variant (29, 89, 179). Mutation on Tyr-1173 residue significantly reduced the phosphorylation levels of the receptor, with the reciprocal mutation of all autophosphorylation sites except Tyr-1173, having a less profound impact (273). Mutations involving all three major phosphorylation sites, Tyr-1173, Tyr-1148 and Tyr-1068 (29, 179), very substantially reduce the levels of autophosphorylation and the tumorigenesis-enhancing characteristic of EGFRvIII (179). Additional studies on the role of these major phosphorylation sites in tumorigenesis of GBMs have indicated a redundant effect on the activation of downstream signaling pathways, suggesting that multiple tyrosine phosphorylation residues on the receptor may serve to recruit similar adaptor proteins (29). We therefore wanted to investigate the impact of the ectodomain mutations on the phosphorylation status of the receptor and downstream signaling events.

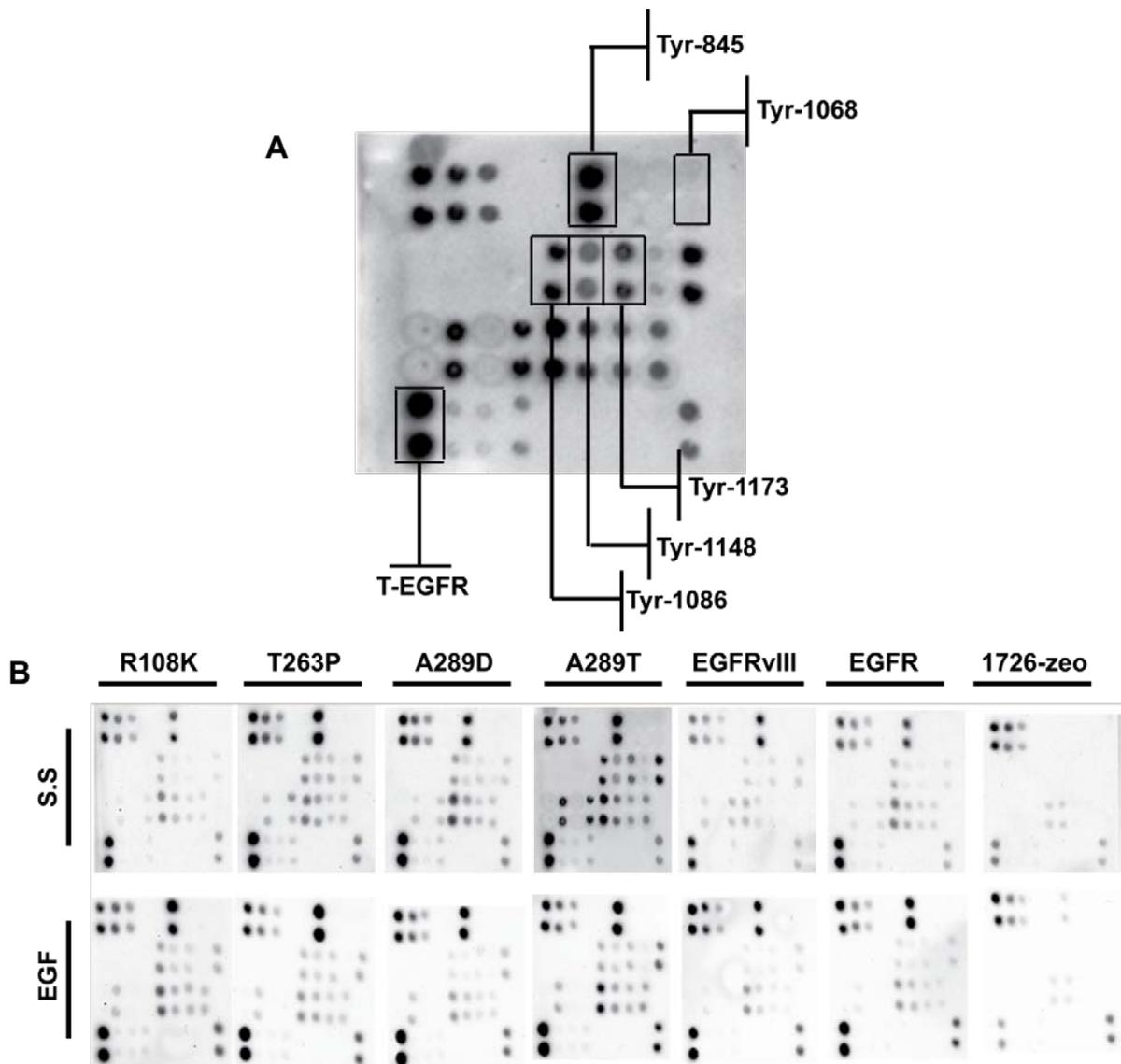
In our study, we generated different glioblastoma cell lines (U87, LNZ308 and LN428) and CHO cells stably expressing the EGFR ectodomain mutants R108K, T263P, A289D and A289T as well as EGFRvIII and EGFR. The expression of EGFR ectodomain mutations in the different glioblastoma and CHO cell lines was confirmed



using western blot (Figure 2).

### Figure 2 EGFR ectodomain mutants overexpressed in stable cell lines

Western blot analysis confirms the expression of ectodomain mutants including EGFRvIII, EGFR and 1726-zeo (expressing the empty retrovirus) after transduction of the mutants in A-U87, B-LNZ308, C-LN428 and D-CHO cell lines.



### Figure 3 EGFR phosphorylation antibody array analysis

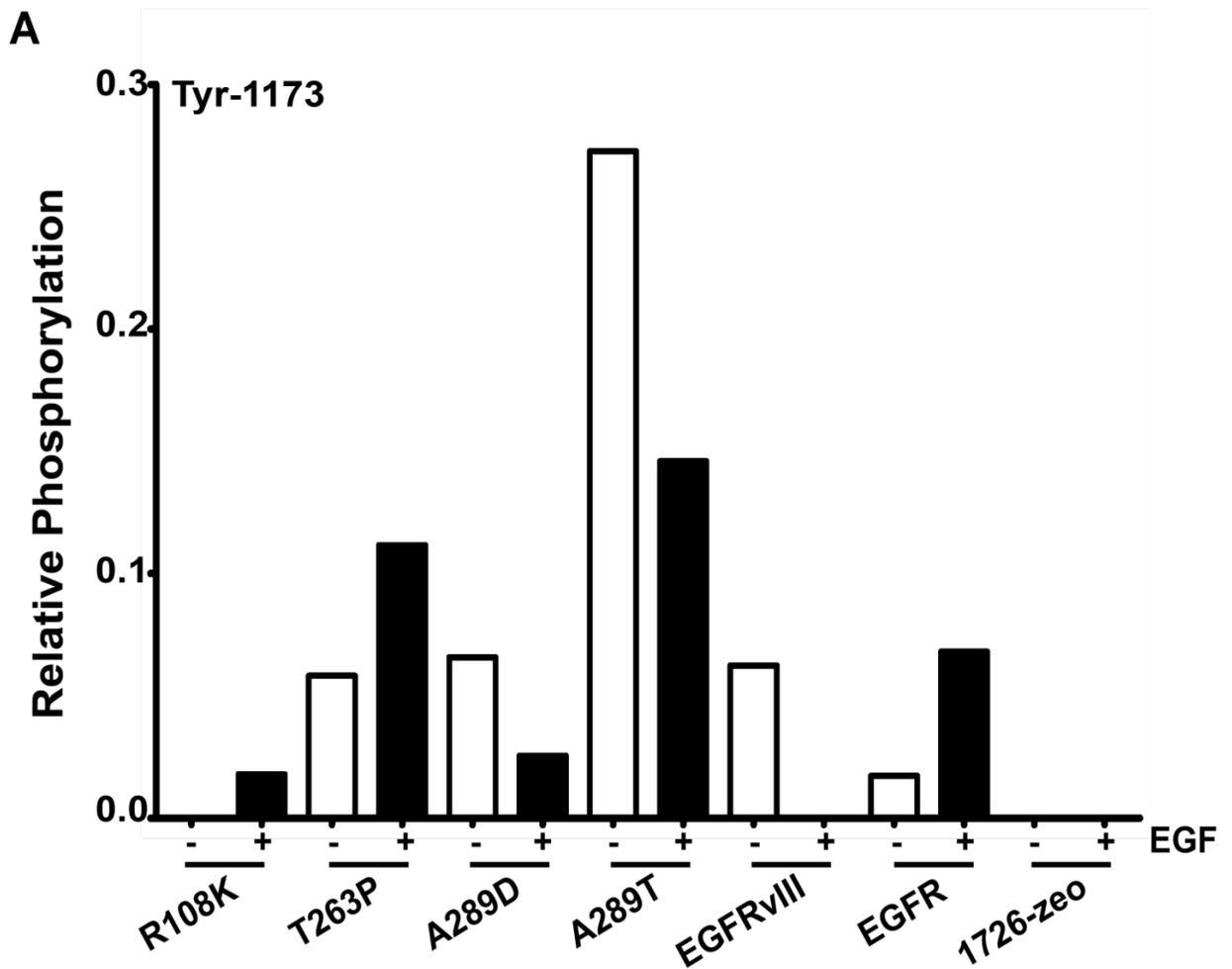
A. Map of the antibody array showing membrane localization of the critical tyrosine residues B. Shows antibody array profiles from U87 cells overexpressing EGFR ectodomain mutants including EGFRvIII, EGFR and the negative control, 1726-zeo in both serum starved (S.S) and ligand stimulated (EGF) conditions.

We used U87 cells stably overexpressing the ectodomain mutants to analyze the

tyrosine phosphorylation profile of the ectodomain mutants using the human EGFR phosphorylation antibody array technique. This array simultaneously detects relative levels of different phosphorylation sites across the ErbB family using site specific antibodies. We mapped and quantified relative autophosphorylation levels on tyrosine sites (Tyr-1173, Tyr-1148, Tyr-1068, Tyr-845) after normalization with EGFR total fraction and biotinylated protein controls. The phosphorylation status was assessed in two different conditions: serum starved condition and after EGF stimulation using 15 ng/ml for 5 minutes (**Figure 3**).

In our analysis we compared the phosphorylation of the ectodomain mutations with either EGFRvIII or EGFR primarily at the tyrosine residues. Additionally, validation for residues Tyr-1173, Tyr-1068 and Tyr-845 using conventional western blot technique was also performed. In general, the same trend was observed for the mutants in our western blot analysis as well as the array analysis in terms of tyrosine phosphorylation levels under both serum deprived and EGF stimulated conditions. Our findings suggest different profiles of auto-phosphorylation for EGFR ectodomain mutations in the tyrosine sites and are enumerated below:

Tyr-1173. It has been reported that the Tyr-1173 residue has a critical function as an activator of the Ras/Raf/MAPK pathway after EGF stimulation resulting in mitogenic and transforming activities (89, 93, 179). This residue also provides a secondary binding site for the SH2 containing adaptor protein, Shc. This tyrosine site is the major phosphorylation site of EGFRvIII and is also considered as a critical phosphorylation site in tumorigenesis of GBM.(274). When we assessed for the relative levels of phosphorylation for Tyr-1173 site in serum starved condition, we detected basal

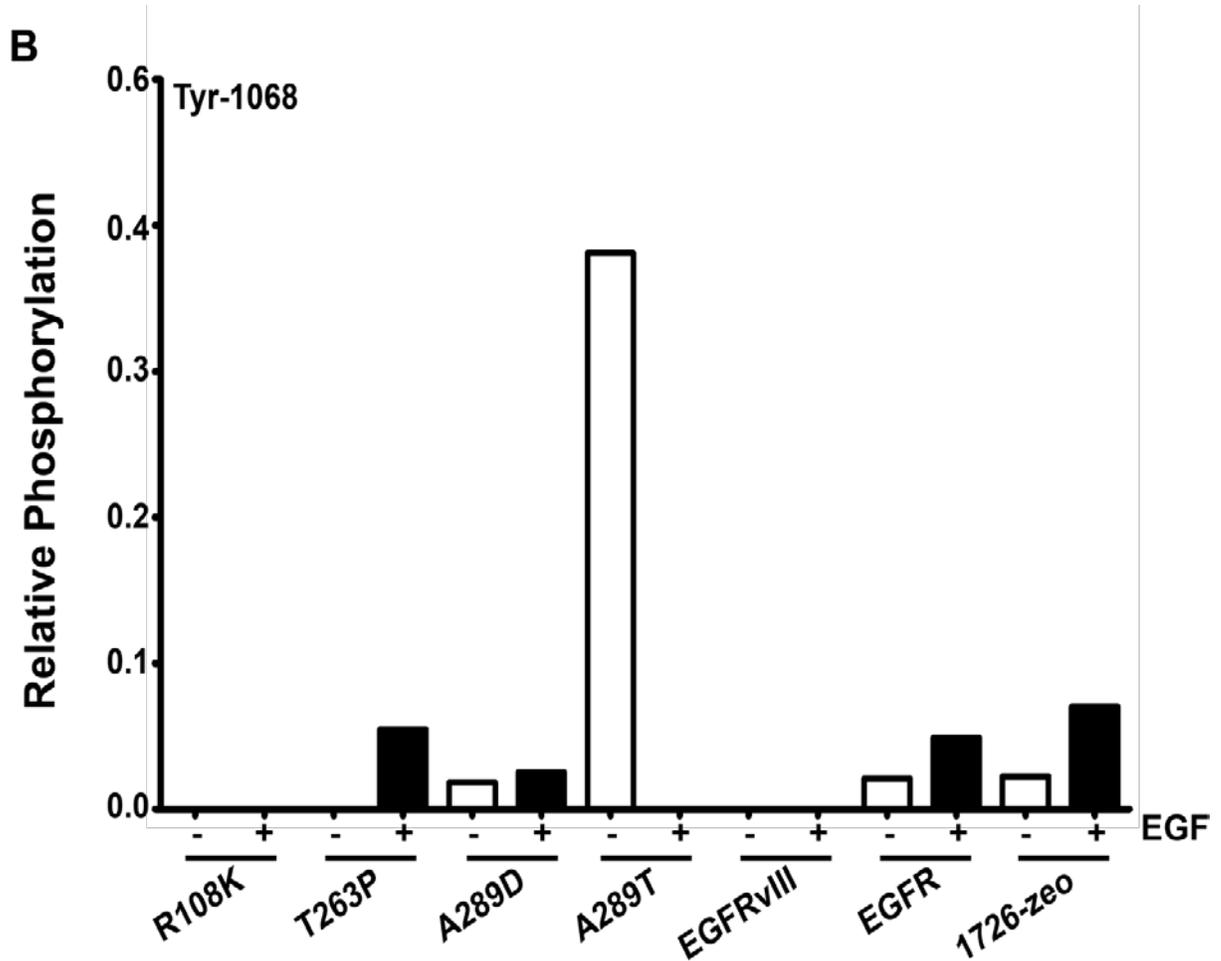


**Figure 4 Differential phosphorylation profiles for Tyr-1173 across EGFR ectodomain mutants**

U87 cells overexpressing ectodomain mutants and also EGFRvIII, EGFR and the negative control (1726-zeo) in serum starved and after EGF stimulation were subjected to array analysis and relative levels of phosphorylation after normalization to total EGFR and biotinylated controls were mapped for Tyr-1173. Data from a single experiment.

activation across all the mutants except for the mutation R108K which did not show

detectable levels of phosphorylation. The highest level of phosphorylation was observed for the A289T mutant. After EGF stimulation, we found that ectodomain mutations R108K and T263P showed robust increases in the levels of Tyr-1173



**Figure 5 Differential phosphorylation profiles for Tyr-1068 phosphorylation across EGFR ectodomain mutants**

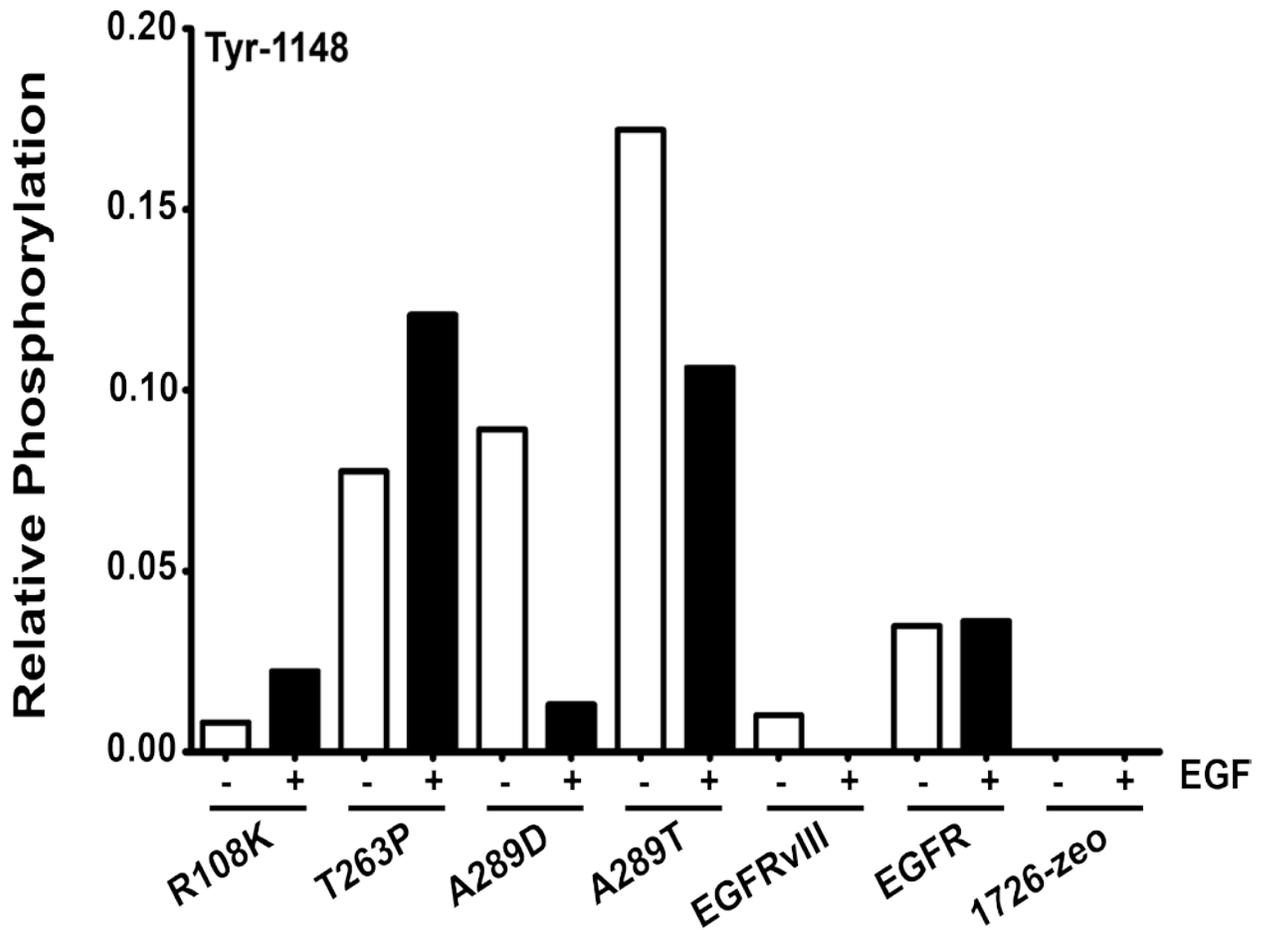
U87 cells stably overexpressing ectodomain mutants and also EGFRvIII, EGFR and the negative control (1726-zeo) in serum starved and after EGF stimulation were subjected to array analysis and relative levels of phosphorylation after normalization to total EGFR and biotinylated controls were mapped for Tyr-1068. Graph illustrates data from a single experiment.

phosphorylation which resembles the profile observed for EGFR. In contrast, the mutations A289T and A289D exhibited similar characteristics as that of EGFRvIII, which does not respond to the EGF ligand, and if anything showed lower signal after EGF stimulation (**Figure 4**).

Tyr-1068. Previous investigations have found that this residue interacts with the adaptor protein Grb2 and activates Erk1/2 through SOS as well as Ras (93, 182, 275). This residue has also been shown to be activated in GBMs (179, 276). In our study, we were unable to detect Tyr-1068 phosphorylation in R108K and EGFRvIII overexpressing cells in both serum starved and EGF stimulated conditions. However, mutants T263P and A289D showed increased phosphorylation after stimulation with EGF a profile similar to EGFR. In serum starved condition, the mutant A289T showed substantial levels of Tyr-1068 phosphorylation. In contrast, marginal phosphorylation was observed in the A289D mutant (**Figure 5**).

Tyr-1148. Early investigations have proposed this site as the major binding site for the adaptor protein Shc, which has an important role in the activation of MAPK pathway (274). It is considered to be among the conserved sites that are activated in tumor development in GBMs (276) (179). Our analysis indicated significant basal levels of phosphorylation in serum starved condition for Tyr-1148 particularly for the mutants T263P, A289D and A289T and to a minor degree for the mutants R108K and EGFRvIII. EGFR also showed basal activation at this site. Further, we observed a slight increase in the levels of phosphorylation for Tyr-1148 after EGF ligand stimulation for EGFR and similarly the trend was maintained for the mutants R108K and T263P. In contrast, A289D and A289T mutants showed no obvious response to

C

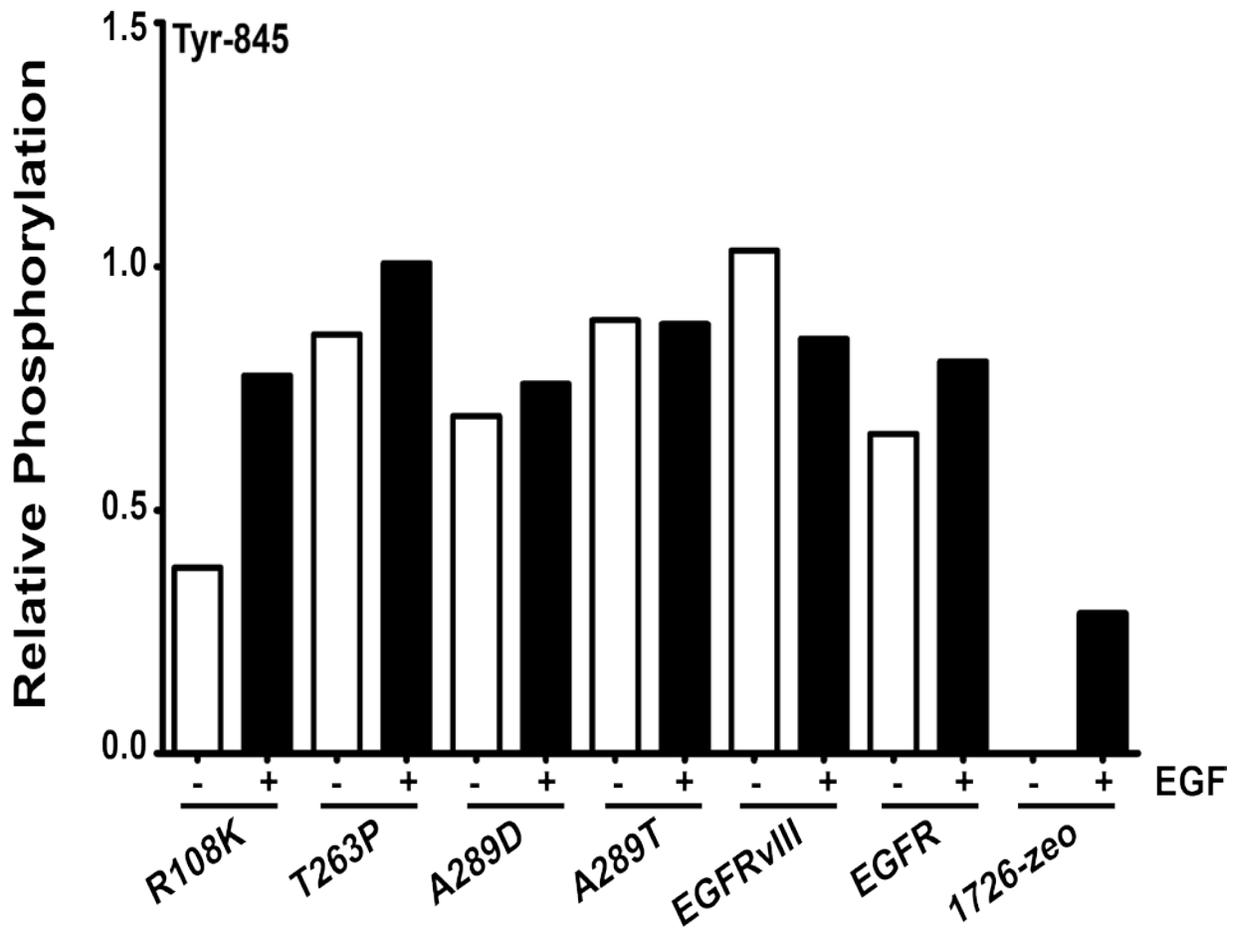


**Figure 6 Differential profiles of phosphorylation for Tyr-1148 across EGFR ectodomain mutants**

U87 cells overexpressing ectodomain mutants and also EGFRvIII, EGFR and the negative control (1726-zeo) in serum starved and after EGF stimulation. Graph shows the relative levels of phosphorylation for Tyr-1148 after array analysis, data belongs to a single experiment and was normalized to total EGFR and biotinylated controls.

EGF ligand stimulation resembling the trend seen in EGFRvIII (**Figure 6**).

D



**Figure 7 Differential phosphorylation profiles for Tyr-845 across EGFR ectodomain mutants**

U87 cells overexpressing ectodomain mutants and also EGFRvIII, EGFR and the negative control (1726-zeo) in serum starved and after EGF stimulation were subjected to array analysis and relative levels of phosphorylation after normalization to total EGFR and biotinylated controls were mapped for Tyr-845. Graph represents data from a single experiment.

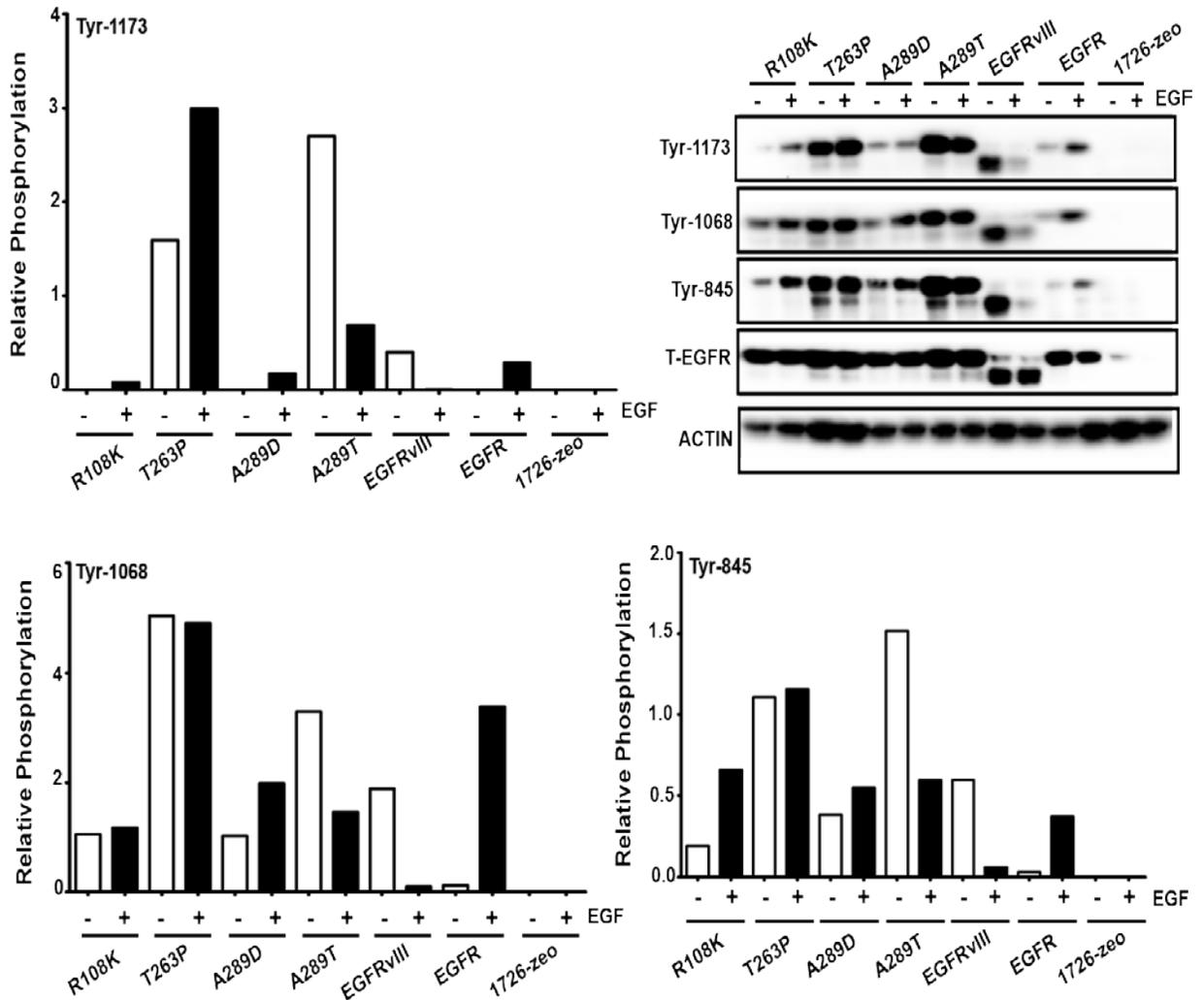
Tyr-845. The Tyr-845 site is phosphorylated by c-Src. Previous studies have demonstrated that this site is of vital importance in cellular proliferation and cell cycle progression (29, 277). This site has also been implicated in stabilizing the activation loop necessary for EGFR'S enzymatic activity (278).

Additional studies have indicated the importance of this activated residue in stimulating additional pathways such as the transcription factor STAT5b (277). This site is also required for the optimal activation of EGFRvIII. Mutation of Y845 site in EGFRvIII leads to decrease in autophosphorylated levels of EGFRvIII and suppression of downstream signaling as well as cell growth (29). We observed in general that all the EGFR ectodomain mutants as well as EGFRvIII and EGFR showed high basal phosphorylation levels on Tyr-845 in serum starved condition, and that in general these levels were not profoundly altered by EGF. One exception was R108K, which displayed a marked increased phospho-levels of Tyr-845 after EGF stimulation **(Figure 7)**.

To provide experimental support for these results from antibody array analysis, we performed validation using western blot for the sites Tyr-1173, Tyr-845 and Tyr-1068.

Similar trends were seen across all the mutations for Tyr-845 after validation. For Tyr-1173 site, mutants R108K, T263P and A289T showed comparable profiles in the validation to those seen in the array analysis. Similarly, tendencies seen in Ray-Bio analysis for mutations R108K, A289D and A289T for Tyr-1068 were confirmed after

validation analysis (**Figure 8**).

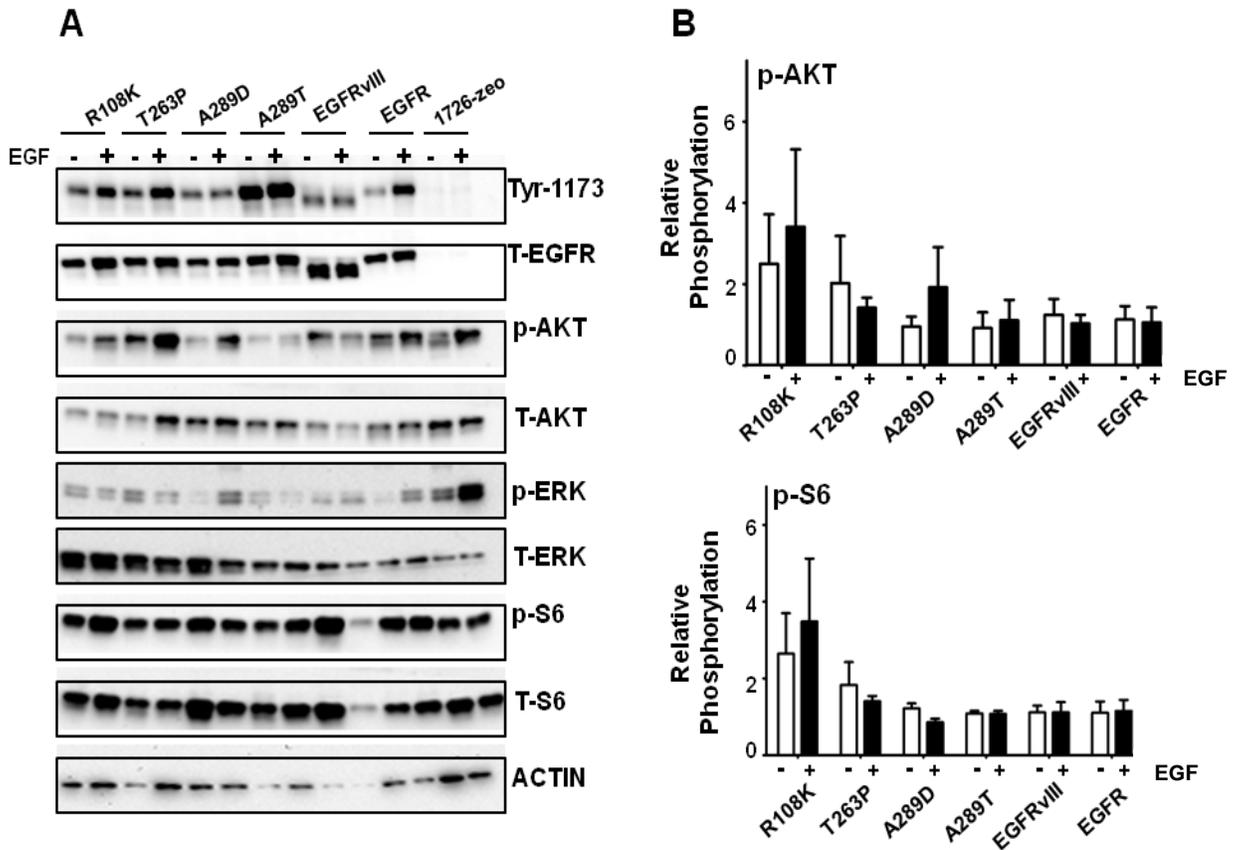


**Figure 8 Western blots validation for selected tyrosine residues**

Signaling densities from the western blots were normalized to total EGFR and actin values and then mapped. Graphs show relative phosphorylation levels from a single representative experiment for Tyr-1173, Tyr-1068 and Tyr-845.

Overall, this analysis showed that individual residues of EGFR mutants showed complex and variable levels of basal and ligand-stimulated activity, with the

ectodomain mutants showing some response to ligand. Of note in our analysis, the A289T mutant displayed persistent elevated basal levels of phosphorylation across the multiple tyrosine sites in the antibody array and this pattern was consistent after

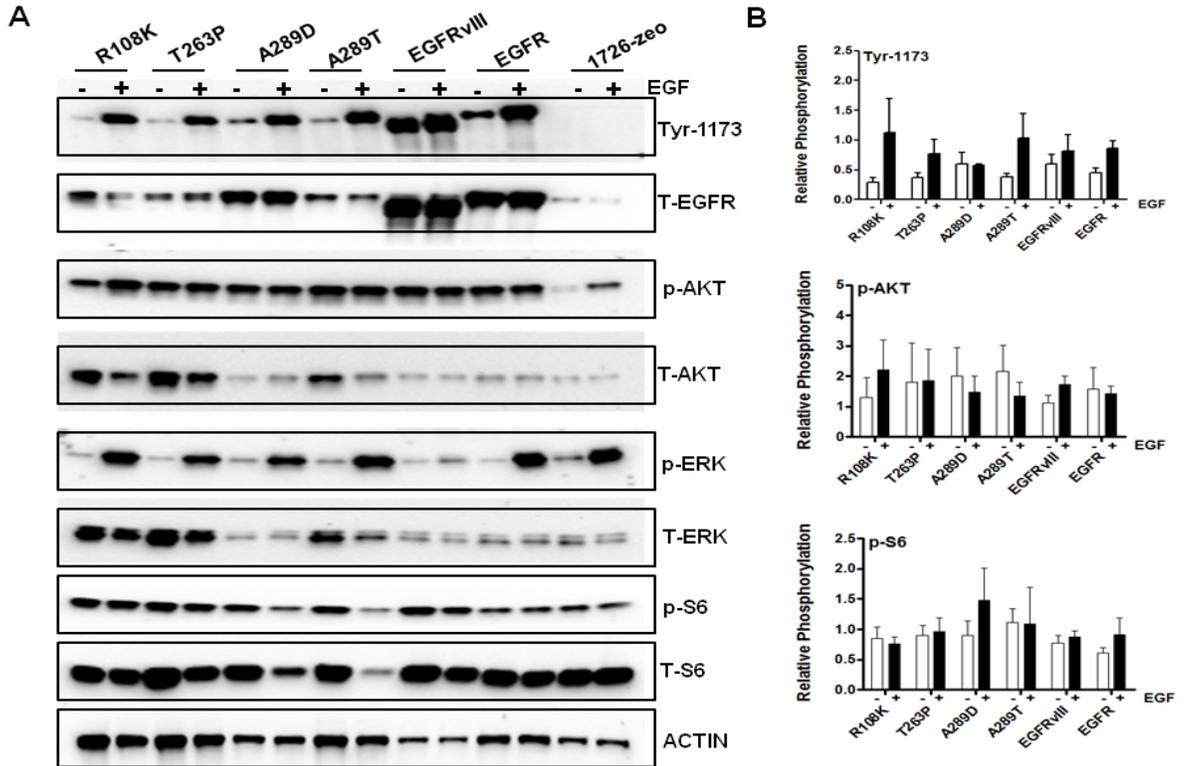


**Figure 9 Downstream signaling of ectodomain mutants overexpressing U87 cells**

A. Western blots show phospho EGFR-Tyr-1173, p-AKT-S473, p-ERK (p-MAPK Thr202/Tyr204) and p-S6-Ser235/26 kinase activation in serum starved and after EGF stimulation of ectodomain mutants overexpressed in U87 cells. B. Graphs depict relative levels of phosphorylation after normalization to total protein for AKT, and S6 and actin fractions. Data from three independent biological experiments. Error bars indicate standard deviation of the mean.

validation using western blot technique, and so resembled EGFRvIII most closely.

As a next step, we examined the impact of ectodomain mutations on downstream signaling, by assessing for active forms of AKT and ribosomal S6 kinases in serum



**Figure 10 Downstream signaling of EGFR ectodomain mutants overexpressing LNZ308 cells**

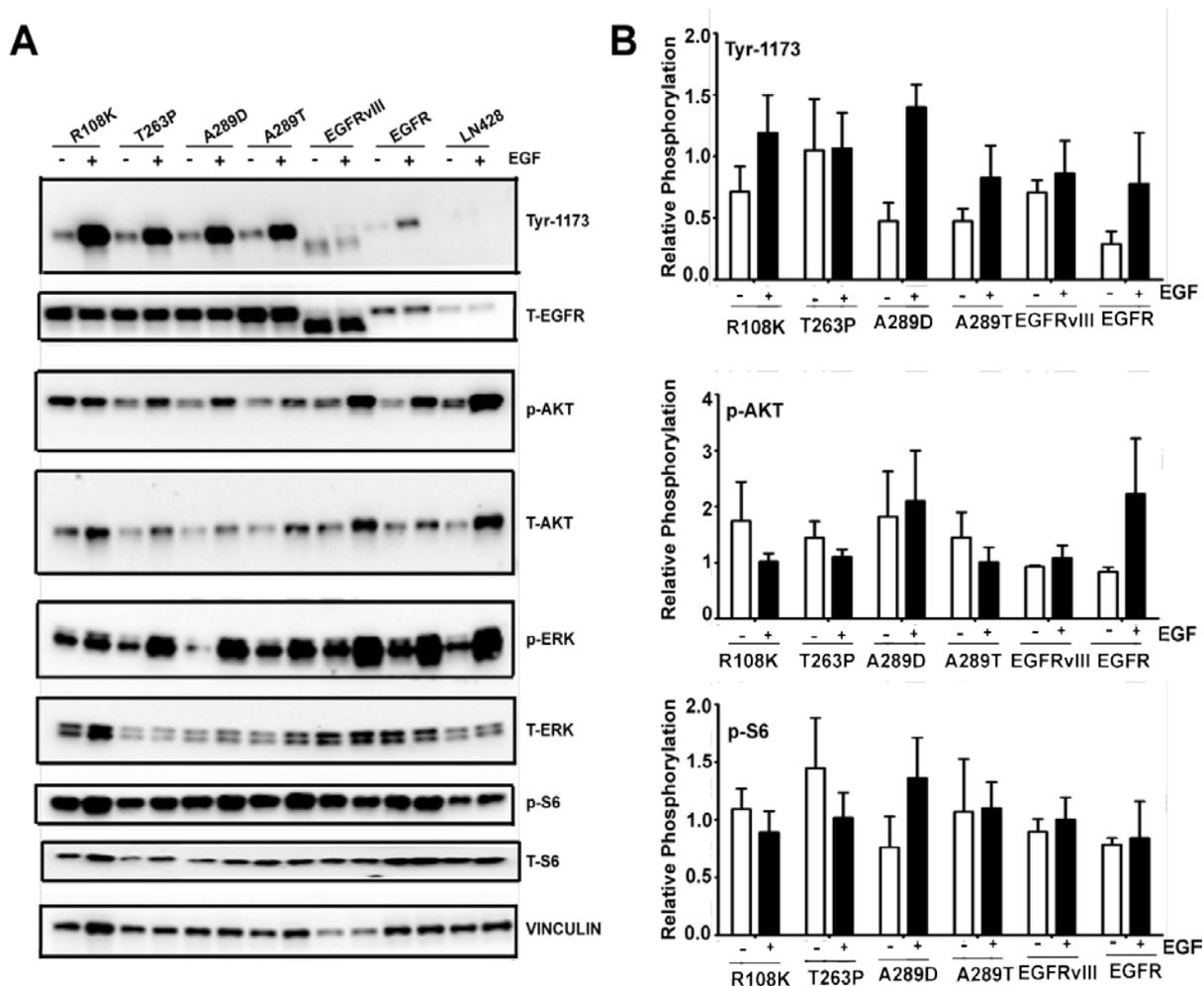
A. Westerns show phosphotyrosine profile and downstream kinase activation of canonical pathway components of EGFR ectodomain mutants overexpressed in LNZ308 cells in serum starved condition and after EGF stimulation. B. Graphs depict relative phosphorylation levels from three independent biological experiments for EGFR-Tyr-1173 and kinase activation for AKT and S6 after normalization to total protein fractions and actin levels. Error bars are standard deviation of the means.

starved and EGF stimulated conditions. In U87 cells which are PTEN null, the findings

did not show significant differences in activation across the mutations in the two pathways in both conditions, which may represent an overall deregulation of the PI3K pathway in these cells (279, 280) **(Figure 9)**.

In addition, we also assessed the contribution of the ectodomain mutations in different molecular backgrounds using stable LNZ308, which are p53 and PTEN mutated as well as LN428 cell lines which contain intact p53 and PTEN. These two panels of cells overexpressing EGFR ectodomain mutations were evaluated in serum starved and after EGF stimulated conditions. Our findings suggest differences in basal activation in both contexts:

In LNZ308 cells, the level of basal phosphorylation for Tyr-1173 was very weak especially for the mutants R108K, T263P and A289T. The mutant A289D showed slightly higher levels of phosphorylation. Examination of the phosphorylation levels for AKT and ribosomal S6 suggested no significant changes in activation of the two pathways in both conditions, again probably related to a loss of PTEN function **(Figure 10)**.



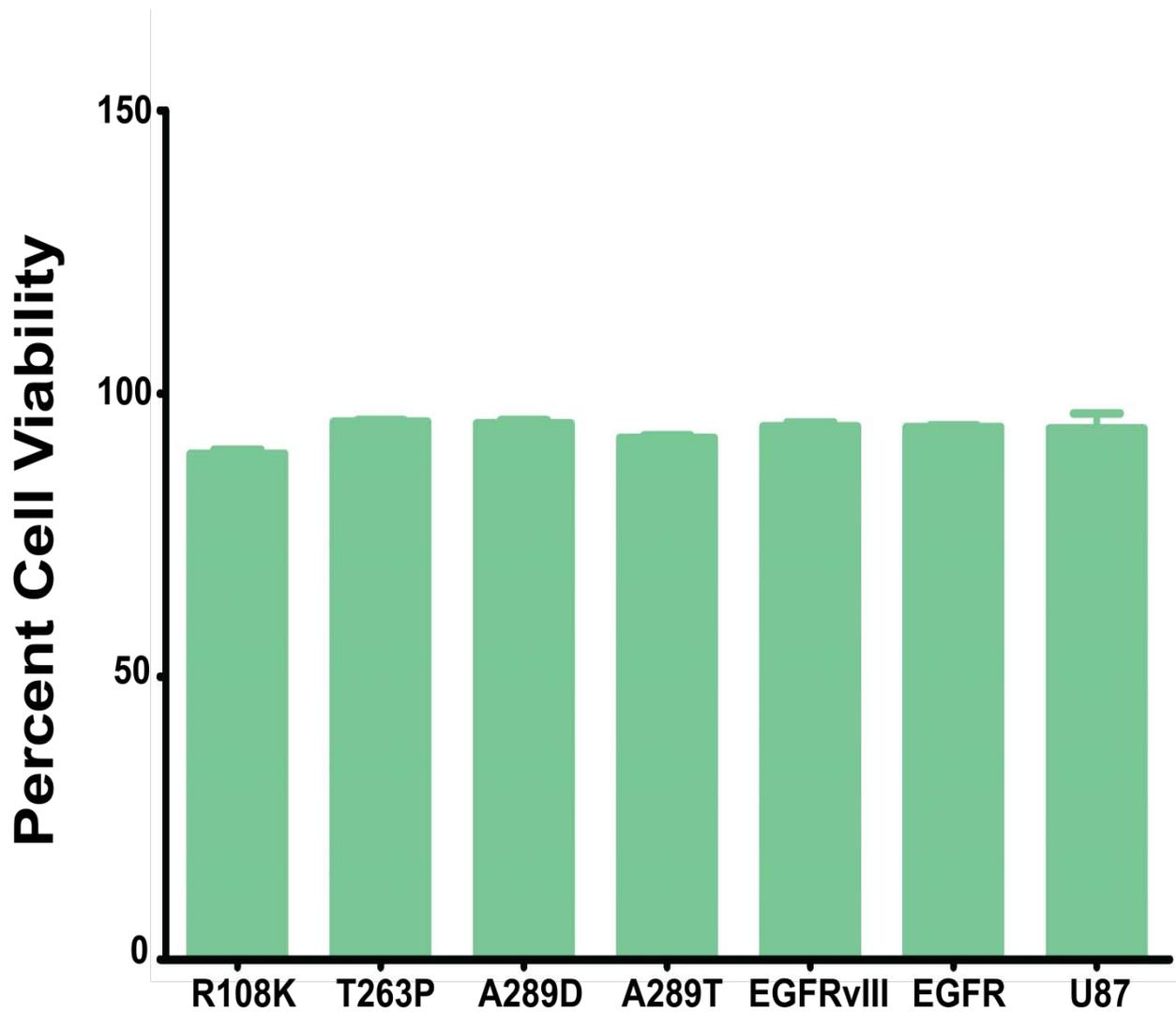
**Figure 11 Basal of EGFR ectodomain mutants overexpressed in LN428 cells**

A. Shows western blot panels for EGFR-Tyr-1173, p-AKT-S473, p-ERK (p-MAPK Thr202/Tyr204) and p-S6-Ser235/26 kinase activation of EGFR ectodomain mutants overexpressed in LN428 cells in serum deprivation and after EGF stimulation conditions. B. Graphs display relative phosphorylation levels after normalization of signaling densities to total proteins and actin levels. Data from three independent biological replicates. Error bars indicate standard deviation of the mean.

In the context of LN428 cells, the basal activation for Tyr-1173 residue was more robust across all the mutants than the pattern seen in LNZ308 cells. We also evaluated the effect on activation of downstream signaling events originating from these mutants. While we observed EGF-induced increases in Akt and S6 phosphorylation, we also found differences in total levels of these proteins, which correlated. This may reflect a difference in the stability of these proteins after activation. As a result, we were not able to report statistically significant differences for Akt or ribosomal S6 kinase activity in either condition (**Figure 11**).

### **3.3 Impact of EGFR ectodomain mutations on cell viability and proliferation**

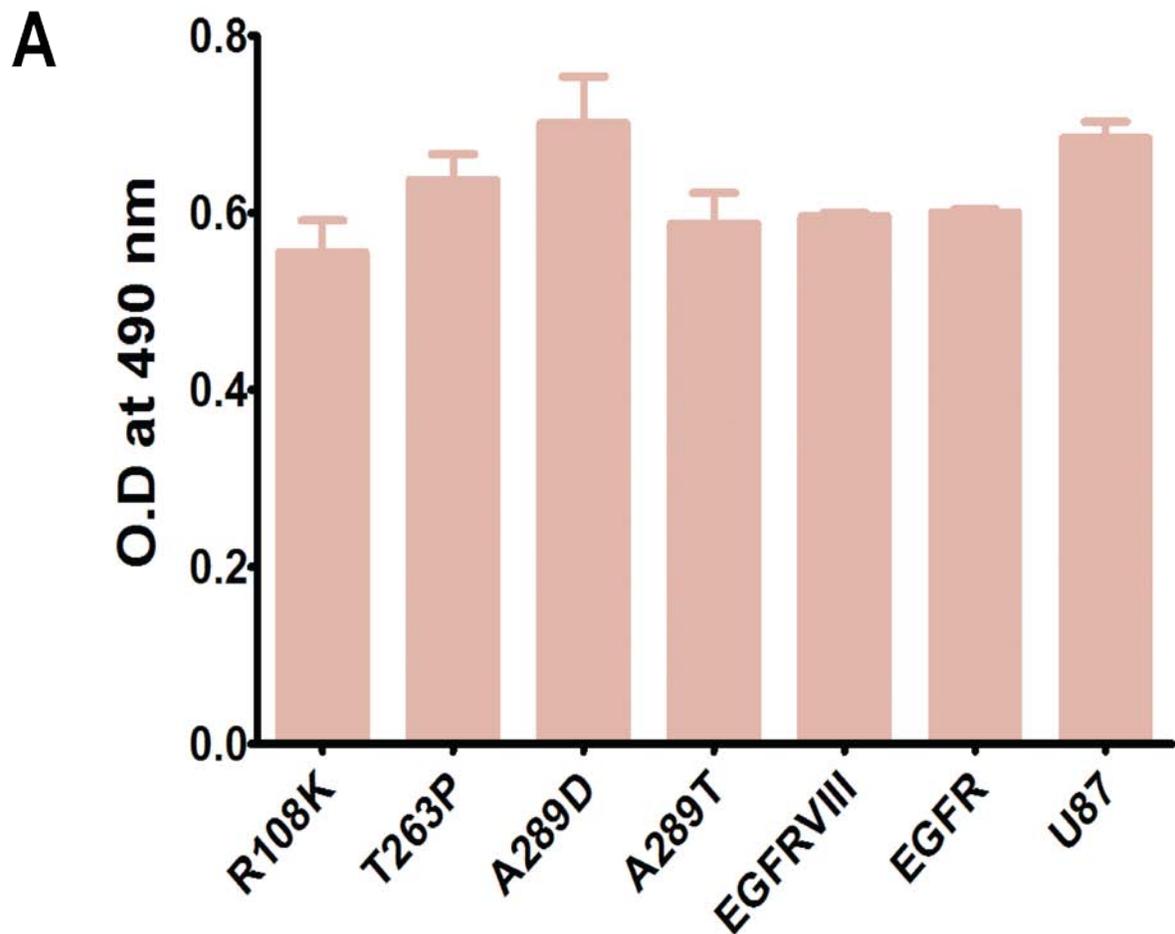
We investigated the effect of these mutants on cell viability using U87 cells overexpressing EGFR ectodomain mutants. Upon comparison among the different ectodomain mutants, EGFRvIII and EGFR we did not find significant differences in the cell viability. The extent of viable cells across all the mutations including EGFRvIII and EGFR were very similar (>90%) (**Figure 12**).

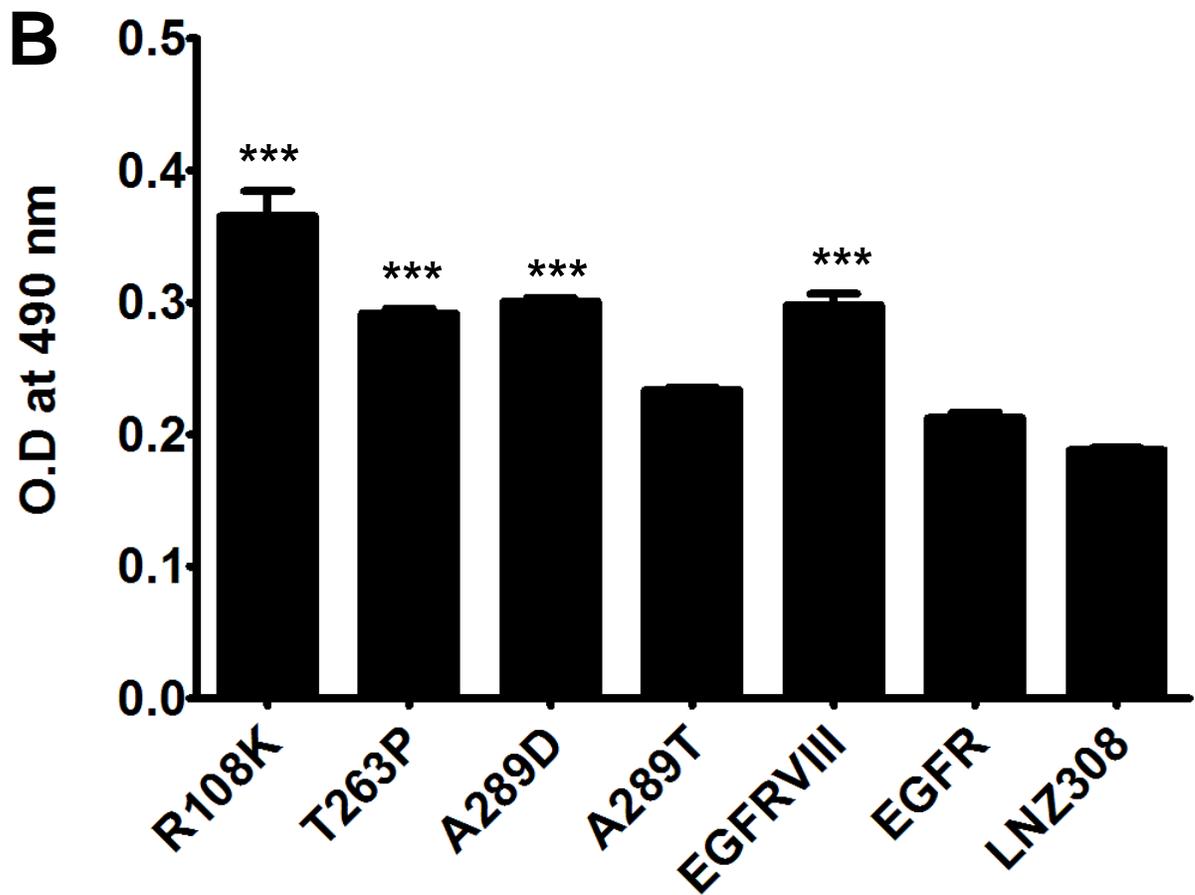


**Figure 12 Effect of EGFR ectodomain mutations overexpressed in U87 cells on cell viability**

Graph illustrates percentage of viable cells from the different ectodomain mutants in U87 cells after 72 hours of incubation under standard conditions. Data was obtained from the automated cell viability analyzer from Beckman (trypan blue exclusion test). Error bars are standard deviation of the means from two replicates in the same biological experiment. No statistically differences were detected by one-way ANOVA test ( $p > 0.05$ ).

In addition, we examined the effect of these mutants on cellular proliferation with a WST1 assay, using two different glioma cell lines: U87 and LNZ308 overexpressing ectodomain mutants as well as EGFRvIII and EGFR. We did not observe a significant impact of the ectodomain mutations overexpressed in U87. In contrast, significant differences were observed in the context of LNZ308 cells, where the mutants R108K, T263, A289D and EGFRvIII showed increase in the bioreduction levels as compared to EGFR (Figure 13).





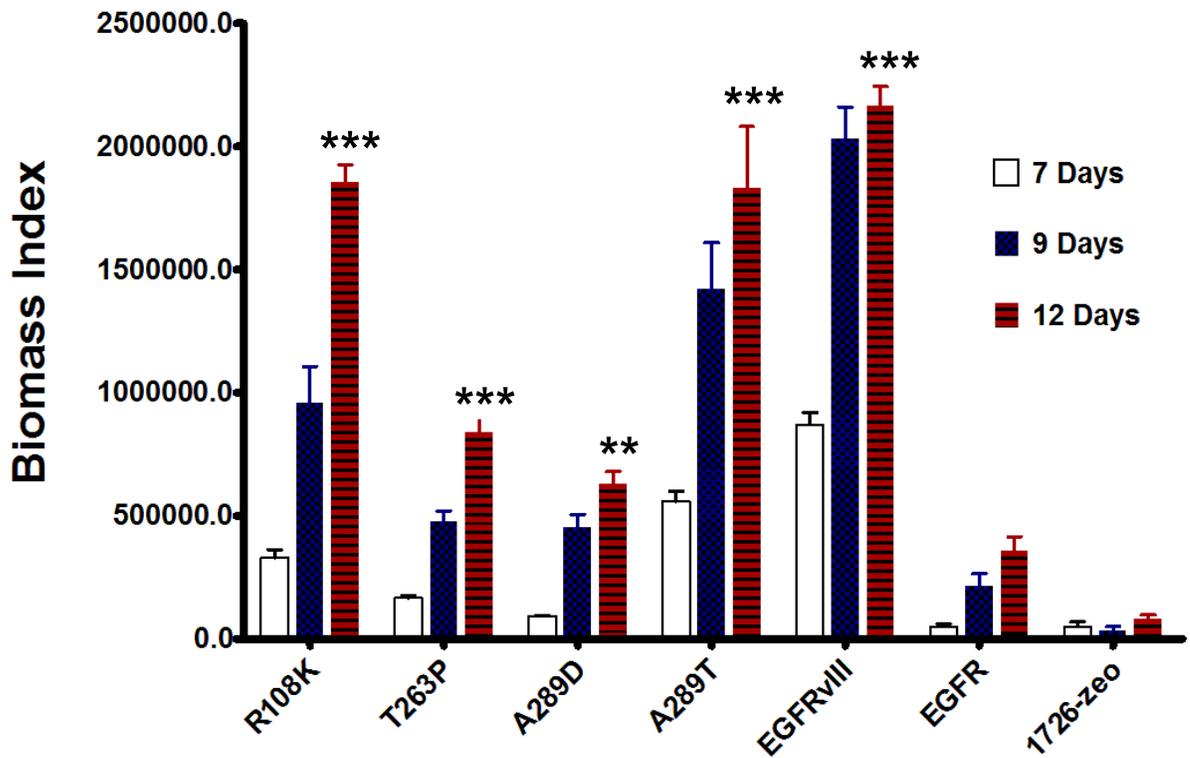
**Figure 13 Cellular proliferation effect of EGFR ectodomain mutants overexpressed in different glioma cell lines**

A. The graph shows bioreduction levels in absorbance units after WST-1 assay of the ectodomain mutants overexpressed in U87 cells after 72 hour of incubation and B. same analysis in LNZ308 cells. Error bars are standard deviation of the means from three replicates in the same biological experiment. No statistically differences were detected in U87 cells overexpressing ectodomain mutants by one-way ANOVA test ( $p > 0.0001$ ). In contrast, statistically differences were observed in the context of LNZ308 for some of the mutants and LNZ308 cells overexpressing EGFR ( $p < 0.0001$ ).

Assays more relevant to determining the capacity of a mutant to promote oncogenicity are: anchorage independent growth and growth rate in xenografts. Furthermore, the literature (91, 116, 174, 281) suggests that EGFRvIII has a profound effect on tumorigenic capabilities of GBM cells as assessed by anchorage independent assays and xenograft studies but not their growth on plastic such as those performed above. Therefore, we decided to analyze the tumorigenic capacities of the ectodomain mutations in these more biologically relevant assays.

### **3.4 Expression of ectodomain mutations enhances anchorage independent colony formation in different glioma cells**

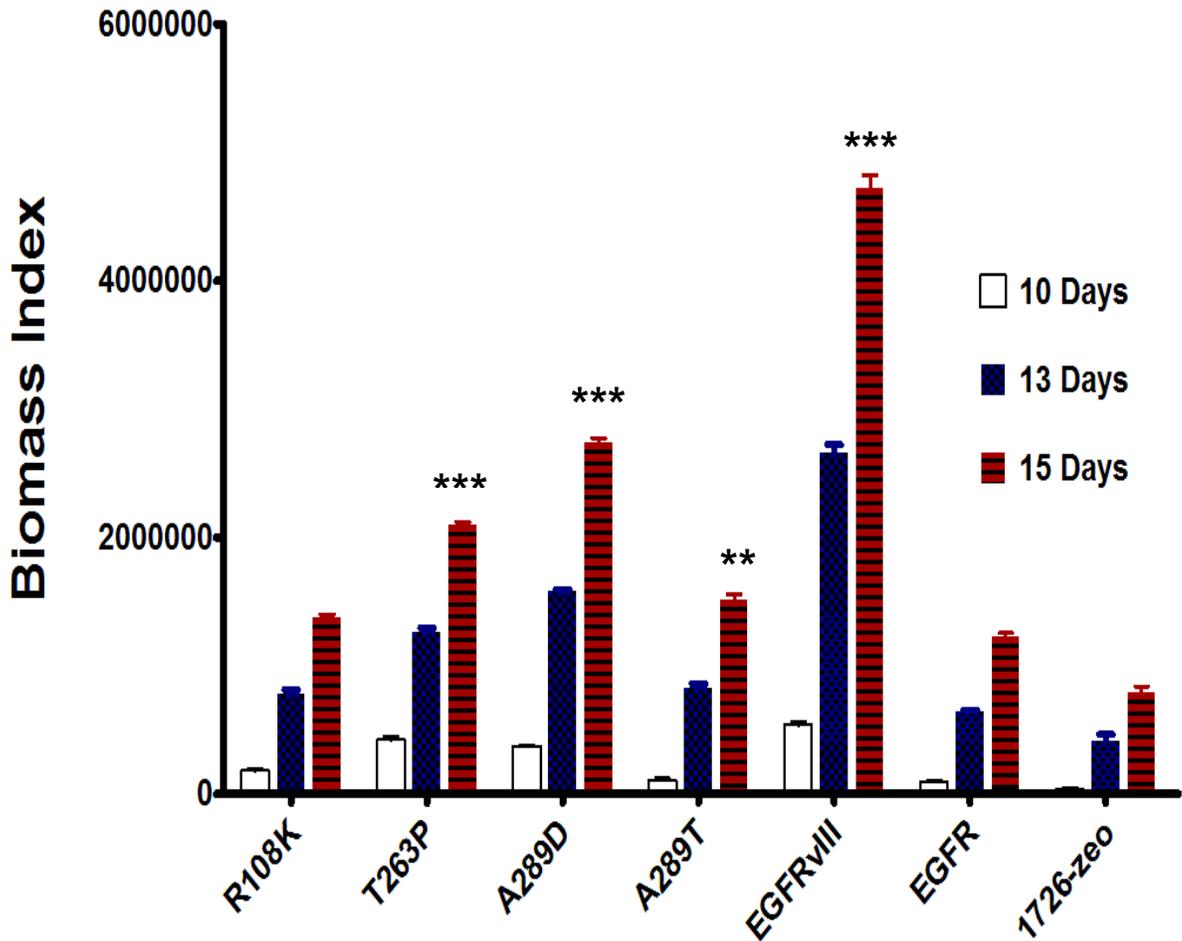
To assess the ability of ectodomain mutations to promote anchorage independent colony formation, we carried out 3-dimensional growth studies on agarose using three different glioblastoma cell lines U87, LN2308 and LN428 that stably overexpressed ectodomain mutations, EGFRvIII and EGFR. The results observed in the set of experiments using stable U87 cells showed an increase in anchorage independent colony formation induced by the ectodomain mutants. It was demonstrated by higher total biomass indices in all the ectodomain mutants as compared to EGFR after twelve days of culture. Among the mutants, R108K, A289T, T263P and EGFRvIII demonstrated the ability to induce higher levels of colony formation in the assay **(Figure 14)**.



**Figure 14 Contribution of U87 cells overexpressing ectodomain mutants in anchorage independent colony growth**

Graph shows total biomass indices of U87 cells overexpressing EGFR ectodomain mutants after 7, 9 and 12 days of incubation. Error bars are standard deviation of the means from three independent biological experiments. Statistically differences were detected by one-way ANOVA test  $P < 0.0001$ .

Extension of these experiments to LNZ308 and LN428 cells showed that ectodomain mutants confer a growth-enhancing phenotype in anchorage independent colony formation in these cell lines as well. In LNZ308 cells, EGFRvIII enhanced the

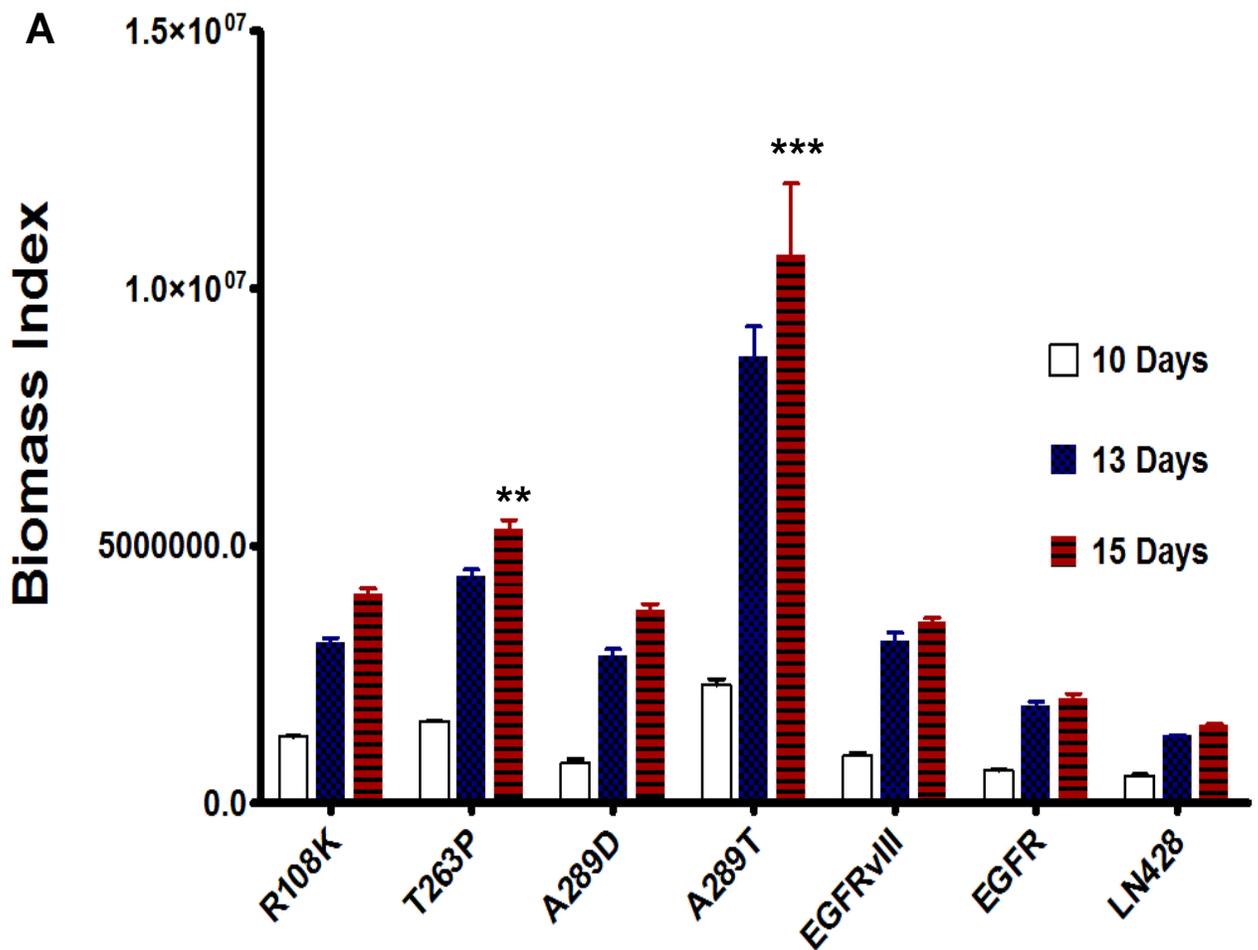


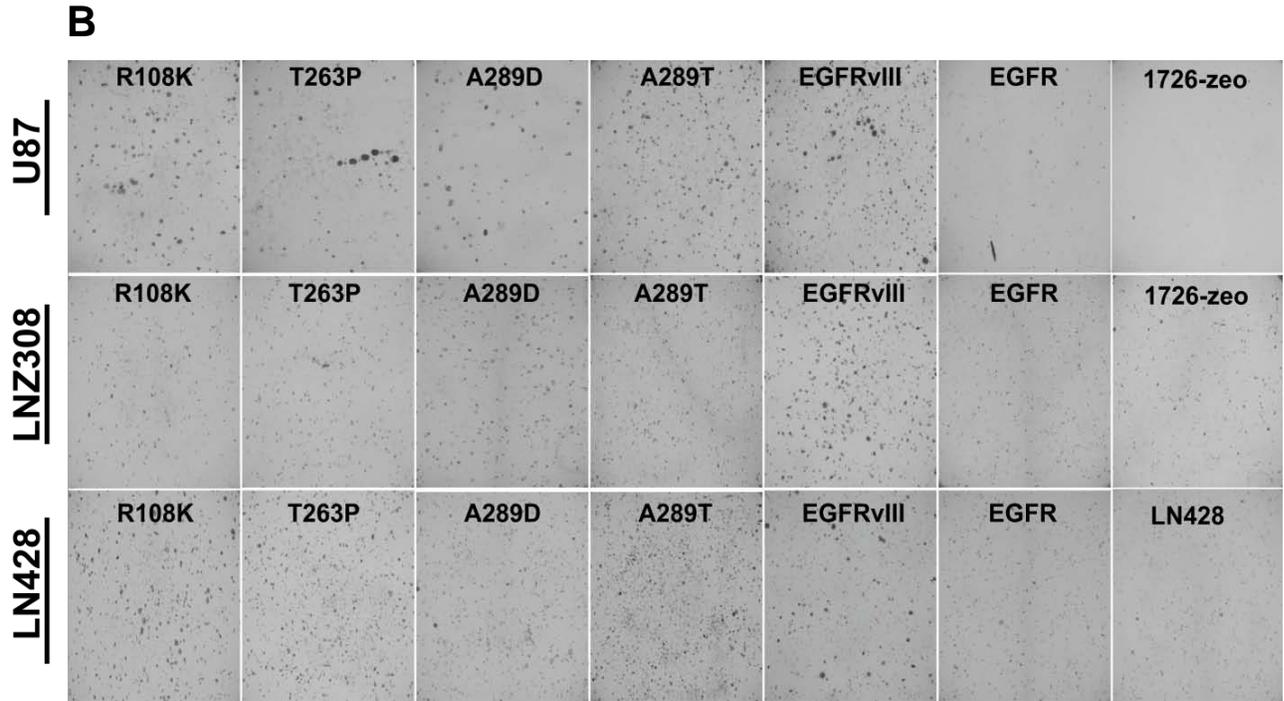
**Figure 15 Contribution of LNZ308 cells overexpressing ectodomain mutants in anchorage independent colony growth**

Graph shows total biomass indices for LNZ308 cells overexpressing EGFR ectodomain mutants after 10, 13 and 15 days of incubation. Error bars are standard deviation of the means from three independent biological experiments. Statistically differences were detected by one-way ANOVA test  $P < 0.0001$ .

growth of the cells to a higher extent (**Figure 15**).

In contrast, in the context of LN428 cells, the mutant A289T exhibited the most substantial increase in anchorage independent growth (**Figure 16**). Overall, the EGFR mutants provided an enhancement of growth in soft-agarose over EGFR, suggesting that they have significant transformation-enhancing properties, consistent with being oncogenes.



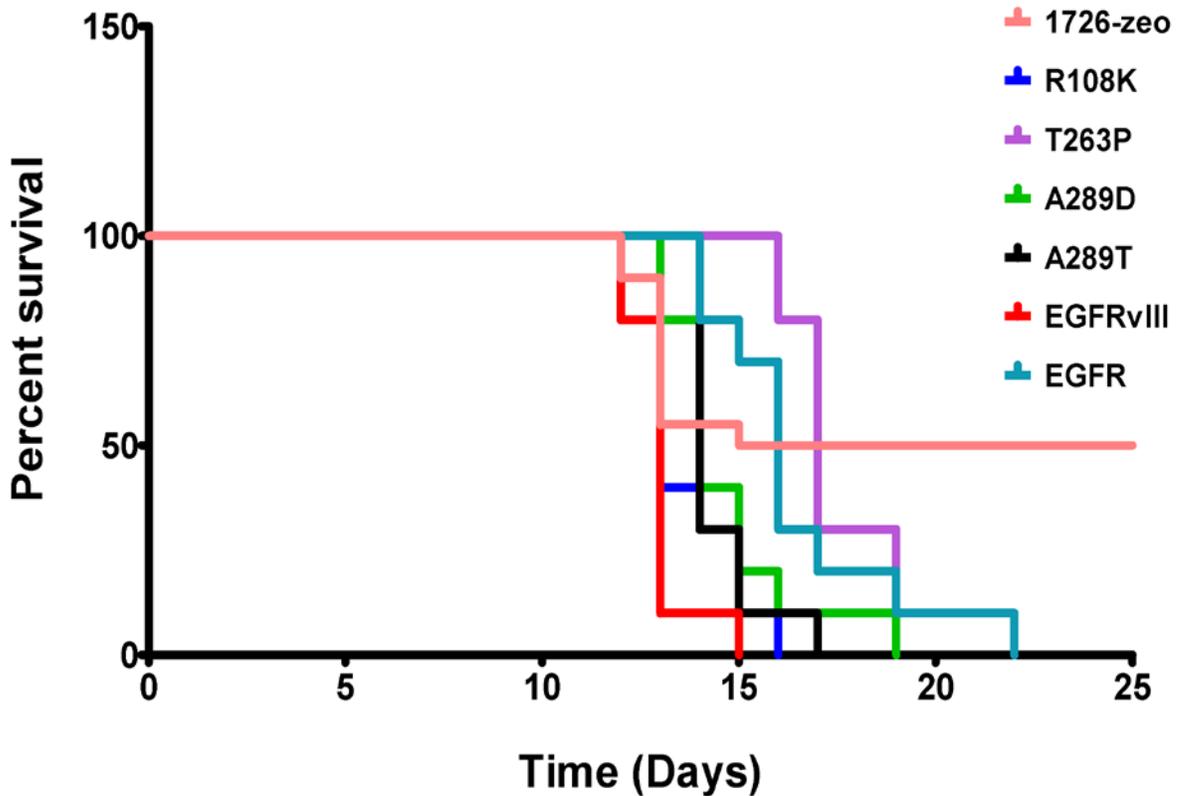


**Figure 16 Contribution of LN428 overexpressing ectodomain mutants in anchorage independent colony growth**

A. Graph shows total biomass indices for LN428 cells overexpressing EGFR ectodomain mutants after 10, 13 and 15 days of incubation. Error bars are standard deviation of the means from three independent biological experiments. Statistically differences were detected by one-way ANOVA test  $P < 0.0001$ . Panel B. Depicts representative images of colony formation obtained from the GelCount instrument for experiments using U87, LNZ308 and LN428.

### 3.5 Expression of EGFR ectodomain mutations decreased tumor latency period in xenograft mice

To correlate *in vitro* results with the ability of these ectodomain mutations to affect



**Figure 17 Kaplan Meier survival analysis in xenograft groups implanted with EGFR ectodomain mutants**

The Kaplan Meier survival analysis graph shows the death of mice in days which indicates the survival function in mice implanted with U87 cells overexpressing the ectodomain mutants, EGFRvIII, EGFR and control group (1726-zeo). All animals that did not die by day 42 were sacrificed. Animal experiment was carried out with the help of the animal core at the Brain Tumor Center (MD Anderson Cancer Center).

tumor formation *in vivo*, we performed xenograft studies in nude mice. U87 stable cell lines overexpressing the ectodomain mutations were implanted intracranially into nude mice and tumor latency periods were recorded as the times at which animals developed neurological symptoms that triggered their euthanasia, according to the IACUC protocol. We evaluated survival time across the different groups of mice using the Kaplan-Meier survival analysis. A decrease in tumor latency period in the group of mice implanted with the EGFRvIII (median survival-13 days) in comparison to EGFR (median survival-16 days) was observed as has been reported (174, 182). Similarly, shorter median survival times were seen in for mice implanted with cells overexpressing mutants R108K (13 days), A289D (14 days) and A289T (14 days). In contrast, mice implanted with cells overexpressing T263P mutant exhibited longer tumor latency (median survival-17 days), which was similar to median survival observed for mice implanted with cells overexpressing EGFR (**Figure 17**).

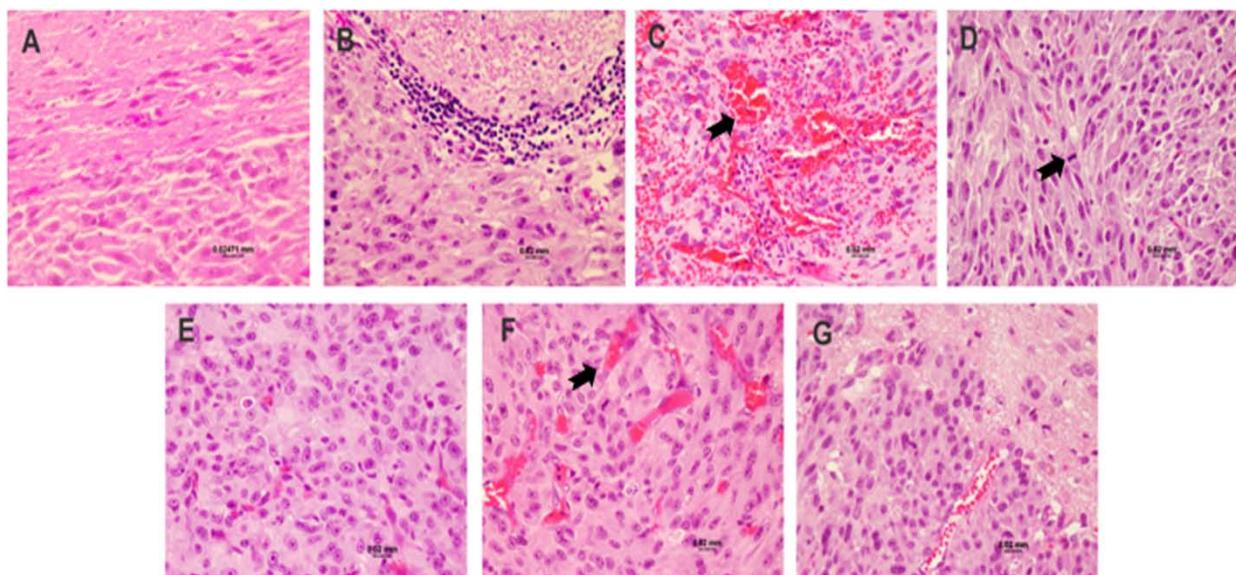
We found that the median survival time for mice implanted with U87 cells overexpressing R108K, A289T and A289D were similar to that seen in mice implanted with cells overexpressing EGFRvIII (13 or 14 days median survival) and significantly shorter than mice implanted with cells expressing EGFR. In contrast, mice implanted with stable cells overexpressing T263P mutant showed similar median survival to that seen in mice implanted with stable cells overexpressing EGFR (**Table 4**)

EGFR Ectodomain Mutations	P Values		Median Survival	
	Mutations Vs EGFR	Mutations vs EGFRvIII	EGFR 16 Days	EGFRvIII 13 days
R108K	0.0017	0.060	13	
T263P	0.068	0.0001	17	
A289D	0.030	0.0005	14	
A289T	0.012	0.0005	14	

**Table 4 Significant differences in survival curves of xenograft groups implanted with U87 cells overexpressing EGFR ectodomain mutants**

Table summarizes the results of the assessment of similarities between the groups of mice implanted with EGFR ectodomain mutants and EGFRvIII or EGFR using the Gehan-Breslow-Wilcoxon test. P-values<0.05 are considered as significant. The last column shows median survival time (in days) for all the groups of mice.

Examination of tissue sections from these mice, after hematoxylin and eosin staining,



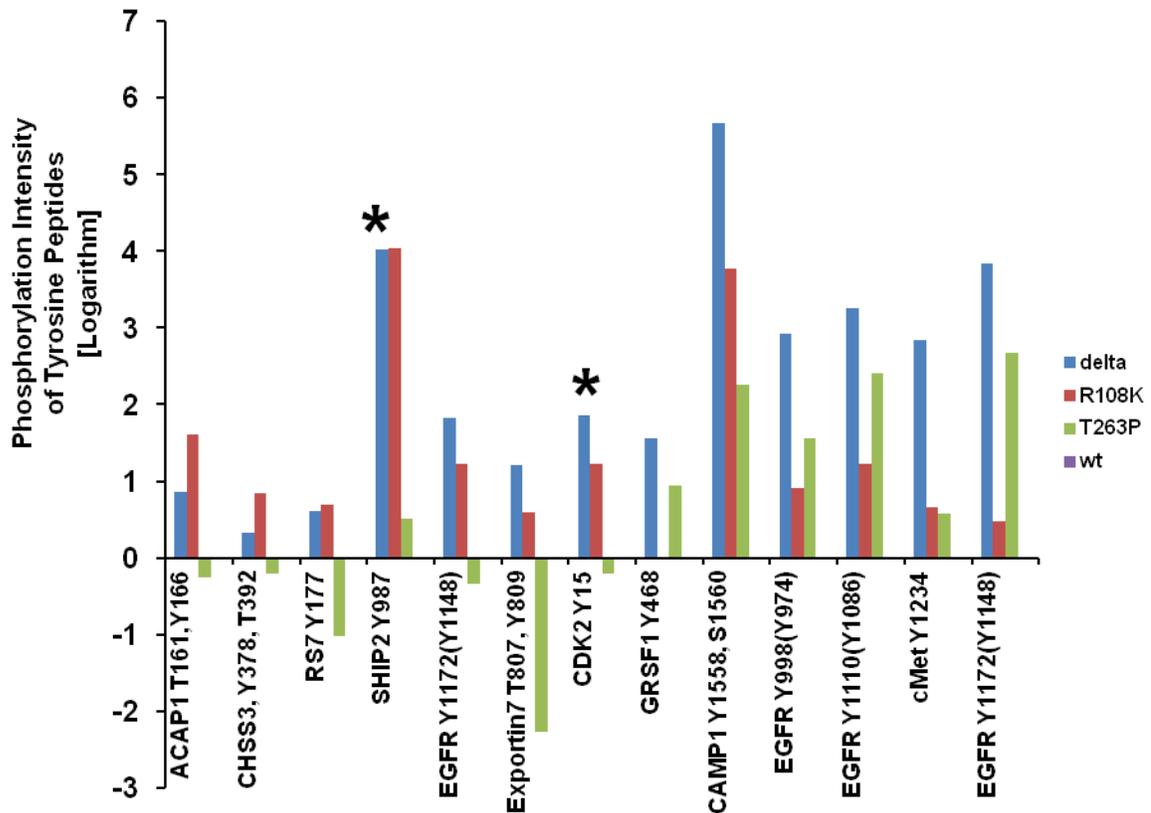
**Figure 18 Histological analysis of xenograft brain tumors**

Brain tumor sections from xenografts were stained with hematoxylin-eosin. Microphotographs show characteristic hypercellularity across all the xenograft tumor sections. Arrows indicate necrotic tissue (C), mitotic figure (D) and neovascularization of the tissue (F). (Scale Bars: 0.02 mm.). Tissue sections were stained at histology core from the division of surgery, MD Anderson Cancer Center.

showed tumors with features characteristic of glioblastoma: hypercellularity, mitotic features as well as necrosis (**Figure 18**).

### **3.6 Phosscan phospho-tyrosine analysis.**

In order to find differences in protein phosphorylation that may form the mechanistic basis for the differences in oncogenic impact of the different EGFR mutants, we performed an unbiased, tyrosine-directed and mass spectrometry based



**Figure 19 Phosphoscan analysis of EGFR ectodomain mutants**

U87 cells overexpressing R108K, T263P, EGFRvIII (delta) and EGFR (wt) were serum starved and the lysates were subjected to treatment with phosphotyrosine antibody to enrich fractions for phosphotyrosine peptides. Graph shows intensity of 13 selected phosphopeptides from a cohort of ~150 phospho-tyrosine peptides obtained after liquid chromatography and mass spectrometry analysis (LC-MS/MS). Asterisks show phosphopeptides with high common relative levels of phosphorylation to EGFRvIII and R108K mutants but different to T263P mutant. Phosphoscan performed, analyzed and graphed by Dr. Chumbalkar, a collaborator from Dr. Bögler's laboratory.

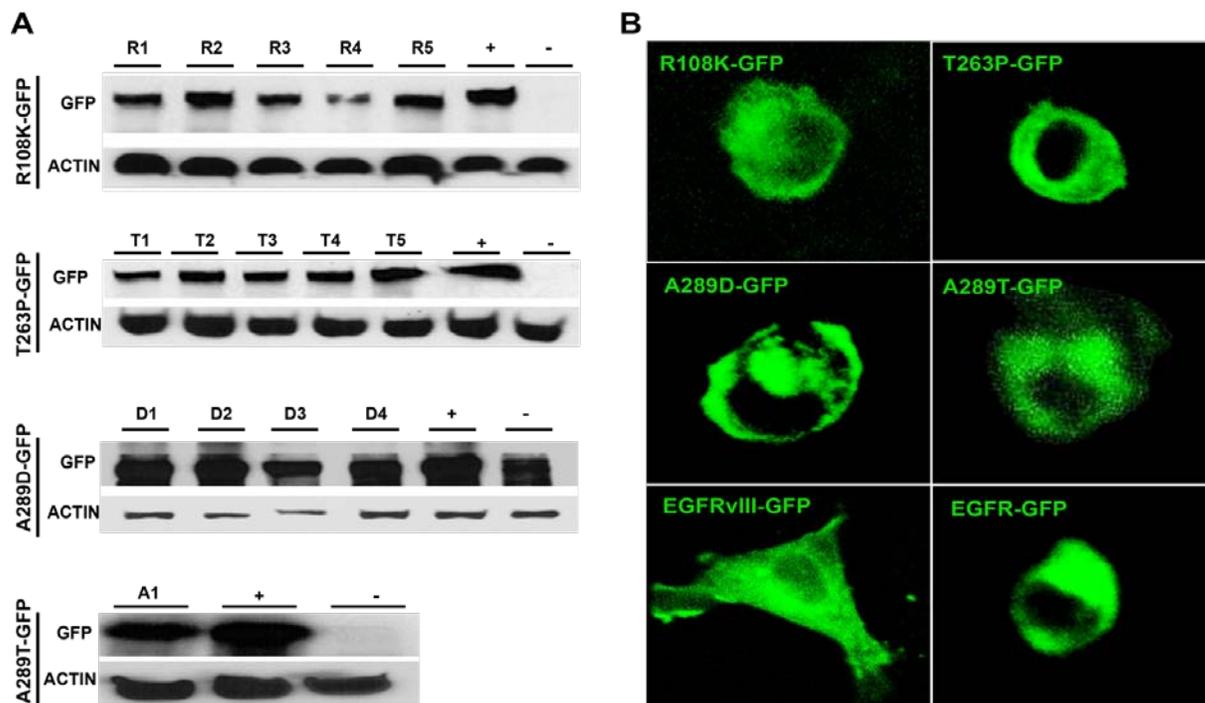
screen using Phospho-Scan analysis on U87 cells stably overexpressing the ectodomain mutations R108K, T263P, EGFRvIII and EGFR that were serum starved for 24 hours. We selected this particular set of mutations because the mutations R108K and T263P showed similar profile of tyrosine phosphorylation with EGFR in the Ray-Bio analysis, but the biological phenotype for these mutations was different. R108K mutant exhibited similar characteristics to EGFRvIII, but the T263P mutation exhibited more similarities with EGFR. In this analysis we identified about 150 phosphopeptides totally. After the filtering of the data set, we obtained a set of 13 phosphopeptides that varied between the two groups (Group I: R108K and EGFRvIII. Group II: T263P and EGFR) (**Figure 19**).

Of the peptides identified, one was common to R108K and EGFRvIII and exhibited substantial levels of relative phosphorylation: SHIP2 Tyr-987. SHIP2 stands for SH2 domain containing inositol 5-phosphatase 2. It is a tyrosine phosphorylated protein (282), which is widely expressed including fibroblasts, non hematopoietic cancer cells and insulin target tissues (283-285) This protein is tyrosine phosphorylated in response to platelet-derived grow factor (PDGF), EGF and insulin-like grow factor 1(IGF-1) in Sh-SY5Y cells or after nerve grow factor (NGF) stimulus in PC-12 cells or in response to insulin in 3T3L1 adipocytes (286). Although the role of tyrosine phosphorylation on SHIP2 is still not clear, mutational studies have indicated an antiproliferative effect of SHIP2 Y987F mutant in pre-adipocytes (26), suggesting that Y987 phosphorylation is important for proliferation. We are in the process of validating SHIP2 Tyr-987 phosphorylation and its role in EGFRvIII / R108K- induced tumorigenesis.

### 3-7 Cellular distribution of ectodomain mutations

Typically, EGFR is a plasma membrane bound receptor, where it activates downstream signaling cascade to elicit its biological response. Recently nuclear localization of EGFR has been demonstrated in various studies in different cancers (92, 287-289). Significantly, nuclear localization of EGFR predicts poor prognosis in different types of cancer (290-292) The role of nuclear EGFR primarily involves transcriptional activation of cyclin D1, B-Myb, iNOS and Aurora-A through binding of ATRS sequences on their promoters, and protein-protein interactions with transcription factors such as, STAT3, STAT5, E2F1 and RHA (92, 290, 293). Studies have also reported nuclear expression of the constitutively activated EGFRvIII (294). More recently, EGFRvIII was shown to be associated with STAT3 (186) to transcriptionally activate Cox2 gene (295).

To examine the subcellular localization of ectodomain mutations, we carried out preliminary confocal microscopic analysis using CHO cells, which do not contain endogenous EGFR. These cells were transiently transfected with GFP-Tagged EGFR mutants as well as EGFR and EGFRvIII and maintained in standard culture media (10% of serum). After microscopic evaluation, our findings showed a similar distribution profile of the EGFRvIII with R108K and A289T mutants, which showed significant cytoplasmic distribution, but also nuclear localization. In contrast, mutants T263P and A289D exhibited more similarity with the pattern showed by EGFR, which is mostly located in the cytoplasmic compartment (**Figure 20**).

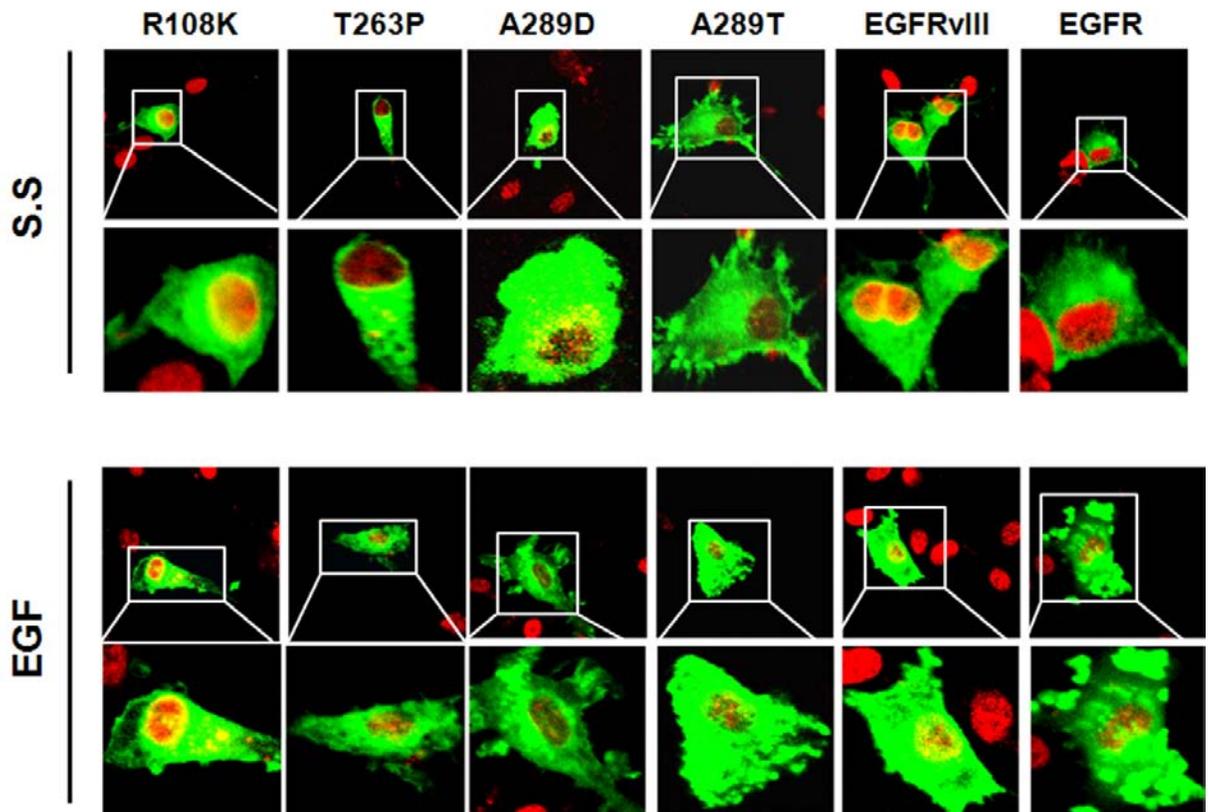


**Figure 20 Cellular distributions of GFP-tagged EGFR ectodomain mutants in CHO cells**

A. shows western blot analysis that confirms the expression of multiple GFP-tagged ectodomain mutants after transient transfection in CHO cells. Sample with the best expression for every mutant was selected for microscopic confocal analysis. B. Results from confocal microscopic analysis show different distribution profiles across the mutants with some of them resembling EGFRvIII or EGFR profiles.

Further we also assed the profile of distribution in glioma cell lines for which we used GFP-Tagged EGFR mutants, which were transiently transfected in the U87 cell line. The profile of distribution was examined in both serum starved condition and after EGF stimulation. In serum starved condition, the receptor was observed in both nuclear and cytoplasmic compartments for R108K, A289D and A289T mutants, which were similar

to the subcellular distribution seen for EGFRvIII. However, the T263P mutant was almost completely restricted to the cytoplasm resembling the profile seen for EGFR. After EGF stimulation, the distribution of all the EGFR ectodomain mutants showed similar subcellular profile of distribution with localization at both the nuclear and also

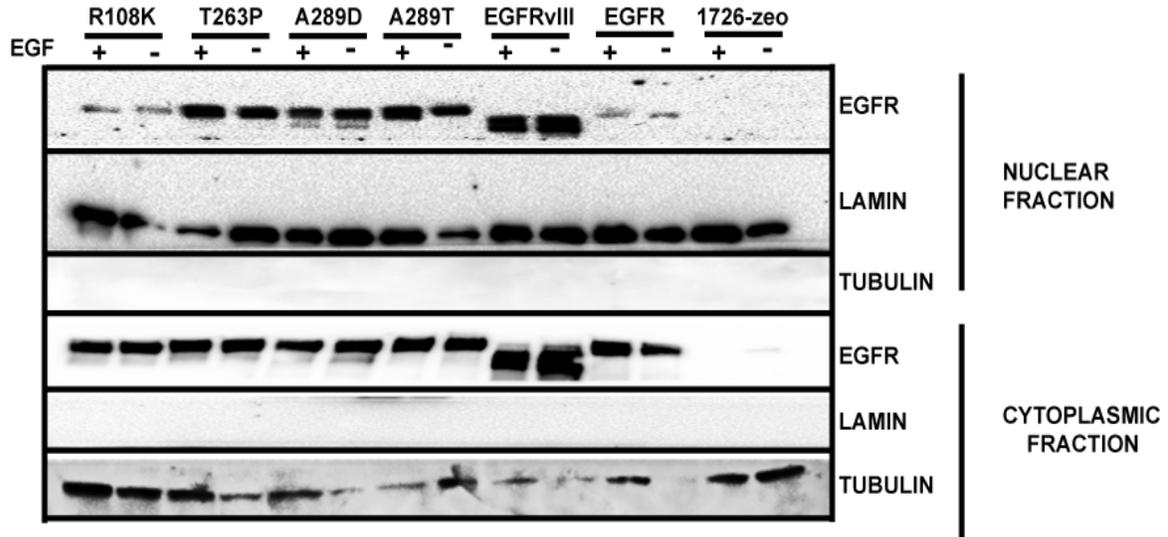


**Figure 21 Profile of distribution of GFP-tagged EGFR ectodomain mutants in U87 cells**

Confocal microscopic images show pattern of distribution of EGFR ectodomain mutants in glioma cells (U87) after transient transfection using GFP-tagged mutants: A. In serum starved condition and B. After EGF stimulation cells were counterstained using Topro3 (blue).

cytoplasmic level (**Figure 21**).

To provide validation that supports the confocal analysis, we carried out western blot analysis. We fractionated stable U87 cell lines overexpressing ectodomain mutations in serum starved and EGF stimulated conditions into nuclear and



**Figure 22 Nuclear and cytoplasmic expression of EGFR ectodomain mutants in U87 stable cells**

Western blot analysis shows nuclear and cytoplasmic expression of EGFR ectodomain mutants, EGFRvIII, wild type EGFR and negative control 1726-zeo in serum starved condition and after EGF stimulation. Western blot performed by Dr. Anupama Gururaj a collaborator from Böglers' Laboratory.

cytoplasmic fractions. After western blot analysis, all the mutants showed, in general a strong cytoplasmic localization in both serum starved and after EGF stimulated conditions. However, the nuclear localization pattern was differential in different conditions. Robust nuclear levels of the mutants T263P, A289D and A289T were observed in serum starved condition resembling the expression pattern seen for

EGFRvIII. In contrast, lower amounts of the R108K mutant were observed in the nucleus resembling the profile seen for EGFR. After EGF stimulation, the mutant A289T showed increased amounts in the nuclear compartment, which was similar to the pattern seen for EGFR. The remaining mutants did not exhibit significant differences in levels in the nucleus after EGF stimulation (**Figure 22**).

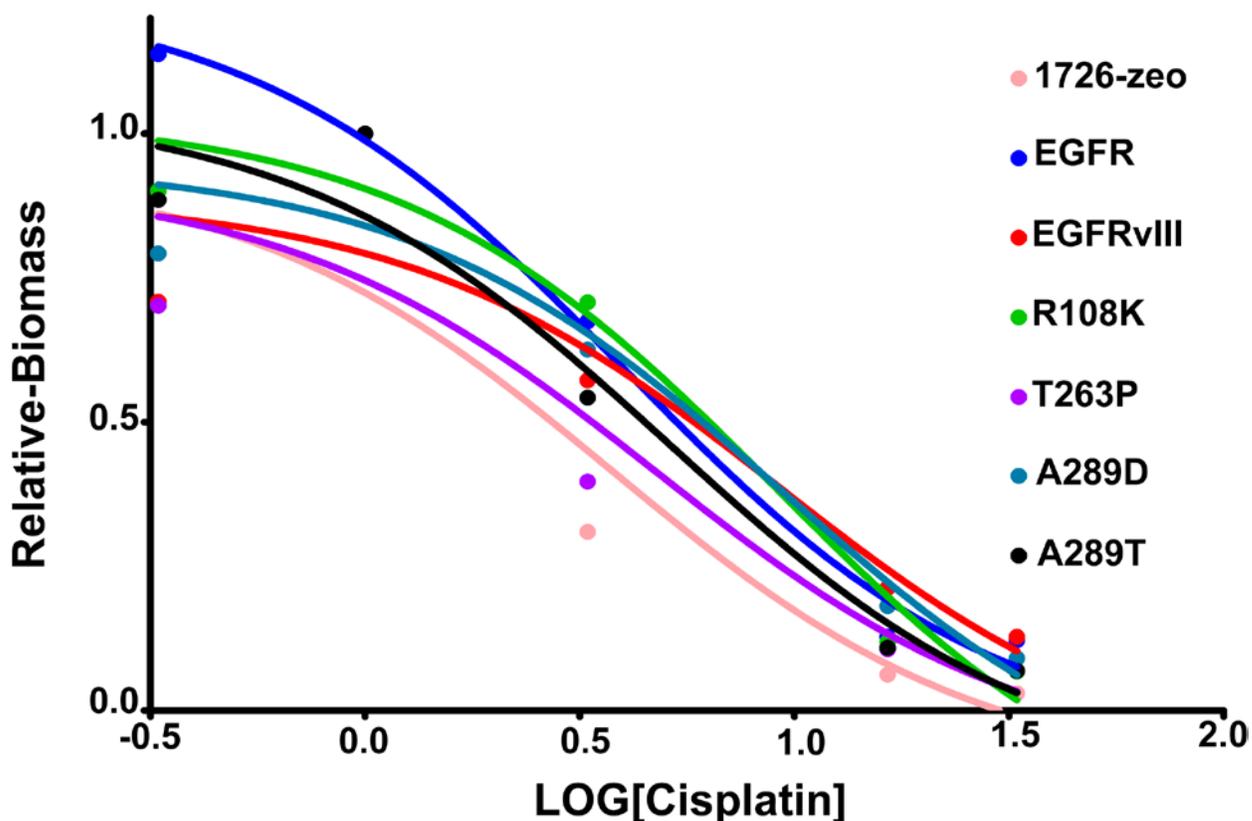
### **3-8 EGFR ectodomain mutants determine response to standard therapy**

Previous clinical studies have shown association of EGFRvIII and PTEN expression with clinical response to TKIs in patients with recurrent malignant gliomas (207). More recently, Lee's studies also have indicated that erlotinib a small molecule TKI, was able to induce cell death in Ba/F3 cells overexpressing EGFR missense mutations. The IC<sub>50</sub> values were between 50-150 nM suggesting that EGFR ectodomain mutations could sensitize GBM cells to TKIs such as erlotinib (9). Similar results from previous investigations that establish association of intact PTEN and expression of EGFRvIII with response to TKIs have been obtained in studies performed by Sarkaria *et al.*, where glioblastoma xenografts which express wild type PTEN in combination with EGFRvIII showed sensitivity to erlotinib treatment (210). The above studies provide support for the relevance of obtaining individual molecular phenotype for tumors and using this knowledge as part of the decision-making process to treat patients with GBMs and also extend this model to other types of cancer.

In our study we tested the sensitivity of U87 cells overexpressing the ectodomain mutations to chemotherapeutic agents such as cisplatin and TMZ. In addition, we also evaluated the effect of EGFR TKI, tarceva on anchorage independent growth of these cells overexpressing ectodomain mutations as well as EGFRvIII and EGFR.

### 3.8.1 Cisplatin treatment:

We determined IC<sub>50</sub> values for cisplatin in U87 cells overexpressing ectodomain mutants, EGFRvIII and EGFR after 5, 7 and 9 days of treatment. We also included control cells 1726-zeo which carries the empty vector and thus expresses endogenous



**Figure 23 Response of U87 cells expressing various EGFR mutants to cisplatin**

Dose effect of cisplatin on relative biomass (soft agarose colony formation assay) for ectodomain mutants stably overexpressed in U87 cells and treated with 4 different cisplatin doses 0.33, 3.3, 16.5 and 33  $\mu$ M. The graph depicts dose effect after 7 days of treatment. Data from three independent biological experiments.

levels of EGFR (**Figure 23**).

After seven days of cisplatin treatment, we found that the T263P mutation showed a substantial response to cisplatin showing an IC<sub>50</sub> of 3.5 µM similar to the response

IC50 (µM) for cisplatin days of drug exposure			
Mutant	<u>5</u>	<u>7</u>	<u>9</u>
R108K	6.6	9.4	5.0
T263P	5.3	3.5	5.1
A289D	6.5	10.5	13.0
A289T	5.8	4.6	8.3
EGFRvIII	7.7	11.0	21.4
EGFR	4.2	3.9	3.8
1726-zeo	3.0	2.1	2.4

**Table 5 Cisplatin IC<sub>50</sub> values for ectodomain mutants overexpressed in U87 cells**

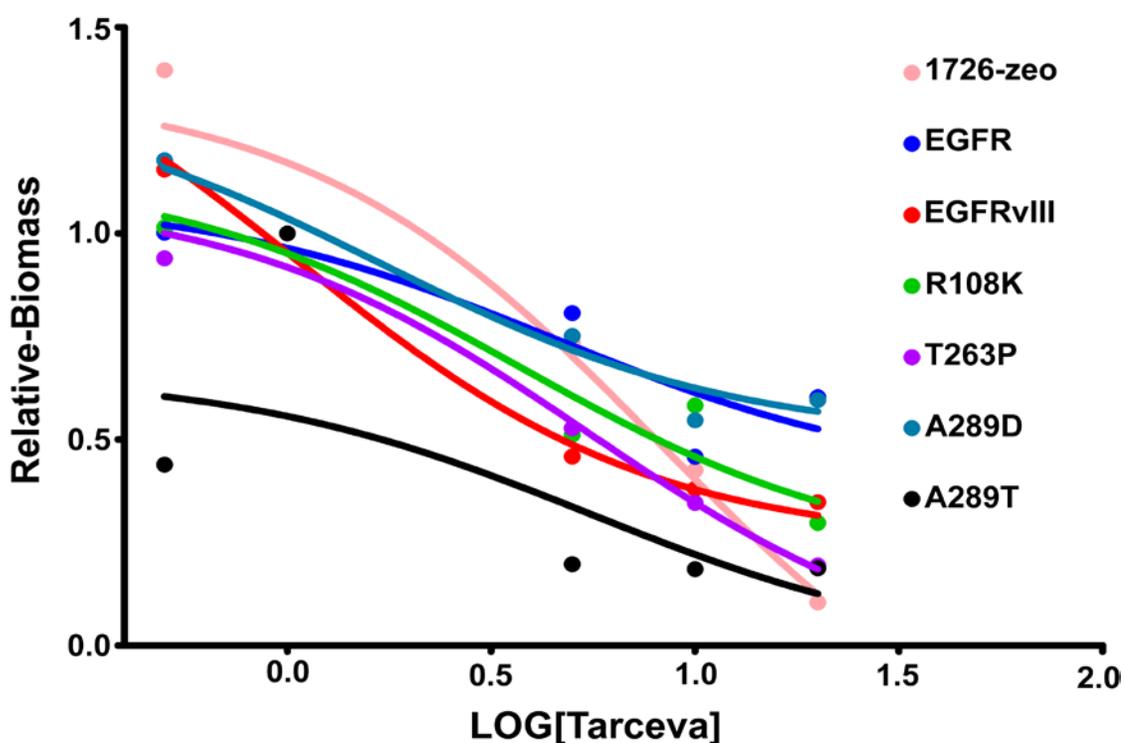
The table summarizes IC<sub>50</sub> values from three independent experiments after 5, 7 and 9 days of cisplatin treatment at 4 different concentrations of 0.3, 3.3, 16.5 and 33 µM. Dose values were transformed using the function  $X = \text{Log}(X)$  and then subjected to analysis using the graphpad prism software program.

seen for EGFR and control group with IC<sub>50</sub> of 3.9 µM and 2.1 µM respectively. In

contrast, mutations R108K, A289D, A289T and EGFRvIII exhibited a protective phenotype to the treatment showing higher IC<sub>50</sub> values 9.4 μM, 10.5 μM, 4.6 μM and 11.0 μM respectively. The cisplatin IC<sub>50</sub> values for ectodomain mutants, EGFRvIII and EGFR overexpressed in U87 cells are summarized in (Table 5).

### 3.8.2 Tarceva treatment:

We determined IC<sub>50</sub> values for tarceva as performed previously for cisplatin using



**Figure 24 Effect of tarceva treatment on ectodomain mutants overexpressed in U87 cells**

The graph shows dose effect on the relative biomass indices of ectodomain mutants (soft agarose colony formation assay), EGFRvIII and EGFR overexpressed in U87 cells at different concentrations of tarceva (0.5, 5.0, 10 and 20 μM) after 9 days of treatment. Data from three independent biological experiments.

4 different concentrations of the inhibitor (0.5, 5.0, 10.0 and 20  $\mu\text{M}$ ). After nine days of treatment, we analyzed the effect of tarceva on relative biomass of ectodomain mutations, EGFRvIII and EGFR overexpressed in U87 cells (**Figure 24**)

IC50 ( $\mu\text{M}$ ) for tarceva			
days of drug exposure			
Mutant	<u>5</u>	<u>7</u>	<u>9</u>
R108K	3.8	46.8	7.9
T263P	38.6	41.3	5.0
A289D	8.7	148.2	11.2
A289T	0.6	310.1	6.4
EGFRvIII	0.5	60.8	3.3
EGFR	0.00008	66.5	16.2
1726-zeo	5.2	29.0	3.5

**Table 6 Tarceva IC<sub>50</sub> values for ectodomain mutants overexpressed in U87 cells**

Table shows the different IC<sub>50</sub> values for ectodomain mutants, EGFRvIII and EGFR overexpressed in U87 cells after day 5, 7 and 9 of tarceva treatment using 4 different doses, soft agarose colony formation assay was carried out. The data was analyzed as is described above for cisplatin treatment and represent three independent experiments.

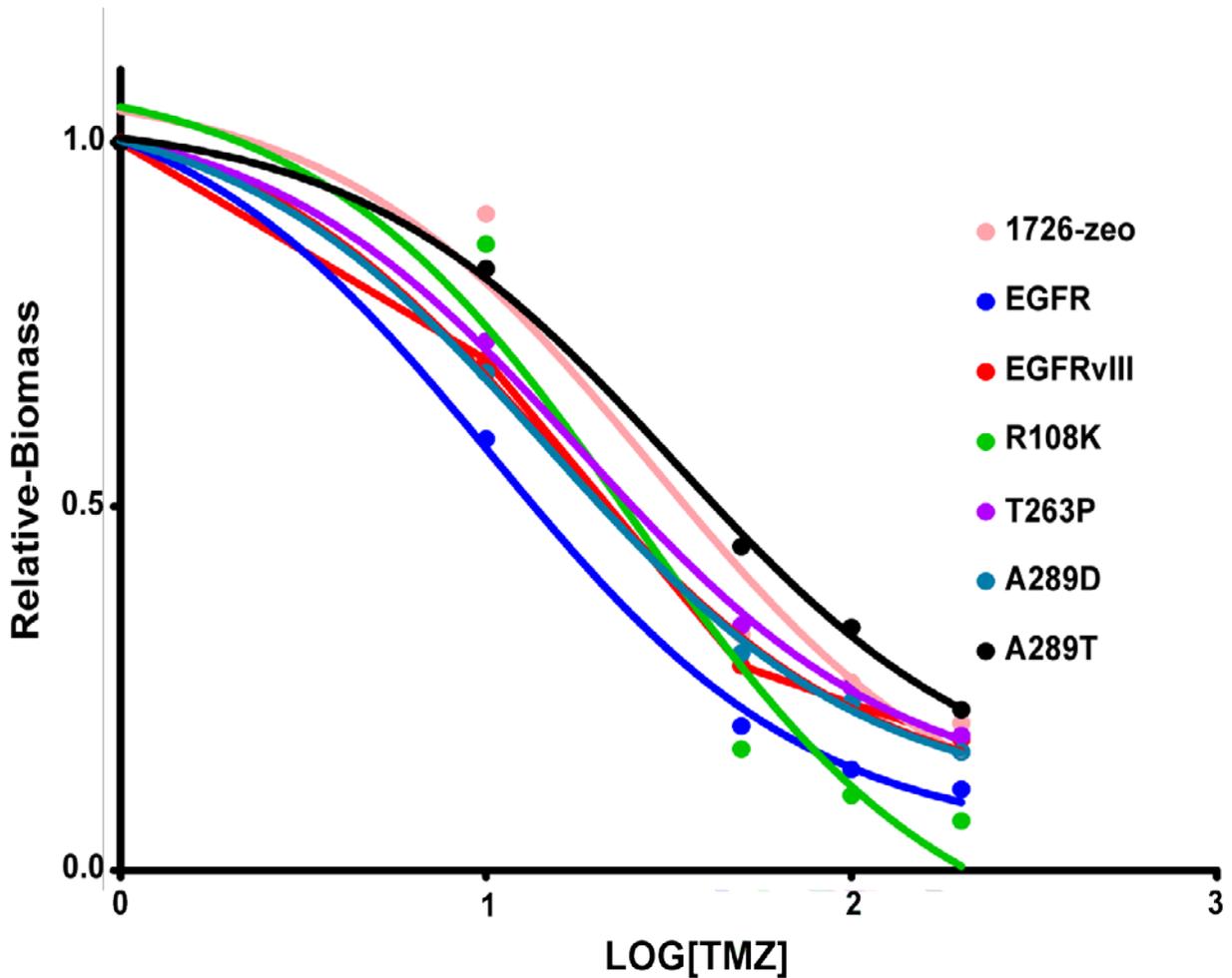
Our findings indicate a response to tarceva for the mutants T263P, A289T and EGFRvIII which showed low IC<sub>50</sub> values of the inhibitor (5.0 μM, 6.4 μM and 3.3 μM) respectively. In contrast, higher IC<sub>50</sub> values were observed for mutants R108K and A289D (7.9 μM and 11.2 μM) respectively, thus indicating a protective phenotype to tarceva treatment. In our analysis EGFR exhibited the highest IC<sub>50</sub> value (16.2 μM), indicating a significant resistance to tarceva treatment. Tarceva IC<sub>50</sub> values for the ectodomain mutations, EGFRvIII and EGFR are summarized in the **(Table 6)**.

### **3.8.3 Temozolomide treatment:**

We established IC<sub>50</sub> values for TMZ treatment in both U87 and LN428 cells overexpressing ectodomain mutants as was performed previously for cisplatin and tarceva. For the analysis, we used data from day 7 and 9 of treatment using four different concentrations. Our findings indicated that the mutants overexpressed in U87 cells showed significant response to TMZ after 7 days of treatment as reflected by lower IC<sub>50</sub> indices **(Figure 25)**.

Thus, after 9 days of treatment the mutants R108K, A289D and EGFRvIII showed lower IC<sub>50</sub> values (0.01 μM, 9.7 μM and 2.8 μM) respectively, mutant T263P exhibited a minor response to the treatment reflecting an IC<sub>50</sub> of (11.9 μM). The mutant A289T was less sensitive to TMZ showing an elevated IC<sub>50</sub> value (26.4 μM). **(Table 7)** shows

IC<sub>50</sub> for the mutants in the U87 cell context.



**Figure 25 Effect of temozolomide treatment on ectodomain mutants overexpressed in U87 cells**

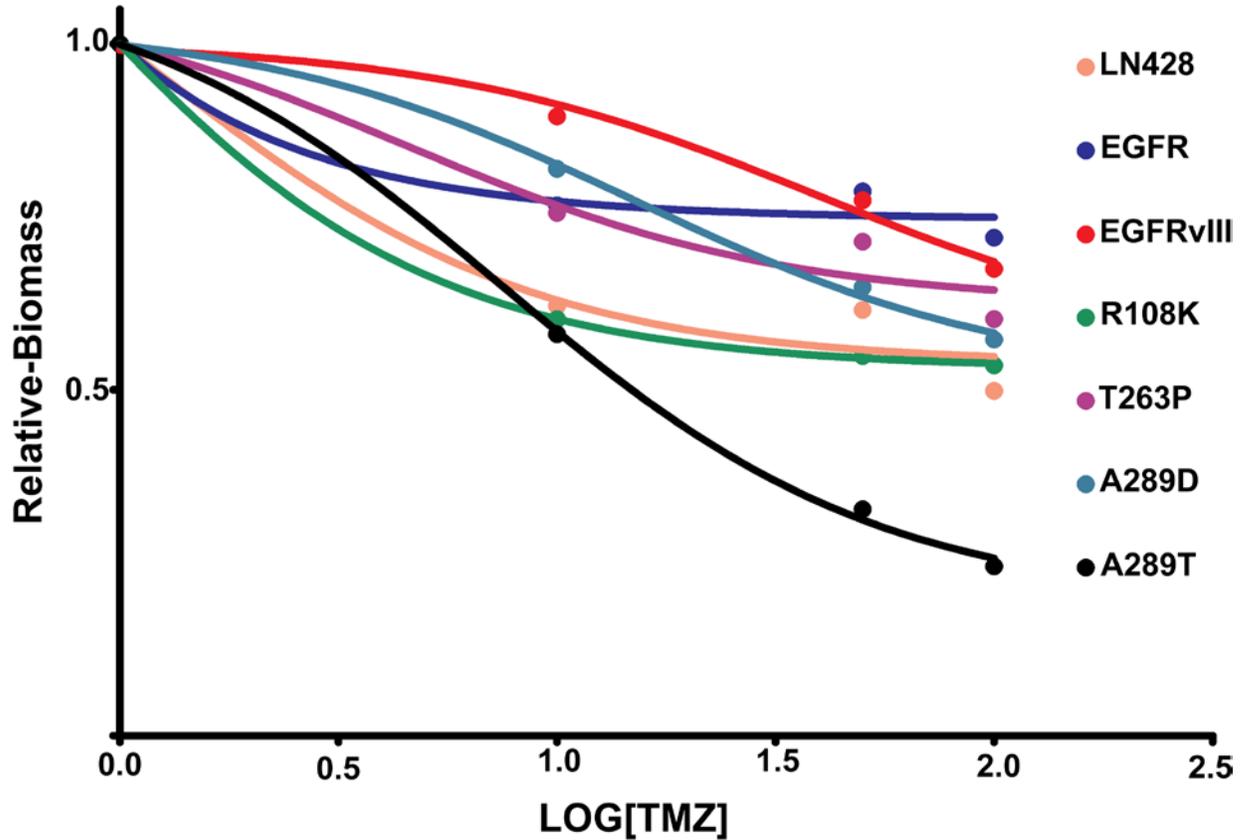
The graph depicts the effect on soft agarose colony formation after treatment using different TMZ doses. Graph illustrates the relative biomass indices across all the EGFR ectodomain mutants after 7 days of treatment. Same analysis described previously for the other inhibitors was performed using data from three independent experiments. Experiments performed by Dr. Takashi Shingu from Dr. Bögler Laboratory.

U87 Cells	IC50 (μM) for temozolomide days of drug exposure	
	<u>7</u>	<u>9</u>
<u>Mutant</u>		
R108K	~0.001	~0.01
T263P	12.5	11.9
A289D	17.0	9.7
A289T	9.6	26.4
EGFRvIII	1.5	2.8
EGFR	6.2	1.8
1726-zeo	1.2	1.1

**Table 7 Temozolomide IC<sub>50</sub> across EGFR ectodomain mutants overexpressed in U87 cells**

The table shows IC<sub>50</sub> values for ectodomain mutants in the context of U87 cells. Data comes from the analysis of 4 different TMZ doses after day 7 and 9 of treatment three independent experiments (soft agarose colony formation assay). The GraphPad Prism software program was used for the analysis.

In contrast, we found a prevalent resistance across the ectodomain mutations overexpressed in LN428 cells after 9 days of treatment (**Figure 26**).



**Figure 26 Dose response effect of temozolomide on ectodomain mutants overexpressed in LN428 cells**

The graph shows TMZ effect in 4 different doses (1.0, 10, 50 and 100  $\mu\text{M}$ ) after 9 days of treatment on the relative biomass indices (soft agarose colony formation assay) across all the ectodomain mutants EGFRvIII and EGFR overexpressed in LN428. Data from three independent experiments. Experiments performed by Dr. Takashi Shingu from Dr. Bögler's Laboratory.

However, the mutant A289T exhibited a marginal response after 9 days of TMZ treatment showing IC<sub>50</sub> values of (21.7 μM). Mutants T263P, A289D, R108K and EGFRvIII exhibited a protective phenotype as evidenced by undetermined IC<sub>50</sub> values. IC<sub>50</sub> for ectodomain mutants overexpressed in LN428 cells are illustrated in **(Table 8)**.

LN428 Cells	IC <sub>50</sub> (μM) for Temozolomide	
	Days of Drug Exposure	
<u>Mutant</u>	<u>7</u>	<u>9</u>
R108K	Undetermined	Undetermined
T263P	Undetermined	Undetermined
A289D	Undetermined	Undetermined
A289T	55.03	21.7
EGFRvIII	Undetermined	Undetermined
EGFR	Undetermined	Undetermined
1726-zeo	Undetermined	Undetermined

**Table 8 Temozolomide IC<sub>50</sub> for ectodomain mutants overexpressed in LN428 cells**

The IC<sub>50</sub> values illustrated in the table were obtained using the relative biomass indices from soft agarose colony formation assay after use 4 different TMZ doses post day 7 and 9 of treatment. Similar analysis performed previously was carried out. Table includes data from three individual experiments.

## **CHAPTER 4 DISCUSSION, SUMMARY, AND FUTURE**

### **DIRECTIONS**

## Discussion

EGFR is a critical driver in the tumorigenesis of GBM patients, where its amplification occurs in 40% to 70% of the primary GBMs (2, 172). Mutations in EGFR have been widely recognized as being part of the multiple mechanisms of deregulation of the receptor. Further, TCGA has reported EGFR as one of the most highly mutated genes in GBMs (10). These mutations coexist in a significant percentage (~ 80%) of tumors that show amplification of the receptor. EGFR mutations have been cataloged from ectodomain mutations or deletions to deletions in the cytoplasmic tail of the receptor (9, 86, 217, 220, 221), where ectodomain mutations represent the most common subset of mutations in EGFR. The most common and widely studied mutation is the type III EGFR variant deletion mutant (EGFRvIII). This variant is has a deletion of exons 2 to 7; it is expressed in about 50 to 60% of GBM tumors overexpressing EGFR (171-173). Typically, this variant shows consistent enhancement in tumorigenesis and clinical studies have indicated a correlation between the expression of EGFRvIII and poor prognosis (175, 176).

More recently, a novel class of missense mutations has been identified in GBMs samples, where the majority of these mutations are located in the ectodomain region of EGFR with some of them located in the deleted area of EGFRvIII. They are found in ~14% of GBM tumors (9), which has been also documented by information obtained by TCGA (10). Further characterization of these mutants in mouse fibroblast has demonstrated a transforming and tumorigenic capacity (9). In addition, some of these mutants exhibited tyrosine kinase activity for the residues Tyr-1068 and Tyr-845 in serum starved condition as seen for EGFRvIII, in BaF3 cells context, which do not

contain endogenous EGFR and stronger tyrosine kinase activation was seen after EGF stimulation in these cells (9). Thus, these mutants respond to EGF similar to EGFR. However, the molecular mechanisms by which ectodomain mutations drive EGFR activation remains to be studied.

In our study we have identified EGFR ectodomain mutations using information contained in the TCGA database. Selecting ones that are located in the deleted area of EGFRvIII (R108K, T263P, A289D and A289T). Previous studies (9) have demonstrated oncogenic capacity for some of them (R108K and T263P) in mouse fibroblast. Therefore, to gain molecular understanding for which these mutants act to activate the receptor, we interrogated whether our cohort of mutants exhibited similar molecular characteristics with EGFRvIII or EGFR. The knowledge acquired in our study may contribute for a better understanding of the molecular mechanisms by which these mutants trigger particular signaling pathways leading to biological contributions in the tumorigenesis of GBMs. Subsequently this information would be useful for the customization of treatments where groups of patients expressing these particular mutants could receive more effective therapies.

In the tumorigenesis of GBM critical sites of phosphorylation on the cytoplasmic tail of EGFR have been determined in previous studies *in vitro* and *in vivo* using U87 cells and xenograft models. These indicate Tyr-1048, Tyr-1068, Tyr-1173 and Tyr-845 as relevant residues for tumor development in GBM (29, 93, 179). In our study, after we tested phosphorylation levels for these residues across all the EGFR ectodomain mutations, we found different profiles of activation. In the context of U87 cells (PTEN mutated / WT-p53), the mutants R108K and T263P showed a similar profile to EGFR,

where these mutants showed basal activation and characteristically responded to the ligand stimulus. In contrast, the mutant A289T exhibited very strong basal activation across all the tyrosine sites but did not show response to the ligand stimulus resembling the profile seen for EGFRvIII. The mutant A289D showed characteristics from both EGFR and EGFRvIII. In the context of LN428 cells the ectodomain mutants showed more robust basal activation than in LNZ308 cell context. These observations suggest potential differences in downstream signaling activation. Previous studies have pointed out a decrease in signaling activation after reduction or loss of phosphorylation levels in critical EGFR tyrosine residues (29). In addition, previous investigations have indicated a distinct signaling pattern of activation, where for EGFRvIII the PI3K pathway could be predominantly activated over the MAPK and STAT3 pathways (89). Therefore, we raised the question as to whether these mutants exhibit characteristic patterns of kinase activation in downstream canonical pathways AKT and ribosomal S6. However, our signaling analysis did not show significant differences in basal or ligand stimulation conditions across all the ectodomain mutations and EGFRvIII and EGFR. After examination of the signaling in different genetic backgrounds from LNZ308 (PTEN/p53 mutated) and LN428 (WT-PTEN / WT-p53) cell lines, similar tendencies were observed. In addition, a strong and consistent basal activation for Tyr-1173 site was observed across all the ectodomain mutants in U87 cells context and to a low extent in LN428 cells. Interestingly, in LNZ308 cells, basal phosphorylation was reduced to almost undetectable levels, suggesting that molecular background could help to define molecular characteristics of these mutants. These observations lead us to hypothesize that molecular context could have an important effect in the biological contribution of these mutants in GBMs. This was

borne out by our results demonstrating the contribution of these mutants in anchorage independent growth in different genetic backgrounds. In general, higher enhancements in anchorage independent growth from the ectodomain mutants as compared to EGFR were observed in all the three stable cells. In the context of U87 cells, mutants R108K, A289T and EGFRvIII showed more significant contribution after comparison to EGFR. In contrast, in stable LN428 all the ectodomain mutations showed greater enhancement than EGFRvIII as well as EGFR. In the context of LNZ308 cells, greater enhancements were seen for the mutants A289D and EGFRvIII as compared to EGFR. These observations also suggest that different genetic backgrounds might have a role in the biological inputs of the ectodomain mutants. However, differences in downstream signaling triggered by these mutants remain elusive.

According to previous studies, cell distribution of the receptor can lead to upregulation of alternative pathways associated with cell cycle progression (92, 293, 295, 296). In our study we assessed the cellular distribution profile of ectodomain mutations. After confocal microscopic examination of transiently expressed ectodomain mutations in U87 cell lines, we found in serum starved condition nuclear and cytoplasmic distribution for mutants R108K, A289T and A289D resembling the pattern seen in EGFRvIII. In contrast, a predominantly cytoplasmic distribution was observed for the mutant T263P similar to the profile seen in EGFR. These findings also correlate with our preliminary confocal examination of transient expressed ectodomain mutations in CHO cell lines. However, in the western blot analysis using stable U87 cells overexpressing ectodomain mutations, we were able to detect the T263P mutant in the nucleus under serum starved conditions. The differences

observed could be due to the different expression systems. The flux of the molecules between the nucleus and the cytoplasm could be potentially different in transiently expressed and stably expressed systems as has been reported earlier in other studies (297, 298). Our observations open the question whether alternative pathways could be upregulated by these mutants in the nuclear compartment. The profile of distribution seen in U87 cells after transient transfection might correlate with results from the anchorage independent growth, where the mutants with more aggressive phenotype were those that showed nuclear and cytoplasmic distribution. These results were also in agreement with our xenograft studies, where the groups of mice implanted with U87 cells overexpressing R108K, A289T and A289D mutants showed shorter tumor latency period similar to the group of mice implanted with EGFRvIII. In contrast, group of mice implanted with U87 cells overexpressing T263P exhibited longer tumor latency period similar to the group of mice implanted with EGFR.

Taking all our data together, we stratify these ectodomain mutants into three groups: A first group, characterized by similarity in the profile of tyrosine phosphorylation to EGFRvIII, more robust enhancement in anchorage independent growth and shorter tumor latency period, where we include the mutant A289T. A second group, which displays a similar profile of tyrosine phosphorylation with EGFR and leads to less enhancement in anchorage independent growth and longer tumor latency period, where we include the mutant T263P. A third group, where we include the mutant A289D which displays characteristics from both EGFR and EGFRvIII **(Table 9)**.

EGFR	Cellular Distribution U87		Cellular Distribution CHO	Independent Anchorage Colony Formation (Biomass-Folds)	Median Survival Xenograft Mice
	S.S	EGF	10% Serum		
A289T	C-N	C-N	C-N	22.3	14
EGFRvIII	C-N	C-N	C-N	26.4	13
R108K	C-N	C-N	C-N	22.6	13
A289D	C-N	C-N	C-N	7.7	14
EGFR	C	C-N	C	4.3	16
T263P	C	C-N	C	10.2	17
1726-zeo	—	—	—	1.0	36

**Table 9 Stratification of EGFR ectodomain mutants according to the biologic characteristics**

Table shows three main groups: First group (in red) which resembles the biology of EGFRvIII. Second group (in blue) showing similar characteristics with EGFR. A third group (in green), which shares characteristics from both groups. C denotes cytoplasmic and N nuclear distribution.

However, the mutant R108K did not fit well in this classification as there was no agreement between the aggressive phenotype and the molecular profile observed for this mutant (**Table 10**).

Mutation	Tyr-1068	Tyr-1148	Tyr-1173	Tyr-845
R108K	NR	EGFR	EGFR	EGFR
T263P	EGFR	EGFR	EGFR	EGFR
A289D	EGFR	EGFRvIII	EGFR	EGFR
A289T	EGFRvIII	EGFRvIII	EGFRvIII	EGFRvIII

**Table 10 Categorization of EGFR ectodomain mutants according to tyrosine phosphorylation profiles**

Table shows identification of three main groups: First group showing similar profile to EGFR (T263P and R108K mutants). A second group with similar pattern to EGFRvIII (A289T mutant) and a third group showing similarities with both tyrosine profiles (A289D mutant). Mutant R108K did not show activity for the residue Tyr-1068 (NR).

We propose that alternative pathways might play a role in the biological contribution of this particular mutant. In our phosphoscan analysis, we identified the phosphopeptide SHIP2-Tyr-987, which appears as a potential target. Mutational

studies indicate that mutation in Tyr-987 residue on SHIP2 leads to an anti-proliferative effect (26). The phosphoscan analysis allowed us to identify significant cohort of phosphopeptides without bias that can help us to understand the biological contribution of these EGFR ectodomain mutations from the molecular point of view. One of the most important advantages of this method is the broad spectrum of phosphopeptides that could be identified. Also, this method enabled us to quantify and detect the EGFR phosphopeptides with high resolution and sensitivity. However, one of the disadvantages is that the analysis is limited to tyrosine residues because of the enrichment method used; in our case we enriched the samples using anti-tyrosine antibodies that allowed us just to identify the affected downstream tyrosine phosphopeptides. We consider that additional and complementary proteomic analyses such Reverse Phase Protein Arrays (RPPA) need to be done, in order to have a more comprehensive spectrum of potential targets that might have important role in the biological contribution of these mutants and subsequently an impact in therapeutics.

In our study we demonstrated that some of these mutants are highly oncogenic. Therefore, we asked the question whether these ectodomain mutants could have a prognostic value in therapy. Our findings showed that the mutants A289T, T263P and EGFRvIII responded well to tarceva treatment. Previous studies have associated EGFRvIII signature with responsiveness to EGFR TKIs (207). In agreement with these results, we found a significant response of EGFRvIII to tarceva treatment. Also previous studies from Lee et al have indicated sensitivity of missense mutations overexpressed in Ba/F3 to TKIs cells suggesting that these mutants can sensitize transformed cells to TKIs (9). In contrast, a different tendency was observed after cisplatin treatment, where persistent resistance was observed in almost all the

ectodomain mutations except the mutant T263P which showed a significant response. Previous studies have reported that nuclear EGFR is a requirement to increase DNA repair, thus, reducing DNA damage of EGFR expressing cells and leading to cisplatin resistance (299). The results from our studies also showed a correlation with the subcellular distribution after transient transfection of the mutants in serum starved condition, where all the ectodomain mutants, except the mutant T263P, exhibited nuclear localization. However, additional studies need to be done to establish potential interactions of ectodomain mutants and proliferating cell nuclear antigen (PCNA) and also DNA dependent kinase (DNA-PK) upregulation, which are EGFR nuclear interacting partners (300-303) and are necessary for DNA repair in EGFR expressing cells.

The ectodomain mutants showed a significant response to TMZ, which was evidenced by  $IC_{50}$  values after seven days of treatment in the context of U87 cells. However, same treatment in cells with a different genetic background (LN428) displayed a protective phenotype across all the mutants, except for the mutant A289T and R108K which showed a marginal response. One of the major determinants for favorable response to TMZ is the status of MGMT promoter methylation (304, 305). However, according to the literature, both the cell lines U87 and LN428 show similar status of MGMT methylation (306), suggesting that others factors contribute to TMZ resistance observed in the context of LN428 cells such as BER activation (307). However, further studies that help to explain the marked sensitivity of A289T mutant to TMZ in the context of LN428 need to be done.

In summary, our results present new information that would contribute to the

acquisition of a more comprehensive understanding of the biology and molecular characteristics of EGFR ectodomain mutants in GBM. In addition, *in vitro* inhibitory studies bring valuable findings, which might be useful as predictive parameters in therapeutic response of GBM patients expressing these mutants.

## **Summary**

- In our study, we showed that in general, EGFR ectodomain mutants in glioma cells can be active even in the absence of the ligand particularly in the context of U87 cells and are thus constitutively active.
- The overexpression of ectodomain mutants enhanced transformation and decreased tumor latency period in xenograft mice as compared to EGFR.
- Based on the molecular characterization and biological phenotype, the ectodomain point mutants that we studied can be stratified in three main groups: A first group similar to EGFRvIII, a second group that resembles EGFR and a third group that shows characteristics from both the first and second category.
- In terms of tyrosine activation profile, the mutant R108K behaves similar to EGFR but biological phenotype is like EGFRvIII suggesting alternative mechanism of activation for this mutant.
- Inhibitory studies using TKI (tarceva) indicated that expression of the mutants T263P, A289T and EGFRvIII could be indicative of sensitivity of glioma cells to this particular treatment.
- Ectodomain mutants exhibited different pattern of response to TMZ treatment in glioma cells with different molecular context, suggesting that genetic background has a significant role in TMZ response.

- In general ectodomain mutants showed a protective phenotype after cisplatin treatment except for the mutant T263P.

### **Future Directions**

In our study we have provided significant information that contributes to the molecular and biologic characterization of the EGFR ectodomain mutants in the context of gliomas. However, we think that numerous questions still need to be addressed. First, a more exhaustive proteomic analysis such as RPPA needs to be done in order to delineate molecular pathways that could be triggered by these ectodomain mutants which are critical in the tumorigenesis of GBMs. In addition, potential targets identified in proteomics analysis require validation as well as establish their contribution in tumorigenesis and significance in therapeutics *in vitro* and *in vivo*.

Further, EGFR nuclear distribution has been associated with poor prognosis in different malignancies. Our findings indicated a predominant cytoplasmic and nuclear distribution across the majority of these mutants, raising the question whether these ectodomain mutants are able to activate transcriptional machinery in the nucleus leading to activation of additional pathways that could potentiate the tumorigenic phenotype and also have implication in responses to therapies.

In our study we evidenced that genetic backgrounds may help to define the biological contribution and therapeutic response of ectodomain mutants. However, clear mechanisms that take place in the definition of these characteristics are unknown. We think that a more comprehensive understanding of the role of the different molecular contexts could be useful to find explanations why particular

treatments are more or less effective in different cohorts of patients. Therefore, the elucidation of all these questions would be useful in the customization of treatments for GBM patients.

## BIBLIOGRAPHY

1. McCarthy, B. J., J. M. Propp, F. G. Davis, and P. C. Burger. 2008. Time trends in oligodendroglial and astrocytic tumor incidence. *Neuroepidemiology* 30: 34-44 PM:18259099.
2. Ohgaki, H., P. Dessen, B. Jourde, S. Horstmann, T. Nishikawa, P. L. Di Patre, C. Burkhard, D. Schuler, N. M. Probst-Hensch, P. C. Maiorka, N. Baeza, P. Pisani, Y. Yonekawa, M. G. Yasargil, U. M. Lutolf, and P. Kleihues. 2004. Genetic pathways to glioblastoma: a population-based study. *Cancer Res.* 64: 6892-6899 PM:15466178.
3. Davis, F. G., B. J. McCarthy, and M. S. Berger. 1999. Centralized databases available for describing primary brain tumor incidence, survival, and treatment: Central Brain Tumor Registry of the United States; Surveillance, Epidemiology, and End Results; and National Cancer Data Base. *Neuro. Oncol.* 1: 205-211 PM:11554389.
4. Louis, D. N., H. Ohgaki, O. D. Wiestler, W. K. Cavenee, P. C. Burger, A. Jouvett, B. W. Scheithauer, and P. Kleihues. 2007. The 2007 WHO classification of tumours of the central nervous system. *Acta Neuropathol.* 114: 97-109 PM:17618441.
5. Furnari, F. B., T. Fenton, R. M. Bachoo, A. Mukasa, J. M. Stommel, A. Stegh, W. C. Hahn, K. L. Ligon, D. N. Louis, C. Brennan, L. Chin, R. A. Depinho, and

- W. K. Cavenee. 2007. Malignant astrocytic glioma: genetics, biology, and paths to treatment. *Genes Dev.* 21: 2683-2710 PM:17974913.
6. Gurney, J. G., and N. Kadan-Lottick. 2001. Brain and other central nervous system tumors: rates, trends, and epidemiology. *Curr. Opin. Oncol.* 13: 160-166 PM:11307058.
  7. Propp, J. M., B. J. McCarthy, F. G. Davis, and S. Preston-Martin. 2006. Descriptive epidemiology of vestibular schwannomas. *Neuro. Oncol.* 8: 1-11 PM:16443943.
  8. He, J., G. Reifenberger, L. Liu, V. P. Collins, and C. D. James. 1994. Analysis of glioma cell lines for amplification and overexpression of MDM2. *Genes Chromosomes. Cancer* 11: 91-96 PM:7529554.
  9. Lee, J. C., I. Vivanco, R. Beroukhim, J. H. Huang, W. L. Feng, R. M. DeBiasi, K. Yoshimoto, J. C. King, P. Nghiemphu, Y. Yuza, Q. Xu, H. Greulich, R. K. Thomas, J. G. Paez, T. C. Peck, D. J. Linhart, K. A. Glatt, G. Getz, R. Onofrio, L. Ziaugra, R. L. Levine, S. Gabriel, T. Kawaguchi, K. O'Neill, H. Khan, L. M. Liau, S. F. Nelson, P. N. Rao, P. Mischel, R. O. Pieper, T. Cloughesy, D. J. Leahy, W. R. Sellers, C. L. Sawyers, M. Meyerson, and I. K. Mellinghoff. 2006. Epidermal growth factor receptor activation in glioblastoma through novel missense mutations in the extracellular domain. *PLoS. Med.* 3: e485 PM:17177598.
  10. 2008. Comprehensive genomic characterization defines human glioblastoma genes and core pathways. *Nature* 455: 1061-1068 PM:18772890.

11. Nigro, J. M., S. J. Baker, A. C. Preisinger, J. M. Jessup, R. Hostetter, K. Cleary, S. H. Bigner, N. Davidson, S. Baylin, P. Devilee, and . 1989. Mutations in the p53 gene occur in diverse human tumour types. *Nature* 342: 705-708  
PM:2531845.
12. von, D. A., D. N. Louis, A. K. von, I. Petersen, T. Hoell, R. Y. Chung, R. L. Martuza, D. A. Schoenfeld, M. G. Yasargil, O. D. Wiestler, and . 1992. Association of epidermal growth factor receptor gene amplification with loss of chromosome 10 in human glioblastoma multiforme. *J. Neurosurg.* 77: 295-301  
PM:1320666.
13. Duerr, E. M., B. Rollbrocker, Y. Hayashi, N. Peters, B. Meyer-Puttlitz, D. N. Louis, J. Schramm, O. D. Wiestler, R. Parsons, C. Eng, and D. A. von. 1998. PTEN mutations in gliomas and glioneuronal tumors. *Oncogene* 16: 2259-2264  
PM:9619835.
14. Ohgaki, H., and P. Kleihues. 2007. Genetic pathways to primary and secondary glioblastoma. *Am. J. Pathol.* 170: 1445-1453 PM:17456751.
15. Abo-Dalo, B., K. Kutsche, V. Mautner, and L. Kluwe. 2010. Large intragenic deletions of the NF2 gene: breakpoints and associated phenotypes. *Genes Chromosomes. Cancer* 49: 171-175 PM:19924781.
16. Louis, D. N. 1994. The p53 gene and protein in human brain tumors. *J. Neuropathol. Exp. Neurol.* 53: 11-21 PM:8301315.
17. Yip, S., J. Miao, D. P. Cahill, A. J. Iafrate, K. Aldape, C. L. Nutt, and D. N. Louis. 2009. MSH6 mutations arise in glioblastomas during temozolomide therapy and

- mediate temozolomide resistance. Clin. Cancer Res. 15: 4622-4629  
PM:19584161.
18. Thiel, G., K. Marczynek, R. Neumann, R. Witkowski, D. A. Marchuk, and P. Nurnberg. 1995. Somatic mutations in the neurofibromatosis 1 gene in gliomas and primitive neuroectodermal tumours. Anticancer Res. 15: 2495-2499  
PM:8669813.
  19. Zhu, Y., F. Guignard, D. Zhao, L. Liu, D. K. Burns, R. P. Mason, A. Messing, and L. F. Parada. 2005. Early inactivation of p53 tumor suppressor gene cooperating with NF1 loss induces malignant astrocytoma. Cancer Cell 8: 119-130 PM:16098465.
  20. Esteller, M., J. Garcia-Foncillas, E. Andion, S. N. Goodman, O. F. Hidalgo, V. Vanaclocha, S. B. Baylin, and J. G. Herman. 2000. Inactivation of the DNA-repair gene MGMT and the clinical response of gliomas to alkylating agents. N. Engl. J. Med. 343: 1350-1354 PM:11070098.
  21. Hegi, M. E., A. C. Diserens, T. Gorlia, M. F. Hamou, T. N. de, M. Weller, J. M. Kros, J. A. Hainfellner, W. Mason, L. Mariani, J. E. Bromberg, P. Hau, R. O. Mirimanoff, J. G. Cairncross, R. C. Janzer, and R. Stupp. 2005. MGMT gene silencing and benefit from temozolomide in glioblastoma. N. Engl. J. Med. 352: 997-1003 PM:15758010.
  22. Muleris, M., A. Almeida, A. M. Dutrillaux, E. Pruchon, F. Vega, J. Y. Delattre, M. Poisson, B. Malfoy, and B. Dutrillaux. 1994. Oncogene amplification in human

- gliomas: a molecular cytogenetic analysis. *Oncogene* 9: 2717-2722  
PM:8058336.
23. Galanis, E., J. Buckner, D. Kimmel, R. Jenkins, B. Alderete, J. O'Fallon, C. H. Wang, B. W. Scheithauer, and C. D. James. 1998. Gene amplification as a prognostic factor in primary and secondary high-grade malignant gliomas. *Int. J. Oncol.* 13: 717-724 PM:9735401.
  24. Mischel, P. S., S. F. Nelson, and T. F. Cloughesy. 2003. Molecular analysis of glioblastoma: pathway profiling and its implications for patient therapy. *Cancer Biol. Ther.* 2: 242-247 PM:12878856.
  25. Hermanson, M., K. Funa, M. Hartman, L. Claesson-Welsh, C. H. Heldin, B. Westermark, and M. Nister. 1992. Platelet-derived growth factor and its receptors in human glioma tissue: expression of messenger RNA and protein suggests the presence of autocrine and paracrine loops. *Cancer Res.* 52: 3213-3219 PM:1317261.
  26. Artemenko, Y., A. Gagnon, S. Ibrahim, and A. Sorisky. 2007. Regulation of PDGF-stimulated SHIP2 tyrosine phosphorylation and association with Shc in 3T3-L1 preadipocytes. *J. Cell Physiol* 211: 598-607 PM:17219406.
  27. Westermark, B., C. H. Heldin, and M. Nister. 1995. Platelet-derived growth factor in human glioma. *Glia* 15: 257-263 PM:8586462.
  28. Di, R. F., R. S. Carroll, J. Zhang, and P. M. Black. 1998. Platelet-derived growth factor and its receptor expression in human oligodendrogliomas. *Neurosurgery* 42: 341-346 PM:9482185.

29. Huang, P. H., E. R. Miraldi, A. M. Xu, V. A. Kundukulam, A. M. Del Rosario, R. A. Flynn, W. K. Cavenee, F. B. Furnari, and F. M. White. 2010. Phosphotyrosine signaling analysis of site-specific mutations on EGFRvIII identifies determinants governing glioblastoma cell growth. *Mol. Biosyst.* 6: 1227-1237 PM:20461251.
30. Hawkins, P. T., K. E. Anderson, K. Davidson, and L. R. Stephens. 2006. Signalling through Class I PI3Ks in mammalian cells. *Biochem. Soc. Trans.* 34: 647-662 PM:17052169.
31. Knobbe, C. B., and G. Reifenberger. 2003. Genetic alterations and aberrant expression of genes related to the phosphatidylinositol-3'-kinase/protein kinase B (Akt) signal transduction pathway in glioblastomas. *Brain Pathol.* 13: 507-518 PM:14655756.
32. Samuels, Y., Z. Wang, A. Bardelli, N. Silliman, J. Ptak, S. Szabo, H. Yan, A. Gazdar, S. M. Powell, G. J. Riggins, J. K. Willson, S. Markowitz, K. W. Kinzler, B. Vogelstein, and V. E. Velculescu. 2004. High frequency of mutations of the PIK3CA gene in human cancers. *Science* 304: 554 PM:15016963.
33. Gallia, G. L., V. Rand, I. M. Siu, C. G. Eberhart, C. D. James, S. K. Marie, S. M. Oba-Shinjo, C. G. Carlotti, O. L. Caballero, A. J. Simpson, M. V. Brock, P. P. Massion, B. S. Carson, Sr., and G. J. Riggins. 2006. PIK3CA gene mutations in pediatric and adult glioblastoma multiforme. *Mol. Cancer Res.* 4: 709-714 PM:17050665.

34. Bader, A. G., S. Kang, and P. K. Vogt. 2006. Cancer-specific mutations in PIK3CA are oncogenic in vivo. *Proc. Natl. Acad. Sci. U. S. A* 103: 1475-1479 PM:16432179.
35. Huang, C. H., D. Mandelker, O. Schmidt-Kittler, Y. Samuels, V. E. Velculescu, K. W. Kinzler, B. Vogelstein, S. B. Gabelli, and L. M. Amzel. 2007. The structure of a human p110alpha/p85alpha complex elucidates the effects of oncogenic PI3Kalpha mutations. *Science* 318: 1744-1748 PM:18079394.
36. Mizoguchi, M., C. L. Nutt, G. Mohapatra, and D. N. Louis. 2004. Genetic alterations of phosphoinositide 3-kinase subunit genes in human glioblastomas. *Brain Pathol.* 14: 372-377 PM:15605984.
37. Wang, H., H. Wang, W. Zhang, H. J. Huang, W. S. Liao, and G. N. Fuller. 2004. Analysis of the activation status of Akt, NFkappaB, and Stat3 in human diffuse gliomas. *Lab Invest* 84: 941-951 PM:15184909.
38. Ahn, J. Y., Y. Hu, T. G. Kroll, P. Allard, and K. Ye. 2004. PIKE-A is amplified in human cancers and prevents apoptosis by up-regulating Akt. *Proc. Natl. Acad. Sci. U. S. A* 101: 6993-6998 PM:15118108.
39. Knobbe, C. B., A. Trampe-Kieslich, and G. Reifenberger. 2005. Genetic alteration and expression of the phosphoinositol-3-kinase/Akt pathway genes PIK3CA and PIKE in human glioblastomas. *Neuropathol. Appl. Neurobiol.* 31: 486-490 PM:16150119.
40. Maira, S. M., I. Galetic, D. P. Brazil, S. Kaech, E. Ingley, M. Thelen, and B. A. Hemmings. 2001. Carboxyl-terminal modulator protein (CTMP), a negative

regulator of PKB/Akt and v-Akt at the plasma membrane. *Science* 294: 374-380  
PM:11598301.

41. Knobbe, C. B., J. Reifenberger, B. Blaschke, and G. Reifenberger. 2004. Hypermethylation and transcriptional downregulation of the carboxyl-terminal modulator protein gene in glioblastomas. *J. Natl. Cancer Inst.* 96: 483-486  
PM:15026474.
42. Gao, T., F. Furnari, and A. C. Newton. 2005. PHLPP: a phosphatase that directly dephosphorylates Akt, promotes apoptosis, and suppresses tumor growth. *Mol. Cell* 18: 13-24 PM:15808505.
43. Skeen, J. E., P. T. Bhaskar, C. C. Chen, W. S. Chen, X. D. Peng, V. Nogueira, A. Hahn-Windgassen, H. Kiyokawa, and N. Hay. 2006. Akt deficiency impairs normal cell proliferation and suppresses oncogenesis in a p53-independent and mTORC1-dependent manner. *Cancer Cell* 10: 269-280 PM:17045205.
44. Malumbres, M., and M. Barbacid. 2009. Cell cycle, CDKs and cancer: a changing paradigm. *Nat. Rev. Cancer* 9: 153-166 PM:19238148.
45. Pei, X. H., and Y. Xiong. 2005. Biochemical and cellular mechanisms of mammalian CDK inhibitors: a few unresolved issues. *Oncogene* 24: 2787-2795  
PM:15838515.
46. Lee, M. H., and H. Y. Yang. 2001. Negative regulators of cyclin-dependent kinases and their roles in cancers. *Cell Mol. Life Sci.* 58: 1907-1922  
PM:11766887.

47. Swiss, V. A., and P. Casaccia. 2010. Cell-context specific role of the E2F/Rb pathway in development and disease. *Glia* 58: 377-390 PM:19795505.
48. Chen, H. Z., S. Y. Tsai, and G. Leone. 2009. Emerging roles of E2Fs in cancer: an exit from cell cycle control. *Nat. Rev. Cancer* 9: 785-797 PM:19851314.
49. Du, W., and J. S. Searle. 2009. The rb pathway and cancer therapeutics. *Curr. Drug Targets*. 10: 581-589 PM:19601762.
50. Ichimura, K., E. E. Schmidt, H. M. Goike, and V. P. Collins. 1996. Human glioblastomas with no alterations of the CDKN2A (p16INK4A, MTS1) and CDK4 genes have frequent mutations of the retinoblastoma gene. *Oncogene* 13: 1065-1072 PM:8806696.
51. Nakamura, M., Y. Yonekawa, P. Kleihues, and H. Ohgaki. 2001. Promoter hypermethylation of the RB1 gene in glioblastomas. *Lab Invest* 81: 77-82 PM:11204276.
52. Merlo, A. 2003. Genes and pathways driving glioblastomas in humans and murine disease models. *Neurosurg. Rev.* 26: 145-158 PM:12783270.
53. Nishikawa, R., F. B. Furnari, H. Lin, W. Arap, M. S. Berger, W. K. Cavenee, and H. J. Su Huang. 1995. Loss of P16INK4 expression is frequent in high grade gliomas. *Cancer Res.* 55: 1941-1945 PM:7728764.
54. Nakamura, M., T. Watanabe, U. Klangby, C. Asker, K. Wiman, Y. Yonekawa, P. Kleihues, and H. Ohgaki. 2001. p14ARF deletion and methylation in genetic pathways to glioblastomas. *Brain Pathol.* 11: 159-168 PM:11303791.

55. Aylon, Y., and M. Oren. 2011. New plays in the p53 theater. *Curr. Opin. Genet. Dev.* 21: 86-92 PM:21317061.
56. Rivlin, N., R. Brosh, M. Oren, and V. Rotter. 2011. Mutations in the p53 Tumor Suppressor Gene: Important Milestones at the Various Steps of Tumorigenesis. *Genes Cancer* 2: 466-474 PM:21779514.
57. Reifenberger, G., J. Reifenberger, K. Ichimura, P. S. Meltzer, and V. P. Collins. 1994. Amplification of multiple genes from chromosomal region 12q13-14 in human malignant gliomas: preliminary mapping of the amplicons shows preferential involvement of CDK4, SAS, and MDM2. *Cancer Res.* 54: 4299-4303 PM:8044775.
58. Quelle, D. E., F. Zindy, R. A. Ashmun, and C. J. Sherr. 1995. Alternative reading frames of the INK4a tumor suppressor gene encode two unrelated proteins capable of inducing cell cycle arrest. *Cell* 83: 993-1000 PM:8521522.
59. Izumoto, S., N. Arita, T. Ohnishi, S. Hiraga, T. Taki, and T. Hayakawa. 1995. Homozygous deletions of p16INK4A/MTS1 and p15INK4B/MTS2 genes in glioma cells and primary glioma tissues. *Cancer Lett.* 97: 241-247 PM:7497469.
60. Stupp, R., W. P. Mason, M. J. van den Bent, M. Weller, B. Fisher, M. J. Taphoorn, K. Belanger, A. A. Brandes, C. Marosi, U. Bogdahn, J. Curschmann, R. C. Janzer, S. K. Ludwin, T. Gorlia, A. Allgeier, D. Lacombe, J. G. Cairncross, E. Eisenhauer, and R. O. Mirimanoff. 2005. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *N. Engl. J. Med.* 352: 987-996 PM:15758009.

61. Deorah, S., C. F. Lynch, Z. A. Sibenaller, and T. C. Ryken. 2006. Trends in brain cancer incidence and survival in the United States: Surveillance, Epidemiology, and End Results Program, 1973 to 2001. *Neurosurg. Focus.* 20: E1 PM:16709014.
62. Adamson, C., O. O. Kanu, A. I. Mehta, C. Di, N. Lin, A. K. Mattox, and D. D. Bigner. 2009. Glioblastoma multiforme: a review of where we have been and where we are going. *Expert. Opin. Investig. Drugs* 18: 1061-1083 PM:19555299.
63. Stevens, M. F., J. A. Hickman, R. Stone, N. W. Gibson, G. U. Baig, E. Lunt, and C. G. Newton. 1984. Antitumor imidazotetrazines. 1. Synthesis and chemistry of 8-carbamoyl-3-(2-chloroethyl)imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one, a novel broad-spectrum antitumor agent. *J. Med. Chem.* 27: 196-201 PM:6694168.
64. Stevens, M. F., J. A. Hickman, S. P. Langdon, D. Chubb, L. Vickers, R. Stone, G. Baig, C. Goddard, N. W. Gibson, J. A. Slack, and . 1987. Antitumor activity and pharmacokinetics in mice of 8-carbamoyl-3-methyl-imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one (CCRG 81045; M & B 39831), a novel drug with potential as an alternative to dacarbazine. *Cancer Res.* 47: 5846-5852 PM:3664486.
65. Newlands, E. S., G. R. Blackledge, J. A. Slack, G. J. Rustin, D. B. Smith, N. S. Stuart, C. P. Quarterman, R. Hoffman, M. F. Stevens, M. H. Brampton, and . 1992. Phase I trial of temozolomide (CCRG 81045; M&B 39831; NSC 362856). *Br. J. Cancer* 65: 287-291 PM:1739631.

66. Brada, M., K. Hoang-Xuan, R. Rampling, P. Y. Dietrich, L. Y. Dirix, D. Macdonald, J. J. Heimans, B. A. Zonnenberg, J. M. Bravo-Marques, R. Henriksson, R. Stupp, N. Yue, J. Bruner, M. Dugan, S. Rao, and S. Zaknoen. 2001. Multicenter phase II trial of temozolomide in patients with glioblastoma multiforme at first relapse. *Ann. Oncol.* 12: 259-266 PM:11300335.
67. Fukushima, T., H. Takeshima, and H. Kataoka. 2009. Anti-glioma therapy with temozolomide and status of the DNA-repair gene MGMT. *Anticancer Res.* 29: 4845-4854 PM:20032445.
68. Pegg, A. E. 1990. Mammalian O6-alkylguanine-DNA alkyltransferase: regulation and importance in response to alkylating carcinogenic and therapeutic agents. *Cancer Res.* 50: 6119-6129 PM:2205376.
69. Lefranc, F., J. Brotchi, and R. Kiss. 2005. Possible future issues in the treatment of glioblastomas: special emphasis on cell migration and the resistance of migrating glioblastoma cells to apoptosis. *J. Clin. Oncol.* 23: 2411-2422 PM:15800333.
70. Jiang, Z., N. Pore, G. J. Cerniglia, R. Mick, M. M. Georgescu, E. J. Bernhard, S. M. Hahn, A. K. Gupta, and A. Maity. 2007. Phosphatase and tensin homologue deficiency in glioblastoma confers resistance to radiation and temozolomide that is reversed by the protease inhibitor nelfinavir. *Cancer Res.* 67: 4467-4473 PM:17483362.

71. Trivedi, R. N., K. H. Almeida, J. L. Fornsglio, S. Schamus, and R. W. Sobol. 2005. The role of base excision repair in the sensitivity and resistance to temozolomide-mediated cell death. *Cancer Res.* 65: 6394-6400 PM:16024643.
72. Friedman, H. S., S. P. Johnson, Q. Dong, S. C. Schold, B. K. Rasheed, S. H. Bigner, F. Ali-Osman, E. Dolan, O. M. Colvin, P. Houghton, G. Germain, J. T. Drummond, S. Keir, S. Marcelli, D. D. Bigner, and P. Modrich. 1997. Methylator resistance mediated by mismatch repair deficiency in a glioblastoma multiforme xenograft. *Cancer Res.* 57: 2933-2936 PM:9230204.
73. Liu, L., P. Taverna, C. M. Whitacre, S. Chatterjee, and S. L. Gerson. 1999. Pharmacologic disruption of base excision repair sensitizes mismatch repair-deficient and -proficient colon cancer cells to methylating agents. *Clin. Cancer Res.* 5: 2908-2917 PM:10537360.
74. Tentori, L., I. Portarena, F. Torino, M. Scerrati, P. Navarra, and G. Graziani. 2002. Poly(ADP-ribose) polymerase inhibitor increases growth inhibition and reduces G(2)/M cell accumulation induced by temozolomide in malignant glioma cells. *Glia* 40: 44-54 PM:12237842.
75. Murat, A., E. Migliavacca, T. Gorlia, W. L. Lambiv, T. Shay, M. F. Hamou, T. N. de, L. Regli, W. Wick, M. C. Kouwenhoven, J. A. Hainfellner, F. L. Heppner, P. Y. Dietrich, Y. Zimmer, J. G. Cairncross, R. C. Janzer, E. Domany, M. Delorenzi, R. Stupp, and M. E. Hegi. 2008. Stem cell-related "self-renewal" signature and high epidermal growth factor receptor expression associated with resistance to concomitant chemoradiotherapy in glioblastoma. *J. Clin. Oncol.* 26: 3015-3024 PM:18565887.

76. 1991. Induction chemotherapy plus radiation compared with surgery plus radiation in patients with advanced laryngeal cancer. The Department of Veterans Affairs Laryngeal Cancer Study Group. *N. Engl. J. Med.* 324: 1685-1690 PM:2034244.
77. Adelstein, D. J., Y. Li, G. L. Adams, H. Wagner, Jr., J. A. Kish, J. F. Ensley, D. E. Schuller, and A. A. Forastiere. 2003. An intergroup phase III comparison of standard radiation therapy and two schedules of concurrent chemoradiotherapy in patients with unresectable squamous cell head and neck cancer. *J. Clin. Oncol.* 21: 92-98 PM:12506176.
78. Samant, S., P. Kumar, J. Wan, C. Hanchett, F. Vieira, T. Murry, F. S. Wong, and K. T. Robbins. 1999. Concomitant radiation therapy and targeted cisplatin chemotherapy for the treatment of advanced pyriform sinus carcinoma: disease control and preservation of organ function. *Head Neck* 21: 595-601 PM:10487945.
79. Huncharek, M., and J. Muscat. 1998. Treatment of recurrent high grade astrocytoma; results of a systematic review of 1,415 patients. *Anticancer Res.* 18: 1303-1311 PM:9615807.
80. Yung, W. K., L. Mechtler, and M. J. Gleason. 1991. Intravenous carboplatin for recurrent malignant glioma: a phase II study. *J. Clin. Oncol.* 9: 860-864 PM:1849986.
81. D'Atri, S., G. Graziani, P. M. Lacal, V. Nistico, S. Gilberti, I. Faraoni, A. J. Watson, E. Bonmassar, and G. P. Margison. 2000. Attenuation of O(6)-

- methylguanine-DNA methyltransferase activity and mRNA levels by cisplatin and temozolomide in jurkat cells. *J. Pharmacol. Exp. Ther.* 294: 664-671  
PM:10900246.
82. Brandes, A. A., U. Basso, M. Reni, F. Vastola, A. Tosoni, G. Cavallo, L. Scopece, A. J. Ferreri, M. G. Panucci, S. Monfardini, and M. Ermani. 2004. First-line chemotherapy with cisplatin plus fractionated temozolomide in recurrent glioblastoma multiforme: a phase II study of the Gruppo Italiano Cooperativo di Neuro-Oncologia. *J. Clin. Oncol.* 22: 1598-1604 PM:15117981.
83. Siddik, Z. H. 2003. Cisplatin: mode of cytotoxic action and molecular basis of resistance. *Oncogene* 22: 7265-7279 PM:14576837.
84. Ogiso, H., R. Ishitani, O. Nureki, S. Fukai, M. Yamanaka, J. H. Kim, K. Saito, A. Sakamoto, M. Inoue, M. Shirouzu, and S. Yokoyama. 2002. Crystal structure of the complex of human epidermal growth factor and receptor extracellular domains. *Cell* 110: 775-787 PM:12297050.
85. Hunter, T. 1984. The epidermal growth factor receptor gene and its product. *Nature* 311: 414-416 PM:6090939.
86. Zandi, R., A. B. Larsen, P. Andersen, M. T. Stockhausen, and H. S. Poulsen. 2007. Mechanisms for oncogenic activation of the epidermal growth factor receptor. *Cell Signal.* 19: 2013-2023 PM:17681753.
87. Hynes, N. E., K. Horsch, M. A. Olayioye, and A. Badache. 2001. The ErbB receptor tyrosine family as signal integrators. *Endocr. Relat Cancer* 8: 151-159  
PM:11566606.

88. Huang, P. H., W. K. Cavenee, F. B. Furnari, and F. M. White. 2007. Uncovering therapeutic targets for glioblastoma: a systems biology approach. *Cell Cycle* 6: 2750-2754 PM:17986864.
89. Huang, P. H., A. Mukasa, R. Bonavia, R. A. Flynn, Z. E. Brewer, W. K. Cavenee, F. B. Furnari, and F. M. White. 2007. Quantitative analysis of EGFRvIII cellular signaling networks reveals a combinatorial therapeutic strategy for glioblastoma. *Proc Natl Acad Sci U S A* 104: 12867-12872 PM:17646646.
90. Schulze, W. X., L. Deng, and M. Mann. 2005. Phosphotyrosine interactome of the ErbB-receptor kinase family. *Mol. Syst. Biol.* 1: 2005 PM:16729043.
91. Moscatello, D. K., R. B. Montgomery, P. Sundareshan, H. McDanel, M. Y. Wong, and A. J. Wong. 1996. Transformational and altered signal transduction by a naturally occurring mutant EGF receptor. *Oncogene* 13: 85-96 PM:8700557.
92. Lin, S. Y., K. Makino, W. Xia, A. Matin, Y. Wen, K. Y. Kwong, L. Bourguignon, and M. C. Hung. 2001. Nuclear localization of EGF receptor and its potential new role as a transcription factor. *Nat. Cell Biol.* 3: 802-808 PM:11533659.
93. Batzer, A. G., D. Rotin, J. M. Urena, E. Y. Skolnik, and J. Schlessinger. 1994. Hierarchy of binding sites for Grb2 and Shc on the epidermal growth factor receptor. *Mol. Cell Biol.* 14: 5192-5201 PM:7518560.
94. Meir Erwin G. 2009. *Cancer Drug Discovery and Development*.

95. Miettinen, P. J., J. E. Berger, J. Meneses, Y. Phung, R. A. Pedersen, Z. Werb, and R. Derynck. 1995. Epithelial immaturity and multiorgan failure in mice lacking epidermal growth factor receptor. *Nature* 376: 337-341 PM:7630400.
96. Threadgill, D. W., A. A. Dlugosz, L. A. Hansen, T. Tennenbaum, U. Lichti, D. Yee, C. LaMantia, T. Mourton, K. Herrup, R. C. Harris, and . 1995. Targeted disruption of mouse EGF receptor: effect of genetic background on mutant phenotype. *Science* 269: 230-234 PM:7618084.
97. Miettinen, P. J., D. Warburton, D. Bu, J. S. Zhao, J. E. Berger, P. Minoo, T. Koivisto, L. Allen, L. Dobbs, Z. Werb, and R. Derynck. 1997. Impaired lung branching morphogenesis in the absence of functional EGF receptor. *Dev. Biol.* 186: 224-236 PM:9205141.
98. Miettinen, P. J., J. R. Chin, L. Shum, H. C. Slavkin, C. F. Shuler, R. Derynck, and Z. Werb. 1999. Epidermal growth factor receptor function is necessary for normal craniofacial development and palate closure. *Nat. Genet.* 22: 69-73 PM:10319864.
99. Adamson, E. D. 1990. EGF receptor activities in mammalian development. *Mol. Reprod. Dev.* 27: 16-22 PM:2271181.
100. Sibilina, M., J. P. Steinbach, L. Stingl, A. Aguzzi, and E. F. Wagner. 1998. A strain-independent postnatal neurodegeneration in mice lacking the EGF receptor. *EMBO J.* 17: 719-731 PM:9450997.
101. Sibilina, M., and E. F. Wagner. 1995. Strain-dependent epithelial defects in mice lacking the EGF receptor. *Science* 269: 234-238 PM:7618085.

102. Kornblum, H. I., R. Hussain, J. Wiesen, P. Miettinen, S. D. Zurcher, K. Chow, R. Derynck, and Z. Werb. 1998. Abnormal astrocyte development and neuronal death in mice lacking the epidermal growth factor receptor. *J. Neurosci. Res.* 53: 697-717 PM:9753198.
103. Lazar, L. M., and M. Blum. 1992. Regional distribution and developmental expression of epidermal growth factor and transforming growth factor-alpha mRNA in mouse brain by a quantitative nuclease protection assay. *J. Neurosci.* 12: 1688-1697 PM:1578263.
104. Wilcox, J. N., and R. Derynck. 1988. Localization of cells synthesizing transforming growth factor-alpha mRNA in the mouse brain. *J. Neurosci.* 8: 1901-1904 PM:2455024.
105. Seroogy, K. B., S. Numan, C. M. Gall, D. C. Lee, and H. I. Kornblum. 1994. Expression of EGF receptor mRNA in rat nigrostriatal system. *Neuroreport* 6: 105-108 PM:7703395.
106. Seroogy, K. B., C. M. Gall, D. C. Lee, and H. I. Kornblum. 1995. Proliferative zones of postnatal rat brain express epidermal growth factor receptor mRNA. *Brain Res.* 670: 157-164 PM:7719717.
107. Kornblum, H. I., R. J. Hussain, J. M. Bronstein, C. M. Gall, D. C. Lee, and K. B. Seroogy. 1997. Prenatal ontogeny of the epidermal growth factor receptor and its ligand, transforming growth factor alpha, in the rat brain. *J. Comp Neurol.* 380: 243-261 PM:9100135.

108. Schroeder, J. A., and D. C. Lee. 1998. Dynamic expression and activation of ERBB receptors in the developing mouse mammary gland. *Cell Growth Differ.* 9: 451-464 PM:9663464.
109. Fowler, K. J., F. Walker, W. Alexander, M. L. Hibbs, E. C. Nice, R. M. Bohmer, G. B. Mann, C. Thumwood, R. Maglitto, J. A. Danks, and . 1995. A mutation in the epidermal growth factor receptor in waved-2 mice has a profound effect on receptor biochemistry that results in impaired lactation. *Proc. Natl. Acad. Sci. U. S. A* 92: 1465-1469 PM:7533293.
110. Salomon, D. S., R. Brandt, F. Ciardiello, and N. Normanno. 1995. Epidermal growth factor-related peptides and their receptors in human malignancies. *Crit Rev. Oncol. Hematol.* 19: 183-232 PM:7612182.
111. Arteaga, C. L. 2001. The epidermal growth factor receptor: from mutant oncogene in nonhuman cancers to therapeutic target in human neoplasia. *J. Clin. Oncol.* 19: 32S-40S PM:11560969.
112. Sporn, M. B., and G. J. Todaro. 1980. Autocrine secretion and malignant transformation of cells. *N. Engl. J. Med.* 303: 878-880 PM:7412807.
113. Rusch, V., J. Mendelsohn, and E. Dmitrovsky. 1996. The epidermal growth factor receptor and its ligands as therapeutic targets in human tumors. *Cytokine Growth Factor Rev.* 7: 133-141 PM:8899291.
114. Tang, P., P. A. Steck, and W. K. Yung. 1997. The autocrine loop of TGF-alpha/EGFR and brain tumors. *J. Neurooncol.* 35: 303-314 PM:9440027.

115. von, B. P., J. Sallaba, K. Dietzmann, M. Warich-Kirches, and E. Kirches. 1998. Correlation of TGF-alpha and EGF-receptor expression with proliferative activity in human astrocytic gliomas. *Pathol. Res. Pract.* 194: 141-147 PM:9587931.
116. Ekstrand, A. J., C. D. James, W. K. Cavenee, B. Seliger, R. F. Pettersson, and V. P. Collins. 1991. Genes for epidermal growth factor receptor, transforming growth factor alpha, and epidermal growth factor and their expression in human gliomas in vivo. *Cancer Res* 51: 2164-2172 PM:2009534.
117. Nister, M., T. A. Libermann, C. Betsholtz, M. Pettersson, L. Claesson-Welsh, C. H. Heldin, J. Schlessinger, and B. Westermark. 1988. Expression of messenger RNAs for platelet-derived growth factor and transforming growth factor-alpha and their receptors in human malignant glioma cell lines. *Cancer Res.* 48: 3910-3918 PM:2454731.
118. Gerosa, M. A., D. Talarico, C. Fognani, E. Raimondi, M. Colombatti, G. Tridente, C. L. De, and V. G. Della. 1989. Overexpression of N-ras oncogene and epidermal growth factor receptor gene in human glioblastomas. *J. Natl. Cancer Inst.* 81: 63-67 PM:2908920.
119. Samuels, V., J. M. Barrett, S. Bockman, C. G. Pantazis, and M. B. Allen, Jr. 1989. Immunocytochemical study of transforming growth factor expression in benign and malignant gliomas. *Am. J. Pathol.* 134: 894-902 PM:2705509.
120. Schlegel, U., P. L. Moots, M. K. Rosenblum, H. T. Thaler, and H. M. Furneaux. 1990. Expression of transforming growth factor alpha in human gliomas. *Oncogene* 5: 1839-1842 PM:2284103.

121. Yung, W. K., X. Zhang, P. A. Steck, and M. C. Hung. 1990. Differential amplification of the TGF-alpha gene in human gliomas. *Cancer Commun.* 2: 201-205 PM:2165797.
122. Ramnarain, D. B., S. Park, D. Y. Lee, K. J. Hatanpaa, S. O. Scoggin, H. Otu, T. A. Libermann, J. M. Raisanen, R. Ashfaq, E. T. Wong, J. Wu, R. Elliott, and A. A. Habib. 2006. Differential gene expression analysis reveals generation of an autocrine loop by a mutant epidermal growth factor receptor in glioma cells. *Cancer Res.* 66: 867-874 PM:16424019.
123. Grandis, J. R., and J. C. Sok. 2004. Signaling through the epidermal growth factor receptor during the development of malignancy. *Pharmacol. Ther.* 102: 37-46 PM:15056497.
124. El-Obeid, A., G. Hesselager, B. Westermark, and M. Nister. 2002. TGF-alpha-driven tumor growth is inhibited by an EGF receptor tyrosine kinase inhibitor. *Biochem. Biophys. Res. Commun.* 290: 349-358 PM:11779176.
125. Mishima, K., S. Higashiyama, A. Asai, K. Yamaoka, Y. Nagashima, N. Taniguchi, C. Kitanaka, T. Kirino, and Y. Kuchino. 1998. Heparin-binding epidermal growth factor-like growth factor stimulates mitogenic signaling and is highly expressed in human malignant gliomas. *Acta Neuropathol.* 96: 322-328 PM:9796995.
126. Goldman, C. K., J. Kim, W. L. Wong, V. King, T. Brock, and G. Y. Gillespie. 1993. Epidermal growth factor stimulates vascular endothelial growth factor

- production by human malignant glioma cells: a model of glioblastoma multiforme pathophysiology. *Mol. Biol. Cell* 4: 121-133 PM:7680247.
127. Watanabe, K., O. Tachibana, K. Sata, Y. Yonekawa, P. Kleihues, and H. Ohgaki. 1996. Overexpression of the EGF receptor and p53 mutations are mutually exclusive in the evolution of primary and secondary glioblastomas. *Brain Pathol.* 6: 217-223 PM:8864278.
128. Kleinschmidt-DeMasters, B. K., K. O. Lillehei, and M. Varella-Garcia. 2005. Glioblastomas in the older old. *Arch. Pathol. Lab Med.* 129: 624-631 PM:15859633.
129. Okada, Y., E. E. Hurwitz, J. M. Esposito, M. A. Brower, C. L. Nutt, and D. N. Louis. 2003. Selection pressures of TP53 mutation and microenvironmental location influence epidermal growth factor receptor gene amplification in human glioblastomas. *Cancer Res.* 63: 413-416 PM:12543796.
130. Hatanpaa, K. J., S. Burma, D. Zhao, and A. A. Habib. 2010. Epidermal growth factor receptor in glioma: signal transduction, neuropathology, imaging, and radioresistance. *Neoplasia.* 12: 675-684 PM:20824044.
131. Nicholson, R. I., J. M. Gee, and M. E. Harper. 2001. EGFR and cancer prognosis. *Eur. J. Cancer* 37 Suppl 4: S9-15 PM:11597399.
132. Lopez-Gines, C., R. Gil-Benso, R. Ferrer-Luna, R. Benito, E. Serna, J. Gonzalez-Darder, V. Quilis, D. Monleon, B. Celda, and M. Cerda-Nicolas. 2010. New pattern of EGFR amplification in glioblastoma and the relationship of gene

copy number with gene expression profile. *Mod. Pathol.* 23: 856-865

PM:20305620.

133. Pauletti, G., S. Dandekar, H. Rong, L. Ramos, H. Peng, R. Seshadri, and D. J. Slamon. 2000. Assessment of methods for tissue-based detection of the HER-2/neu alteration in human breast cancer: a direct comparison of fluorescence in situ hybridization and immunohistochemistry. *J. Clin. Oncol.* 18: 3651-3664  
PM:11054438.
134. Mischel, P. S., R. Shai, T. Shi, S. Horvath, K. V. Lu, G. Choe, D. Seligson, T. J. Kremen, A. Palotie, L. M. Liau, T. F. Cloughesy, and S. F. Nelson. 2003. Identification of molecular subtypes of glioblastoma by gene expression profiling. *Oncogene* 22: 2361-2373 PM:12700671.
135. Herold-Mende, C., M. M. Mueller, M. M. Bonsanto, H. P. Schmitt, S. Kunze, and H. H. Steiner. 2002. Clinical impact and functional aspects of tenascin-C expression during glioma progression. *Int. J. Cancer* 98: 362-369  
PM:11920587.
136. Shtiegman, K., and Y. Yarden. 2003. The role of ubiquitylation in signaling by growth factors: implications to cancer. *Semin. Cancer Biol.* 13: 29-40  
PM:12507554.
137. Lipkowitz, S. 2003. The role of the ubiquitination-proteasome pathway in breast cancer: ubiquitin mediated degradation of growth factor receptors in the pathogenesis and treatment of cancer. *Breast Cancer Res.* 5: 8-15  
PM:12559039.

138. Waterman, H., G. Levkowitz, I. Alroy, and Y. Yarden. 1999. The RING finger of c-Cbl mediates desensitization of the epidermal growth factor receptor. *J. Biol. Chem.* 274: 22151-22154 PM:10428778.
139. Dikic, I., and S. Giordano. 2003. Negative receptor signalling. *Curr. Opin. Cell Biol.* 15: 128-135 PM:12648667.
140. Andoniou, C. E., C. B. Thien, and W. Y. Langdon. 1994. Tumour induction by activated abl involves tyrosine phosphorylation of the product of the cbl oncogene. *EMBO J.* 13: 4515-4523 PM:7925293.
141. Levkowitz, G., H. Waterman, S. A. Ettenberg, M. Katz, A. Y. Tsygankov, I. Alroy, S. Lavi, K. Iwai, Y. Reiss, A. Ciechanover, S. Lipkowitz, and Y. Yarden. 1999. Ubiquitin ligase activity and tyrosine phosphorylation underlie suppression of growth factor signaling by c-Cbl/Sli-1. *Mol. Cell* 4: 1029-1040 PM:10635327.
142. Waterman, H., M. Katz, C. Rubin, K. Shtiegman, S. Lavi, A. Elson, T. Jovin, and Y. Yarden. 2002. A mutant EGF-receptor defective in ubiquitylation and endocytosis unveils a role for Grb2 in negative signaling. *EMBO J.* 21: 303-313 PM:11823423.
143. Fischer, O. M., S. Hart, A. Gschwind, and A. Ullrich. 2003. EGFR signal transactivation in cancer cells. *Biochem. Soc. Trans.* 31: 1203-1208 PM:14641026.
144. van, d., V, S. Oliveira, R. M. Schiffelers, G. Storm, van Bergen En Henegouwen PM, and R. C. Roovers. 2009. Crosstalk between epidermal growth factor

- receptor- and insulin-like growth factor-1 receptor signaling: implications for cancer therapy. *Curr. Cancer Drug Targets*. 9: 748-760 PM:19754359.
145. Jin, Q., and F. J. Esteva. 2008. Cross-talk between the ErbB/HER family and the type I insulin-like growth factor receptor signaling pathway in breast cancer. *J. Mammary. Gland. Biol. Neoplasia*. 13: 485-498 PM:19034632.
146. Karamouzis, M. V., P. A. Konstantinopoulos, and A. G. Papavassiliou. 2009. Targeting MET as a strategy to overcome crosstalk-related resistance to EGFR inhibitors. *Lancet Oncol*. 10: 709-717 PM:19573800.
147. Mueller, K. L., Z. Q. Yang, R. Haddad, S. P. Ethier, and J. L. Boerner. 2010. EGFR/Met association regulates EGFR TKI resistance in breast cancer. *J. Mol. Signal*. 5: 8 PM:20624308.
148. Chumbalkar, V., K. Latha, Y. Hwang, R. Maywald, L. Hawley, R. Sawaya, L. Diao, K. Baggerly, W. K. Cavenee, F. B. Furnari, and O. Bogler. 2011. Analysis of phosphotyrosine signaling in glioblastoma identifies STAT5 as a novel downstream target of DeltaEGFR. *J Proteome. Res* 10: 1343-1352 PM:21214269.
149. Moro, L., M. Venturino, C. Bozzo, L. Silengo, F. Altruda, L. Beguinot, G. Tarone, and P. Defilippi. 1998. Integrins induce activation of EGF receptor: role in MAP kinase induction and adhesion-dependent cell survival. *EMBO J*. 17: 6622-6632 PM:9822606.
150. Moro, L., L. Dolce, S. Cabodi, E. Bergatto, E. E. Boeri, M. Smeriglio, E. Turco, S. F. Retta, M. G. Giuffrida, M. Venturino, J. Godovac-Zimmermann, A. Conti,

- E. Schaefer, L. Beguinot, C. Tacchetti, P. Gaggini, L. Silengo, G. Tarone, and P. Defilippi. 2002. Integrin-induced epidermal growth factor (EGF) receptor activation requires c-Src and p130Cas and leads to phosphorylation of specific EGF receptor tyrosines. *J. Biol. Chem.* 277: 9405-9414 PM:11756413.
151. Bill, H. M., B. Knudsen, S. L. Moores, S. K. Muthuswamy, V. R. Rao, J. S. Brugge, and C. K. Miranti. 2004. Epidermal growth factor receptor-dependent regulation of integrin-mediated signaling and cell cycle entry in epithelial cells. *Mol. Cell Biol.* 24: 8586-8599 PM:15367678.
152. Daub, H., C. Wallasch, A. Lankenau, A. Herrlich, and A. Ullrich. 1997. Signal characteristics of G protein-transactivated EGF receptor. *EMBO J.* 16: 7032-7044 PM:9384582.
153. Gschwind, A., N. Prenzel, and A. Ullrich. 2002. Lysophosphatidic acid-induced squamous cell carcinoma cell proliferation and motility involves epidermal growth factor receptor signal transactivation. *Cancer Res.* 62: 6329-6336 PM:12414665.
154. Lui, V. W., S. M. Thomas, Q. Zhang, A. L. Wentzel, J. M. Siegfried, J. Y. Li, and J. R. Grandis. 2003. Mitogenic effects of gastrin-releasing peptide in head and neck squamous cancer cells are mediated by activation of the epidermal growth factor receptor. *Oncogene* 22: 6183-6193 PM:13679857.
155. Thomas, S. M., J. R. Grandis, A. L. Wentzel, W. E. Gooding, V. W. Lui, and J. M. Siegfried. 2005. Gastrin-releasing peptide receptor mediates activation of

- the epidermal growth factor receptor in lung cancer cells. *Neoplasia*. 7: 426-431  
PM:15967120.
156. Chen, L., R. R. Hodges, C. Funaki, D. Zoukhri, R. J. Gaivin, D. M. Perez, and D. A. Dartt. 2006. Effects of alpha1D-adrenergic receptors on shedding of biologically active EGF in freshly isolated lacrimal gland epithelial cells. *Am. J. Physiol Cell Physiol* 291: C946-C956 PM:16760267.
157. Huang, J., J. Hu, X. Bian, K. Chen, W. Gong, N. M. Dunlop, O. M. Howard, and J. M. Wang. 2007. Transactivation of the epidermal growth factor receptor by formylpeptide receptor exacerbates the malignant behavior of human glioblastoma cells. *Cancer Res*. 67: 5906-5913 PM:17575160.
158. Casas-Gonzalez, P., A. Ruiz-Martinez, and J. A. Garcia-Sainz. 2003. Lysophosphatidic acid induces alpha1B-adrenergic receptor phosphorylation through G beta gamma, phosphoinositide 3-kinase, protein kinase C and epidermal growth factor receptor transactivation. *Biochim. Biophys. Acta* 1633: 75-83 PM:12880866.
159. Zhao, Y., D. He, B. Saatian, T. Watkins, E. W. Spannhake, N. J. Pyne, and V. Natarajan. 2006. Regulation of lysophosphatidic acid-induced epidermal growth factor receptor transactivation and interleukin-8 secretion in human bronchial epithelial cells by protein kinase Cdelta, Lyn kinase, and matrix metalloproteinases. *J. Biol. Chem*. 281: 19501-19511 PM:16687414.
160. Neaud, V., J. G. Duplantier, C. Mazzocco, W. Kisiel, and J. Rosenbaum. 2004. Thrombin up-regulates tissue factor pathway inhibitor-2 synthesis through a

- cyclooxygenase-2-dependent, epidermal growth factor receptor-independent mechanism. *J. Biol. Chem.* 279: 5200-5206 PM:14623891.
161. Kanda, Y., K. Mizuno, Y. Kuroki, and Y. Watanabe. 2001. Thrombin-induced p38 mitogen-activated protein kinase activation is mediated by epidermal growth factor receptor transactivation pathway. *Br. J. Pharmacol.* 132: 1657-1664 PM:11309236.
162. Beaucage, P., and P. Moreau. 2004. EGF receptor transactivation in angiotensin II and endothelin control of vascular protein synthesis in vivo. *J. Cardiovasc. Pharmacol.* 44 Suppl 1: S20-S23 PM:15838279.
163. Spinella, F., L. Rosano, G. Elia, C. Di, V, P. G. Natali, and A. Bagnato. 2004. Endothelin-1 stimulates cyclooxygenase-2 expression in ovarian cancer cells through multiple signaling pathways: evidence for involvement of transactivation of the epidermal growth factor receptor. *J. Cardiovasc. Pharmacol.* 44 Suppl 1: S140-S143 PM:15838264.
164. Keely, S. J., S. O. Calandrella, and K. E. Barrett. 2000. Carbachol-stimulated transactivation of epidermal growth factor receptor and mitogen-activated protein kinase in T(84) cells is mediated by intracellular  $Ca^{2+}$ , PYK-2, and p60(src). *J. Biol. Chem.* 275: 12619-12625 PM:10777553.
165. McCole, D. F., S. J. Keely, R. J. Coffey, and K. E. Barrett. 2002. Transactivation of the epidermal growth factor receptor in colonic epithelial cells by carbachol requires extracellular release of transforming growth factor- $\alpha$ . *J. Biol. Chem.* 277: 42603-42612 PM:12202486.

166. Newton, J. S. 2002. Angiotensin and epidermal growth factor receptor cross talk goes up and down. *J. Hypertens.* 20: 597-598 PM:11910289.
167. De, P. P., A. Porcellini, C. Savoia, A. Lombardi, B. Gigante, G. Frati, S. Rubattu, B. Musumeci, and M. Volpe. 2002. Functional cross-talk between angiotensin II and epidermal growth factor receptors in NIH3T3 fibroblasts. *J. Hypertens.* 20: 693-699 PM:11910305.
168. Madarame, J., S. Higashiyama, H. Kiyota, A. Madachi, F. Toki, T. Shimomura, N. Tani, Y. Oishi, and N. Matsuura. 2003. Transactivation of epidermal growth factor receptor after heparin-binding epidermal growth factor-like growth factor shedding in the migration of prostate cancer cells promoted by bombesin. *Prostate* 57: 187-195 PM:14518027.
169. Porcile, C., A. Bajetto, F. Barbieri, S. Barbero, R. Bonavia, M. Biglieri, P. Pirani, T. Florio, and G. Schettini. 2005. Stromal cell-derived factor-1alpha (SDF-1alpha/CXCL12) stimulates ovarian cancer cell growth through the EGF receptor transactivation. *Exp. Cell Res.* 308: 241-253 PM:15921680.
170. Schwechheimer, K., S. Huang, and W. K. Cavenee. 1995. EGFR gene amplification--rearrangement in human glioblastomas. *Int J Cancer* 62: 145-148 PM:7622287.
171. Steck, P. A., P. Lee, M. C. Hung, and W. K. Yung. 1988. Expression of an altered epidermal growth factor receptor by human glioblastoma cells. *Cancer Res.* 48: 5433-5439 PM:3416300.

172. Ekstrand, A. J., N. Sugawa, C. D. James, and V. P. Collins. 1992. Amplified and rearranged epidermal growth factor receptor genes in human glioblastomas reveal deletions of sequences encoding portions of the N- and/or C-terminal tails. *Proc. Natl. Acad. Sci. U. S. A* 89: 4309-4313 PM:1584765.
173. Libermann, T. A., H. R. Nusbaum, N. Razon, R. Kris, I. Lax, H. Soreq, N. Whittle, M. D. Waterfield, A. Ullrich, and J. Schlessinger. 1985. Amplification, enhanced expression and possible rearrangement of EGF receptor gene in primary human brain tumours of glial origin. *Nature* 313: 144-147 PM:2981413.
174. Nagane, M., F. Coufal, H. Lin, O. Bogler, W. K. Cavenee, and H. J. Huang. 1996. A common mutant epidermal growth factor receptor confers enhanced tumorigenicity on human glioblastoma cells by increasing proliferation and reducing apoptosis. *Cancer Res* 56: 5079-5086 PM:8895767.
175. Schlegel, J., A. Merdes, G. Stumm, F. K. Albert, M. Forsting, N. Hynes, and M. Kiessling. 1994. Amplification of the epidermal-growth-factor-receptor gene correlates with different growth behaviour in human glioblastoma. *Int. J. Cancer* 56: 72-77 PM:8262681.
176. Hurtt, M. R., J. Moossy, M. Donovan-Peluso, and J. Locker. 1992. Amplification of epidermal growth factor receptor gene in gliomas: histopathology and prognosis. *J. Neuropathol. Exp. Neurol.* 51: 84-90 PM:1311022.
177. O'Rourke, D. M., E. J. Nute, J. G. Davis, C. Wu, A. Lee, R. Murali, H. T. Zhang, X. Qian, C. C. Kao, and M. I. Greene. 1998. Inhibition of a naturally occurring

- EGFR oncoprotein by the p185neu ectodomain: implications for subdomain contributions to receptor assembly. *Oncogene* 16: 1197-1207 PM:9528862.
178. Voldborg, B. R., L. Damstrup, M. Spang-Thomsen, and H. S. Poulsen. 1997. Epidermal growth factor receptor (EGFR) and EGFR mutations, function and possible role in clinical trials. *Ann. Oncol.* 8: 1197-1206 PM:9496384.
179. Huang, H. S., M. Nagane, C. K. Klingbeil, H. Lin, R. Nishikawa, X. D. Ji, C. M. Huang, G. N. Gill, H. S. Wiley, and W. K. Cavenee. 1997. The enhanced tumorigenic activity of a mutant epidermal growth factor receptor common in human cancers is mediated by threshold levels of constitutive tyrosine phosphorylation and unattenuated signaling. *J Biol Chem* 272: 2927-2935 PM:9006938.
180. Brennan, P. J., T. Kumagai, A. Berezov, R. Murali, and M. I. Greene. 2000. HER2/neu: mechanisms of dimerization/oligomerization. *Oncogene* 19: 6093-6101 PM:11156522.
181. Burgess, A. W., H. S. Cho, C. Eigenbrot, K. M. Ferguson, T. P. Garrett, D. J. Leahy, M. A. Lemmon, M. X. Sliwkowski, C. W. Ward, and S. Yokoyama. 2003. An open-and-shut case? Recent insights into the activation of EGF/ErbB receptors. *Mol. Cell* 12: 541-552 PM:14527402.
182. Prigent, S. A., M. Nagane, H. Lin, I. Huvar, G. R. Boss, J. R. Feramisco, W. K. Cavenee, and H. S. Huang. 1996. Enhanced tumorigenic behavior of glioblastoma cells expressing a truncated epidermal growth factor receptor is

- mediated through the Ras-Shc-Grb2 pathway. *J. Biol. Chem.* 271: 25639-25645  
PM:8810340.
183. Choe, G., S. Horvath, T. F. Cloughesy, K. Crosby, D. Seligson, A. Palotie, L. Inge, B. L. Smith, C. L. Sawyers, and P. S. Mischel. 2003. Analysis of the phosphatidylinositol 3'-kinase signaling pathway in glioblastoma patients in vivo. *Cancer Res.* 63: 2742-2746 PM:12782577.
184. Zhu, H., J. Acquaviva, P. Ramachandran, A. Boskovitz, S. Woolfenden, R. Pfannl, R. T. Bronson, J. W. Chen, R. Weissleder, D. E. Housman, and A. Charest. 2009. Oncogenic EGFR signaling cooperates with loss of tumor suppressor gene functions in gliomagenesis. *Proc. Natl. Acad. Sci. U. S. A* 106: 2712-2716 PM:19196966.
185. Weil, R. J. 2008. Incorporating molecular tools into early-stage clinical trials. *PLoS. Med.* 5: e21 PM:18215108.
186. De, I., I. G. Konopka, S. V. Puram, J. A. Chan, R. M. Bachoo, M. J. You, D. E. Levy, R. A. Depinho, and A. Bonni. 2008. Identification of a PTEN-regulated STAT3 brain tumor suppressor pathway. *Genes Dev.* 22: 449-462  
PM:18258752.
187. Li, B., M. Yuan, I. A. Kim, C. M. Chang, E. J. Bernhard, and H. K. Shu. 2004. Mutant epidermal growth factor receptor displays increased signaling through the phosphatidylinositol-3 kinase/AKT pathway and promotes radioresistance in cells of astrocytic origin. *Oncogene* 23: 4594-4602 PM:15077177.

188. Moscatello, D. K., M. Holgado-Madruga, D. R. Emlet, R. B. Montgomery, and A. J. Wong. 1998. Constitutive activation of phosphatidylinositol 3-kinase by a naturally occurring mutant epidermal growth factor receptor. *J. Biol. Chem.* 273: 200-206 PM:9417065.
189. Wu, C. J., D. M. O'Rourke, G. S. Feng, G. R. Johnson, Q. Wang, and M. I. Greene. 2001. The tyrosine phosphatase SHP-2 is required for mediating phosphatidylinositol 3-kinase/Akt activation by growth factors. *Oncogene* 20: 6018-6025 PM:11593409.
190. Boise, L. H., M. Gonzalez-Garcia, C. E. Postema, L. Ding, T. Lindsten, L. A. Turka, X. Mao, G. Nunez, and C. B. Thompson. 1993. *bcl-x*, a *bcl-2*-related gene that functions as a dominant regulator of apoptotic cell death. *Cell* 74: 597-608 PM:8358789.
191. Mizoguchi, M., R. A. Betensky, T. T. Batchelor, D. C. Bernay, D. N. Louis, and C. L. Nutt. 2006. Activation of STAT3, MAPK, and AKT in malignant astrocytic gliomas: correlation with EGFR status, tumor grade, and survival. *J. Neuropathol. Exp. Neurol.* 65: 1181-1188 PM:17146292.
192. Grandal, M. V., R. Zandi, M. W. Pedersen, B. M. Willumsen, D. B. van, and H. S. Poulsen. 2007. EGFRvIII escapes down-regulation due to impaired internalization and sorting to lysosomes. *Carcinogenesis* 28: 1408-1417 PM:17372273.

193. Davies, G. C., P. E. Ryan, L. Rahman, M. Zajac-Kaye, and S. Lipkowitz. 2006. EGFRvIII undergoes activation-dependent downregulation mediated by the Cbl proteins. *Oncogene* 25: 6497-6509 PM:16702950.
194. Schmidt, M. H., F. B. Furnari, W. K. Cavenee, and O. Bogler. 2003. Epidermal growth factor receptor signaling intensity determines intracellular protein interactions, ubiquitination, and internalization. *Proc Natl Acad Sci U S A* 100: 6505-6510 PM:12734385.
195. Nagane, M., A. Levitzki, A. Gazit, W. K. Cavenee, and H. J. Huang. 1998. Drug resistance of human glioblastoma cells conferred by a tumor-specific mutant epidermal growth factor receptor through modulation of Bcl-XL and caspase-3-like proteases. *Proc Natl Acad Sci U S A* 95: 5724-5729 PM:9576951.
196. Hussein, M. R., R. M. El-Ghorori, and Y. G. El-Rahman. 2006. Alterations of p53, BCL-2, and hMSH2 protein expression in the normal brain tissues, gliosis, and gliomas. *Int. J. Exp. Pathol.* 87: 297-306 PM:16875495.
197. Green, D. R., and G. Kroemer. 2004. The pathophysiology of mitochondrial cell death. *Science* 305: 626-629 PM:15286356.
198. Strik, H., M. Deininger, J. Streffer, E. Grote, J. Wickboldt, J. Dichgans, M. Weller, and R. Meyermann. 1999. BCL-2 family protein expression in initial and recurrent glioblastomas: modulation by radiochemotherapy. *J. Neurol. Neurosurg. Psychiatry* 67: 763-768 PM:10567494.

199. Wick, W., S. Wagner, S. Kerkau, J. Dichgans, J. C. Tonn, and M. Weller. 1998. BCL-2 promotes migration and invasiveness of human glioma cells. *FEBS Lett.* 440: 419-424 PM:9872414.
200. Wick, W., C. Grimm, C. Wild-Bode, M. Platten, M. Arpin, and M. Weller. 2001. Ezrin-dependent promotion of glioma cell clonogenicity, motility, and invasion mediated by BCL-2 and transforming growth factor-beta2. *J. Neurosci.* 21: 3360-3368 PM:11331365.
201. Wick, W., C. Wild-Bode, B. Frank, and M. Weller. 2004. BCL-2-induced glioma cell invasiveness depends on furin-like proteases. *J. Neurochem.* 91: 1275-1283 PM:15584904.
202. Shinojima, N., K. Tada, S. Shiraishi, T. Kamiryo, M. Kochi, H. Nakamura, K. Makino, H. Saya, H. Hirano, J. Kuratsu, K. Oka, Y. Ishimaru, and Y. Ushio. 2003. Prognostic value of epidermal growth factor receptor in patients with glioblastoma multiforme. *Cancer Res.* 63: 6962-6970 PM:14583498.
203. Aldape, K. D., K. Ballman, A. Furth, J. C. Buckner, C. Giannini, P. C. Burger, B. W. Scheithauer, R. B. Jenkins, and C. D. James. 2004. Immunohistochemical detection of EGFRvIII in high malignancy grade astrocytomas and evaluation of prognostic significance. *J. Neuropathol. Exp. Neurol.* 63: 700-707 PM:15290895.
204. Heimberger, A. B., R. Hlatky, D. Suki, D. Yang, J. Weinberg, M. Gilbert, R. Sawaya, and K. Aldape. 2005. Prognostic effect of epidermal growth factor

- receptor and EGFRvIII in glioblastoma multiforme patients. *Clin. Cancer Res.* 11: 1462-1466 PM:15746047.
205. Pelloski, C. E., K. V. Ballman, A. F. Furth, L. Zhang, E. Lin, E. P. Sulman, K. Bhat, J. M. McDonald, W. K. Yung, H. Colman, S. Y. Woo, A. B. Heimberger, D. Suki, M. D. Prados, S. M. Chang, F. G. Barker, J. C. Buckner, C. D. James, and K. Aldape. 2007. Epidermal growth factor receptor variant III status defines clinically distinct subtypes of glioblastoma. *J. Clin. Oncol.* 25: 2288-2294 PM:17538175.
206. Pedersen, M. W., N. Pedersen, L. H. Ottesen, and H. S. Poulsen. 2005. Differential response to gefitinib of cells expressing normal EGFR and the mutant EGFRvIII. *Br. J. Cancer* 93: 915-923 PM:16189524.
207. Mellinghoff, I. K., M. Y. Wang, I. Vivanco, D. A. Haas-Kogan, S. Zhu, E. Q. Dia, K. V. Lu, K. Yoshimoto, J. H. Huang, D. J. Chute, B. L. Riggs, S. Horvath, L. M. Liau, W. K. Cavenee, P. N. Rao, R. Beroukhim, T. C. Peck, J. C. Lee, W. R. Sellers, D. Stokoe, M. Prados, T. F. Cloughesy, C. L. Sawyers, and P. S. Mischel. 2005. Molecular determinants of the response of glioblastomas to EGFR kinase inhibitors. *N. Engl. J Med* 353: 2012-2024 PM:16282176.
208. Haas-Kogan, D. A., M. D. Prados, T. Tihan, D. A. Eberhard, N. Jelluma, N. D. Arvold, R. Baumber, K. R. Lamborn, A. Kapadia, M. Malec, M. S. Berger, and D. Stokoe. 2005. Epidermal growth factor receptor, protein kinase B/Akt, and glioma response to erlotinib. *J. Natl. Cancer Inst.* 97: 880-887 PM:15956649.

209. Pelloski, C. E., K. V. Ballman, A. F. Furth, L. Zhang, E. Lin, E. P. Sulman, K. Bhat, J. M. McDonald, W. K. Yung, H. Colman, S. Y. Woo, A. B. Heimberger, D. Suki, M. D. Prados, S. M. Chang, F. G. Barker, J. C. Buckner, C. D. James, and K. Aldape. 2007. Epidermal growth factor receptor variant III status defines clinically distinct subtypes of glioblastoma. *J. Clin. Oncol.* 25: 2288-2294 PM:17538175.
210. Sarkaria, J. N., L. Yang, P. T. Grogan, G. J. Kitange, B. L. Carlson, M. A. Schroeder, E. Galanis, C. Giannini, W. Wu, E. B. Dinca, and C. D. James. 2007. Identification of molecular characteristics correlated with glioblastoma sensitivity to EGFR kinase inhibition through use of an intracranial xenograft test panel. *Mol. Cancer Ther.* 6: 1167-1174 PM:17363510.
211. Mellinghoff, I. K., T. F. Cloughesy, and P. S. Mischel. 2007. PTEN-mediated resistance to epidermal growth factor receptor kinase inhibitors. *Clin. Cancer Res.* 13: 378-381 PM:17255257.
212. Wang, M. Y., K. V. Lu, S. Zhu, E. Q. Dia, I. Vivanco, G. M. Shackleford, W. K. Cavenee, I. K. Mellinghoff, T. F. Cloughesy, C. L. Sawyers, and P. S. Mischel. 2006. Mammalian target of rapamycin inhibition promotes response to epidermal growth factor receptor kinase inhibitors in PTEN-deficient and PTEN-intact glioblastoma cells. *Cancer Res* 66: 7864-7869 PM:16912159.
213. Heimberger, A. B., G. E. Archer, L. E. Crotty, R. E. McLendon, A. H. Friedman, H. S. Friedman, D. D. Bigner, and J. H. Sampson. 2002. Dendritic cells pulsed with a tumor-specific peptide induce long-lasting immunity and are effective

against murine intracerebral melanoma. *Neurosurgery* 50: 158-164  
PM:11844246.

214. Heimberger, A. B., L. E. Crotty, G. E. Archer, K. R. Hess, C. J. Wikstrand, A. H. Friedman, H. S. Friedman, D. D. Bigner, and J. H. Sampson. 2003. Epidermal growth factor receptor VIII peptide vaccination is efficacious against established intracerebral tumors. *Clin. Cancer Res.* 9: 4247-4254 PM:14519652.
215. Choi, B. D., G. E. Archer, D. A. Mitchell, A. B. Heimberger, R. E. McLendon, D. D. Bigner, and J. H. Sampson. 2009. EGFRvIII-targeted vaccination therapy of malignant glioma. *Brain Pathol.* 19: 713-723 PM:19744042.
216. Sampson, J. H., A. B. Heimberger, G. E. Archer, K. D. Aldape, A. H. Friedman, H. S. Friedman, M. R. Gilbert, J. E. Herndon, R. E. McLendon, D. A. Mitchell, D. A. Reardon, R. Sawaya, R. J. Schmittling, W. Shi, J. J. Vredenburgh, and D. D. Bigner. 2010. Immunologic escape after prolonged progression-free survival with epidermal growth factor receptor variant III peptide vaccination in patients with newly diagnosed glioblastoma. *J. Clin. Oncol.* 28: 4722-4729  
PM:20921459.
217. Frederick, L., X. Y. Wang, G. Eley, and C. D. James. 2000. Diversity and frequency of epidermal growth factor receptor mutations in human glioblastomas. *Cancer Res.* 60: 1383-1387 PM:10728703.
218. Pines, G., P. H. Huang, Y. Zwang, F. M. White, and Y. Yarden. 2010. EGFRvIV: a previously uncharacterized oncogenic mutant reveals a kinase autoinhibitory mechanism. *Oncogene* 29: 5850-5860 PM:20676128.

219. Wykosky, J., D. M. Gibo, C. Stanton, and W. Debinski. 2005. EphA2 as a novel molecular marker and target in glioblastoma multiforme. *Mol. Cancer Res.* 3: 541-551 PM:16254188.
220. Wong, A. J., J. M. Ruppert, S. H. Bigner, C. H. Grzeschik, P. A. Humphrey, D. S. Bigner, and B. Vogelstein. 1992. Structural alterations of the epidermal growth factor receptor gene in human gliomas. *Proc Natl Acad Sci U S A* 89: 2965-2969 PM:1557402.
221. Humphrey, P. A., L. M. Gangarosa, A. J. Wong, G. E. Archer, M. Lund-Johansen, R. Bjerkvig, O. D. Laerum, H. S. Friedman, and D. D. Bigner. 1991. Deletion-mutant epidermal growth factor receptor in human gliomas: effects of type II mutation on receptor function. *Biochem. Biophys. Res. Commun.* 178: 1413-1420 PM:1678600.
222. Peschard, P., and M. Park. 2003. Escape from Cbl-mediated downregulation: a recurrent theme for oncogenic deregulation of receptor tyrosine kinases. *Cancer Cell* 3: 519-523 PM:12842080.
223. Ullrich, A., L. Coussens, J. S. Hayflick, T. J. Dull, A. Gray, A. W. Tam, J. Lee, Y. Yarden, T. A. Libermann, J. Schlessinger, and . 1984. Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermoid carcinoma cells. *Nature* 309: 418-425 PM:6328312.
224. Libermann, T. A., N. Razon, A. D. Bartal, Y. Yarden, J. Schlessinger, and H. Soreq. 1984. Expression of epidermal growth factor receptors in human brain tumors. *Cancer Res.* 44: 753-760 PM:6318976.

225. Sato, J. D., T. Kawamoto, A. D. Le, J. Mendelsohn, J. Polikoff, and G. H. Sato. 1983. Biological effects in vitro of monoclonal antibodies to human epidermal growth factor receptors. *Mol. Biol. Med.* 1: 511-529 PM:6094961.
226. Kawamoto, T., J. D. Sato, A. Le, J. Polikoff, G. H. Sato, and J. Mendelsohn. 1983. Growth stimulation of A431 cells by epidermal growth factor: identification of high-affinity receptors for epidermal growth factor by an anti-receptor monoclonal antibody. *Proc. Natl. Acad. Sci. U. S. A* 80: 1337-1341 PM:6298788.
227. Gill, G. N., T. Kawamoto, C. Cochet, A. Le, J. D. Sato, H. Masui, C. McLeod, and J. Mendelsohn. 1984. Monoclonal anti-epidermal growth factor receptor antibodies which are inhibitors of epidermal growth factor binding and antagonists of epidermal growth factor binding and antagonists of epidermal growth factor-stimulated tyrosine protein kinase activity. *J. Biol. Chem.* 259: 7755-7760 PM:6330079.
228. Baselga, J. 2001. The EGFR as a target for anticancer therapy--focus on cetuximab. *Eur. J. Cancer* 37 Suppl 4: S16-S22 PM:11597400.
229. Goldstein, N. I., M. Prewett, K. Zuklys, P. Rockwell, and J. Mendelsohn. 1995. Biological efficacy of a chimeric antibody to the epidermal growth factor receptor in a human tumor xenograft model. *Clin. Cancer Res.* 1: 1311-1318 PM:9815926.
230. Sobrero, A. F., J. Maurel, L. Fehrenbacher, W. Scheithauer, Y. A. Abubakr, M. P. Lutz, M. E. Vega-Villegas, C. Eng, E. U. Steinhauer, J. Prausova, H. J. Lenz,

- C. Borg, G. Middleton, H. Kroning, G. Luppi, O. Kisker, A. Zobel, C. Langer, J. Kopit, and H. A. Burris, III. 2008. EPIC: phase III trial of cetuximab plus irinotecan after fluoropyrimidine and oxaliplatin failure in patients with metastatic colorectal cancer. *J. Clin. Oncol.* 26: 2311-2319 PM:18390971.
231. Bonner, J. A., P. M. Harari, J. Giralt, N. Azarnia, D. M. Shin, R. B. Cohen, C. U. Jones, R. Sur, D. Raben, J. Jassem, R. Ove, M. S. Kies, J. Baselga, H. Youssoufian, N. Amellal, E. K. Rowinsky, and K. K. Ang. 2006. Radiotherapy plus cetuximab for squamous-cell carcinoma of the head and neck. *N. Engl. J. Med.* 354: 567-578 PM:16467544.
232. Butts, C. A., D. Bodkin, E. L. Middleman, C. W. Englund, D. Ellison, Y. Alam, H. Kreisman, P. Graze, J. Maher, H. J. Ross, P. M. Ellis, W. McNulty, E. Kaplan, V. Pautret, M. R. Weber, and F. A. Shepherd. 2007. Randomized phase II study of gemcitabine plus cisplatin or carboplatin [corrected], with or without cetuximab, as first-line therapy for patients with advanced or metastatic non small-cell lung cancer. *J. Clin. Oncol.* 25: 5777-5784 PM:18089875.
233. Thienelt, C. D., P. A. Bunn, Jr., N. Hanna, A. Rosenberg, M. N. Needle, M. E. Long, D. L. Gustafson, and K. Kelly. 2005. Multicenter phase I/II study of cetuximab with paclitaxel and carboplatin in untreated patients with stage IV non-small-cell lung cancer. *J. Clin. Oncol.* 23: 8786-8793 PM:16246975.
234. Pirker, R., J. R. Pereira, A. Szczesna, P. J. von, M. Krzakowski, R. Ramlau, I. Vynnychenko, K. Park, C. T. Yu, V. Ganul, J. K. Roh, E. Bajetta, K. O'Byrne, M. F. de, W. Eberhardt, T. Goddemeier, M. Emig, and U. Gatzemeier. 2009. Cetuximab plus chemotherapy in patients with advanced non-small-cell lung

- cancer (FLEX): an open-label randomised phase III trial. *Lancet* 373: 1525-1531 PM:19410716.
235. Lynch, T. J., T. Patel, L. Dreisbach, M. McCleod, W. J. Heim, R. C. Hermann, E. Paschold, N. O. Iannotti, S. Dakhil, S. Gorton, V. Pautret, M. R. Weber, and D. Woytowicz. 2010. Cetuximab and first-line taxane/carboplatin chemotherapy in advanced non-small-cell lung cancer: results of the randomized multicenter phase III trial BMS099. *J. Clin. Oncol.* 28: 911-917 PM:20100966.
236. Molli, P. R., L. Adam, and R. Kumar. 2008. Therapeutic IMC-C225 antibody inhibits breast cancer cell invasiveness via Vav2-dependent activation of RhoA GTPase. *Clin. Cancer Res.* 14: 6161-6170 PM:18829495.
237. Eller, J. L., S. L. Longo, M. M. Kyle, D. Bassano, D. J. Hicklin, and G. W. Canute. 2005. Anti-epidermal growth factor receptor monoclonal antibody cetuximab augments radiation effects in glioblastoma multiforme in vitro and in vivo. *Neurosurgery* 56: 155-162 PM:15617598.
238. Patel, D., A. Lahiji, S. Patel, M. Franklin, X. Jimenez, D. J. Hicklin, and X. Kang. 2007. Monoclonal antibody cetuximab binds to and down-regulates constitutively activated epidermal growth factor receptor VIII on the cell surface. *Anticancer Res.* 27: 3355-3366 PM:17970081.
239. Belda-Iniesta, C., J. C. Carpeno, E. C. Saenz, M. Gutierrez, R. Perona, and M. G. Baron. 2006. Long term responses with cetuximab therapy in glioblastoma multiforme. *Cancer Biol. Ther.* 5: 912-914 PM:16929166.

240. Van, C. E., C. H. Kohne, E. Hitre, J. Zaluski, C. R. Chang Chien, A. Makhson, G. D'Haens, T. Pinter, R. Lim, G. Bodoky, J. K. Roh, G. Folprecht, P. Ruff, C. Stroh, S. Tejpar, M. Schlichting, J. Nippgen, and P. Rougier. 2009. Cetuximab and chemotherapy as initial treatment for metastatic colorectal cancer. *N. Engl. J. Med.* 360: 1408-1417 PM:19339720.
241. Saltz, L. B., H. J. Lenz, H. L. Kindler, H. S. Hochster, S. Wadler, P. M. Hoff, N. E. Kemeny, E. M. Hollywood, M. Gonen, M. Quinones, M. Morse, and H. X. Chen. 2007. Randomized phase II trial of cetuximab, bevacizumab, and irinotecan compared with cetuximab and bevacizumab alone in irinotecan-refractory colorectal cancer: the BOND-2 study. *J. Clin. Oncol.* 25: 4557-4561 PM:17876013.
242. Hasselbalch, B., U. Lassen, S. Hansen, M. Holmberg, M. Sorensen, M. Kosteljanetz, H. Broholm, M. T. Stockhausen, and H. S. Poulsen. 2010. Cetuximab, bevacizumab, and irinotecan for patients with primary glioblastoma and progression after radiation therapy and temozolomide: a phase II trial. *Neuro. Oncol.* 12: 508-516 PM:20406901.
243. Halatsch, M. E., E. E. Gehrke, V. I. Vougioukas, I. C. Botefur, F. Borhani, T. Efferth, E. Gebhart, S. Domhof, U. Schmidt, and M. Buchfelder. 2004. Inverse correlation of epidermal growth factor receptor messenger RNA induction and suppression of anchorage-independent growth by OSI-774, an epidermal growth factor receptor tyrosine kinase inhibitor, in glioblastoma multiforme cell lines. *J. Neurosurg.* 100: 523-533 PM:15035290.

244. Prados, M. D., K. R. Lamborn, S. Chang, E. Burton, N. Butowski, M. Malec, A. Kapadia, J. Rabbitt, M. S. Page, A. Fedoroff, D. Xie, and S. K. Kelley. 2006. Phase 1 study of erlotinib HCl alone and combined with temozolomide in patients with stable or recurrent malignant glioma. *Neuro. Oncol.* 8: 67-78 PM:16443950.
245. Krishnan, S., P. D. Brown, K. V. Ballman, J. B. Fiveash, J. H. Uhm, C. Giannini, K. A. Jaeckle, F. J. Geoffroy, L. B. Nabors, and J. C. Buckner. 2006. Phase I trial of erlotinib with radiation therapy in patients with glioblastoma multiforme: results of North Central Cancer Treatment Group protocol N0177. *Int. J. Radiat. Oncol. Biol. Phys.* 65: 1192-1199 PM:16626884.
246. Doherty, L., D. C. Gigas, S. Kesari, J. Drappatz, R. Kim, J. Zimmerman, L. Ostrowsky, and P. Y. Wen. 2006. Pilot study of the combination of EGFR and mTOR inhibitors in recurrent malignant gliomas. *Neurology* 67: 156-158 PM:16832099.
247. Rich, J. N., D. A. Reardon, T. Peery, J. M. Dowell, J. A. Quinn, K. L. Penne, C. J. Wikstrand, L. B. Van Duyn, J. E. Dancey, R. E. McLendon, J. C. Kao, T. T. Stenzel, B. K. Ahmed Rasheed, S. E. Tourt-Uhlig, J. E. Herndon, J. J. Vredenburgh, J. H. Sampson, A. H. Friedman, D. D. Bigner, and H. S. Friedman. 2004. Phase II trial of gefitinib in recurrent glioblastoma. *J. Clin. Oncol.* 22: 133-142 PM:14638850.
248. Lassman, A. B., M. R. Rossi, J. J. Raizer, L. E. Abrey, F. S. Lieberman, C. N. Grefe, K. Lamborn, W. Pao, A. H. Shih, J. G. Kuhn, R. Wilson, N. J. Nowak, J. K. Cowell, L. M. DeAngelis, P. Wen, M. R. Gilbert, S. Chang, W. A. Yung, M.

- Prados, and E. C. Holland. 2005. Molecular study of malignant gliomas treated with epidermal growth factor receptor inhibitors: tissue analysis from North American Brain Tumor Consortium Trials 01-03 and 00-01. *Clin. Cancer Res.* 11: 7841-7850 PM:16278407.
249. Paez, J. G., P. A. Janne, J. C. Lee, S. Tracy, H. Greulich, S. Gabriel, P. Herman, F. J. Kaye, N. Lindeman, T. J. Boggon, K. Naoki, H. Sasaki, Y. Fujii, M. J. Eck, W. R. Sellers, B. E. Johnson, and M. Meyerson. 2004. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science* 304: 1497-1500 PM:15118125.
250. Lynch, T. J., D. W. Bell, R. Sordella, S. Gurubhagavatula, R. A. Okimoto, B. W. Brannigan, P. L. Harris, S. M. Haserlat, J. G. Supko, F. G. Haluska, D. N. Louis, D. C. Christiani, J. Settleman, and D. A. Haber. 2004. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N. Engl. J. Med.* 350: 2129-2139 PM:15118073.
251. Marie, Y., A. F. Carpentier, A. M. Omuro, M. Sanson, J. Thillet, K. Hoang-Xuan, and J. Y. Delattre. 2005. EGFR tyrosine kinase domain mutations in human gliomas. *Neurology* 64: 1444-1445 PM:15851741.
252. Iwata. 2002. *Proc. Am. Soc. Clin. Oncol.*
253. Vogelbaum. 2003. *Soc. Neuro Oncol.* 2003, 309, Vogelbaum abs ET-47.
254. Heimberger, A. B., C. A. Learn, G. E. Archer, R. E. McLendon, T. A. Chewing, F. L. Tuck, J. B. Pracyk, A. H. Friedman, H. S. Friedman, D. D. Bigner, and J. H. Sampson. 2002. Brain tumors in mice are susceptible to blockade of

epidermal growth factor receptor (EGFR) with the oral, specific, EGFR-tyrosine kinase inhibitor ZD1839 (iressa). *Clin. Cancer Res.* 8: 3496-3502

PM:12429640.

255. Asahina, H., K. Yamazaki, I. Kinoshita, N. Sukoh, M. Harada, H. Yokouchi, T. Ishida, S. Ogura, T. Kojima, Y. Okamoto, Y. Fujita, H. Dosaka-Akita, H. Isobe, and M. Nishimura. 2006. A phase II trial of gefitinib as first-line therapy for advanced non-small cell lung cancer with epidermal growth factor receptor mutations. *Br. J. Cancer* 95: 998-1004 PM:17047648.
256. Inoue, A., K. Kobayashi, K. Usui, M. Maemondo, S. Okinaga, I. Mikami, M. Ando, K. Yamazaki, Y. Saijo, A. Gemma, H. Miyazawa, T. Tanaka, K. Ikebuchi, T. Nukiwa, S. Morita, and K. Hagiwara. 2009. First-line gefitinib for patients with advanced non-small-cell lung cancer harboring epidermal growth factor receptor mutations without indication for chemotherapy. *J. Clin. Oncol.* 27: 1394-1400 PM:19224850.
257. Sequist, L. V., R. G. Martins, D. Spigel, S. M. Grunberg, A. Spira, P. A. Janne, V. A. Joshi, D. McCollum, T. L. Evans, A. Muzikansky, G. L. Kuhlmann, M. Han, J. S. Goldberg, J. Settleman, A. J. Iafrate, J. A. Engelman, D. A. Haber, B. E. Johnson, and T. J. Lynch. 2008. First-line gefitinib in patients with advanced non-small-cell lung cancer harboring somatic EGFR mutations. *J. Clin. Oncol.* 26: 2442-2449 PM:18458038.
258. Hoelzinger, D. B., L. Mariani, J. Weis, T. Woyke, T. J. Berens, W. S. McDonough, A. Sloan, S. W. Coons, and M. E. Berens. 2005. Gene expression

- profile of glioblastoma multiforme invasive phenotype points to new therapeutic targets. *Neoplasia*. 7: 7-16 PM:15720813.
259. Salcman, M. 1980. Survival in glioblastoma: historical perspective. *Neurosurgery* 7: 435-439 PM:6255368.
260. Chen, B., S. C. Borinstein, J. Gillis, V. W. Sykes, and O. Bogler. 2000. The glioma-associated protein SETA interacts with AIP1/Alix and ALG-2 and modulates apoptosis in astrocytes. *J. Biol. Chem.* 275: 19275-19281 PM:10858458.
261. Simpson, R. J., L. M. Connolly, J. S. Eddes, J. J. Pereira, R. L. Moritz, and G. E. Reid. 2000. Proteomic analysis of the human colon carcinoma cell line (LIM 1215): development of a membrane protein database. *Electrophoresis* 21: 1707-1732 PM:10870958.
262. Syka, J. E., J. J. Coon, M. J. Schroeder, J. Shabanowitz, and D. F. Hunt. 2004. Peptide and protein sequence analysis by electron transfer dissociation mass spectrometry. *Proc. Natl. Acad. Sci. U. S. A* 101: 9528-9533 PM:15210983.
263. Pitteri, S. J., P. A. Chrisman, J. M. Hogan, and S. A. McLuckey. 2005. Electron transfer ion/ion reactions in a three-dimensional quadrupole ion trap: reactions of doubly and triply protonated peptides with  $\text{SO}_2^+$ . *Anal. Chem.* 77: 1831-1839 PM:15762593.
264. Tsou, C. C., C. F. Tsai, Y. H. Tsui, P. R. Sudhir, Y. T. Wang, Y. J. Chen, J. Y. Chen, T. Y. Sung, and W. L. Hsu. 2010. IDEAL-Q, an automated tool for label-

- free quantitation analysis using an efficient peptide alignment approach and spectral data validation. *Mol. Cell Proteomics*. 9: 131-144 PM:19752006.
265. Lal, S., M. Lacroix, P. Tofilon, G. N. Fuller, R. Sawaya, and F. F. Lang. 2000. An implantable guide-screw system for brain tumor studies in small animals. *J. Neurosurg*. 92: 326-333 PM:10659021.
266. Garcia de Palazzo, I. E., G. P. Adams, P. Sundareshan, A. J. Wong, J. R. Testa, D. D. Bigner, and L. M. Weiner. 1993. Expression of mutated epidermal growth factor receptor by non-small cell lung carcinomas. *Cancer Res*. 53: 3217-3220 PM:8391918.
267. Emrich, J. G., L. W. Brady, T. S. Quang, R. Class, C. Miyamoto, P. Black, and U. Rodeck. 2002. Radioiodinated (I-125) monoclonal antibody 425 in the treatment of high grade glioma patients: ten-year synopsis of a novel treatment. *Am. J. Clin. Oncol*. 25: 541-546 PM:12477994.
268. Moscatello, D. K., M. Holgado-Madruga, A. K. Godwin, G. Ramirez, G. Gunn, P. W. Zoltick, J. A. Biegel, R. L. Hayes, and A. J. Wong. 1995. Frequent expression of a mutant epidermal growth factor receptor in multiple human tumors. *Cancer Res*. 55: 5536-5539 PM:7585629.
269. Wikstrand, C. J., L. P. Hale, S. K. Batra, M. L. Hill, P. A. Humphrey, S. N. Kurpad, R. E. McLendon, D. Moscatello, C. N. Pegram, C. J. Reist, and . 1995. Monoclonal antibodies against EGFRvIII are tumor specific and react with breast and lung carcinomas and malignant gliomas. *Cancer Res*. 55: 3140-3148 PM:7606735.

270. Fernandes, H., S. Cohen, and S. Bishayee. 2001. Glycosylation-induced conformational modification positively regulates receptor-receptor association: a study with an aberrant epidermal growth factor receptor (EGFRvIII/DeltaEGFR) expressed in cancer cells. *J. Biol. Chem.* 276: 5375-5383 PM:11087732.
271. Reva, B., Y. Antipin, and C. Sander. 2007. Determinants of protein function revealed by combinatorial entropy optimization. *Genome Biol.* 8: R232 PM:17976239.
272. Reva, B., Y. Antipin, and C. Sander. 2011. Predicting the functional impact of protein mutations: application to cancer genomics. *Nucleic Acids Res.* PM:21727090.
273. Huang, P. H., E. R. Miraldi, A. M. Xu, V. A. Kundukulam, A. M. Del Rosario, R. A. Flynn, W. K. Cavenee, F. B. Furnari, and F. M. White. 2010. Phosphotyrosine signaling analysis of site-specific mutations on EGFRvIII identifies determinants governing glioblastoma cell growth. *Mol Biosyst.* 6: 1227-1237 PM:20461251.
274. Okabayashi, Y., Y. Kido, T. Okutani, Y. Sugimoto, K. Sakaguchi, and M. Kasuga. 1994. Tyrosines 1148 and 1173 of activated human epidermal growth factor receptors are binding sites of Shc in intact cells. *J. Biol. Chem.* 269: 18674-18678 PM:8034616.
275. Li, N., A. Batzer, R. Daly, V. Yajnik, E. Skolnik, P. Chardin, D. Bar-Sagi, B. Margolis, and J. Schlessinger. 1993. Guanine-nucleotide-releasing factor hSos1

- binds to Grb2 and links receptor tyrosine kinases to Ras signalling. *Nature* 363: 85-88 PM:8479541.
276. Helin, K., T. Velu, P. Martin, W. C. Vass, G. Allevato, D. R. Lowy, and L. Beguinot. 1991. The biological activity of the human epidermal growth factor receptor is positively regulated by its C-terminal tyrosines. *Oncogene* 6: 825-832 PM:1646987.
277. Boerner, J. L., M. L. Demory, C. Silva, and S. J. Parsons. 2004. Phosphorylation of Y845 on the epidermal growth factor receptor mediates binding to the mitochondrial protein cytochrome c oxidase subunit II. *Mol. Cell Biol.* 24: 7059-7071 PM:15282306.
278. Biscardi, J. S., M. C. Maa, D. A. Tice, M. E. Cox, T. H. Leu, and S. J. Parsons. 1999. c-Src-mediated phosphorylation of the epidermal growth factor receptor on Tyr845 and Tyr1101 is associated with modulation of receptor function. *J. Biol. Chem.* 274: 8335-8343 PM:10075741.
279. Furnari, F. B., H. Lin, H. S. Huang, and W. K. Cavenee. 1997. Growth suppression of glioma cells by PTEN requires a functional phosphatase catalytic domain. *Proc. Natl. Acad. Sci. U. S. A* 94: 12479-12484 PM:9356475.
280. Haas-Kogan, D., N. Shalev, M. Wong, G. Mills, G. Yount, and D. Stokoe. 1998. Protein kinase B (PKB/Akt) activity is elevated in glioblastoma cells due to mutation of the tumor suppressor PTEN/MMAC. *Curr. Biol.* 8: 1195-1198 PM:9799739.

281. Tang, C. K., X. Q. Gong, D. K. Moscatello, A. J. Wong, and M. E. Lippman. 2000. Epidermal growth factor receptor VIII enhances tumorigenicity in human breast cancer. *Cancer Res.* 60: 3081-3087 PM:10850460.
282. Lazar, D. F., and A. R. Saltiel. 2006. Lipid phosphatases as drug discovery targets for type 2 diabetes. *Nat. Rev. Drug Discov.* 5: 333-342 PM:16582877.
283. Geier, S. J., P. A. Algate, K. Carlberg, D. Flowers, C. Friedman, B. Trask, and L. R. Rohrschneider. 1997. The human SHIP gene is differentially expressed in cell lineages of the bone marrow and blood. *Blood* 89: 1876-1885 PM:9058707.
284. Liu, Q., F. Shalaby, J. Jones, D. Bouchard, and D. J. Dumont. 1998. The SH2-containing inositol polyphosphate 5-phosphatase, ship, is expressed during hematopoiesis and spermatogenesis. *Blood* 91: 2753-2759 PM:9531585.
285. Muraille, E., X. Pesesse, C. Kuntz, and C. Erneux. 1999. Distribution of the src-homology-2-domain-containing inositol 5-phosphatase SHIP-2 in both non-haemopoietic and haemopoietic cells and possible involvement of SHIP-2 in negative signalling of B-cells. *Biochem. J.* 342 Pt 3: 697-705 PM:10477282.
286. Habib, T., J. A. Hejna, R. E. Moses, and S. J. Decker. 1998. Growth factors and insulin stimulate tyrosine phosphorylation of the 51C/SHIP2 protein. *J. Biol. Chem.* 273: 18605-18609 PM:9660833.
287. Kamio, T., K. Shigematsu, H. Sou, K. Kawai, and H. Tsuchiyama. 1990. Immunohistochemical expression of epidermal growth factor receptors in human adrenocortical carcinoma. *Hum. Pathol.* 21: 277-282 PM:2312105.

288. Lipponen, P., and M. Eskelinen. 1994. Expression of epidermal growth factor receptor in bladder cancer as related to established prognostic factors, oncoprotein (c-erbB-2, p53) expression and long-term prognosis. *Br. J. Cancer* 69: 1120-1125 PM:7911031.
289. Marti, U., C. Ruchti, J. Kampf, G. A. Thomas, E. D. Williams, H. J. Peter, H. Gerber, and U. Burgi. 2001. Nuclear localization of epidermal growth factor and epidermal growth factor receptors in human thyroid tissues. *Thyroid* 11: 137-145 PM:11288982.
290. Lo, H. W., W. Xia, Y. Wei, M. Ali-Seyed, S. F. Huang, and M. C. Hung. 2005. Novel prognostic value of nuclear epidermal growth factor receptor in breast cancer. *Cancer Res.* 65: 338-348 PM:15665312.
291. Psyrrri, A., Z. Yu, P. M. Weinberger, C. Sasaki, B. Haffty, R. Camp, D. Rimm, and B. A. Burtness. 2005. Quantitative determination of nuclear and cytoplasmic epidermal growth factor receptor expression in oropharyngeal squamous cell cancer by using automated quantitative analysis. *Clin. Cancer Res.* 11: 5856-5862 PM:16115926.
292. Xia, W., Y. Wei, Y. Du, J. Liu, B. Chang, Y. L. Yu, L. F. Huo, S. Miller, and M. C. Hung. 2009. Nuclear expression of epidermal growth factor receptor is a novel prognostic value in patients with ovarian cancer. *Mol. Carcinog.* 48: 610-617 PM:19058255.
293. Lo, H. W., S. C. Hsu, M. Ali-Seyed, M. Gunduz, W. Xia, Y. Wei, G. Bartholomeusz, J. Y. Shih, and M. C. Hung. 2005. Nuclear interaction of EGFR

- and STAT3 in the activation of the iNOS/NO pathway. *Cancer Cell* 7: 575-589  
PM:15950906.
294. Edwards, J., P. Traynor, A. F. Munro, C. F. Pirret, B. Dunne, and J. M. Bartlett. 2006. The role of HER1-HER4 and EGFRvIII in hormone-refractory prostate cancer. *Clin. Cancer Res.* 12: 123-130 PM:16397033.
295. Lo, H. W., X. Cao, H. Zhu, and F. Ali-Osman. 2010. Cyclooxygenase-2 is a novel transcriptional target of the nuclear EGFR-STAT3 and EGFRvIII-STAT3 signaling axes. *Mol. Cancer Res.* 8: 232-245 PM:20145033.
296. Hung, L. Y., J. T. Tseng, Y. C. Lee, W. Xia, Y. N. Wang, M. L. Wu, Y. H. Chuang, C. H. Lai, and W. C. Chang. 2008. Nuclear epidermal growth factor receptor (EGFR) interacts with signal transducer and activator of transcription 5 (STAT5) in activating Aurora-A gene expression. *Nucleic Acids Res* 36: 4337-4351
297. Adya, N., T. Stacy, N. A. Speck, and P. P. Liu. 1998. The leukemic protein core binding factor beta (CBFbeta)-smooth-muscle myosin heavy chain sequesters CBFalpha2 into cytoskeletal filaments and aggregates. *Mol. Cell Biol.* 18: 7432-7443 PM:9819429.
298. Devroe, E., A. Engelman, and P. A. Silver. 2003. Intracellular transport of human immunodeficiency virus type 1 integrase. *J. Cell Sci.* 116: 4401-4408  
PM:13130095.

299. Hsu, S. C., S. A. Miller, Y. Wang, and M. C. Hung. 2009. Nuclear EGFR is required for cisplatin resistance and DNA repair. *Am. J. Transl. Res.* 1: 249-258 PM:19956435.
300. Wang, S. C., Y. Nakajima, Y. L. Yu, W. Xia, C. T. Chen, C. C. Yang, E. W. McIntush, L. Y. Li, D. H. Hawke, R. Kobayashi, and M. C. Hung. 2006. Tyrosine phosphorylation controls PCNA function through protein stability. *Nat. Cell Biol.* 8: 1359-1368 PM:17115032.
301. Dittmann, K., C. Mayer, B. Fehrenbacher, M. Schaller, U. Raju, L. Milas, D. J. Chen, R. Kehlbach, and H. P. Rodemann. 2005. Radiation-induced epidermal growth factor receptor nuclear import is linked to activation of DNA-dependent protein kinase. *J. Biol. Chem.* 280: 31182-31189 PM:16000298.
302. Bandyopadhyay, D., M. Mandal, L. Adam, J. Mendelsohn, and R. Kumar. 1998. Physical interaction between epidermal growth factor receptor and DNA-dependent protein kinase in mammalian cells. *J. Biol. Chem.* 273: 1568-1573 PM:9430697.
303. Weterings, E., and D. J. Chen. 2007. DNA-dependent protein kinase in nonhomologous end joining: a lock with multiple keys? *J. Cell Biol.* 179: 183-186 PM:17938249.
304. Brennand, J., and G. P. Margison. 1986. Reduction of the toxicity and mutagenicity of alkylating agents in mammalian cells harboring the *Escherichia coli* alkyltransferase gene. *Proc. Natl. Acad. Sci. U. S. A* 83: 6292-6296 PM:3529080.

305. van Niftherik, K. A., J. van den Berg, W. F. van der Meide, N. Ameziane, L. E. Wedekind, R. D. Steenbergen, S. Leenstra, M. V. Lafleur, B. J. Slotman, L. J. Stalpers, and P. Sminia. 2010. Absence of the MGMT protein as well as methylation of the MGMT promoter predict the sensitivity for temozolomide. *Br. J. Cancer* 103: 29-35 PM:20517307.
306. Aghi, M., S. Rabkin, and R. L. Martuza. 2006. Effect of chemotherapy-induced DNA repair on oncolytic herpes simplex viral replication. *J. Natl. Cancer Inst.* 98: 38-50 PM:16391370.
307. Tang, J. B., E. M. Goellner, X. H. Wang, R. N. Trivedi, C. M. St Croix, E. Jelezcova, D. Svilar, A. R. Brown, and R. W. Sobol. 2010. Bioenergetic metabolites regulate base excision repair-dependent cell death in response to DNA damage. *Mol. Cancer Res.* 8: 67-79 PM:20068071.

## **VITA**

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