EGFRvIII Expression and Signaling in head and neck squamous cell carcinoma

by

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Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer in the United States and is even more prevalent in many developing countries. The mortality rate of ~50% has remained unchanged for decades emphasizing the need to increase our understanding of HNSCC biology. Overexpression of EGFR is found in up to 90% of HNSCC cases and has been implicated in oncogenicity by providing sustained signaling for cell proliferation, anti-apoptosis, angiogenesis and metastasis. Cetuximab (an EGFR specific monoclonal antibody) was FDA-approved for HNSCC in 2006 making it the first new HNSCC treatment in 45 years. However, the clinical response to cetuximab is only 10%, indicating the need to better understand the mechanisms of EGFR biology in HNSCC and investigate the mechanisms of tumor growth in the presence of wtEGFR blockade.

An altered form of EGFR, EGFRvIII, lacks exons 2-7 and is a probable mechanism of cetuximab resistance. We previously reported EGFRvIII to be expressed in ~40% of HNSCC tumors where HNSCC cells expressing EGFRvIII were relatively resistant to cetuximab treatment in xenograft models. The present study was undertaken to determine the prognostic significance of EGFR amplification, mRNA and protein levels in HNSCC and to better understand the biology of EGFRvIII in HNSCC.

We found that EGFR protein levels and phosphorylation status are the best indicators of patient prognosis. Utilizing the Cancer Genome Atlas data and clinical samples we found that

EGFRvIII expression in GBM is often a DNA or transcriptional aberration but in HNSCC it is more likely a splice variant. We developed HNSCC cell lines expressing EGFRvIII and utilizing these models *in vitro* and *in vivo*, we were able to determine that EGFRvIII increases HNSCC invasion and migration via STAT3 and knockdown of STAT3 using siRNA or a transcriptional decoy abrogated invasion and migration. Akt was involved in EGFRvIII-mediated proliferation, but not invasion. SFKs were also found to be involved in EGFRvIII invasion and migration, specifically Lyn. *In vivo* xenograft growth was significantly inhibited by treatment with the SFK inhibitor dasatinib.

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LIST OF ABBREVIATIONS

AR	Amphiregulin
C225	Cetuximab
CI	Confidence interval
CML	Chronic myelogenous leukemia
CRT	Chemoradiotherapy
CT	Chemotherapy
CXCR4	Chemokine (C-X-C motif) receptor 4
DMEM	Dulbecco’s modification of Eagle’s medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNSrc	Dominant negative Src
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EGFRvIII	Epidermal growth factor receptor variant 3
ErbB	Erythroblastic leukemia viral oncogene homolog
FAK	Focal adhesion kinase
FBS	Fetal bovine serum

FDA	United States Food and Drug Administration
FISH	Fluorescence *in situ* hybridization
Fyn	FYN oncogene related to SRC,FGR,YES
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GBM	Glioblastoma multiforme
Grb2	Growth factor receptor bound protein 2
HB-EGF	heparin binding EGF
Her	Heregulin
HIF	Hypoxia inducible factor
HNSCC	Head and neck squamous cell carcinoma
HPV	Human papilloma virus
HR	Hazards ratio
IB	Immunoblotting
IHC	Immunohistochemistry
IP	Immunoprecipitation
Jak	Janus kinase
Lyn	v-yes-1 Yamaguchi sarcoma viral related oncogene homolog
LRT	Log-rank test
MAPK	Mitogen-activated protein kinase
MMP	Matrix metalloproteinase
mRNA	messenger ribonucleic acid
MTT	3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)
NSCLC	Non-small cell lung carcinoma

OS	Overall survival
PFS	Progression free survival
PBS	Phosphate buffered saline
PDK1	Phosphoinositide-dependent protein kinase-1
PI3K	Phosphatidylinositol-3 kinases
pSTAT	phosphorylated STAT
QRT-PCR	Quantitative reverse transcription polymerase chain reaction
RNA	Ribonucleic acid
RPPA	Reverse phase protein array
RT	Radiotherapy
RT-PCR	Reverse transcription polymerase chain reaction
RTK	Receptor tyrosine kinase
SCC	Squamous cell carcinoma
SFK	Src family kinase
SH	Src homology domain
siRNA	Small interfering ribonucleic acid
SNP	Single-nucleotide polymorphism
SOCS	Suppresor of cytokine signaling
Src	v-Src avian sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog
STAT	Signal transducer and activator of transcription
TCGA	The cancer genome atlas project
TGF	Transforming growth factor
TMA	Tissue Microarray

TKI	Tyrosine kinase inhibitor
VEGF	Vascular endothelial growth factor
wtEGFR	wild type epidermal growth factor receptor
Yes	Yamaguchi sarcoma viral (v-yes) oncogene homolog

PREFACE

Two chapters of this dissertation contain published manuscripts on which I am an author (Sarah Morgan-Wheeler):

Wheeler SE, Suzuki S, Thomas SM, Sen M, Leeman-Neill RJ, Chiosea SI, Kuan CT, Bigner DD, Gooding WE, Lai SY, Grandis JR. “Epidermal growth factor receptor variant III mediates head and neck cancer cell invasion via STAT3 activation.” Oncogene 2010 Sep 16; 29(37): 5135-45.

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1.0 INTRODUCTION

1.1 SQUAMOUS CELL CARCINOMA OF THE HEAD AND NECK

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer in the United States [1] and the leading cancer in many developing countries [2]. The mortality rate (~50%) has remained unchanged for decades emphasizing the need to increase our understanding of HNSCC biology. Population-based estimates of 36,540 cases of oral cavity and pharynx cancers and 12,720 laryngeal cancers occurred in the U.S. in 2010 [3]. A primary challenge in the successful treatment of head and neck cancers, more than 90% of which are squamous cell carcinoma (HNSCC), is the high rate of tumor recurrence. Most HNSCC cases are diagnosed as locoregionally advanced disease (stage III or stage IV) [3] where approximately 50% of HNSCC patients treated for a first primary cancer will experience disease recurrence within 5 years, and most will die as a result.

HNSCC is associated with high morbidity due to damage to the upper aerodigestive tract from surgical intervention, chemotherapy, and radiation. Studies have shown that exposure to environmental carcinogens, namely chronic tobacco and alcohol use, are major risk factors in the development of HNSCC [4]. However, most people exposed to these and other carcinogens will not develop HNSCC, indicating that genetic predispositions and alterations contribute to

oncogenicity. Pharyngeal infection with the human papilloma virus (HPV), primarily types 16 and 18 [5, 6], has emerged as a probable cause of a subset of oropharyngeal cancers where HPV-associated HNSCC appear to have an improved outcome independent of treatment approach [7]. Treatment for HNSCC has traditionally included primary chemoradiotherapy (CRT) or surgical resection followed by radiation (or CRT).

1.2 EGFR IN HNSCC

To improve patient care, research has sought to elucidate the mechanisms of HNSCC tumorigenesis and progression. Overexpression of EGFR has been identified as a common characteristic of HNSCC found in up to ~90% of cases as a result of gene amplification and/or transcriptional activation [8, 9]. EGFR signaling is involved in cell proliferation, motility, adhesion, angiogenesis, cell survival and invasion [10]. Overexpression of EGFR has been implicated in oncogenicity by providing sustained signaling for cell proliferation, anti-apoptosis, angiogenesis and metastasis [11]. EGFR overexpression contributes to tumor progression and is an independent predictor of poor prognosis [12]. This dramatic overexpression has led to the development of EGFR-targeted therapies including monoclonal antibodies, tyrosine kinase-specific inhibitors, ligand-linked immunotoxins, and antisense approaches [13]. Cetuximab (an EGFR specific monoclonal antibody) was FDA-approved for HNSCC in 2006 based on the results of a phase III trial of cetuximab in combination with radiation, making it the first new HNSCC treatment in 45 years. [14]. While the success of this molecular targeting strategy was modest (10% clinical response rate in HNSCC [15]) it underscores the importance of understanding the biology of these malignancies. Increased understanding of the molecular

mechanisms contributing to HNSCC tumorigenesis and progression is required to reduce the high mortality rate of this disease. Such an understanding will allow us to optimize therapy for each HNSCC patient based on the molecular characteristics of their tumor.

1.3 BIOLOGY OF EGFR AND OTHER ERBB FAMILY MEMBERS

EGFR is a member of the ErbB growth factor receptor tyrosine kinase family. ErbB receptors are generally found on the cell surface. ErbB receptors contain an extracellular ligand-binding domain, a transmembrane region and an intracellular domain with tyrosine kinase activity (except ErbB3). Upon ligand binding, the receptors dimerize and autophosphorylate thereby initiating a signaling cascade downstream of the dimer. Ligand binding induces a conformation change of the receptor ectodomain (creating an extended and stabilized conformation, except for ErbB2 which constitutively maintains the stabilized conformation and has no known ligand [16]) to facilitate receptor dimerization [17]. ErbB ligands are produced as transmembrane precursors and the ectodomains are processed via proteolysis leading to the shedding of soluble growth factors [18]. In normal tissues this signaling cascade is tightly controlled and regulates processes that include epithelial development and injury response. The major pathways activated by ErbB receptors include Ras/Raf/MAPK; PI3K/AKT; PLCgamma and STATs, all of which lead to the transcription of target genes that may contribute to tumor progression [19]. Regulation of ErbB receptor signaling occurs through temporal and spatial expression of receptor ligands and through receptor endocytosis. Endocytic trafficking leads to receptor recycling or ubiquitination and lysosomal degradation of the receptor [20].

EGFR activation can be induced through autocrine or paracrine ligands. There are six major EGFR ligands that are expressed at the mRNA level in some, but not all, HNSCC cell lines including: heparin binding EGF (HB-EGF), transforming growth factor alpha (TGF-alpha), betacellulin, with amphiregulin (AR), heregulin, and epidermal growth factor (EGF) [21]. TGF-alpha and AR are the primary ligands implicated in autocrine growth signaling [10]. EGFR can homodimerize or heterodimerize with other members of the ErbB receptor family [22].

ErbB2 has no known exogenous ligands that directly bind to it. If ErbB2 is highly overexpressed it can spontaneously dimerize and autoactivate, but it is most commonly activated *via* heterodimerization with other ErbB family members [16]. ErbB3 has no intrinsic tyrosine kinase activity but is transactivated by EGFR and ErbB2. ErbB3 ligands include neuregulins, heregulin and neu differentiation factor [23]. ErbB4 can homodimerize or heterodimerize with other members of the ErbB receptor family. ErbB4 ligands include neuregulins, epiregulin, heregulin, neu differentiation factor, and betacellulin [23].

1.4 ERBB FAMILY MEMBERS IN HNSCC

1.4.1 ErbB Family Expression

Expression of EGFR, ErbB2, ErbB3 and ErbB4 has been reported in HNSCC [10], although data on ErbB4 are conflicting [24-28]. The entire ErbB receptor family has been found to be expressed at increased levels in invasive carcinoma and ErbB1, ErbB2 and ErbB4 have been identified as overexpressed in *in situ* carcinoma [10]. Most cases of overexpression

demonstrated elevated expression of multiple receptors simultaneously, providing indirect evidence for the formation of ErbB receptor heterodimers [29].

1.4.1.1 EGFR

EGFR is the most well studied of the ErbB receptors in HNSCC. Overexpression of EGFR in oral dysplasias compared to normal mucosa has been demonstrated [30, 31]. However, EGFR upregulation in the normal-appearing epithelium adjacent to malignant tissue in HNSCC has also been reported, supporting the idea of field cancerization in HNSCC [32]. EGFR expression has been reported to be lower in laryngeal tumors as compared to tumors of the pharynx and oral cavity [33] indicating that EGFR expression may differ between HNSCC anatomic sites [10]. In HNSCC tumors, 92% have elevated EGFR mRNA levels and 87% have elevated mRNA for its ligand TGF-alpha [32]. Additionally, EGFR protein overexpression has been reported in over 80% of HNSCC cases [10] and EGFR gene amplification has been demonstrated in up to 15% of HNSCC tumors [34]. High levels of EGFR protein expression have been correlated with lower patient survival in HNSCC [35-38].

EGFR dysregulation appears to result from several potential mechanisms, including gene amplification and transcriptional activation [39-41]. Additionally, EGFR mRNA overexpression may result from dysregulated p53, which directly increases EGFR gene transcription in HNSCC [42]. Polymorphisms in dinucleotide repeats in the first intron of the EGFR gene have also been implicated in decreasing the efficiency of EGFR gene transcription. In 12 HNSCC cell lines, cell lines with lower numbers of dinucleotide repeats had increased levels of EGFR mRNA and protein [41, 43]. Transcriptional activation appears to be a common cause of EGFR

overexpression in HNSCC, although the precise mechanisms leading to increased gene transcription are incompletely understood [44].

Another possible mechanism of cellular dysregulation by EGFR is through nuclear localization of EGFR where it can act as a transcription factor. Nuclear localization of EGFR in HNSCC has been reported and is associated with STAT3 interaction and transcriptional activation of inducible nitric oxide synthase in HNSCC [10]. While the precise role of nuclear EGFR is incompletely understood, localization of EGFR to the nucleus suggests that genes may be transactivated by EGFR independent of direct EGFR downstream signaling.

1.4.1.2 ErbB2

ErbB2 is overexpressed in HNSCC compared to levels detected in corresponding normal mucosa. Studies have shown that ErbB2 is overexpressed in ~3-39% of tumors of HNSCC with gene amplification present in approximately 5% of cases [26, 45-51]. Studies indicate that ErbB2 overexpression may correlate with survival. IHC staining has shown that ErbB2 overexpression is significantly correlated with a decrease in disease free survival indicating a possible prognostic value of ErbB2 expression in this cancer [49]. Gene amplification was also studied using a semi-quantitative PCR technique, but genomic amplifications of ErbB2 are not common and did not significantly correlate with patient survival in HNSCC [52]. ErbB2 may also be involved in resistance to 5-fluorouracil, cisplatin and the EGFR inhibitor gefitinib [25, 53]. However, most ErbB2 targeting strategies have not demonstrated clinical efficacy to date in HNSCC [54-56]. One recent report demonstrated preliminary efficacy in HNSCC with lapatinib, a reversible small-molecule inhibitor of EGFR and HER2 and further studies are ongoing [57].

While ErbB2 may heterodimerize with other ErbB family members and contribute to HNSCC tumor progression, the role of heterodimers in HNSCC is incompletely understood [25].

1.4.1.3 ErbB3

ErbB3 is overexpressed at the mRNA and protein levels in a subset of HNSCC cell lines [58] and is overexpressed in 21-81% of HNSCC tumors [26, 45-47]. However, studies vary and are limited by the poor quality of the antibodies available for IHC and immunoblotting. To date, ErbB3 gene amplification has not been detected in HNSCC [59]. ErbB3 appears to be related to the malignant progression of HNSCC in clinical specimens [58] and ErbB3 overexpression in HNSCC is correlated with survival and metastasis in several cohorts [60]. ErbB3 expression and signaling has been correlated with resistance to the EGFR inhibitor gefitinib in lung cancer [25, 61] and the limited HNSCC data suggests that a similar mechanism may also be important in this cancer [25].

1.4.1.4 ErbB4

Expression of ErbB4 is detected in HNSCC (26-69%) [26, 28, 45, 46] but the role of this ErbB family member in these cancers is unclear. Expression of ErbB4 did not correlate with invasion, angiogenesis, metastasis or HNSCC tumor progression [62], although expression of all four ErbB receptors in oral SCC was significantly associated with decreased patient survival [63]. Other evidence indicates that ErbB4 expression may be lost *in vitro* [28]. These findings provide indirect evidence of cooperation among ErbB receptors in HNSCC cancer progression.

1.4.1.5 EGFR Alterations

Unlike non-small cell lung carcinoma, HNSCC is not characterized by mutations of the

EGFR kinase domain [64]. Expression of the EGFR variant III has been identified in up to 42% of HNSCC tumors, but is not expressed in normal tissue [65]. EGFRvIII was originally characterized in glioblastoma where it was thought to be a common somatic mutation associated with EGFR gene amplification leading to gene rearrangement [66, 67]. EGFRvIII lacks exons 2-7 with a novel glycine residue at the exon 1/8 junction. Exons 2-7 comprise the majority of the extracellular ligand binding domain so that cells that express EGFRvIII are likely to bind to EGFR monoclonal antibodies with reduced affinity. Thus, EGFRvIII represents a possible mechanism of cetuximab resistance [65]. EGFRvIII remains largely unstudied in HNSCC, in part due to the difficulty in creating model systems. EGFRvIII expression is lost *in vitro*; consequently HNSCC cells must be stably transfected with an EGFRvIII construct to establish a model for preclinical investigations [68]. We have shown that EGFRvIII expression in HNSCC cell lines leads to increased cell proliferation *in vitro* and increased tumorigenicity *in vivo* as compared to vector-transfected control cells [65].

1.4.2 EGFR Signaling

Upon EGFR autophosphorylation, a variety of protein signaling molecules are recruited to the plasma membrane including growth factor receptor bound protein 2 (Grb2) and Shc. Activation of these proteins initiates the EGFR signaling cascades that lead to transcriptional regulation of target genes. In HNSCC members of the EGFR signaling pathway have been found at increased levels, including MAPK, AKT, STAT3 and STAT5 (Figure 1) [10].

1.4.2.1 MAPK

Following EGFR activation, Grb2 and Sos (adaptor proteins) bind EGFR directly at Y1092 and Y1110 or through Shc (which binds at EGFR Y1172 and Y1197) [69]. This activates Raf-1 initiating a cascade that results in phosphorylated MAPK, which is then translocated to the nucleus where it activates cell proliferation transcription factors [17]. The Ras-Raf-MEK-ERK pathway is the primary MAPK pathway downstream of ErbBs in HNSCC and leads to upregulation of cyclin D1, which induces cell cycle progression (Figure 1.1) [59]. Activated MAPK in HNSCC was found to correlate with EGFR and TGF-alpha overexpression [70]. The formation of an E-cadherin-EGFR intercellular complex between tumor cells is thought to contribute to HNSCC invasion and metastasis. This complex leads to ligand independent activation of EGFR, which activates the MAPK pathway and transcription of Bcl2 allowing the cell to escape apoptosis induced by loss of the extra cellular matrix [71].

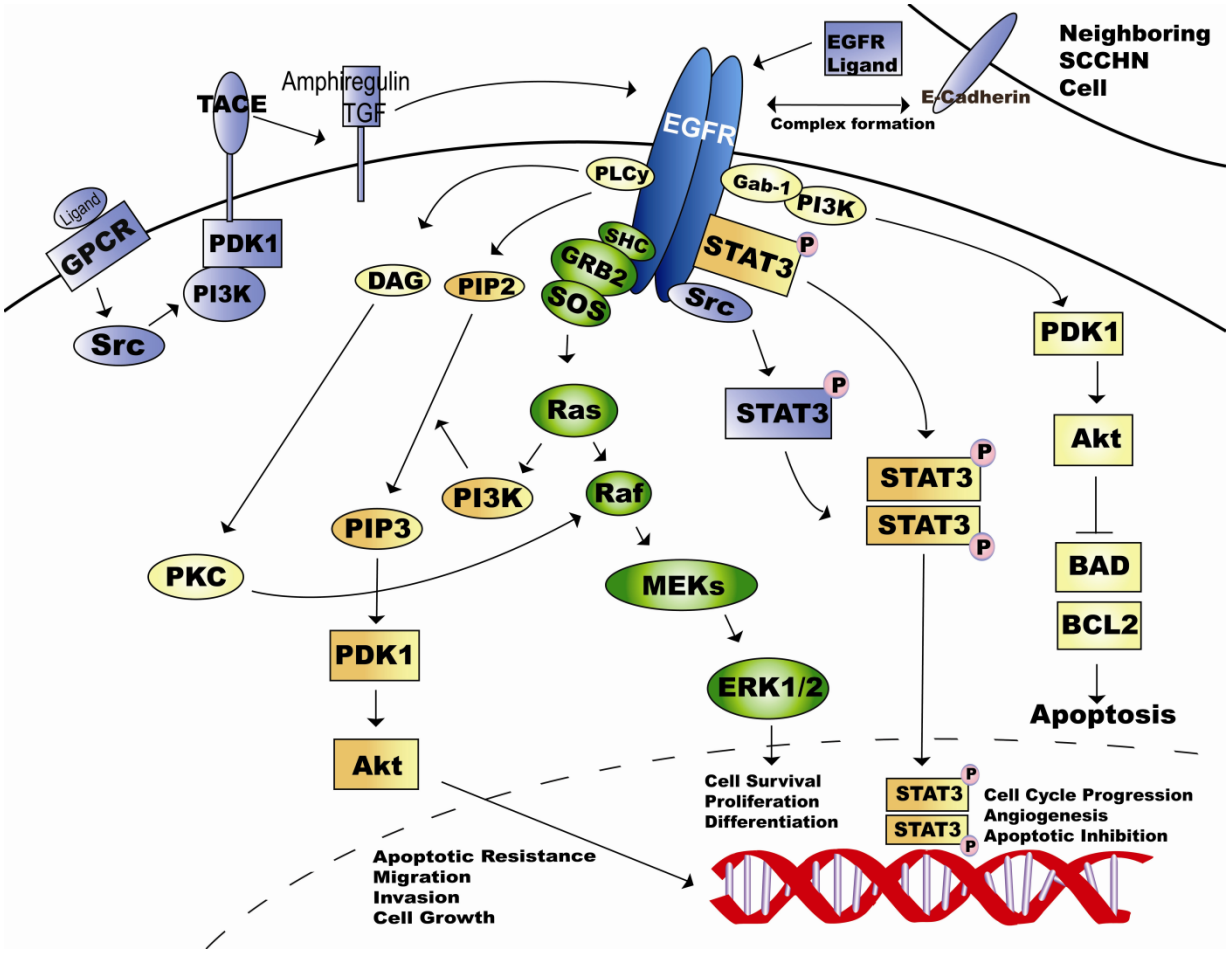


Figure 1.1 EGFR signaling in HNSCC EGFR is activated by ligand binding and subsequent receptor heterodimerization or homodimerization which leads to receptor autophosphorylation. EGFR can be transactivated by G-protein-coupled receptors (GPCR). GRPR activates Src leading to activation of PDK1 and PI3K, whereby TACE cleaves EGFR proligand and activates EGFR. EGFR can also be transactivated by cell adhesion molecules such as E-cadherin from neighboring tumor cells. The EGFR-E-cadherin complex activates EGFR and leads to MAPK signaling allowing for cell survival during the early stages of metastasis. EGFR activation leads to five primary signaling cascades. 1) MAPK: EGFR phosphorylation recruits Grb2 and Sos (adaptor proteins) that bind EGFR at Y1092 and Y1110 or alternately Grb2 and Sos bind via Shc at Y1172 and Y1197. Raf-1 is activated and the MAPK signaling cascade is activated resulting in cell survival, proliferation and differentiation; 2) STATs: STATs can be activated by interacting directly with EGFR or through Src-mediated EGFR signaling. Once phosphorylated STATs homodimerize or heterodimerize and are translocated to the nucleus where they induce the transcription of target genes that lead to cell cycle progression, angiogenesis, and apoptotic inhibition; 3) Src: Can

be activated by binding directly to EGFR at Y915 and Y944 and can also activate EGFR by phosphorylating EGFR at Y845. Downstream of EGFR, Src can activate the STAT pathway or the PI3K pathway. Activation of Src is implicated in cell proliferation, migration, adhesion, angiogenesis and immune function; 4) PLCγ: Binds directly to EGFR and activates the MAPK pathway through PKC and the PI3K pathway leading to AKT activation. This results in migration and invasion; and 5) PI3K: Involved in many pathways and results in activation of AKT that leads to apoptotic resistance, cell growth, invasion and migration.

1.4.2.2 STATs

STAT1, STAT3, and STAT5 [9, 72, 73] are activated in HNSCC and contribute to the transduction of EGFR signaling in the cell. STAT3 and STAT5 are transcription factors and oncogenes that help to regulate cell cycle progression, angiogenesis and apoptosis inhibition through their target genes. STATs can be activated by interacting directly with EGFR through SH2 domains or indirectly through Src-mediated EGFR signaling [74]. After activation, STATs dimerize and are then translocated to the nucleus where they induce the transcription of target genes (Figure 1.1) [74].

Constitutive activation of STAT3 has been reported in HNSCC [74] and STAT3 has been shown to be an oncogene and a mediator of cellular transformation [17, 75]. STAT3 is likely activated in HNSCC through autocrine activation of EGFR by TGF-alpha [74]. The identification of constitutive STAT3 activity in normal mucosa indicates that STAT3 activation may have an early role in HNSCC progression. STAT3 has been demonstrated to be upregulated both with and independent of EGFR upregulation [74]. In HNSCC, targeting STAT3 inhibited

cell growth *in vivo* and *in vitro* [76, 77]. Additionally, combined targeting of STAT3 and EGFR produces enhanced antitumor effects *in vitro* and *in vivo* as compared to EGFR targeting alone [78]. These findings indicate that STAT3 overexpression may contribute to cancer progression. STAT5 is also overexpressed and activated in HNSCC and an antisense blockade of STAT5b inhibited tumor growth [79].

STAT3 is a cytoplasmic transcription factor that regulates gene expression in response to various growth factors and cytokines, including EGFR [19]. STATs are activated via tyrosine phosphorylation which induces STAT dimerization and subsequent translocation to the nucleus. In the nucleus STATs bind regulatory elements and induce transcription of their target genes. Target genes of STAT3 include Bcl-x_L, Bcl-2 (antiapoptosis), c-myc, cyclin D1, cyclin D3 (cell cycle regulators) matrix metalloproteinases (contribute to metastasis), and VEGF (angiogenesis) [19]. Our lab has shown that STAT3 mediates survival, growth, and inhibits apoptosis in HNSCC cell lines and tumor xenograft models [19]. Further, we have previously reported elevated STAT3 activation levels in HNSCC cell lines and tumors when compared to normal oral mucosal epithelial cells [12, 80, 81]. Recently published data in glioblastoma show that STAT3 forms a complex with EGFRvIII (but not wtEGFR) in the nucleus and mediates EGFRvIII induced transformation of glial cells [82]. These studies suggest that STAT3 may be a key molecular mediator in EGFRvIII tumors where targeting STAT3 may overcome cetuximab resistance associated with the truncated ligand binding domain of EGFRvIII [83].

1.4.2.3 Src Family Kinases

Src family kinases (SFKs) are involved in cell proliferation, migration, adhesion, angiogenesis, and immune function [17]. Src is a signal transducer of EGFR signaling and is

also independently activated and leads to the activation of many pathways including STATs and PI3K [17]. In HNSCC cell lines Src family kinases are activated by TGF-alpha stimulation via direct binding to EGFR at Y915 and Y944 [69, 84]. Corresponding normal epithelial cells did not show activation of the SFKs (cSrc, cYes, Fyn, Lyn) [84]. Activated levels of STAT3 and STAT5 were highly correlated with Src phosphotyrosine (activation) levels and coimmunoprecipitation of STAT3 or STAT5 showed interaction with cSrc. cSrc blockade demonstrated reduced STAT3 and STAT5 activation in addition to reduced cell growth in HNSCC cell lines. This indicates that SFKs mediate STAT growth pathways in HNSCC [84]. In HNSCC Src can be activated independently of EGFR and transphosphorylate EGFR at Y845, leading to EGFR receptor activation [85].

Src family kinases have been shown to mediate STAT3 activation in several cancers including HNSCC [86, 87]. Our lab reported that in HNSCC, Src kinases activated by EGFR contribute to STAT3 activation [84]. In another study we demonstrated that c-Src, Fyn, Lyn and Yes were involved in proliferation and invasion of HNSCC cells [88]. Others have reported that c-Src expression in HNSCC is elevated in areas of hyperproliferation as detected by IHC [89]. Due to the prevalence of Src in various forms of cancer there are several Src inhibitors under development. In 2006 the FDA approved the Src inhibitor dasatinib for treatment of CML and other select leukemias. Dasatinib inhibits invasion, induces cell cycle arrest, apoptosis, and downregulates activated Src in HNSCC cell lines [90]. AZD0530 (AstraZeneca) is another Src inhibitor currently under clinical investigation that has been shown to abrogate proliferation and invasion of HNSCC cells in combination with wtEGFR blockade [91, 92]. Src mediation of EGFRvIII signaling has been studied *in vivo* in gliomas where the glioblastoma cell line U87MG

was stably transfected with EGFRvIII and DNSrc. *In vivo* inoculation of mice with EGFRvIII-expressing GBM cells or GMB cells expressing EGFRvIII/DNSrc showed that the DNSrc cell lines have significantly decreased tumor size [83].

1.4.2.4 PLCgamma-1

PLCgamma-1 likely contributes to HNSCC invasion and migration. PLCgamma-1 can interact directly with EGFR and hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP2) to inositol 1,3,5-triphosphate (involved in intracellular calcium release) and 1,2-diacylglycerol (a cofactor in PKC activation). PKC activates Raf, which leads to MAPK activation (figure 1) [17, 93]. PLCgamma-1 is downstream of EGFR and may mediate the invasive and metastatic mechanisms of HNSCC [94]. PLCgamma-1 contributes to tumor cell invasion in *in vitro* HNSCC experiments when activated by EGFR [95]. Activation of PLCgamma through EGFR stimulation with EGF promotes HNSCC migration [95]. In human HNSCC tumor samples, IHC staining showed that the tumor stained higher for phosphorylated PLCgamma-1 than the normal mucosa [95] demonstrating an increase in PLCgamma-1 activity in HNSCC. Other *in vitro* experiments on HNSCC cells demonstrated that chemotaxis and invasion of metastatic HNSCC cells were dependent on PI3K and its substrate PLCgamma-1 although this pathway can be activated through EGFR or chemokine receptor 7 [96].

1.4.2.5 PI3K

PI3K can be activated by EGFR through EGFR heterodimerization with ErbB3, which contains a docking site for the p85 subunit of PI3K, or alternately through Gab-1 binding EGFR. Once the p85 subunit is docked, the p110 subunit of PI3K (containing catalytic activity) generates phosphatidylinositol 3,4,5-triphosphate (PIP3), which phosphorylates and activates

AKT. Activated AKT was found to be overexpressed in 57-81% of HNSCC tumors [95]. This pathway is involved in resistance to apoptosis, cell growth, invasion and migration [17]. Cell migration may also be mediated by the Rho family of GTP-binding proteins [97] and PI3K through activation of Ras and Rac [98]. Other non-receptor tyrosine kinases appear to be possible mediators downstream of EGFR and upstream of PI3K. The non-receptor tyrosine kinase Syk may operate downstream of EGFR to participate in mediating signaling through the PI3K and PLCgamma pathways in HNSCC causing increased cell motility [99].

The PI3K pathway downstream of the ErbB receptors is also a major mechanism of apoptosis evasion in head and neck cancer. PI3K catalyzes the conversion of PIP2 to a lipid second messenger PIP3, which results in recruiting and activating of PKB and AKT through PDK. In head and neck cancers EGFR can lead to PI3K activation directly or through Ras. PI3K then induces downstream amplification of PDK1, which in turn phosphorylates AKT. In HNSCC, AKT regulates cell survival by affecting several downstream targets including the FOXO family of forkhead transcription factors, Bad, caspase 9 and by activating NF-kB. Previous studies in HNSCC showed that PTEN mutations are extremely rare [59], however, recent data in our laboratory indicates that up to 7% of HNSCC harbors PTEN mutations (unpublished data). Indicating that PI3K activation through loss of PTEN function may be a significant factor in dysregulated PI3K signaling in HNSCC.

1.5 EGFRVIII

1.5.1 EGFRvIII signaling

EGFRvIII has been best characterized in gliomas. Differential activation of signaling molecules by EGFRvIII compared with wtEGFR has been reported and suggests that the differences are quantitative rather than qualitative. EGFRvIII signaling likely plays a role in tumorigenesis [100, 101]. EGFRvIII expression has been shown to influence cell survival, proliferation, motility, invasiveness and treatment resistance [102-106]. In glioblastoma, breast cancer and HNSCC cell lines EGFRvIII enhances *in vivo* tumorigenicity [65, 107, 108], within the tumors proliferation is increased and apoptosis is decreased [102, 107].

EGFRvIII is more tumorigenic than EGFRwt [102, 107, 109-111] despite the fact that there is no difference in the cytoplasmic signaling domain of EGFRwt and EGFRvIII. Differential activation of EGFRvIII signaling pathways compared to EGFRwt has been reported [112-115]. Altered oncogenic phenotypes may be attributed to altered signaling kinetics [116, 117]. Ligand binding to wtEGFR results in rapid receptor internalization, dephosphorylation and degradation or recycling of the receptor [118]. EGFRvIII is constitutively tyrosine phosphorylated at lower levels than ligand stimulated wtEGFR [116]. The lower levels of phosphorylation prevent interaction with the Cbl-SETA-endophilin complex that enables receptor internalization of the wtEGFR after ligand binding. EGFRvIII is therefore retained at the plasma membrane and continues to participate in oncogenic signaling pathways [116].

EGFRvIII has been shown *in vitro* and in patient samples to induce constitutive activation of the PI3K/Akt pathway [119-121]. Blockade of this pathway has been shown to reduce the EGFRvIII enhanced oncogenic phenotype [120, 121]. MAPK has been reported to be involved/activated in EGFRvIII expressing models [110, 122, 123] as well as independent/not activated in EGFRvIII expressing models [112, 124, 125]. These differences may indicate that MAPK plays a minor and secondary role in the oncogenic phenotype of EGFRvIII in cancer biology.

Both STAT3 and SFKs have been implicated as key mediators in the EGFRvIII oncogenic phenotype. Mellinghoff et al. [126] found significant correlation between activated STAT3 levels and EGFRvIII (but not wtEGFR) in 82 malignant astrocytic gliomas using immunohistochemistry. Johns et al. [83] reported that abrogation of Src signaling in an *in vivo* EGFRvIII positive xenograft model significantly reduces EGFRvIII mediated tumorigenesis. EGFRvIII signaling in HNSCC has not been extensively investigated.

1.5.2 EGFRvIII targeting strategies

Since EGFRvIII is only expressed in cancer cells, specific targeting strategies would be expected to result in minimal toxicity to normal cells [65]. An EGFRvIII-specific immunotoxin, MR1-1, has recently entered a phase I clinical trial at Duke University for glioblastoma. MR1-1 is composed of a single-chain Fv fragment of an EGFRvIII specific antibody fused to Pseudomonas exotoxin (PE38) which is cytotoxic [127]. MR1-1 has shown success in EGFRvIII positive glioblastoma regression in *in vivo* models, as well as immunizing mice against EGFRvIII positive tumors [128]. In NSCLC, EGFRvIII has been shown to be present in

approximately 5% of tumors [129]. Treatment of EGFRvIII-expressing cells with the reversible tyrosine kinase inhibitors gefitinib and erlotinib was associated with increased resistance to these inhibitors. However, EGFRvIII-expressing NSCLC cells were highly sensitive (IC_{50} 9.4 nM) to an irreversible EGFR/Her2 inhibitor HKI-272 (of the same class as BIBW2992) [129]. A phase 2 clinical trial of BIBW2992 in combination with cetuximab in EGFRvIII-expressing HNSCC is underway at the University of Chicago [130]. A phase I trial with the monoclonal antibody ABT-806 with activity against EGFR and EGFRvIII is also underway (NCT01255657).

1.6 SPECIFIC AIMS

Squamous cell carcinoma of the head and neck (HNSCC) is the sixth most common cancer in the United States [1]. Development of more effective therapies is needed to reduce the high mortality rate seen with this cancer. Epidermal Growth Factor Receptor (EGFR) has been demonstrated to be a therapeutic target for HNSCC treatment. EGFR overexpression is present in up to ~90% of tumors where levels correlate with decreased patient survival [38]. In 2006, cetuximab (Erbitux™; Imclone Systems), an EGFR-specific monoclonal antibody, became the first new FDA-approved treatment for HNSCC in 45 years. Despite ubiquitous expression of EGFR in HNSCC tumors, cetuximab has demonstrated a clinical response rate as a single agent therapy of only ~10% [15]. The biology of EGFR overexpression and appropriate prognostic EGFR markers for clinical use are still incompletely understood in HNSCC. One potential mechanism of resistance to the wild type EGFR blockade is the expression of the constitutively active EGF receptor variant 3 (EGFRvIII). Studies from our laboratory and others have found the presence of EGFRvIII in approximately 40% of HNSCC, where EGFRvIII-expressing HNSCC

cells were resistant to cetuximab *in vitro* [65, 131]. In glioma, (where EGFRvIII has been best characterized) Signal Transducer and Activator of Transcription 3 (STAT3) [132] and Src family kinases (SFKs) [83] have been elucidated as key regulatory proteins in the oncogenic phenotype of EGFRvIII. The mechanism of EGFRvIII expression and the signaling consequences of this altered form of EGFR have not been studied in HNSCC. **I propose to evaluate the clinical relevance of prognostic EGFR biomarkers and elucidate the mechanism of expression of EGFRvIII in HNSCC. I hypothesize that EGFRvIII-specific signaling through STAT3 and SFKs contributes to the oncogenic phenotype of EGFRvIII and inhibition of STAT3 and/or SFKs will abrogate growth and invasion of EGFRvIII-expressing HNSCC.**

1.6.1 Specific Aim 1: Evaluate the clinical relevance of prognostic EGFR biomarkers.

High tumor levels of EGFR as assessed by IHC are associated with poor prognosis [133]. There is discordance in the literature about the relationship between EGFR gene amplification, EGFR mRNA and EGFR protein/phosphoprotein levels and the clinical utility of each molecular characteristic in patient prognosis. I will assess each of these molecular characteristics in a cohort of prospectively collected HNSCC tissue and evaluate the relationship between characteristics and their correlation with patient prognosis. Characterizing the biology of EGFR in HNSCC should improve our understanding of the role it plays in tumor progression as well as better inform treatment selection.

1.6.2 Specific Aim 2: Elucidate the mechanism of EGFRvIII expression in HNSCC.

EGFRvIII may contribute to the EGFR inhibitor resistance observed in HNSCC [65]. The mechanism of EGFRvIII expression in HNSCC has not been defined. Screening of patient HNSCC tumors show the presence of EGFRvIII mRNA transcripts and EGFRvIII protein [65] but my preliminary screen of 2 patient tumors with EGFRvIII at the mRNA level does not detect EGFRvIII at the genomic level. I will determine if EGFRvIII expression is the result of genomic mutations, gene amplification or alternative splicing by using PCR to amplify exons 1,2, 7 and 8 and the adjacent introns in the DNA and pre-mRNA of frozen human tumor specimens that are EGFRvIII positive. These PCR products will then be sequenced and compared to genomic sequences in the NCBI database.

1.6.3 Specific Aim 3: Characterize EGFRvIII-specific signaling pathways that contribute to the oncogenic phenotype.

EGFRvIII is an attractive molecular target for HNSCC therapy as it is present in ~40% of HNSCC but not in normal tissue [65]. Little is known about differential activation of downstream signaling molecules in HNSCC cells expressing wtEGFR and EGFRvIII. I will evaluate the role of STAT3 and Src family kinases (SFKs) in the EGFRvIII oncogenic phenotype. Preliminary studies in our lab indicate a greater degree of STAT3 activation in EGFRvIII-expressing HNSCC cells as compared to vector controls. In order to determine the role of STAT3 and SFKs in mediating HNSCC proliferation and invasion downstream of EGFRvIII I will use siRNA and dominant-negative (DN) constructs to evaluate the effects of STAT3 and SFK abrogation on proliferation, and invasion.

2.0 EGFR AS A PROGNOSTIC INDICATOR IN HNSCC

2.1 INTRODUCTION

2.1.1 Molecular characterization of EGFR as a prognostic indicator in HNSCC

The epidermal growth factor receptor (EGFR) was recognized as a contributing factor in HNSCC development and progression as early as the 1990s, consequently molecular characterization of EGFR in tumors, especially EGFR protein expression and gene amplification, has been a focus of several studies intending to define prognostic markers for HNSCC [63, 64, 133-146].

2.1.1.1 EGFR amplification, mRNA and protein levels in HNSCC and their prognostic value

High levels of EGFR mRNA and protein have been found in 92% and 40 - 90% of HNSCC, respectively [133, 147, 148]. It has been shown that high EGFR protein evaluated by quantitative immunohistochemistry (IHC) was a significant indicator of poor patient prognosis [133], and high tumor levels EGFR as assessed by IHC have generally been found to be associated with poorer prognosis [63, 133, 135, 137, 146]. EGFR gene amplification has been reported to be associated with reduced progression-free survival [64, 144-146]. However, some studies found that EGFR gene amplification was not associated with increased EGFR mRNA or protein levels

[144, 145] while others reported a correlation between EGFR gene amplification and EGFR expression levels [64, 146]. The relationships between tumor EGFR gene amplification, EGFR gene expression and EGFR protein expression have not been clearly defined. Some reports have found a correlation between amplification and transcript/protein levels [64, 146, 149] while others have found no correlation [145, 150, 151]. These reports have distinct methodologies and cohorts, making direct comparison difficult.

2.1.1.2 Phosphorylated EGFR in HNSCC prognosis

Few and conflicting studies about the role of phosphorylated EGFR (pEGFR) in HNSCC exist. pEGFR and pEGFR PY1092 (detected using immunoblotting methods) have been associated with advanced disease stage and early relapse respectively [152, 153]. Another study using IHC to evaluate pEGFR found that it does not correlate with relapse free or overall survival [134]. Phosphorylation status of EGFR is particularly important in tyrosine kinase inhibitor studies. One such study found that decreased pEGFR in patient tumors after 1 week of Erlotinib treatment was significantly associated with increased time to progression and OS [154].

2.1.1.3 EGFR mRNA as a prognostic indicator in HNSCC

We could find no reports on the prognostic value of EGFR mRNA in HNSCC. One report in HNSCC found that EGFR mRNA levels did not correlate with EGFR gene amplification; gene amplification was found to have prognostic value but mRNA levels were not assessed for prognostic significance [145]. EGFR mRNA has been studied in other cancers, such as the related aerodigestive tract cancer NSCLC, where there is variation in the reports. In NSCLC no correlation between EGFR mRNA levels and OS [155] and a trend towards

correlation [156] have both been reported. Clarification of the value of EGFR mRNA remains to be determined in HNSCC.

2.1.1.4 Surrogate tissue in HNSCC prognosis

Tissue samples

Surrogate tissue that can reflect the state of the tumor itself has been a specific area of study for some years in aerodigestive tract cancers due to the frequent field cancerization effect of smoking in these tissues. In HNSCC normal oral mucosa frequently harbors the same chromosomal abberations and DNA methylation patterns found in the tumor itself [157-160]. EGFR mRNA is proposed to be increased in pre-malignant and malignant lesion brush biopsies compared to normal healthy controls [161]. Likewise, in NSCLC normal bronchial epithelium and oral mucosa have been found to have similar genetic and epigenetic patterns as the tumor itself [162-164].

Fluid samples

EGFR protein levels in saliva of HNSCC patients did not appear to differ from healthy controls and did not correlate with tumor EGFR levels [165]. Circulating tumor cells (CTCs) from HNSCC patient blood samples may have prognostic value. Specific levels of EGFR have not been evaluated, however, EGFR has been used in combination with other proteins as a cell surface marker to detect or confirm CTCs [166, 167] Recent studies have shown that presence of CTCs in HNSCC, through RT-PCR detection of markers such as cytokeratin 20 or negative depletion methodologies, are indicative of reduced disease-free survival [168-170].

2.1.2 Rationale for studying EGFR molecular characteristics for HNSCC prognosis

These clinical and biological disparities have led us to undertake an analysis to define the relationship between EGFR gene amplification, EGFR mRNA and EGFR protein and phosphoprotein levels in prospectively collected tumor tissues from a North American cohort of HNSCC patients treated with surgery with curative intent. Access to the primary HNSCC tumor can be limited, consequently, we also sought to evaluate buccal mucosa as a surrogate tissue for HNSCC by characterizing the prognostic value of buccal EGFR mRNA and evaluating the correlation between buccal and tumor EGFR mRNA. Characterizing these many facets of EGFR is anticipated to improve our understanding of the role of EGFR in HNSCC biology and further define prognostic molecular characteristics in order to better inform treatment selection.

2.2 MATERIALS AND METHODS

2.2.1 Study Subjects and Tissue Samples:

Surgical patients who were treated with curative intent for pathologically-confirmed squamous cell carcinoma of the oral cavity, oropharynx, hypopharyx, or larynx (HNSCC) were enrolled in this Early Detection Research Network- (EDRN-) sponsored study prior to surgery (n=154). Patients gave written informed consent, donated tumor and buccal tissues for study and answered an administered questionnaire regarding tobacco use. Tumor specimens from 58 of these HNSCC patients, 50 with paired adjacent histologically normal adjacent mucosa, were used to construct a tissue microarray (TMA), which has been previously described [171]. Fresh-

frozen tissues (67 HNSCC tumors and 49 adjacent mucosal tissues) from this same cohort were evaluated using reverse-phase protein array (RPPA) and 50 fresh-frozen HNSCC tumors were used for quantitative real-time PCR (QRTPCR). RNA isolated from buccal mucosal swabs was available for 40 patients (30 of the 52 patients whose tumors were evaluated using QRTPCR). Tissues were collected under the auspices of a tissue bank protocol approved by the University of Pittsburgh Institutional Review Board. A summary of cases enrolled in this study and subjects with tumor specimens incorporated into TMAs, RPPA or QRTPCR is provided in Table 1.

2.2.2 Evaluation of arrayed tissues using immunohistochemical staining

2.2.2.1 Tissue microarry (TMA) construction.

Construction of this TMA has been previously described [171]. For TMA quality assessment and morphological confirmation of tumor, one hematoxylin- and eosin-stained slide was evaluated for every ten tissue sections.

2.2.2.2 IHC staining and quantification.

P16 immunohistochemical (IHC) staining of deparaffinized TMA tumor core sections was performed (p16INK4 mAb, BD Pharmingen, San Jose, CA, 1:200 dilution), followed by antigen retrieval with microwave and citrate buffer. EGFR staining was done without antigen retrieval (EGFR M3563, Dako; 1:500 dilution). Signal amplification was performed using an antibody-conjugated proprietary micropolymer peroxidase (ImmPRESS^{TM}, Vector, Burlingame, CA). Immunoreactive cells were visualized following incubation with diaminobenzidine (DAB) chromogenic substrate. Sections were counterstained with hematoxylin and lithium carbonate to

provide morphologic detail. IHC staining was scored semi-quantitatively for each core. The percentage of immunoreactive cells was recorded to the nearest 5^{th} percentile. Intensity was scored as 0 (none), 1+ (weak), 2+ (moderate), or 3+ (strong). A composite score (IHC Score) was derived from the product of the percentage and intensity of staining.

2.2.3 Fluorescence *In Situ* Hybridization (FISH)

Dual color FISH analysis was performed using a Spectrum Green-labeled chromosome 7 centromeric probe (CEP7) and a Spectrum Orange-labeled EGFR probe (Vysis, Inc., Downers Grove, IL) and nuclei were counterstained with DAPI/antifade (Vysis, Inc.). Only individual and well-delineated cells were scored. At least 60 cells were scored for each triplicate tissue section. Gene amplification was present if ratio of EGFR to CEP7 probe signals was greater than 2.0.

2.2.4 HPV evaluation

HPV status was assessed using an HPV pan-specific DNA probe (Dako, Wide Spectrum HPV DNA Probe Cocktail, Biotinylated), which recognizes HPV subtypes 6, 11, 16, 18, 31, 33, 35, 45, 51 and 52, and bright field *in situ* hybridization.

2.2.5 Quantification of EGFR protein in tissues by reverse-phase protein array (RPPA)

RPPA, a high throughput adaptation of immunoblotting, was used to quantify tumor proteins and phosphoproteins. Briefly, seven 2-fold dilutions of tumor protein lysate were spotted onto nitrocellulose-coated FAST slides. Commercially available antibodies (EGFR SC-03 1:1000 [Santa Cruz Biotechnology, Inc.; Santa Cruz, CA], EGFR PY-992 Cell Signaling #2235 1:100 and EGFR PY-1068 Cell Signaling #2234 1:100 [Cell Signaling Technology; Danvers, MA) and Catalyzed Signal Amplification (CSA) System (DakoCytomation) were used to detect these specific proteins and phosphoproteins. Proteins/phosphoproteins were measured using a nonparametric algorithm to quantify computerized optical densities detected using local background adjustment (MicroVigene Software) [172].

2.2.6 Immunoblotting

Tumors were lysed in lysis buffer containing 1% TritonX, 10 mM Tris-HCl, 5 mM EDTA, 5 0mM NaCl, and supplemented with Complete mini protease inhibitor cocktail tablets (Roche; Mannheim, Germany) and PhosSTOP phosphatase inhibitor cocktail tablets (Roche). Protein levels were determined using the Bio-Rad protein assay method (Bio-Rad Laboratories, Hercules, CA). Forty μg of total protein were separated on 8% SDS-PAGE gels and transferred to nitrocellulose membranes using a semi-dry transfer machine (BioRad Laboratories, Hercules, CA). Membranes were blocked with Odyssey blocking buffer (Li-Cor Biosciences; Lincoln, NE), probed with primary and subsequently secondary antibodies and visualized using Odyssey Infrared Imaging System (Li-Cor Biosciences) according to manufacturer's instructions.

Primary antibodies used for blotting included β-actin, phospho-Src (Y416), phospho-STAT3 (Y705XP), STAT3, phospho-EGFR Y992, and phospho-EGFR Y1068 from Cell Signaling Technology, Beverly, MA, Src B-12 (Santa Cruz Biotechnology; Santa Cruz, CA), and EGFR (BD Transduction Laboratories; San Jose, CA). Secondary antibodies used for blotting included goat anti rabbit IRDye 680 or goat anti mouse IRDye 800CW (Li Cor Biosciences).

2.2.7 Quantitative RTPCR analysis of tumor and buccal specimens

Tumor RNA was isolated using the RNeasy kit (Qiagen; Valencia, CA). Buccal cell RNA was isolated using TRIZOL (Life Technologies, Inc.) according to the manufacturer's instructions. cDNA was synthesized from RNA using SuperScript First-Strand Synthesis System (Invitrogen; Carlsbad, CA) for buccal cells and Superscript III First-Strand kit (Invitrogen) for tumor samples in the presence of random hexamer deoxynucleotides according to manufacturers' instructions. Taqman real-time PCR quantification of EGFR and beta-glucuronidase (β-Gus) gene expression was performed in duplicate using a 7700 Sequence Detector (Applied Biosystems Inc., Foster City, CA) with an initial 12-minute denaturation at 95°C, followed by 40 cycles of denaturation at 95°C and 60 seconds of annealing and extension at 60°C. Samples were prepared using Applied Biosystems TaqMan Universal PCR Master Mix (Foster City, CA), 48 ng of cDNA, 1.0 uM primers and 0.4 uM probes. PCR amplifications of EGFR were performed using a single pair of primers (forward primer: 5'-ATACGCGGCAGGACCAAG-3'; reverse primer: 5'-GGAGCGTAATCCCAAGGATGT-3'). PCR amplifications of β-Gus were performed using the forward primer 5'-CTCATTTGGAATTTTGCCGATT-3' and reverse primer 5'-CCGAGTGAAGATCCCCTTTTTA-3'. The fluorescent probes (EGFR: 5'-

CATGGTCAGTTTTCTCTTGCAGTCGTC-3' and β-Gus: TGAACAGTCACCGACGAGAGTGCTGG) were designed to hybridize to the antisense strand of the respective PCR products. Both probes were labeled with 6-carboxy-fluorescein phosphoramidite at the 5' end, and as a quencher, 5-carboxy-tetramethyl-rhodamine was incorporated at the 3' of both probes. EGFR expression was measured relative to the endogenous control gene β -Gus, using the comparative CT method.

2.2.8 Statistical analysis:

Statistical analyses were performed using STATA V9 (Statacorp, College Station, TX), SPSS V14.0 (Chicago, IL) and Graphpad Prism (La Jolla, CA). Statistical methods used for each figure are listed in the figure legend. Significance was defined as $P<0.05$. Progression-free survival (PFS) time was defined as time from first treatment to first subsequent upper aerodigestive cancer, metastasis, death or last follow-up. Overall survival (OS) time was defined as time from first treatment to death or last follow-up. Univariable and Cox proportional hazards models (CPH) were used to estimate the hazards ratio (HR) for each molecular marker associated with PFS by log rank tests. EGFR characteristics found to be associated with survival in univariable CPH were evaluated for association with survival in multivariable CPH adjusted for candidate prognostic variables found to be significantly associated with survival in this cohort (Wald $P<0.05$). Candidate prognostic variables included the following: age, sex, tumor HPV status [173], AJCC disease stage, smoking status at first treatment, PY category, cancer type and adjuvant treatment as defined in Table 1. PFS and OS multivariable CPH were developed and

evaluated independently. The assumption of proportional hazards was tested by evaluation of Schoenfeld residuals.

Table 1. HNSCC Cohort Description and Analysis

	All Cases		TMA Cases			RPPA Cases			QPCR Cases		
	n=154		n=58		P*	n=67		P*	n=50		P*
Gender											
Male	110	71.4%	41	70.7%	0.88‡	48	71.6%	0.96‡	36	72.0%	0.91‡
Female	44	28.6%	17	29.3%		19	28.4%		14	28.0%	
Ethnicity											
White	148	96.1%	57	98.3%	0.41‖	65	97.0%	0.69‖	51	98.1%	0.66‖
Non-white	6	4.4%	1	4.4%		2	4.4%		1	4.4%	
Age											
Median (Range)	58 (23-89)		61 (23-80)		0.42§	58 (23-80)		0.76§	60 (23-80)		0.44§
Smoking Status											
Never smoker	24	15.6%	6	10.3%		9	13.4%		6	12.0%	
Former smoker	50	32.5%	23	39.7%	0.20‡	23	34.3%	0.79‡	16	32.0%	0.65‡
Active Smoker	80	51.9%	29	50.0%		35	52.2%		28	56.0%	
Cigarette Pack-Years											
0 py	24	15.6%	6	10.3%		9	13.4%		6	12.0%	
1-59 py	57	37.0%	18	31.0%	0.16‡	21	31.3%	0.42‡	17	34.0%	0.45‡
≥60 py	58	37.7%	26	44.8%		28	41.8%		22	44.0%	
Unknown	15	9.7%	8	13.8%		9	13.4%		5	10.0%	
Alcohol Drinker											
Never drinker	40	26.0%	16	27.6%	0.72‡	18	26.9%	0.82‡	14	28.0%	0.69‡
Ever drinker	114	74.8%	42	74.8%		49	74.8%		36	74.8%	
Alcohol Quantity†											
0	40	26.0%	16	27.6%		18	26.9%		14	28.0%	
1-4	48	31.2%	16	27.6%	0.85‡	18	26.9%	0.43‡	9	18.0%	0.08‡
≥5	46	29.9%	19	32.8%		24	35.8%		20	40.0%	
Unknown	20	13.0%	7	12.1%		7	10.4%		7	14.0%	
Tumor Type											
Metastasis	2	1.3%	2	3.4%		2	3.0%		1	2.0%	
New Primary	11	7.1%	2	3.4%	.15‖	3	4.5%	.30‖	1	2.0%	.28‖
Primary	128	83.1%	50	86.2%		57	85.1%		43	86.0%	
Recurrence	13	8.4%	4	6.9%		5	7.5%		5	10.0%	
Tumor Site											
Oral Cavity	70	45.5%	23	39.7%		27	40.3%		16	32.0%	
Oropharynx	29	18.8%	12	20.7%		12	17.9%		12	24.0%	
Hypopharynx	11	7.1%	2	3.4%	.32‖	3	4.5%	.23‖	2	4.0%	.06‖
Larynx	40	26.0%	19	32.8%		22	32.8%		18	36.0%	
Other	4	2.6%	2	3.4%		3	4.5%		2	4.0%	
Disease Stage											
I	26	16.9%	6	10.3%		6	9.0%		5	10.0%	
II	21	13.6%	11	19.0%		13	19.4%		7	14.0%	
III	30	19.5%	7	12.1%	.09‖	10	14.9%	.049‖	8	16.0%	.45‖
IV	61	39.6%	27	46.6%		30	44.8%		23	46.0%	
Recurrence/Metastasis	15	9.7%	6	10.3%		7	10.4%		6	12.0%	
Unknown	1	0.6%	1	1.7%		1	1.5%		1	2.0%	

Table 1 Continued

Treatment												
RT Only	41	26.8%	19	32.8%		20	29.0%		18	36.0%		
CRT	27	17.6%	10	17.2%	.43‖	11	15.9%	.73‖	9	18.0%	.16‖	
No CRT	79	51.6%	27	46.6%		35	50.7%		21	42.0%		
Unknown	6	3.9%	2	3.4%		3	4.3%		2	4.0%		
Vital Status												
Alive	79	51.3%	26	44.8%	0.15‡	30	44.8%	0.12‡	20	40.0%	0.04‡	
Dead	73	47.4%	32	55.2%		37	55.2%		30	60.0%		
Unknown	2	1.3%	0	0.0%		0	0.0%		0	0.0%		
Time to Death												
Median (Range)	44.6 (1.5-112.3)		42.2 (1.5-112.3)		0.79§	42.5 (1.5-112.3)		0.82§	44.2 (1.5-112.3)		0.76§	
Disease Progression Status												
No disease progression	64	41.6%	22	37.9%	0.41‡	26	38.8%	0.46‡	16	32.0%	0.08‡	
Disease Progression	88	57.1%	36	62.1%		41	61.2%		34	68.0%		
Unknown	2	1.3%	0	0.0%		0	0.0%		0	0.0%		
Progression Free Survival												
Median (Range)	33.8 (1.2-108.4)		26.4 (1.2-106.9)		0.62§	26.8 (1.2-106.9)		0.75§	24.8 (1.2-106.9)		0.92§	

†Typical number of alcohol drinks in 2 week period

‡ Chi square test

§ = rank sum test

‖=Fisher's exact test

* Analyzed versus unanalyzed cases

2.3 RESULTS

2.3.1 Analyzed tumor subsets were representative of the EDRN cohort

Cases available for molecular analysis were based upon incorporation of tumor tissues into a TMA (TMA studies) or availability of fresh-frozen tissues (RPPA and QRTPCR studies). Of the 58 tumors arrayed on the TMA, 54 were also analyzed by RPPA and 42 were evaluated by QRTPCR. A total of 67 tumors were available for RPPA studies, and of these 48 were evaluated in QRTPCR studies. Overall, 40 tumor samples were analyzed using all methods. Patient and tumor characteristics of analyzed tumors were compared to the entire EDRN cohort (Table 1). For all subsets of evaluations, patients and tumors that were analyzed for molecular characteristics did not differ significantly from subjects not analyzed by sex, age, smoking/drinking histories, tumor type, or tumor site. Cases analyzed by RPPA did differ from non-analyzed cases with respect to disease stage (TMA and QRT-PCR cases did not). This is not unexpected as advanced stage tumors usually yield more tissue for evaluation (Table 1).

Tumors incorporated into the TMA were evaluated for p16 and HPV status (Table 2). Only 4 of the evaluated 49 tumors were found to be positive for HPV: 2 oropharyngeal, 1 laryngeal and 1 neck metastasis (9 of the 58 TMA tumor specimens could not be evaluated for

HPV or P16 status). Therefore, 25% of the TMA arrayed oropharyngeal cases were HPV positive. All HPV positive tumors by ISH also had high p16 levels.

Table 2 HPV Description and Analysis

	HPV Negative n=45		HPV Positive n=4		p-value†
Gender					
Male	32	94.1%	2	5.9%	0.357
Female	13	86.7%	2	13.3%	
Age					
Median (Range)	61 (23-80)		55 (46-56)		0.044
Pack Years					
Never smoker	3	60.0%	2	40.0%	0.084
1-59 py	15	100.0%	0	.0%	
≥60 py	20	90.9%	2	9.1%	
Unknown	7	100.0%	0	.0%	
Alcohol Quantity					
Never drinker	12	100.0%	0	.0%	0.557
1-4 per 2 weeks	12	85.7%	2	14.3%	
≥5 per 2 weeks	15	93.8%	1	6.3%	
Unknown	6	85.7%	1	14.3%	
Tumor Type					
Metastasis	1	50.0%	1	50.0%	0.359
New Primary	1	100.0%	0	.0%	
Primary	41	93.2%	3	6.8%	
Recurrence	2	100.0%	0	.0%	
Tumor Site					
Oral Cavity	22	100.0%	0	.0%	0.032
Oropharynx	6	75.0%	2	25.0%	
Hypopharynx	2	100.0%	0	.0%	
Larynx	14	93.3%	1	6.7%	
Neck	1	50.0%	1	50.0%	
Disease Stage					
0-1	4	100.0%	0	.0%	0.515
II	11	100.0%	0	.0%	
III	6	100.0%	0	.0%	
IV	21	87.5%	3	12.5%	
Recurrence/Metastasis	3	75.0%	1	25.0%	
Vital Status					
Alive	19	86.4%	3	13.6%	0.231
Dead	26	96.3%	1	3.7%	
Time to Death					
Median (Range)	40 (2-112)		63 (43-74)		0.057
Disease Progression Status					
No disease progression	16	84.2%	3	15.8%	0.155
Disease progression	29	96.7%	1	3.3%	
Progression Free Survival					
Median (Range)	18.3 (1.5-106.9)		62.6 (18.7-73.5)		0.027

2.3.2 Correlation of cohort characteristics with survival

We evaluated our cohort for associations between overall survival (OS) and demographic factors, tobacco use, disease stage or tumor site in order to generally compare our cohort to previously described surgical cohorts. The analysis of our cohort indicated that increasing age by category (<55, 55-65, or >65 years) tended to be associated with reduced OS (p=0.09; log rank test (LRT)). The ratio of men to women in our cohort was approximately 2.5:1, which was similar to other HNSCC case cohorts [136, 174]. Patient sex, however, was not associated with differential OS (P=0.77; LRT). Increasing number of pack-years (PY) of cigarette smoking history by PY category and active smoking status at first treatment were associated with significantly reduced OS (P=0.002 and P=0.03, respectively; LRT). Patients with higher AJCC tumor stage had shorter OS (P=0.0004; LRT) as did patients with nodal disease (P=0.02; LRT). OS differed depending upon whether or not a patient received adjuvant CT or RT (P=0.001; LRT) with patients who received RT adjuvant treatment only in addition to surgery having shorter OS. Tumor site was not significantly associated with OS (P=0.69; LRT). Tumor type was associated with OS: patients with recurrent tumors had significantly shorter survival (P=0.004)

HPV-positive/ P16 high tumors tended to be associated with improved survival, but this did not reach statistical significance (P=0.27). This lack of statistically significant association likely reflects the low prevalence of HPV-positive tumors in our surgical cohort (8%) and our study's diminished power regarding this association. Median OS for patients with HPV-negative tumors was 45.1 months while the median survival time was not reached for patients with HPV-positive tumors. Of the 4 patients with HPV-positive tumors, one patient died 42.6 months after treatment. This patient was 1 of 2 HPV-positive patients who were active smokers at the time of

first treatment. The 3 patients with HPV-positive tumors who were alive at last follow-up had an average follow-up time of 66.2 months (Table 2).

There were no statistical differences between cases evaluated for molecular characteristics and those not evaluated with regard to any of these candidate prognostic factors (Table 1). Fresh-frozen tumor tissue was required for RPPA studies. Tumors evaluated by RPPA were of more advanced disease stages than those not evaluated by RPPA (Table 1), likely reflecting the availability of tissue. Overall, our patient cohort characteristics are typical of a surgical HNSCC cohort with regard to patient demographics, tobacco and alcohol use histories and tumor site distributions. In general, our cohort also exhibited the typical associations with reduced survival including higher disease stage, presence of nodal disease and heavier tobacco use histories.

2.3.3 EGFR protein levels by IHC but not by RPPA were higher in tumors with amplified EGFR

We previously reported that elevated tumor EGFR levels in EDRN TMA-arrayed tumors tended to be associated with reduced PFS (HR= 1.95, P=0.06) [171]. We assessed tumor EGFR gene amplification status in order characterize relationships between tumor EGFR gene amplification and EGFR protein levels.

We evaluated EGFR gene amplification status using FISH analysis with a focus on the number and percentage of tumors with (1) EGFR gene amplification, (2) hyperploidy without

EGFR gene amplification and (3) no hyperploidy or EGFR gene amplification (Figure 1A). Previous studies have evaluated increased EGFR gene copy number defined as either EGFR amplification or hyperploidy. We found that 31/57 tumors had increased EGFR gene copy number. EGFR protein levels by IHC were significantly higher in HNSCC tumors with EGFR gene amplification (Figure 1B). Of the 13 tumors with amplified EGFR, 11 tumors had high EGFR by IHC analysis and the remaining 2 samples had intermediate EGFR expression; no tumor with amplified EGFR had low levels of EGFR protein by IHC analysis. Of the 56 tumors successfully evaluated for EGFR protein by IHC, 19 tumors had high levels of EGFR protein by IHC, defined as having IHC scores in the highest tertile. Of these 19 tumors, 58% had EGFR amplification, 21% were hyperploid and 21% were neither hyperploid nor had amplified EGFR, indicating that EGFR amplification was not the only process through which high tumor EGFR levels resulted. Of the 4 HPV-positive tumors, none had EGFR gene amplification nor were hyperploid, and all exhibited low EGFR protein levels by IHC (Table 3).

Table 3 HPV Molecular Analysis

	HPV Negative n=45		HPV Positive n=4		p-value†
EGFR Amplification by FISH					
Not Amplified	34	89.5%	4	10.5%	0.348
Amplified	11	100.0%	0	.0%	
Hyperploidy by FISH					
No Hyperploidy	25	86.2%	4	13.8%	0.112
Hyperploidy	20	100.0%	0	.0%	
EGFR Protein by IHC					
Low EGFR	19	82.6%	4	17.4%	0.042
High EGFR	26	100.0%	0	.0%	
EGFR Protein by RPPA					
Low EGFR	23	100.0%	0	.0%	0.054
High EGFR	19	82.6%	4	17.4%	
pEGFR Y1068 by RPPA					
Low pEGFR Y1068	20	83.3%	4	16.7%	0.065
High pEGFR Y1068	22	100.0%	0	.0%	
pEGFR Y992 by RPPA					
Low pEGFR Y992	22	91.7%	2	8.3%	0.662
High pEGFR Y992	20	90.9%	2	9.1%	
EGFR mRNA by QPCR					
Low EGFR	15	83.3%	3	16.7%	0.323
High EGFR	16	94.1%	1	5.9%	
EGFRvIII mRNA by QPCR					
Low EGFRvIII	24	85.7%	4	14.3%	0.391
High EGFRvIII	7	100.0%	0	.0%	

Tumor EGFR protein as measured by RPPA did not differ by EGFR amplification or tumor hyperploid status (Figure 1C). To validate our RPPA results 10 available tumors evaluated by RPPA with either high or low EGFR levels were assayed by immunoblot for EGFR protein levels. We found that tumors defined as having high EGFR protein levels by RPPA also had significantly higher EGFR protein levels as determined by immunoblotting than those tumors defined as having low EGFR by RPPA (Figure 1E). We found no correlation between EGFR protein levels as assessed by IHC analysis and EGFR protein by RPPA (rho=0.16, P=0.26).

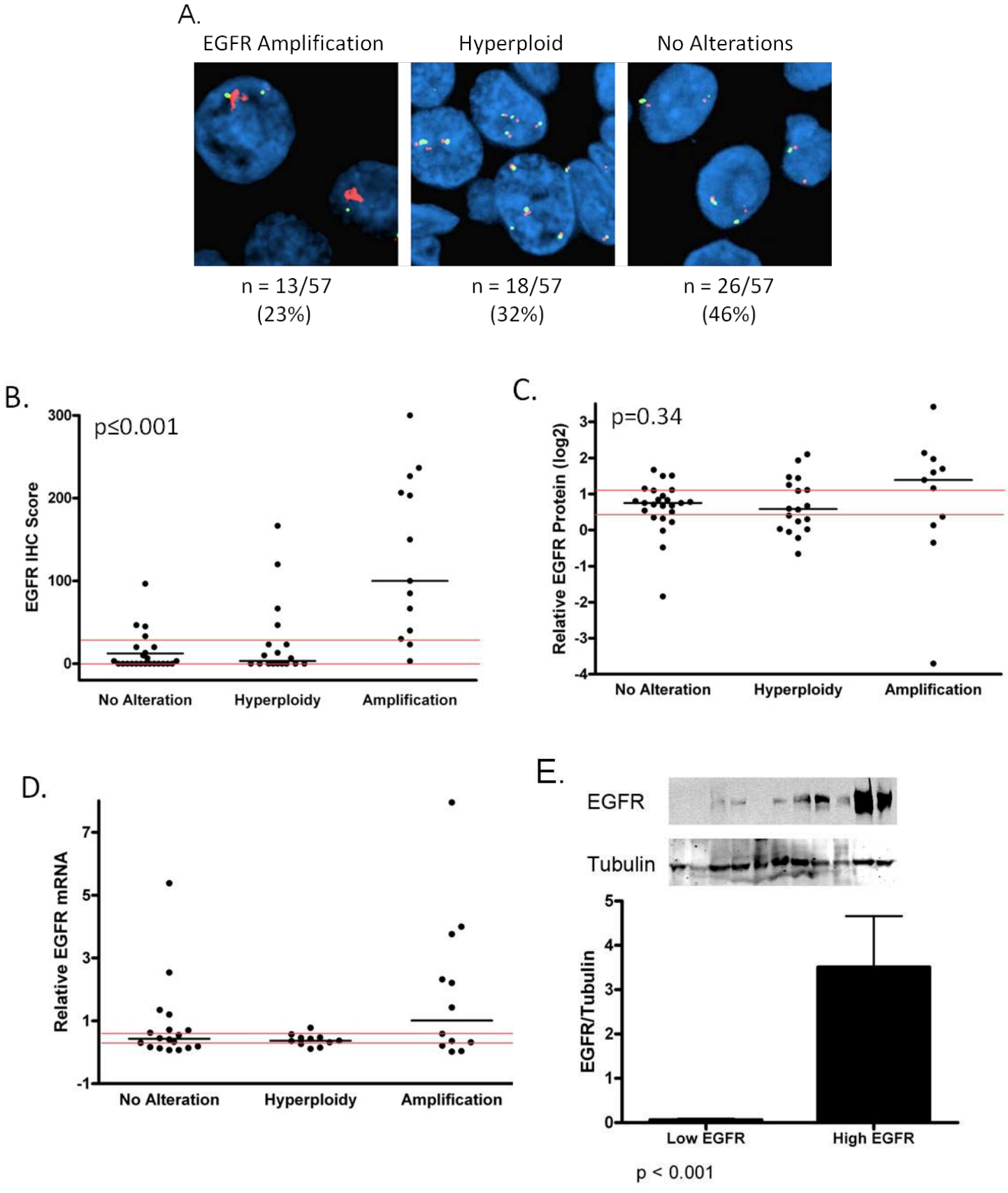

A.
EGFR Amplification
Hyperploid
No Alterations
n = 13/57
(23%)
n = 18/57
(32%)
n = 26/57
(46%)
B.
p≤0.001
EGFR IHC Score
300
200
100
0
No Alteration
Hyperploidy
Amplification
C.
p=0.34
Relative EGFR Protein (log2)
3
2
1
0
-1
-2
-3
-4
No Alteration
Hyperploidy
Amplification
D.
Relative EGFR mRNA
7
5
3
1
-1
No Alteration
Hyperploidy
Amplification
E.
EGFR
Tubulin
EGFR/Tubulin
5
4
3
2
1
0
Low EGFR
High EGFR
p < 0.001

Figure 2.1 Evaluation of EGFR tumor protein and mRNA levels categorized by EGFR gene amplification and ploidy status.

(A) Representative EGFR (red) and chromosome 7 (green) FISH for tumors with EGFR gene amplification (top panel, n=13/57), hyperploidy without EGFR gene amplification (middle panel, n=18/57) and neither EGFR gene amplification nor hyperploidy (bottom panel, n=26/57). (B) EGFR protein levels determined by IHC staining categorized by tumor EGFR gene amplification and ploidy status. (C) Tumor EGFR mRNA level by EGFR gene amplification and ploidy status. (D) EGFR protein levels determined by reverse phase protein array (RPPA) categorized by EGFR gene amplification and ploidy status. Medians (black horizontal bars), overall tertiles (red horizontal bars) and P values (Kruskal-Wallis tests) are provided. (E) Available fresh-frozen tumor tissues with either high or low levels of EGFR by RPPA were quantified using immunoblot densitometry and levels normalized to actin. Averages, standard deviations and associated p values (rank sum tests) are provided.

2.3.4 Tumor EGFR mRNA levels did not differ by EGFR gene amplification status.

To the best of our knowledge EGFR mRNA levels in HNSCC have not been evaluated for prognostic significance. In NSCLC reports of EGFR mRNA level association with prognosis and survival is still controversial [155, 156]. An evaluation of overall EGFR mRNA levels found that EGFR mRNA levels were higher in HNSCC compared to normal and were decreased in the tumor following cisplatin exposure (no analysis for prognostic significance was performed) [175]. EGFR mRNA (by southern blot) was reported to be increased in HNSCC compared to normal specimens [32] but EGFR mRNA (by microarray) did not differ between EGFR FISH positive and FISH negative groups [145]. EGFR mRNA did correlate with protein

by automated IHC evaluation but not by traditional interpretation methods [8]. We measured tumor EGFR mRNA levels by QRT-PCR and found tumor EGFR mRNA levels did not differ by EGFR amplification or hyperploid status (Figure 1C), and we found no correlation between tumor EGFR protein levels by IHC analysis and EGFR mRNA levels (rho=0.28, P=0.12). Similarly, there was no correlation between tumor EGFR protein levels by RPPA and tumor EGFR mRNA levels (rho=0.04; P=0.81).

2.3.5 High tumor EGFR protein by IHC, but not tumor EGFR protein by RPPA, gene amplification, or mRNA, was associated with reduced survival

For this study, we reanalyzed EGFR tumor protein levels assessed by IHC analysis using tertiles to define high, intermediate and low EGFR expressing tumors in order to represent the data in more detail. We found that high tumor EGFR levels by IHC were associated with significantly reduced PFS compared to tumors with low EGFR levels (Figure 2A). In our cohort, EGFR amplification was not a significant indicator of patient progression free survival (PFS) (Figure 2B). When tumors with high EGFR copy number, defined as having either amplified EGFR or hyperploidy [145], were compared to tumors without either EGFR amplification or hyperploidy, we observed no significant difference in PFS (p=0.09). Levels of EGFR protein by RPPA and EGFR mRNA were also not significant predictors of PFS (Figures 2C and 2D, respectively).

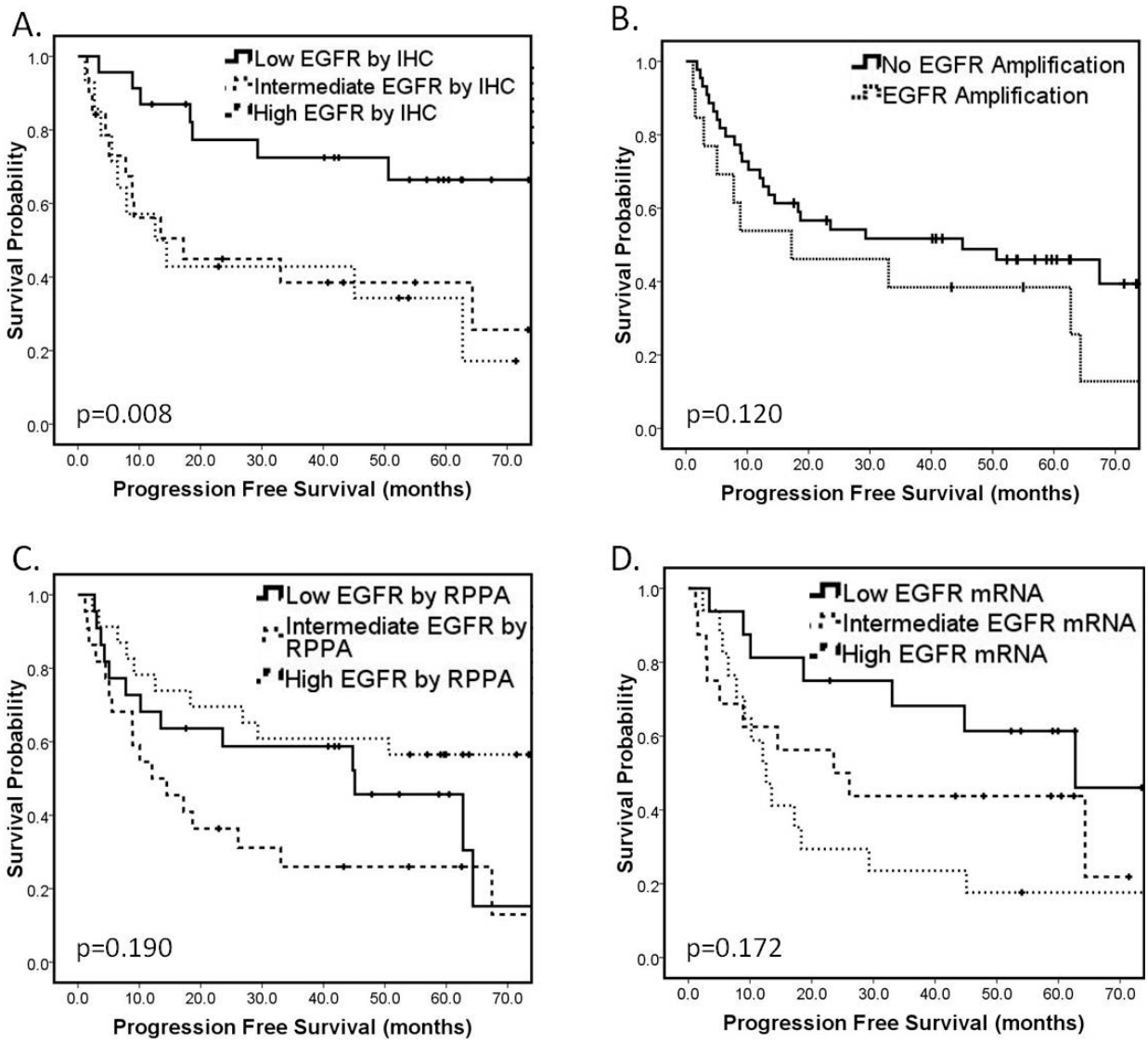


Figure 2.2 Progression-free survival by tumor EGFR gene amplification and hyperploidy status and EGFR mRNA and protein levels. Kaplan-Meier progression-free survival plots by (A) EGFR amplification status, (B) hyperploidy or amplification versus no alteration, (C) EGFR mRNA level, and (D) EGFR protein level by RPPA. Log rank tests compare high versus low (C & D), associated P values are provided.

2.3.6 EGFR mRNA levels in buccal cells did not reflect tumor biology nor associate with survival

We measured EGFR mRNA expression in buccal swabs in order to assess whether the oral mucosa could provide information regarding EGFR mRNA expression in tumors within the upper aerodigestive mucosa and to determine whether EGFR mRNA expression in buccal cells provided prognostic information. Buccal cells from 40 EDRN cases were successfully evaluated for EGFR mRNA levels. Of these 40 patients with EGFR mRNA expression measured in buccal cells, tumor specimens were also analyzed for EGFR protein by IHC analysis (n=33), EGFR protein by RPPA (n=39) and EGFR tumor mRNA levels by QRTPCR (n=30). We found that EGFR mRNA levels in buccal cells were not correlated with EGFR mRNA levels in tumors (Figure 3A), and buccal cell EGFR mRNA levels were not associated with PFS (Figure 3B).

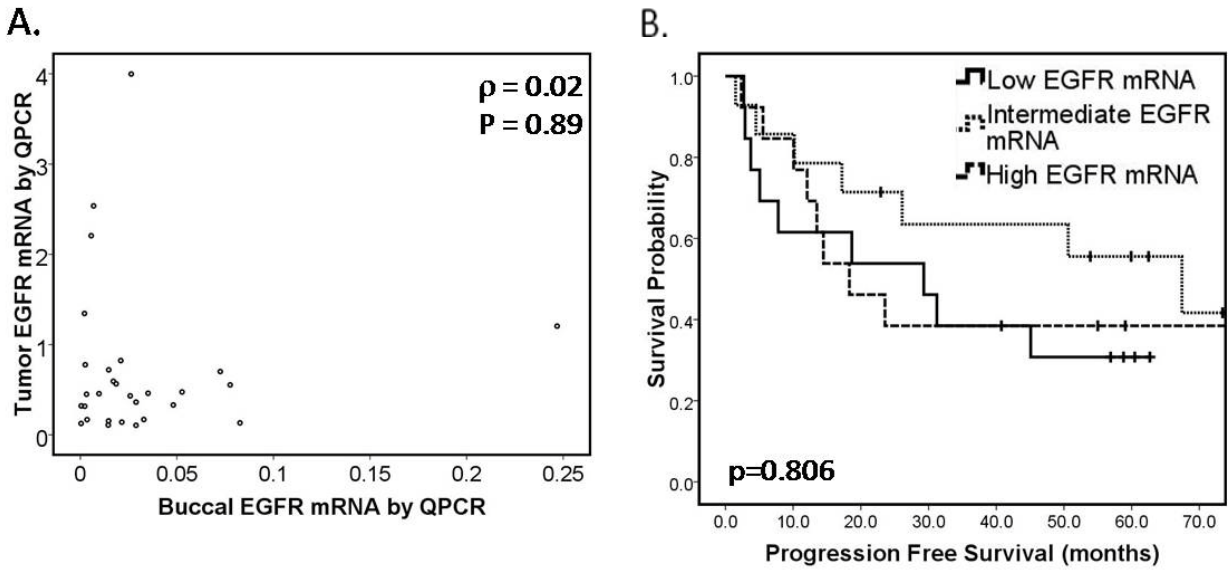


Figure 2.3 Scatter plots of buccal EGFR mRNA levels by (A) tumor EGFR mRNA levels. Spearman's correlation coefficients and associated P values are provided. (B) Kaplan-Meier progression-free survival plot by EGFR buccal mRNA tertile level. Log rank test P value for high versus low is provided.

2.3.7 Elevated levels of site-specific pEGFR at Y1068 but not Y992 were associated with reduced survival

RPPA was used to measure site-specific phosphorylation of EGFR at sites Y1068 and Y992. RPPA results were validated by immunoblotting analysis of 10 available tumors with high and low expression levels of pEGFR P-Y1068 or P-Y992 by RPPA. We confirmed that tumors with high pEGFR at sites Y1068 and Y992 by RPPA had significantly higher levels of these specific EGFR phosphoproteins (Figures 2.4A and B). High tumor levels of EGFR P-Y1068 were associated with significantly reduced PFS compared to tumors with low levels (Figure 2.4C). Tumor levels of EGFR P-Y992 were not significantly associated with PFS (Figure 2.4D). All four HPV positive tumors had low pEGFR P-Y1068 levels while two of these tumors had high and two had low levels of pEGFR P-Y992 (Table 3).

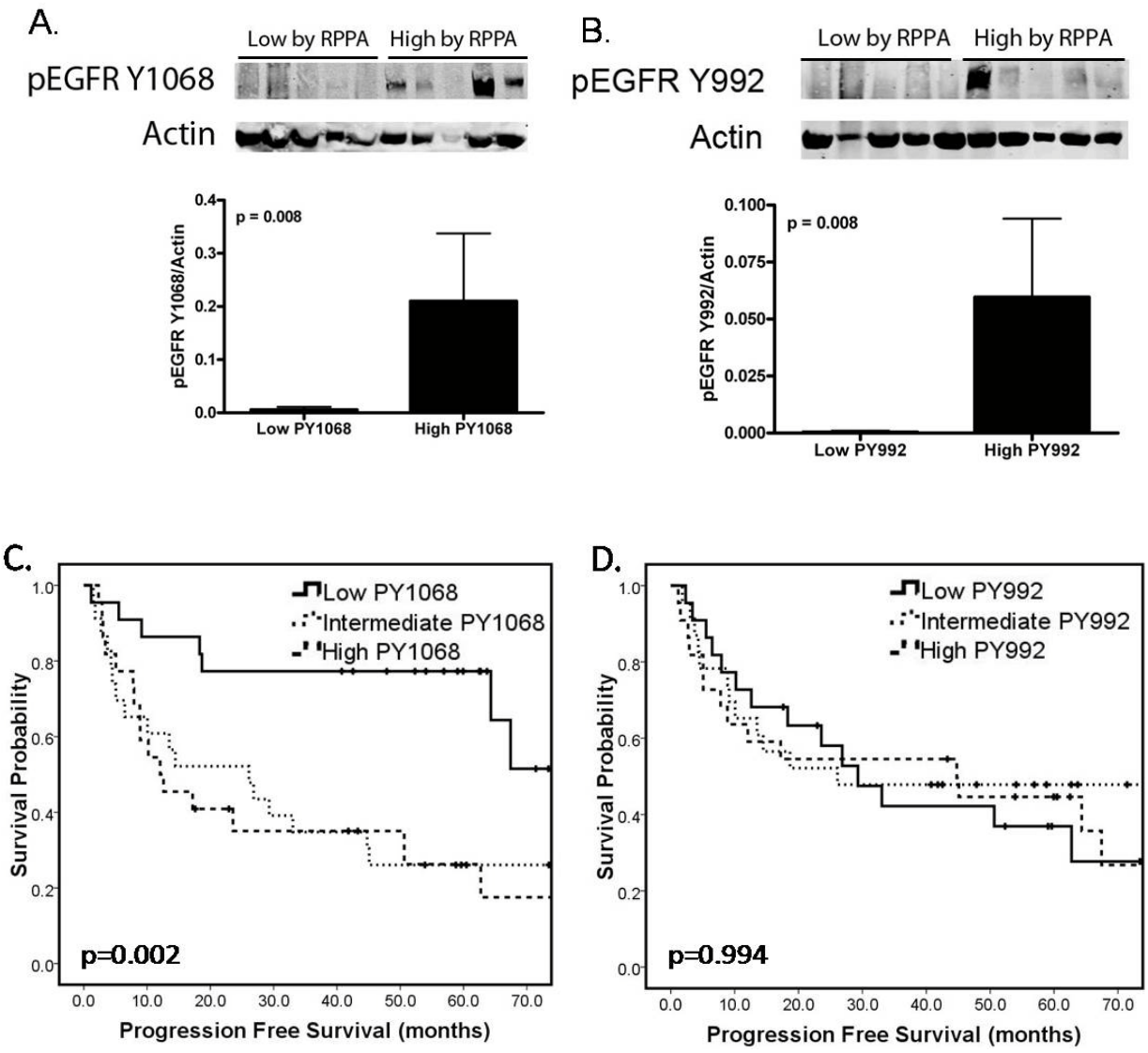


Figure 2.4 Phosphorylation at EGFR PY-1068 was associated with reduced progression-free survival. Available fresh-frozen tumor tissues with either high or low levels of (A) pEGFR Y1068 or (B) pEGFR Y992 by RPPA were quantified using immunoblot densitometry and levels normalized to actin. Averages, standard deviations and associated p values (rank sum tests) are provided.

The difference in prognostic value of the specific pEGFR sites led us to question if the downstream signaling of these sites was activated. With the limited remaining tissue we immunoblotted for pSFK P-Y416 and STAT3 P-Y705 levels normalized to total protein levels in 5 tumors ranked high in pEGFR P-Y1068 and 5 tumors ranked low in pEGFR P-Y1068 (Figure 2.5A and B). Patient tumors with high EGFR P-Y1068 showed no significant difference in pSFK P-Y416 levels compared to tumors with low EGFR P-Y1068. Tumors expressing high levels of EGFR P-Y1068 did have higher levels of pSTAT3 P-Y705 compared low expressors.

To determine whether tumor levels of pEGFR P-Y1068 had prognostic significance even after considering tumor EGFR protein levels as assessed by IHC analysis, we constructed a Cox proportional hazards model to assess high versus low levels of tumor EGFR protein by IHC and EGFR P-Y1068, with the median defining high versus low for each marker. In this model, high tumor EGFR levels by IHC and high tumor EGFR P-Y1068 levels by RPPA were both independently statistically associated reduced PFS (HR= 2.68; 95% CI=1.22-5.87 and HR=3.23; 95% CI = 1.30-7.99, respectively), indicating that both contributed significantly as prognostic indicators. We then combined EGFR and EGFR P-Y1068 levels into three groups, low for both EGFR and EGFR P-Y1068, high in either or high in both (Figure 2.5C). High tumor EGFR protein by IHC and high tumor EGFR P-Y1068 was associated with significantly reduced PFS compared to low tumor levels of EGFR protein by IHC and pEGFR P-Y1068 (HR= 4.79; 95% CI = 1.39 – 16.5) even in multivariable Cox proportional hazards models adjusted for nodal stage and patient age (HR=5.52; 95% CI = 1.41-21.67).

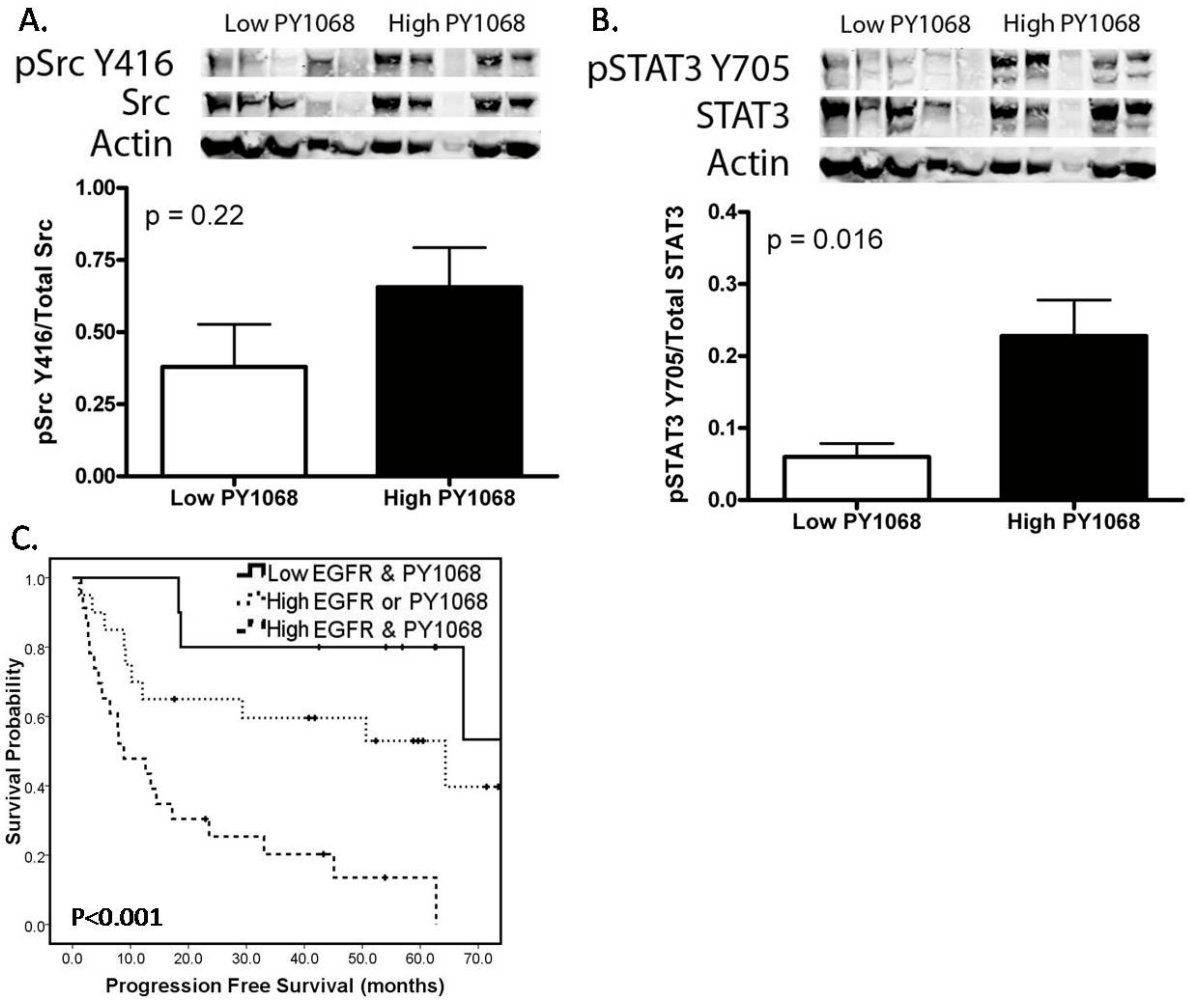


Figure 2.5 pEGFR Y1068 correlates with increased pSTAT3 and combined with EGFR IHC levels identifies HNSCC subgroup with high risk disease. (A and B) Representative tumor samples expressing either high or low pEGFR Y1068 (identical to those in Figure 2.4A) were evaluated using immunoblot densitometry for levels of (A) pSrc Y416 and c-Src and (B) pSTAT3 and STAT3. Actin is included for visual reference and was not included in calculations. Averages, standard deviations and rank sum test-associated p values are indicated. Relative levels of phosphoproteins were normalized to corresponding unphosphorylated protein. (C) EGFR intermediate and high protein levels by IHC combined with pEGFR Y1068 intermediate and high levels versus lowest tertile reference. Hazards ratios are listed in the text, log rank test used for p-value comparing low in EGFR and Y1068 to high in EGFR and Y1068.

2.4 DISCUSSION

HPV screening of the TMA showed that 4 of 49 cases were positive for HPV with 3 of these having no disease progression. It is also notable that all 4 of these tumors did not demonstrate EGFR amplification or hyperploidy. While there are certainly too few samples to draw definitive conclusions, these data support the previous findings that HPV positive HNSCC may have fewer genome-wide copy number alterations than HPV negative HNSCC [176, 177]. Consistent with previous reports HPV positive tumors were also low in EGFR by IHC [178]. Recent studies have shown that HPV is highly associated with high p16 levels [179-182] and we found that all HPV cases had high levels of p16.

EGFR gene amplification and ploidy status were evaluated and related to EGFR protein and mRNA levels. EGFR amplification correlated significantly with EGFR protein by IHC but not with relative EGFR protein by RPPA or mRNA transcript. While EGFR amplification leads to high levels of EGFR as detected by IHC, high levels of EGFR are also detected in cases with no amplification or hyperploidy. This indicates that biologically there are other mechanisms of high EGFR expression and demonstrates that EGFR amplification status does not yield more information for potential patient prognosis in addition to IHC in this cohort.

The discordance between EGFR as detected by IHC and RPPA is not unexpected, although a study of caveolin-1 and p70S6k in NSCLC found trend agreement between RPPA and

IHC [183] there are few studies directly comparing IHC and RPPA. Due to the difference in basic tissue processing and assay design these methods evaluate different populations of EGFR. EGFR detected by IHC in this study is limited to cytoplasmic and membrane EGFR of tumor cells, while RPPA detects cytoplasmic, membrane and nuclear EGFR in both tumor and up to 30% stromal tissues. EGFR mRNA levels did not significantly correlate with either RPPA or IHC. This was not unexpected due to the difference in half-life of mRNA transcript and protein as well as the multiple layers of regulation involved in translation. We did find that of the 12 cases that had EGFR gene amplification and overexpression of EGFR by IHC 11 were evaluated for EGFR mRNA and 7 (64%) had high levels of EGFR transcript by QRTPCR.

There is precedence for both concordance and discordance between copy-number alterations, mRNA and protein expression in cancer tissues [184, 185]. It is possible that the detection methods employed in our study also contribute to the discontinuity we have observed. A study of 189 patients with non-small-cell lung cancer evaluated 2 IHC IGF1R antibodies and found that one correlated with mRNA expression but the other did not [186], indicating that current reagents and techniques are still limited and flawed. Biologically, protein overexpression can come from gene amplification, mRNA stabilization, protein modifications and other signaling alterations. In HNSCC there are mechanisms beyond gene amplification that must be considered to fully understand the biological mechanism of EGFR protein overexpression.

We previously published that high EGFR expression by IHC is associated with reduced PFS in this cohort [171]. Other studies on EGFR gene amplification in HNSCC have grouped EGFR gene amplification and hyperploidy when analyzing the prognostic significance of EGFR

DNA alterations [145]. As these two mechanisms of DNA alteration are biologically distinct we first evaluated if EGFR gene amplification alone was sufficient to provide prognostic information about PFS. EGFR gene amplification did not correlate with significantly reduced PFS. EGFR gene amplification combined with hyperploidy was also not significantly indicative of reduced PFS. This is in contrast to findings by Chung et al. who found that EGFR gene amplification combined with hyperploidy was associated with reduced PFS but not with protein expression [145]. This discordance in findings is likely due to differences in cohort characteristics as well as methodological differences. Chung et al. had 83% of cases with stage III-IV tumors while our cohort had 57%, their cohort also had only 8% of tumors with no hyperploidy or amplification while we found that 46% of tumors had no EGFR amplification or hyperploidy. It is possible that the increased alterations found in the Chung et al. cohort compared to our cohort are partially attributable to the increased percentage of late stage tumors found in the cohort. Additionally, the statistical analysis of our cohort had PFS data for over 5 years while the PFS data for Chung et al. evaluated for 36 months. Finally, our cohort was uniformly treated with surgery with curative intent while the Chung et al. cohort had heterogeneous treatment (some patients received surgery while others received radiation). Together, these factors likely impact the differential findings between these two studies.

Analysis of high versus low EGFR mRNA levels or high versus low EGFR protein levels by RPPA also showed no significant decrease in PFS with high EGFR mRNA or protein levels. It seems that mRNA and total protein as determined by RPPA are poor indicators of patient prognosis and not recommended for use as future HNSCC biomarkers.

HNSCC tumors are frequently difficult to access and biopsy can therefore be relatively invasive. Due to the common field cancerization found in HNSCC [187, 188] it is possible that a reliable biomarker may be identified in histologically normal mucosa. Buccal cells are easily collected with an oral swab and provide sufficient material for mRNA analysis of genes of interest [161]. We analyzed patient buccal cells for EGFR mRNA expression and found no difference in PFS between high and low EGFR expression. We considered that possibly EGFR mRNA levels may correlate with EGFR by IHC which is indicative of patient PFS and could provide a less invasive first step in patient testing. Buccal cell EGFR mRNA did not, however, correlate with tumor EGFR by IHC and further did not correlate with tumor EGFR mRNA. EGFR levels in buccal cells do not appear to correlate with tumor EGFR levels, or prognosis. There is little justification for assessment of EGFR in buccal cells to guide treatment decisions.

There have been very limited reports on the phosphorylation status of specific tyrosines on EGFR in a cohort of HNSCC. Utilizing reverse phase protein arrays (RPPA) we were able for the first time to determine the phosphorylation status of EGFR P-Y1068 and P-Y992 in a prospective cohort of HNSCC cases and test for correlation with patient PFS. High levels of EGFR P-Y1068 but not EGFR P-Y992 significantly correlated with reduced PFS. These findings suggest that there may be biologically relevant differences between phosphorylation at these sites that could impact patient PFS. Combining EGFR by IHC and EGFR P-Y1068 increased the HR from 2.86 and 3.32 respectively to 7.49 for cases with high levels of EGFR and EGFR P-Y1068 combined. This may indicate that a small, high risk population of HNSCC can be defined by high levels of both EGFR and EGFR P-Y1068.

In addition to modulating receptor activity, specific EGFR phosphorylated tyrosines are known to recruit signal transduction mediators of different pathways. For example, Grb2 binds EGFR P-Y1068 [189, 190], leading to the phosphorylation of STAT3 and Src through Ras/MAPK pathway activation [191]. EGFR P-Y992 has been reported to bind PLC-γ leading to the activation of second messengers 1,2-diacylglycerol and inositol 1,3,5-triphosphate (IP3). IP3 can signal for the release of intracellular calcium, thereby activating many calcium-dependent pathways including NFkB activation [192]. Therefore, the evaluation of tumor levels of site specific EGFR phosphorylation not only provides an indication of the relevance of EGFR kinase activity for clinical outcome but also provides insights into which of the many signaling pathways initiated by EGFR may be most relevant to poorer clinical outcomes. *In vitro* it has been found that gefitinib effectively inhibits phosphorylation of EGFR at Y1068 in HNSCC cell lines [193]. This suggests that EGFR P-Y1068 may be an important marker to test in the context of EGFR targeted therapy sensitivity and resistance.

Due to the strong correlation of PFS with EGFR P-Y1068 we probed into the possible molecular implications of phosphorylation at this site. Src has been implicated in the control of STAT3 mediated phenotypes [19] and STAT3 has been found to be constitutively active in both HNSCC cell lines and tumors [19]. Phosphorylation of Src at the activation site Y416 (normalized to total Src) was not significantly different between tumors with high Y1068 by RPPA and tumors with low Y1068. Src is involved in many signaling pathways and even large changes in one pathway may be unlikely to be visible via immunoblot in the context of the many other signaling cascades. Phosphorylation of STAT3 at Y705 (normalized to total STAT3) was significantly higher in the tumors with high levels of Y1068 compared to low levels of Y1068.

This is a very small number of tumors from the cohort and no definitive molecular signaling can be obtained from such a small number, but it supports previous findings that activation of STAT3 may play a role in HNSCC progression [19]. As follow up data become available from cohorts treated with EGFR targeting therapies EGFR P-Y1068 and STAT3 P-Y705 may be promising biomarkers to probe for treatment sensitivity versus resistance.

This cohort was characteristic of a surgical cohort and therefore may not provide adequate representation of oropharyngeal cancers treated with chemoradiation, as noted by the low incidence of HPV positive cases. This work does represent, to the best of our knowledge, the first North American surgical cohort with HNSCC analyzed for EGFR biomarkers with full 5-year follow-up data.

2.4.1 Conclusions

We present here data indicating that EGFR amplification and hyperploidy in HNSCC add no new prognostic information to EGFR IHC of patient tumors. Further, EGFR transcript in buccal cells is a poor biomarker for HNSCC. Phosphorylation of EGFR at P-Y1068 does appear to be a promising biomarker for HNSCC prognosis and could be useful in future work to determine molecular correlates of EGFR targeted therapy resistance and sensitivity.

3.0 EGFRVIII MECHANISM OF EXPRESSION

3.1 INTRODUCTION

3.1.1 EGFRvIII Biology

EGFRvIII contains a deletion of exons 2-7 of EGFR which results in an in-frame deletion of 267 amino acids from the extracellular ligand binding domain. EGFRvIII appears to be unable to bind ligand, signals constitutively and is coexpressed with wtEGFR in several solid tumors including glioblastoma (GBM) and HNSCC [67, 194]. EGFRvIII was first described in GBM and has been best studied in this model system [67].

EGFRvIII signaling likely plays a role in tumorigenesis [100, 101]. EGFRvIII expression has been shown to influence cell survival, proliferation, motility, invasiveness and treatment resistance [102-106]. In glioblastoma, breast cancer and HNSCC cell lines EGFRvIII enhances *in vivo* tumorigenicity [65, 107, 108], within the tumors proliferation is increased and apoptosis is decreased [102, 107].

3.1.2 EGFRvIII Incidence

EGFRvIII is a promising therapeutic target in cancer as it is expressed only in cancerous tissue [195]. EGFRvIII has been detected in cancer of the brain, breast, lung [195, 196], prostate [197], and head and neck [65]. The most common EGFR alteration in GBM and HNSCC is EGFRvIII [65, 67, 198]. EGFRvIII is present in ~40% of HNSCC [65, 131]. In glioblastoma EGFR gene amplification is present in up to 36-40% of glioblastoma [199, 200], EGFRvIII is present in 41% of GBM with EGFR gene amplification [67], and only about 5% of GBMs express EGFRvIII without gene amplification (12% of anaplastic astrocytomas) [201]. One study has evaluated the origin of EGFRvIII expression in EGFR gene amplified glioma and proposed that it results from intragenic rearrangements during EGFR gene amplification where Alu repeat sequences in the introns surrounding exons 2 and 7 mediate loss of exons 2-7 [202]. In HNSCC the correlation of EGFRvIII expression with EGFR gene amplification is relatively unexplored. One recent study in HNSCC indicated that EGFR gene amplification was not correlated with EGFRvIII expression [131]. These contrasting incidences of EGFRvIII warrant further investigation into the mechanism of EGFRvIII expression. Increased understanding the biology of EGFRvIII expression will aid in the treatment of tumors harboring this alteration.

3.2 MATERIALS AND METHODS

3.2.1 Tissue samples

3.2.1.1 EGFRvIII incidence in EGFR amplified tumors:

Surgical patients who were treated with curative intent for pathologically-confirmed squamous cell carcinoma of the oral cavity, oropharynx, hypopharyx, or larynx (HNSCC) were enrolled in this Early Detection Research Network- (EDRN-) sponsored study prior to surgery (n=154). Patients gave written informed consent, donated tumor and buccal tissues for study and answered an administered questionnaire regarding tobacco use. Tumor specimens from 58 of these HNSCC patients, 50 with paired adjacent histologically normal adjacent mucosa, were used to construct a tissue microarray (TMA), which has been previously described [171] and evaluated for EGFR gene amplification. Fresh-frozen tissues (25 HNSCC tumors) from this same cohort were evaluated using RT-PCR for EGFRvIII expression as previously described [65].

3.2.1.2 Glioma and HNSCC tissues evaluated for DNA and RNA alterations:

Surgical patients gave written informed consent to donate tumor tissue. Tissue from 6 glioma cases with EGFR gene amplification and 5 HNSCC cases was collected as fresh frozen tissue and used in sequencing analyses.

All tissues were collected under the auspices of a tissue bank protocol approved by the University of Pittsburgh Institutional Review Board.

3.2.2 Fluorescence *In Situ* Hybridization (FISH)

Dual color FISH analysis was performed using a Spectrum Green-labeled chromosome 7 centromeric probe (CEP7) and a Spectrum Orange-labeled EGFR probe (Vysis, Inc., Downers Grove, IL). TMA sections were de-parafinized, dehydrated in ethanol, and air-dried. Following protease K digestions, slides were denatured with formamide and dehydrated in ethanol. Probe hybridization was performed overnight at 37^{o}C, and nuclei were counterstained with DAPI/antifade (Vysis, Inc.). Only individual and well-delineated cells were scored. At least 60 cells were scored for each triplicate tissue section. Gene amplification was present if ratio of EGFR to CEP7 probe signals was greater than 2.0.

3.2.3 EGFRvIII PCR detection

Presence of EGFRvIII was detected by RT-PCR as previously described [65]. Briefly, total RNA was isolated using the RNeasy kit (Qiagen, Valencia, CA) and RT-PCR was performed using the Titanium One-Step RT-PCR kit (Clontech; Mountain View, CA) according to manufacturer's instructions. 100 ng of RNA was used per reaction with primers designed to flank the exon 2-7 deletion (5′-ATGCGACCCTCCGGGACG-3′ and 5′-ATTCCGTTACACACTTTGCGGC-3′). Reverse transcription was done at 50°C for 60 minutes followed by enzyme inactivation and hot-start PCR at 94°C for 5 minutes. Denaturation, annealing, and extension were done at 94°C (30 seconds), 66°C (30 seconds), and 68°C (1 minute), respectively, for a total of 40 cycles. The reaction was completed with an extension period at 68°C for 2 minutes. PCR products were visualized on a 1.5% agarose gel containing GelRed Nucleic Acid Stain (Biotium, Inc.; Hayward, CA). Confirmatory cDNA sequencing was

performed on all resultant EGFRvIII amplified products. The agarose-fractionated amplicon corresponding to the EGFR mutant band was excised and purified according to manufacturer's instructions using the Qiagen Gel Extraction kit. The DNA product was sequenced BigDye Terminator sequencing premix (Applied Biosystems, Inc.; Carlsbad, CA) with the Applied Biosystems, Inc. 3730xl or 3130xl DNA Analyzer by the DNA Core Facility at the University of Pittsburgh School of Medicine.

3.2.4 Exon junction sequencing

Total RNA and total DNA were each isolated from fresh-frozen tumors using the Qiagen Allprep DNA/RNA kit according to manufacturer's instructions. RNA was reverse transcribed using the SuperScript III Reverse Transcriptase kit (Invitrogen; Carlsbad, CA) according to manufacturer's instructions with 2.5 ug of RNA input. PCR was performed on cDNA and DNA with primers designed to flank the end of EGFR exon 1 and the following intron (Fwd 5' CCAGTATTGATCGGGAGAGC 3'; Rev 5' CGCAGCTGATCTCAAGGAAA 3'), the beginning of exon 2 and the previous intron (Fwd 5' TGGACCTTGAGGGATTGTTT 3'; Rev 5' CTTCAAGTGGAATTCTGCCC 3'), the end of exon 7 and the following intron (Fwd 5' GCTTTCTGACGGGAGTCAAC 3'; Rev 5' AGACAGAGCGGGACAAGGAT 3'), the beginning of exon 8 and the previous intron (Fwd 5' CTTTCCATCACCCCTCAAGA

3'; Rev 5' CTCAGCAGCCGAGAACAAG 3') and primers located in exon 1 and in the intron following exon 8 for detection of EGFRvIII at the genomic and unspliced mRNA levels (Fwd 5' CAGTATTGATCGGGAGAGCC 3'; Rev 5' CACAACCTTCAGTGCCTTCC 3'). A schematic of these primer designs are displayed in Figure 2. GAPDH was used as the control gene for DNA/RNA integrity (Fwd 5' TGGAATTTGCCATGGGTG 3'; Rev 5'

GTGAAGGTCGGAGTCAAC 3'). PCR was performed using the Phusion High Fidelity PCR master mix (New England BioLabs, Inc.; Ipswich, MA) with 500 ng DNA or 375 ng cDNA. PCR for all primers excluding exon 1 was performed with an initial start of 98°C for 30 seconds. Denaturation, annealing, and extension were done at 98°C (10 seconds), 56°C (30 seconds), and 72°C (30 seconds), respectively, for a total of 38 cycles. The reaction was completed with an extension period at 72°C for 10 minutes. PCR for exon 1 was performed with an initial start of 98°C for 30 seconds. Denaturation, annealing, and extension were done at 98°C (10 seconds), 60°C (30 seconds), and 72°C (30 seconds), respectively, for a total of 40 cycles. The reaction was completed with an extension period at 72°C for 10 minutes. The PCR products were separated on 1.5% agarose gels, excised, purified and sequenced as noted above. Sequencing results were compared with the standard NCBI EGFR sequence NC_000007.13 for DNA and NM_201284 for mRNA via the basic local alignment search tool (Nucleotide BLAST; NCBI).

3.2.5 TCGA Data Analysis

The Cancer Genome Atlas Project is sponsored by the National Cancer Institute. In the first phase of this project 200 glioblastoma samples were collected under an institutional review board protocol at the TCGA Biospecimens Core Resource at the International Genomics Consortium (Phoenix, AZ) [203]. All specimens were assayed on three different gene expression microarray platforms: Affymetrix Human Exon 1.0 ST GeneChips, Affymetrix HT-HG-U133A GeneChips, and custom designed Agilent 244,000 feature gene expression microarrays as well as the Affymetrix Genome-Wide Human SNP Array 6.0 [203]. Additionally Applied Biosystems Sequence data was available and used in this work for EGFR [203].

TCGA data was analyzed using the Integrated Genomics Viewer (IGV) designed by the Broad Institute of Massachusetts Institute of Technology and Harvard University and is available publicly [204]. The IGV allows users to load and view compiled, corrected and normalized TCGA data from all glioblastoma samples. To view and analyze this data the IGV version 1.5 was used and all TCGA (hg18) GBM data was loaded directly from the servers. Tracks were sorted by linking_id (cases) and manually evaluated for copy number loss in the exon 2-7 area in gene expression and/or SNP array data. Loss of >1 in any gene expression or SNP platform was considered EGFRvIII positive.

3.3 RESULTS

3.3.1 EGFRvIII is not exclusively expressed in EGFR amplified HNSCC

Reports in glioma indicated that EGFRvIII is present almost exclusively in tumors with EGFR gene amplification [67, 201]. A recent study in HNSCC indicated that in this tumor type EGFRvIII expression was not correlated with EGFR gene amplification in a small cohort [131]. This discordance led us to evaluate a cohort of HNSCC with and without EGFR gene amplification and determine if there was an increased incidence of EGFRvIII in EGFR gene amplified samples. Tumors without EGFR amplification (n=13) and with EGFR amplification (n=12) were screened via RT-PCR for EGFRvIII expression (see Figure 3.1 for a representation of the RT-PCR screen). We found that 4/12 tumors with EGFR amplification had EGFRvIII expression and 2/13 tumors without EGFR amplification had EGFRvIII expression. This

indicates that EGFR gene amplification is not the only mechanism of EGFRvIII expression in HNSCC.

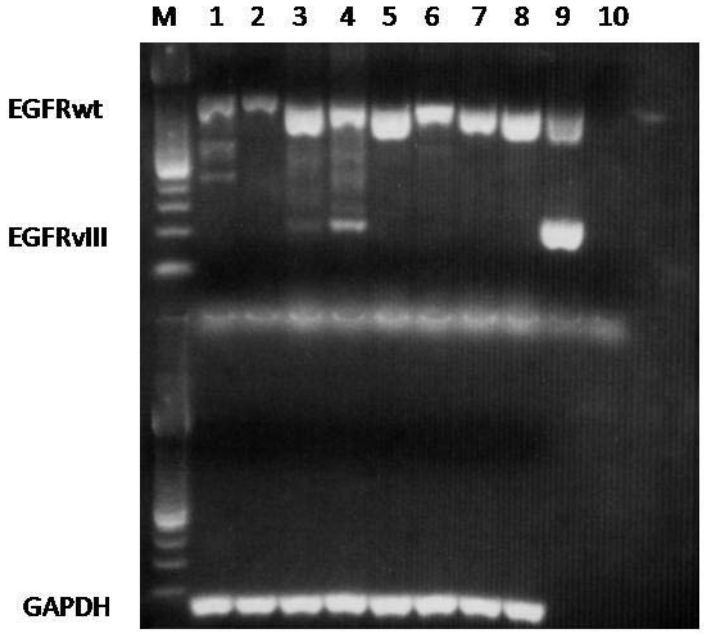


Figure 3.1 EGFRvIII RT-PCR Screen. RNA was isolated from HNSCC tumors with EGFR gene amplification and EGFRwt, EGFRvIII and GAPDH were RT-PCR amplified as described in materials and methods. Lanes 1-8 are EGFR gene amplified tumor samples. Lane M is the 100 bp marker, lane 9 is U87MGvIII used as a positive control, lane 10 is the water control. All EGFRvIII bands were excised and sequenced to verify exon 1 to exon 8 joining.

3.3.2 EGFRvIII does not contain splice donor/acceptor mutations in glioma or HNSCC

EGFRvIII is most frequently detected by mRNA or protein assays and has an in-frame deletion of exons 2-7. We hypothesized that this alteration may often be due to alternative splicing, which would be detected at the mRNA and protein levels but not in unspliced RNA or genomic DNA. Point mutations in the splice donor or acceptor sites in DNA or RNA-editing of unspliced RNA at the splice donor and acceptor sites could each result in alternative splicing. To determine if there were mutations in these sites we amplified the splice donor and acceptor sites in exons 1, 2, 7, and 8 (Schematic in Figure 2) in 4 HNSCC tumors harboring EGFRvIII detected by RT-PCR and 1 HNSCC tumor with EGFRwt only. The PCR products were then sequenced and compared to reference sequences. We found no alterations in the genomic DNA or unspliced RNA splice sites (Table 4).

Previous work with IHC in our laboratory indicated that EGFRvIII is not likely as highly expressed in HNSCC as in glioma (unpublished data). We thought that the high level of EGFRwt found in HNSCC may mask alterations in unspliced RNA which would be more evident in glioma. We obtained 5 fresh frozen glioma that expressed EGFRvIII and 1 glioma that expressed only EGFRwt and tested the DNA and unspliced RNA for splice site alterations. Again, no alterations were found (Table 5).

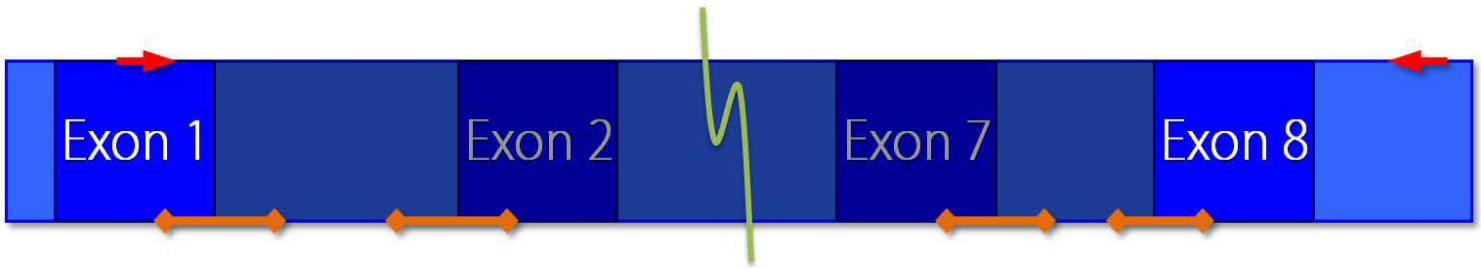


Figure 3.2 Schematic of sequencing primers and areas of interest. Red arrows indicate the location of the primers used to detect EGFRvIII in genomic DNA and unspliced RNA. Orange bars indicate areas amplified for splice donor and acceptor mutations. Shaded area is lost in EGFRvIII.

Table 4 Splice donor and acceptor site mutations in HNSCC

Sample (vIII status)	Exon 1		Exon 2		Exon 7		Exon 8	
	Pre mRNA	DNA	Pre mRNA	DNA	Pre mRNA	DNA	Pre mRNA	DNA
HNSCC 1 (-)	-	-	-	-	-	-	-	-
HNSCC 2 (+)	-	-	-	-	-	-	-	-
HNSCC 3 (+)	-	-	-	-	-	-	-	-
HNSCC 4 (+)	-	-	-	-	-	-	-	-
HNSCC 5 (+)	-	-	-	-	-	-	-	-

Table 5 Splice donor and acceptor site mutations in GBM

Sample (vIII status)	Exon 1		Exon 2		Exon 7		Exon 8	
	Pre mRNA	DNA	Pre mRNA	DNA	Pre mRNA	DNA	Pre mRNA	DNA
GBM 1 (-)	-	-	-	-	-	-	-	-
GBM 2 (+)	-	-	-	-	-	-	-	-
GBM 3 (+)	-	-	-	-	-	-	-	-
GBM 4 (+)	-	-	-	-	-	-	-	-
GBM 5 (+)	-	-	-	-	-	-	-	-
GBM 6 (+)	-	-	-	-	-	-	-	-

Table 6 Unspliced RNA and genomic DNA detection of Exon 1 to Exon 8 joining.

HNSCC			GBM		
	Pre mRNA	DNA		Pre mRNA	DNA
HNSCC 1 (-)	-	-	GBM 1 (-)	-	-
HNSCC 2 (+)	-	-	GBM 2 (+)	vIII	-
HNSCC 3 (+)	-	-	GBM 3 (+)	vIII	-
HNSCC 4 (+)	-	-	GBM 4 (+)	vIII	-
HNSCC 5 (+)	-	-	GBM 5 (+)	vIII	-
			GBM 6 (+)	vIII	-

3.3.3 In HNSCC EGFRvIII is not detectable at the DNA or unspliced RNA level

Having determined that EGFRvIII was not likely to be the consequence of alternative splicing through point mutations we considered that it may be a genomic deletion despite the lack of correlation between EGFRvIII and EGFR gene amplification. It was also possible that it could arise through transcriptional deregulation as well. We explored these hypotheses by designing a forward primer located within exon 1 and a reverse primer in the intron following exon 8 (Figure 3.2). We screened the previous glioma and HNSCC sample DNA and unspliced RNA for deletion of exons 2-7 using the primers as noted. We found that gliomas expressing EGFRvIII at the mRNA level also harbored EGFRvIII in unspliced RNA but not at the genomic level (Table 3). No deletion of exons 2-7 was detected in the DNA or RNA of the glioma that did not express EGFRvIII. In HNSCC deletion of exons 2-7 was not detected at the DNA or unspliced RNA level in any tumors (Table 6).

3.3.4 Relevance of TCGA data for EGFRvIII in glioma

The Cancer Genome Atlas project (TCGA) is sponsored by the NCI and has completed extensive characterization of >200 glioblastoma samples [203] by gene expression arrays, SNP arrays, sequencing, methylation arrays and other assays. In a recent report Verhaak et al. [205] proposed a new method of GBM classification and found that 7/116 of these GBM harbored EGFRvIII in gene expression and SNP data ([205] and personal communication Dr. N Hayes). This group uploaded the TCGA data used in their report to the Integrated Genomics Viewer (IGV 1.5) developed by the Broad Institute [204] which also contains the whole of the TCGA

GBM data. As the Verhaak et al. group had taken the intersection of the RNA and DNA data we chose to review the TCGA data manually for copy changes greater than 1 in either the RNA (gene expression) or DNA (SNP) data. I found 14/116 cases (12%) had a reduced copy number in the Exon 2-7 area of gene expression or SNP data (referred to as EGFRvIII positive for simplicity). Of the 14 EGFRvIII positive cases 4 were positive by RNA but not DNA, 1 was positive by DNA but not RNA and 9 were positive by both RNA and DNA (Table 7). Also of note in the Applied Biosystems sequencing data for EGFR there were only 16 mutations found. All mutations were missense mutations and 7 of the 16 mutations were present in Exon 7.

Table 7 TCGA samples with decreased copy number in the exon 2-7 range.

TCGA Linking ID	Expression (RNA)	SNP (DNA)
02-0009	x	
02-0064	x	x
06-0137	x	x
06-0211	x	x
06-0412	x	
06-0646	x	x
06-0195	x	
08-0244	x	x
08-0354		x
08-0357	x	x
08-0358	x	x
08-0360	x	
08-0518	x	x
08-0529	x	x

3.4 DISCUSSION

EGFRvIII is a variant of EGFR that contains an inframe deletion of exons 2-7 of the external ligand binding domain. EGFRvIII has been shown to be constitutively active in the absence of ligand and does not appear to bind ligand [116]. EGFRvIII has been shown to be tumor specific and as such is an ideal therapeutic target [195]. Trials are on-going testing combination therapies and EGFRvIII targeted therapies in glioma and other cancer types that express EGFRvIII in an attempt to cure cancers harboring this variant. The most promising trials are those underway for the EGFRvIII peptide vaccination. Trials thus far have been small but have shown prolonged progression free survival and overall survival benefits with limited toxicities [206, 207]. Unfortunately, recurrence was still observed in many patients and 82% of these patients had lost EGFRvIII expression [207]. The mechanism of expression and loss of expression of EGFRvIII has not been determined and a further understanding of the biology of EGFRvIII expression and signaling is needed to fully understand how to optimize the treatment of EGFRvIII expressing tumors.

EGFRvIII likely arrises as a genomic deletion, transcriptional aberration or splice variant. We undertook this study to determine which, if any of these mechanisms was most likely the cause of EGFRvIII expression.

Genomic deletion of exons 2-7 through EGFR gene amplification has been widely accepted as the mechanism of EGFRvIII expression [66, 67]. The almost exclusive expression of EGFRvIII in EGFR amplified glioblastomas has lent much support to this hypothesis [67, 201]. However, we found that in HNSCC EGFRvIII expression is common in both EGFR

amplified and unamplified tumors; a finding supported by a previous report in HNSCC [131]. This finding does not preclude that genomic alterations may still be involved in EGFRvIII expression. Intron 1 of many genes, including EGFR, has been shown to have important regulatory function for transcription and translation [208]. This intron in EGFR harbors a downstream enhancer element in close proximity to a polymorphic simple sequence repeat with 14-21 CA dinucleotides with the most frequent allele containing 16 CA repeats [209, 210]. The presence of allelic imbalance in CA repeats may indicate increased genomic instability in this area which may also contribute to a fragile site predisposed for chromosomal strand break [211, 212].

In addition to CA repeats, intron 1 contains 11 short interspersed elements known as Alu elements which are approximately 300 nucleotides in length and have been shown to be involved in non-allelic homologous recombination in several types of cancers including breast, AML and hepatoma [213]. A previous study of EGFRvIII in glioblastoma implicated Alu elements in EGFRvIII expression by characterizing EGFR breakpoints downstream of Alu elements [202]. While not conclusive, this study showed that Alu elements may be involved in intragenic rearrangement of EGFR to express EGFRvIII [202]. Our analysis of TCGA GBM data does show reduced levels of intron 1-intron 7 in 10/14 samples that had reduced copies of exons 2-7. By PCR we did not detect direct joining of exon 1 to exon 8 in genomic DNA, however, a recombination event in intron 1 would likely retain a large intron between exon 1 and 8 and thereby be undetectable in our assays. However, the finding that in GBM direct joining of exon 1 to exon 8/intron 8 was found in unspliced RNA, implicates transcriptional regulation as a mechanism of EGFRvIII expression in GBM. Additionally, analysis of TCGA data showed that

4/14 cases of decreased exon2-7 expression were found at the RNA but not the DNA level supporting the idea that EGFRvIII may arise from both DNA and RNA mechanisms. In HNSCC we did not find exon 1 to exon 8 joining in DNA or unspliced RNA, indicating that there may be different mechanisms of EGFRvIII expression in different cell types and conditions.

Alternative splicing due to point mutations or RNA editing does not appear to be a mechanism of EGFRvIII expression as we found no alterations in splice acceptor and donor sites in genomic DNA and unspliced RNA. There are many known and hypothesized mechanisms of regulating alternative splicing and many of these may be involved in EGFRvIII expression. Interestingly, alternative splicing in general is found to be less common in cancer than in normal tissue [214]. There have been reports, however, that alternative splicing can create oncogenes that drive cell motility in breast and colon cancers [215]. The presence of reduced levels of exons 2-7 in TCGA SNP data indicate that if alternative splicing is a mechanism of EGFRvIII expression, it is not the only mechanism. To determine if alternative splicing is a primary mechanism of EGFRvIII expression DNA and unspliced RNA data should be compared to protein positivity to determine if there are a high number of cases positive by protein assays but negative by DNA or unspliced RNA methodologies.

The data presented here support the idea that EGFRvIII may have variable mechanisms of expression depending on cellular conditions. It appears that in HNSCC EGFRvIII is a splice variant, while the data indicate that in GBM EGFRvIII is present before mRNA splicing occurs.

4.0 EGFRVIII MEDIATES HNSCC CELL INVASION VIA STAT3 ACTIVATION

4.1 INTRODUCTION

4.1.1 EGFR targeting and metastasis in HNSCC

The epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase that regulates crucial cellular signaling pathways contributing to tumor progression. EGFR is frequently amplified and over-expressed in several human solid tumors including in a high percentage of head and neck squamous cell carcinomas (HNSCC). EGFR overexpression in HNSCC has been correlated with tumor progression, resistance to conventional therapy and poor prognosis [216]. Preclinical studies demonstrated the anti-tumor effects of EGFR targeting and the FDA approved the EGFR monoclonal antibody cetuximab for clinical use in HNSCC based on the results of a phase III trial [14]. However, while combining EGFR targeting with radiation prolonged overall survival, it did not reduce the incidence of metastasis. Despite the nearly ubiquitous expression of EGFR in HNSCC, there is only a 13% response rate when cetuximab is administered as a single agent [15]. The tumor features that contribute to resistance to EGFR targeting are incompletely understood.

4.1.2 EGFRvIII

Receptor alterations that influence ligand and antibody binding may play a role in therapeutic resistance. The most common EGFR alteration in several cancers, including HNSCC, consists of a truncation in the extracellular domain known as EGFR variant III (EGFRvIII). This mutation eliminates exons 2-7 resulting in a distorted ligand-binding region [66, 68]. EGFRvIII does not bind ligand but is constitutively activated in a ligand-independent manner. The presence of EGFRvIII in human tumors has been associated with tumor growth, metastasis, and survival in several malignancies including glioma, carcinomas of the breast, lung and HNSCC [217]. Furthermore, EGFRvIII has been reported to increase resistance to anti-tumor agents including EGFR inhibitors [65].

4.1.3 Rationale and hypothesis

We previously reported the expression of EGFRvIII in up to 42% of HNSCC where co-expression of EGFRvIII with wild-type EGFR increased HNSCC cell proliferation *in vitro* and tumor volume *in vivo*. Moreover, EGFRvIII decreased HNSCC cell apoptosis in response to cisplatin and decreased growth inhibition following treatment with cetuximab [65]. While these results support the role of EGFRvIII in mediating tumor growth in response to EGFR targeting, the contribution of EGFRvIII to invasion and the precise downstream pathways that are induced by EGFRvIII are incompletely understood. EGFRvIII expression in glioma has been reported to correlate with expression of phosphotyrosine STAT3 [132]. The lethality of HNSCC is associated with the tendency of these cancers to invade surrounding structures and metastasize.

The present study was undertaken to test the hypothesis that EGFRvIII induces HNSCC invasion and subsequently, metastasis via activation of STAT3 signaling.

4.2 MATERIALS AND METHODS

4.2.1 Cell lines, reagents, and cell culture

C225/cetuximab was purchased from the research pharmacy at the University of Pittsburgh Cancer Institute. For *in vitro* cell stimulation, recombinant human EGF (Sigma-Aldrich Corp., St. Louis, MO) was used. Phosphatidylinositol 3-kinase (PI3K) inhibitor NVP-BEZ235-AN-4 was obtained from Novartis (East Hanover, NJ). EGFRvIII-transfected HNSCC cells (Cal33vIII) and vector control transfected HNSCC cells (Cal33control) were generated as described previously [65]. Cal33 and 686LN cells were a kind gift from Dr. Gerard Milano (Centre Antoine-Lacassagne, Nice, France) and Dr.Georgia Chen (Emory University, Atlanta, GA) respectively. Cal33 cells were maintained in DMEM (Mediatech, Inc., Herndon, VA) with 10% heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA). 686LN cells were maintained in DMEM/F12 (1:1) (Invitrogen, Carlsbad, CA) and 10% heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA). All cells were incubated at 37°C in the presence of 5% CO_2. To establish hypoxic conditions (1% O_2), cells were placed in an InVivo300 hypoxia workstation (Ruskinn Life Sciences Ltd, UK).

4.2.2 Transfection with vector control or EGFRvIII

Transfection with vector control or EGFRvIII was performed as previously described [65]. HNSCC cells were plated at a density of $1x10^6$ cells in a 100mm tissue culture dish. Following 16 hours incubation in complete media cells were transfected using 9ug of the expression vector pLERNL containing the EGFRvIII cDNA or vector control and Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Plasmid DNA were a kind gift from Dr. Frank Furnari, Ludwig Institute for Cancer Research, La Jolla, CA. Transfection media was replaced with complete media after 6 hours and cells were incubated for 24 hours for further transfection or 48 hours for use in assays.

4.2.3 RT-PCR analysis and cDNA sequencing of EGFRvIII

Total RNA was isolated from HNSCC cell lines (5×10^6 cells) using the RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Total RNA (1 μg) was reverse-transcribed with the first-strand cDNA synthesis using SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA). To detect the deleted region of EGFRvIII, standard RT-PCR was performed as described previously [65].

4.2.4 Flow cytometry

Indirect analytic flow cytometry was done on a Becton Dickinson FACS calibur equipped with CellQuest Pro software (Becton Dickinson, San Jose, CA). Assays were done at 4°C; all washes were done with iced medium to facilitate the detection of cell surface receptors without allowing

internalization to occur. All profiles were obtained with cells maintained in ice-cold 1% bovine serum albumin/PBS. The percentage of a population designated as positive was arbitrarily defined as that region in which only the highest fluorescing 10% of the isotype-control stained cells graphed, corrected for background; this is a conservative estimate of the total positive staining population. In order to examine the cell surface expression of EGFRvIII proteins, target cultured cells were stained with anti-EGFRvIII monoclonal antibody L8A4 under nonpermeabilized conditions. Subconfluent cells were detached from culture flasks by incubation with 0.02% EDTA/PBS; 10^6 cells were maintained in 0.5% paraformaldehyde/ PBS for 10 minutes at 4°C, washed, resuspended in 150 mL PBS containing 10% fetal bovine serum, and blocked for 20 minutes at 4°C. After two washes, the samples were reacted with L8A4 monoclonal antibody (10 mg/mL, black line) and irrelevant mouse IgG1k (10 mg/mL, solid gray) in PBS for 60 min. After two additional washes, cells were incubated with FITC-labeled secondary antibody for 30 minutes at 4°C and analyzed on a Becton Dickinson FACS calibur instrument (Becton Dickinson).

4.2.5 Western blotting

Cell lines were lysed in detergent containing 1% NP40, 0.1 mM phenylmethylsulfonyl fluoride, 1 mg/ml leupeptin, and 1 mg/ml aprotinin, and protein levels were determined using the Bio-Rad protein assay method (Bio-Rad Laboratories, Hercules, CA). Forty μg of total protein were separated on 8% SDS-PAGE gels and transferred to nitrocellulose membranes using the semi-dry transfer machine (BioRad Laboratories, Hercules, CA). Membranes were blocked with 5% skim milk/Tris-buffered saline with Tween 20 (TBS-T) solution for 2 hours at room temperature,

and incubated with primary antibodies in 5% skim milk in TBS-T overnight at 4°C. After washing with TBS-T three times, membranes were incubated for 1 hour with HRP-conjugated secondary antibody (Bio-Rad Laboratories, Hercules, CA) 1:3000 diluted in 5% skim milk in TBS-T. The filters were rinsed with TBS-T three times, and the blot was developed using Luminol Regent (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) by autoradiography. Antibodies used for blotting included β-tubulin (Abcam, Cambridge, UK), HIF-1α (BD Transduction Laboratories, San Jose, CA), phospho-AKT (Ser473), AKT, phospho-STAT3 (Tyr705) and STAT3 (Cell Signaling Technology, Beverly, MA).

4.2.6 Luciferase reporter assay

HNSCC cells (4 x 10^5/ml) were plated onto 6-well tissue culture plates. After cells were transiently transfected with vector control or EGFRvIII and incubated in complete medium for 24 hours cells were then co-transfected with pSTAT3TALuc, a generous gift from Dr. Jacqueline Bromberg (Memorial Sloan-Kettering Cancer Center, New York, NY) and pRL-TK (Promega, Madison, WI) a Renilla luciferase construct, using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions [218]. The transfection media was replaced to complete DMEM after 4 h of transfection. Cells were lysed and luciferase assays were performed 24h after the transfection using the Dual-Luciferase Reporter Assay System kit (Promega, Madison, WI). Cell lysates were subjected to protein estimation using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA). Relative light units (RLU) from luciferase was normalized to RLU from renilla luciferase (to account for differences in transfection efficiency) and to micrograms of protein (to account for differences in protein concentrations between samples).

4.2.7 Matrigel invasion assay and cell migration assay

Cell invasion was evaluated *in vitro* using Matrigel-coated semi-permeable modified Boyden inserts with a pore size of 8 µm (Becton Dickinson/Biocoat, Bedford, MA). Cell migration was evaluated *in vitro* using semi-permeable modified Boyden inserts with a pore size of 8 µm (Becton Dickinson/Biocoat, Bedford, MA). For both assays cells were plated in duplicate at a density of 1.3 x 10^4 cells per well in serum free media in the insert. At the same time cells were plated in 24-well plates to serve as loading controls. Both the insert and the holding well were subjected to the same medium composition with the exception of serum. The insert contained no serum, whereas the lower well contained 10% fetal bovine serum (FBS) that served as a chemo-attractant. After 24 h of treatment at 37°C in a 5% CO2 incubator, the cells in the insert were removed by wiping gently with a cotton swab. Cells on the reverse side of the insert were fixed and stained with Hema 3 (Fisher Scientific, Hampton, NH) according to the manufacturer's instructions. Cells plated in 24-well plates were subjected to MTT assays and the cell numbers across the groups were normalized. The number of invading or migrating cells was adjusted accordingly.

4.2.8 STAT3 siRNA and STAT3 decoy transfection

The STAT3 decoy and the mutant control decoy sequences (double-stranded deoxyribonucleotides with phosphorothioate modifications in the first three bases and last three bases of the sequences) were generated as described previously [219]. The mutant control decoy, carrying a single base mutation, that does not abrogate STAT3 DNA binding activity, was used as a control as in previous studies [219, 220]. The siRNA sequences targeting STAT3 human

mRNA (D-003544-01, sense 5'-CCAACGACCUGCAGCAAUAUU-3', and antisense 5'-PUAUUGCUGCAGGUCGUUGGUU-3'; Dharmacon, Lafayette, CO) were transfected into HNSCC cells for STAT3 silencing. The nontargeting siRNA (D-001210-01, sense 5'-UAGCGACUAAACACAUCAAUU-3', antisense 5-UUGAUGUGUUUAGUCGCUAUU-3'; Dharmacon) was used as a control. The siRNA or decoy transfections were performed using the Lipofectamine 2000 (Life Technologies Inc). In brief, HNSCC cells were transfected with vector control or EGFRvIII and following 24 hours incubation in complete media cells were transfected with 800 pmol of STAT3 siRNA or non-targeting control siRNA, or 50.4 pmol of STAT3 decoy or mutant control decoy. The transfection medium was replaced with complete media after 4 h of transfection.

4.2.9 *In vitro* growth assay

To determine if the sensitivity of HNSCC cells to PI3K/AKT inhibition was affected by EGFRvIII expression, vector-transfected and EGFRvIII-transfected HNSCC cells (1.5×10^4) were seeded onto 24 well plates 24 hours after transfection and treated with a PI3K inhibitor NVP-BEZ235-AN-4 (100nM) or DMSO (1ul/ml of media). Each cell population was then assayed every other day for 6 days in triplicate using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). MTT was combined with warm phosphate buffered saline (5mg/ml) and placed on cells for 1 hour at 37°C in a 5% CO_2 incubator. The MTT was removed and replaced with an equal volume of DMSO to solubilize the cells. The absorbance was read on a μQuant spectrophotometer (Bio-Tek Instruments, Inc. Winooski, VT). Percent cell growth was determined by normalizing each cell population to the average day 2 absorbance value for that population.

4.2.10 *In vivo* growth of HNSCC cell expressing EGFRvIII and analysis of HNSCC xenografts

HNSCC cells [Cal33 expressing EGFRvIII (Cal33vIII or empty vector-transfected parental cells (control-1)] were cultured in DMEM containing 10% fetal bovine serum. Cells were trypsinized and cell number and viability were determined using trypan blue dye exclusion. A suspension of 7.5×10^5 HNSCC cells in 50 µL serum free media was injected into the flanks of *nu/nu* athymic nude mice (n = 20; Harlan Sprague-Dawley, Indianapolis, IN) subcutaneously. Cal33vIII was injected into the right flank and control cells were injected into the left flank. Tumor volumes were measured in two dimensions with vernier calipers and calculated using the formula: (length x width^2) x 0.52. At the end of the study, mice were sacrificed by cervical dislocation under anesthesia the tumors surgically excised and snap frozen in dry ice. To evaluate the expression of phosphorylated and total STAT3 in HNSCC xenografts, tumors homogenized, sonicated in detergent containing 1% NP40, 0.1 mM phenylmethylsulfonyl fluoride, 1 mg/ml leupeptin, and 1 mg/ml aprotinin. Forty µg of total protein were separated on 8% SDS-PAGE gels and immunoblotted for phosphorylated and total STAT3 and β-tubulin. Animal use and care was in strict compliance with institutional guidelines established by the Institutional Animal Care and Use Committee at the University of Pittsburgh.

4.2.11 Statistical analysis

For migration and invasion studies, the statistical significance of differences in the number of invading cells or migrated area were assessed by use of Wilcoxon-Mann-Whitney two-tailed exact test.

4.3 RESULTS

4.3.1 Expression of EGFRvIII in engineered HNSCC cells

We previously reported that EGFRvIII is expressed in approximately 40% of HNSCC tumors [65]. For reasons that are incompletely understood, expression of EGFRvIII in human tumors is routinely lost in tissue culture [68]. Therefore, to study the consequences of EGFRvIII in HNSCC, we transfected EGFR vIII into a representative HNSCC cell line (686LN) as described in the materials and methods section. Due to the high level of wild-type EGFR in HNSCC cell lines and tissues, commercially available EGFR antibodies that are reported to detect both EGFRvIII and wild-type EGFR in other tumor systems, are unable to identify EGFRvIII expression in HNSCC. Expression of the EGFRvIII transcript was therefore determined by RT-PCR where the HNSCC cell line Cal33 stably expressing EGFRvIII was used as the control for EGFRvIII. EGFRvIII gene expression was detected in transient transfectants through 6 days (the duration of the experiments used) post-transfection (Figure 4.1A). For the animal experiments, the HNSCC cell line Cal33 was stably transfected with EGFRvIII or vector control as described previously [65]. Flow cytometry was performed to measure the degree of expression of EGFRvIII in the stably transfected Cal33 clone. FACS analysis revealed no EGFRvIII expression in the vector transfected control cells and approximately $5x10^3$ EGFRvIII receptors per cell in the EGFRvIII-transfected Cal33 cells (Figure 4.1B). Quantitative PCR was also performed to determine the relative expression levels of wild-type EGFR and EGFRvIII in these cells and demonstrated a 7-fold higher level of wild-type EGFR compared with EGFRvIII. These results are consistent with findings in human HNSCC where all HNSCC tumors that

express EGFRvIII also express wild-type EGFR, with a higher level of wild-type EGFR compared to EGFRvIII [65].

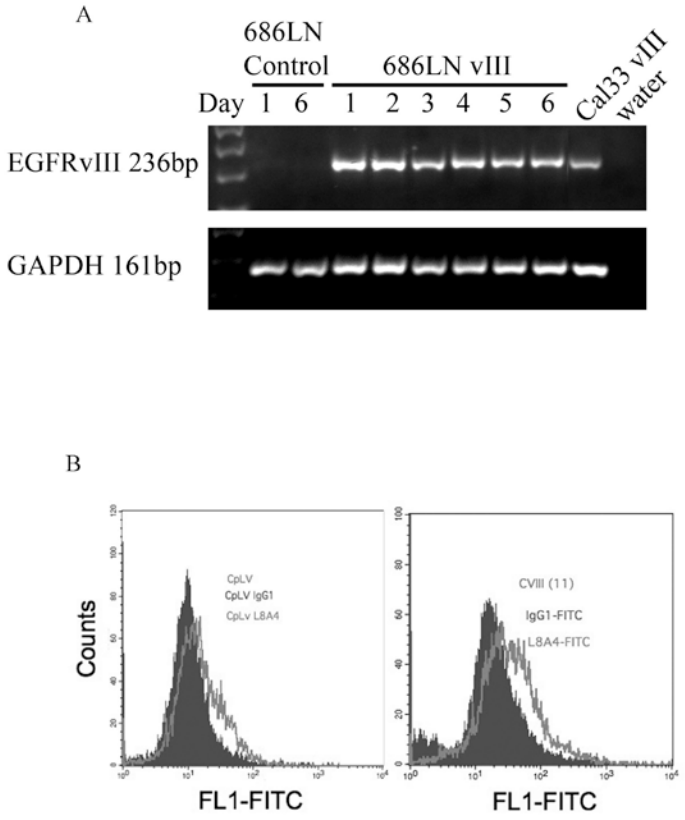


Figure 4.1 EGFRvIII expression in HNSCC-transfected cells. (A) EGFRvIII mRNA levels were examined in HNSCC transiently transfected cells (686LN) by RT-PCR. Representative ethidium bromide-stained gel showing EGFRvIII and GAPDH PCR products from 2 vector control transfections (1 and 6 days post transfection) and EGFRvIII transfected cells (1-6 days post transfection). The EGFR-transfected Cal33vIII cell line was used as positive control for EGFRvIII-transfected cells. Experiments were repeated 3 times with similar results. (B) Flow cytometry results for the Cal33 clone stably transfected with EGFRvIII and its respective vector control clone. Surface EGFRvIII on Cal33vIII was detected by flow cytometry. A vector-transfected control clone (control-1) was used as negative control. Data is represented as Mean Fluorescent Intensity. Experiments were repeated 3 times with similar results.

4.3.2 EGFRvIII increases HNSCC motility and invasion.

We previously reported that EGFRvIII induces HNSCC cell proliferation *in vitro* and tumor growth *in vivo* [65]. EGFRvIII has been shown to induce motility in murine fibroblasts [221]. To determine the consequences of EGFRvIII on directional HNSCC cell motility, cell migration assays were performed using a transwell assay. As shown in Figure 4.2A, HNSCC cell migration was increased in EGFRvIII-expressing cell compared to a vector-transfected control (p=0.03). In order to validate these findings in other HNSCC cell lines, we transiently transfected 1483 and PCI-37A cells with an EGFRvIII expression plasmid and tested the migration of the cells. EGFRvIII expressing cells demonstrated increased migration. We next assessed the consequences of EGFRvIII on HNSCC cell invasion through Matrigel, controlling for proliferation. As shown in Figure 4.2A, EGFRvIII-expressing HNSCC cells were significantly more invasive than vector transfected controls (p=0.03).

Cetuximab was FDA-approved for the treatment of HNSCC in 2006. We previously reported that HNSCC cells expressing EGFRvIII are relatively resistant to the growth inhibitory effects of cetuximab *in vitro* and *in vivo* [65]. Since the addition of cetuximab to radiation did not prevent metastasis in HNSCC patients, we next determined the effects of cetuximab on EGFRvIII-mediated migration and invasion [14]. As shown in Figure 4.2B and C, while cetuximab abrogated EGF-induced migration and invasion of vector control-transfected HNSCC cells, treatment of EGFRvIII-expressing HNSCC cells with cetuximab failed to decrease migration or invasion. While EGF induced migration and invasion of vector-transfected control cells, EGF treatment had no significant effect on the motility or invasive capacity of EGFRvIII cells (which are more motile and invasive than controls in the absence of growth factor

stimulation). These results suggest that EGFRvIII induces HNSCC cell motility and invasion *in vitro*, which are not abrogated by treatment with the only clinically approved EGFR targeting strategy in HNSCC. These results were also validated in a transitional cell carcinoma model (T-24 cells) stably expressing EGFRvIII.

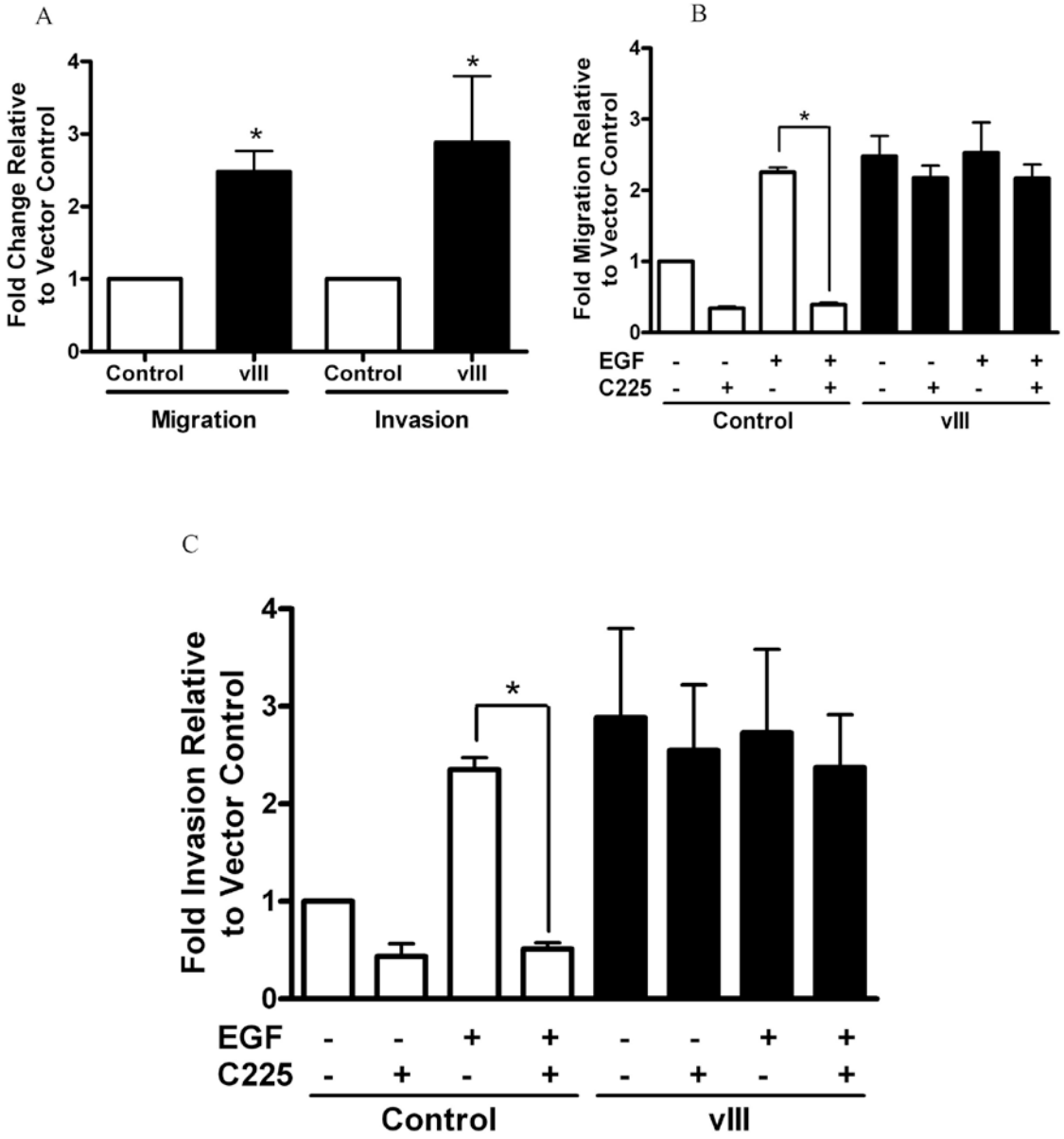


Figure 4.2 EGFRvIII increases HNSCC migration and invasion. (A) The migration of EGFRvIII-transfected 686LN cells (vIII) compared with a vector-transfected control was determined using a Boyden chamber assay. The fold-increase in the EGFRvIII transfected cells was compared with vector-transfected controls. Cumulative results are shown from four independent experiments (**p*=0.03). The invasive capacity of EGFRvIII-expressing 686LN cells (vIII) compared with a vector-transfected control (control-1) was determined using a Boyden chamber Matrigel assay. The fold increase in invasion relative to vector-transfected control is shown. Cumulative results are shown from four independent experiments (**p*=0.03). (B) EGFRvIII-transfected 686LN cells

(vIII) and vector-transfected control cells (control) were subjected to a migration assay in the presence of EGF (10 ng/ml) and/or C225 (7 μg/ml) for 24 hours. The fold-increase in migration relative to untreated vector-transfected control (control) is shown. The migration of EGFRvIII-expressing HNSCC cells was not abrogated by C225 treatment compared with vector-transfected control cells (*p=0.03). (C) The experiment from B was repeated using an invasion assay. The invasion of EGFRvIII-expressing HNSCC (686LN) cells was not abrogated by C225 treatment compared to vector-transfected control cells (p=0.03).

4.3.3 EGFRvIII increases STAT3 activation.

The precise signaling pathways induced by EGFRvIII in the setting of cancer cells that also express wild-type EGFR are incompletely understood. STAT3 is activated downstream of several receptor and non-receptor tyrosine kinases including EGFR. A correlation between EGFRvIII expression and expression of phosphotyrosine STAT3 has been noted in glioblastomas [132]. We previously reported that STAT3 is activated downstream of wild-type EGFR in HNSCC [76]. To determine whether STAT3 is differentially activated by EGFRvIII, we analyzed expression of tyrosine phosphorylated STAT3 by immunoblotting in addition to STAT3 transcriptional activity in the EGFRvIII-expressing HNSCC cells compared with vector-controls. As shown in Figure 4.3A, phosphotyrosine STAT3 was expressed at higher levels in HNSCC cells that contain EGFRvIII compared to vector-transfected controls. Other HNSCC cells (1483 and PCI-37A) transiently transfected with the EGFRvIII cDNA construct and the EGFR vIII stably expressing urothelial line T-24 also demonstrated higher levels of phosphorylated STAT3 (data not shown). In addition, EGFRvIII-expressing HNSCC cells demonstrated increased STAT3 transcriptional activity using an hSIE luciferase reporter assay compared with controls (p<0.001) (Figure 4.3B). To determine the relative expression levels of phosphorylated and total STAT3 in EGFRvIII-expressing HNSCC tumors, xenografts derived

from EGFRvIII-expressing cells were analyzed. As shown in Figure 4.3C, EGFRvIII-expressing tumors contained higher levels of phosphorylated STAT3 compared to tumors derived from vector-transfected control cells (p=0.04). Further, EGFRvIII expression was associated with increased tumor growth in a xenograft model, thus confirming our previous findings [65]. To determine the effects of cetuximab on EGFRvIII-mediated STAT3 activation, HNSCC cells expressing EGFRvIII were treated with cetuximab followed by STAT3 promoter assays. While cetuximab decreased STAT3 promoter activity in vector-transfected control cells cetuximab was unable to abrogate STAT3 activation in HNSCC cells expressing EGFRvIII (Figure 4.3D). Similarly, EGF increased STAT3 promoter activity in vector-transfected controls, while EGF was unable to augment the already elevated levels of STAT3 promoter activation in HNSCC cells expressing EGFRvIII. These results indicate that EGFRvIII enhances STAT3 transcription and phosphorylation in HNSCC, effects that are resistant to treatment with cetuximab.

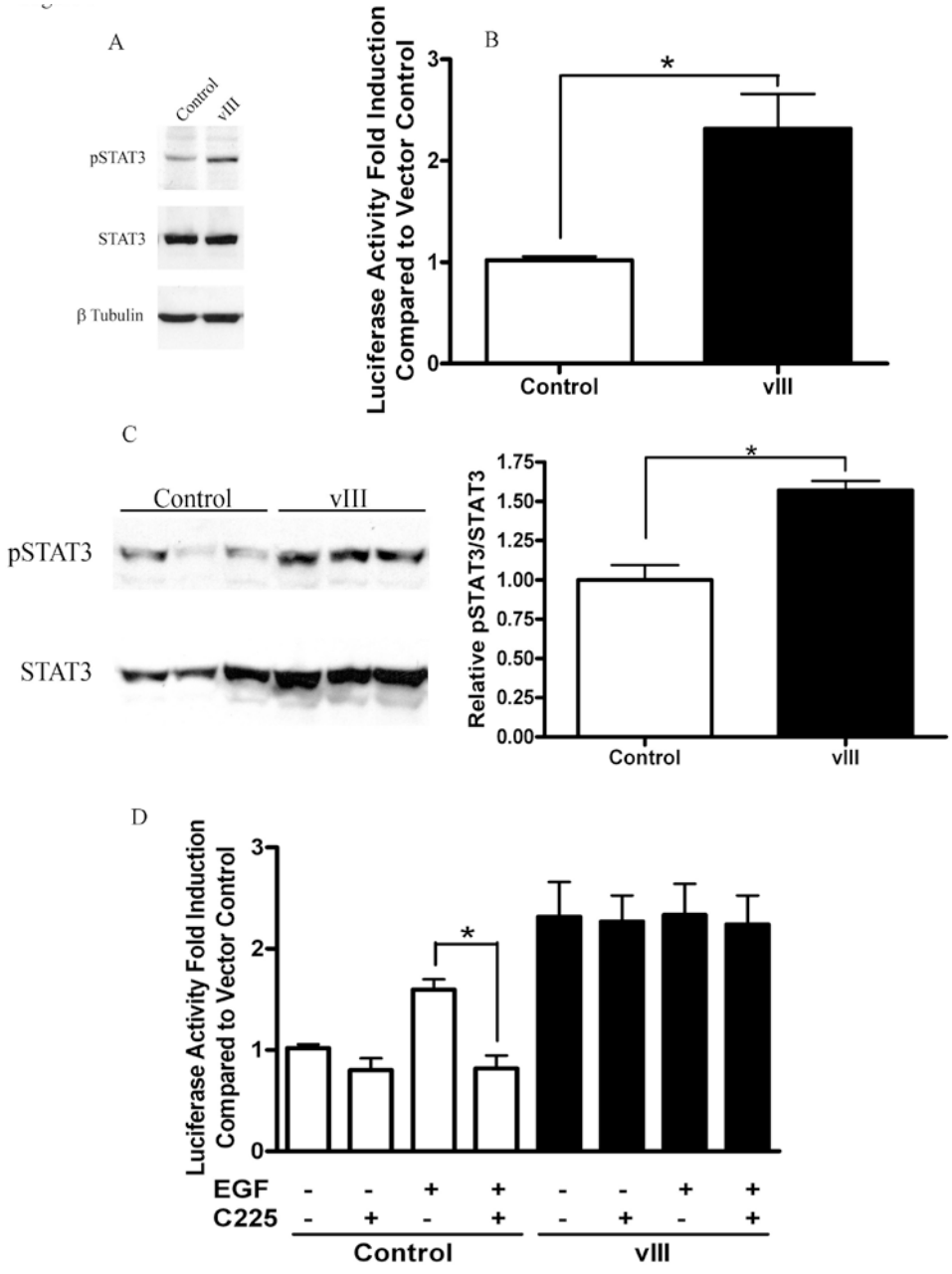


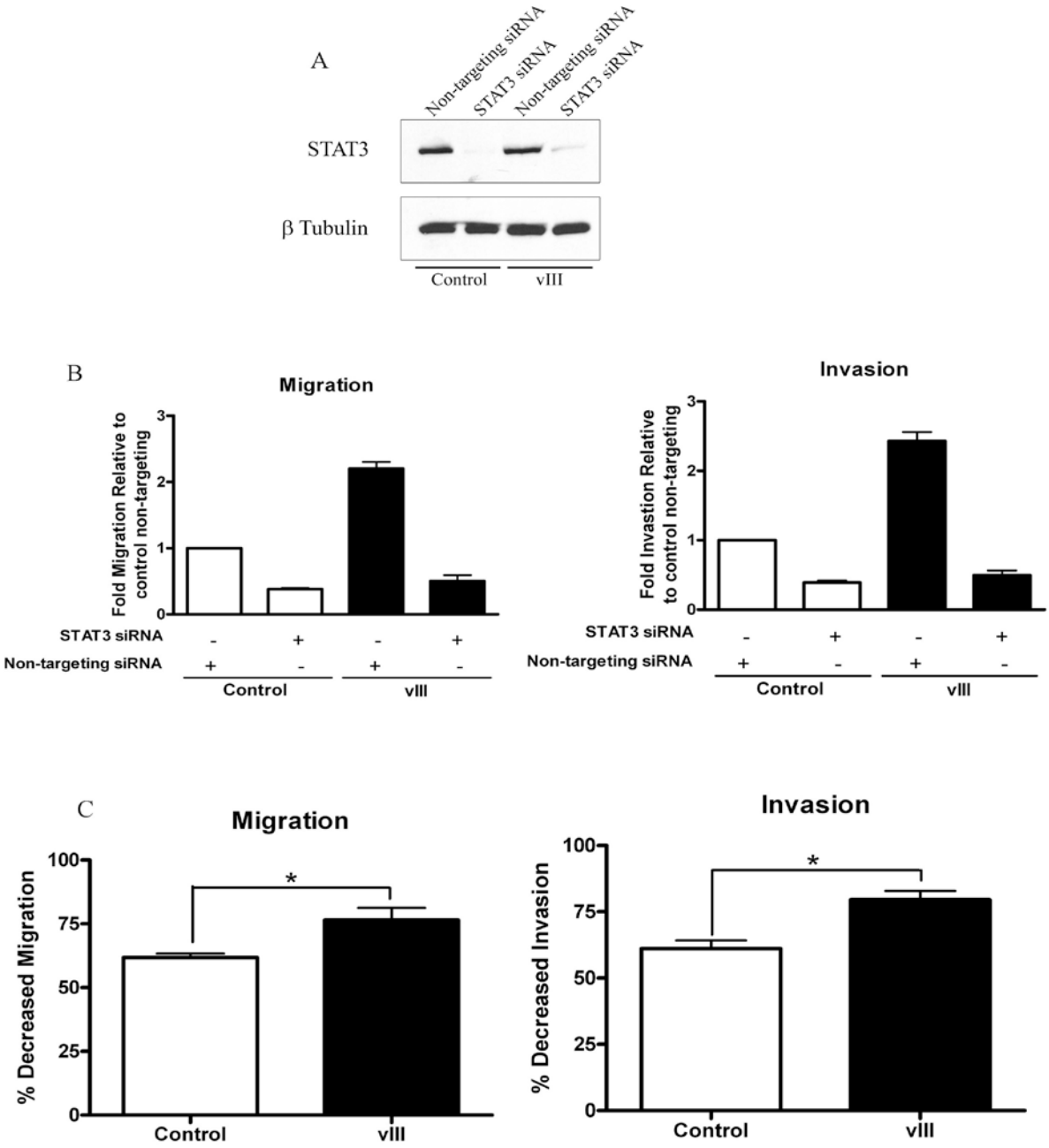
Figure 4.3 EGFRvIII increases STAT3 activation. (A) After serum starvation for 24 hours, cell extracts from vector-transfected control 686LN cells (control) and EGFRvIII-transfected cells (vIII) were analyzed by Western blot analysis. The blot was incubated with phosphotyrosine-STAT3 antibody, stripped and probed for total STAT3 and β-Tubulin to ensure equivalent loading. The experiment was performed 3 times with similar results. (B) Vector-transfected control 686LN cells (control) and EGFRvIII cells (vIII) were transiently co-transfected with a luciferase construct under the control of STAT3 responsive promoter and a Renilla luciferase construct 24 hours

after transfection with vector control or EGFRvIII, incubated in complete media for 24h and assayed for luciferase activity. The fire-fly luciferase activity units (RLU) were normalized to Renilla luciferase RLU and micrograms of total protein and expressed as a fold of the activity of the control in each experiment. Cumulative results from three independent experiments indicate a significant increase in STAT3 promoter activity in 686LN HNSCC cells expressing EGFRvIII (*p<0.001). (C) HNSCC xenografts derived from EGFRvIII-expressing Cal33 cells and xenografts derived from vector-transfected control cells were analyzed for phosphorylated and total STAT3 expression by immunoblotting. A representative immunoblot of pSTAT3 and STAT3 levels in control and vIII xenograft tumors are depicted. Densitometry analysis was performed on immunoblots from all tumors (n=17) and phosphorylated STAT3 expression levels relative to total STAT3 are shown as mean ± SE. EGFRvIII-expressing Cal33 HNSCC cell xenograft tumor (n=10) expressed 1.5-fold higher levels of phosphorylated STAT3 compared to control Cal33 HNSCC xenografts (n=7) (*p=0.043). (D) EGFRvIII-transfected 686LN cells (vIII) and vector-transfected control cells (control) were transiently co-transfected with a hSIE-luciferase construct and Renilla luciferase construct for 4 h. Cells were incubated in serum free media +/- EGF (10ng/ml) and/or cetuximab (C225) (0.7μg/ml) for 24h and assayed for luciferase activity. The luciferase RLU were normalized to Renilla luciferase-RLU and to micrograms of total protein and expressed as a fold of the activity of the vector control (control) in each experiment. Cumulative results are shown from two independent experiments. The hSIE promoter activity of EGFRvIII-expressing 686LN cells was not stimulated by EGF or abrogated by cetuximab treatment. In contrast, cetuximab effectively abrogated EGF induced STAT3 promoter activity in HNSCC cells (*p=0.03).

4.3.4 STAT3 is required for EGFRvIII-mediated motility and invasion.

STAT3 has been implicated in several oncogenic processes including proliferation, survival, and invasion and may represent a therapeutic target for cancer [222]. To determine whether STAT3 is required for EGFRvIII-mediated cell motility and invasion, we performed migration and invasion assays in the presence or absence of siRNA targeting STAT3, under conditions where siRNA did not modulate proliferation. In order to examine the phenotypic effects of EGFRvIII signaling via STAT3, we assessed cell invasion and migration in the absence of EGFR ligand.

STAT3 siRNA effectively abrogated STAT3 levels in vector control and EGFRvIII expressing cells (Figure 4.4A). As shown in Figures 4.4B and 4.4C, knockdown of STAT3 (and phosphotyrosine STAT3) reduced the motility and invasion of EGFRvIII-expressing HNSCC cells, under conditions controlling for proliferation (migration: p=0.03, invasion: p=0.02). In fact, on STAT3 knockdown, the degree of migration and invasion in the EGFRvIII cells were comparable to levels in vector-transfected controls. In addition to downmodulation of STAT3 expression using siRNA, we also blocked STAT3 in the cells with a transcription factor decoy directed against STAT3 as described previously [219]. The STAT3 decoy interferes with STAT3-mediated DNA binding and abrogates STAT3 target gene expression. As shown in Figures 4.4D and 4.4E, treatment with the STAT3 decoy resulted in reduction of both migration and invasion in EGFRvIII expressing cells compared to treatment with a mutant control decoy, under conditions where the decoy did not affect cell growth. In the absence of EGFR ligand there was a significant reduction in the migration or invasion of control cells treated with the STAT3 decoy (migration: p=0.05, invasion: p=0.03). However, the STAT3 decoy abrogated the migration and invasion of EGFRvIII expressing HNSCC cells to a significantly greater degree than the vector transfected control cells (p=0.03 and p=0.05, respectively).


A
Non-targeting siRNA
STAT3 siRNA
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β Tubulin
Control
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vIII
Invasion
Fold Invastion Relative to control non-targeting
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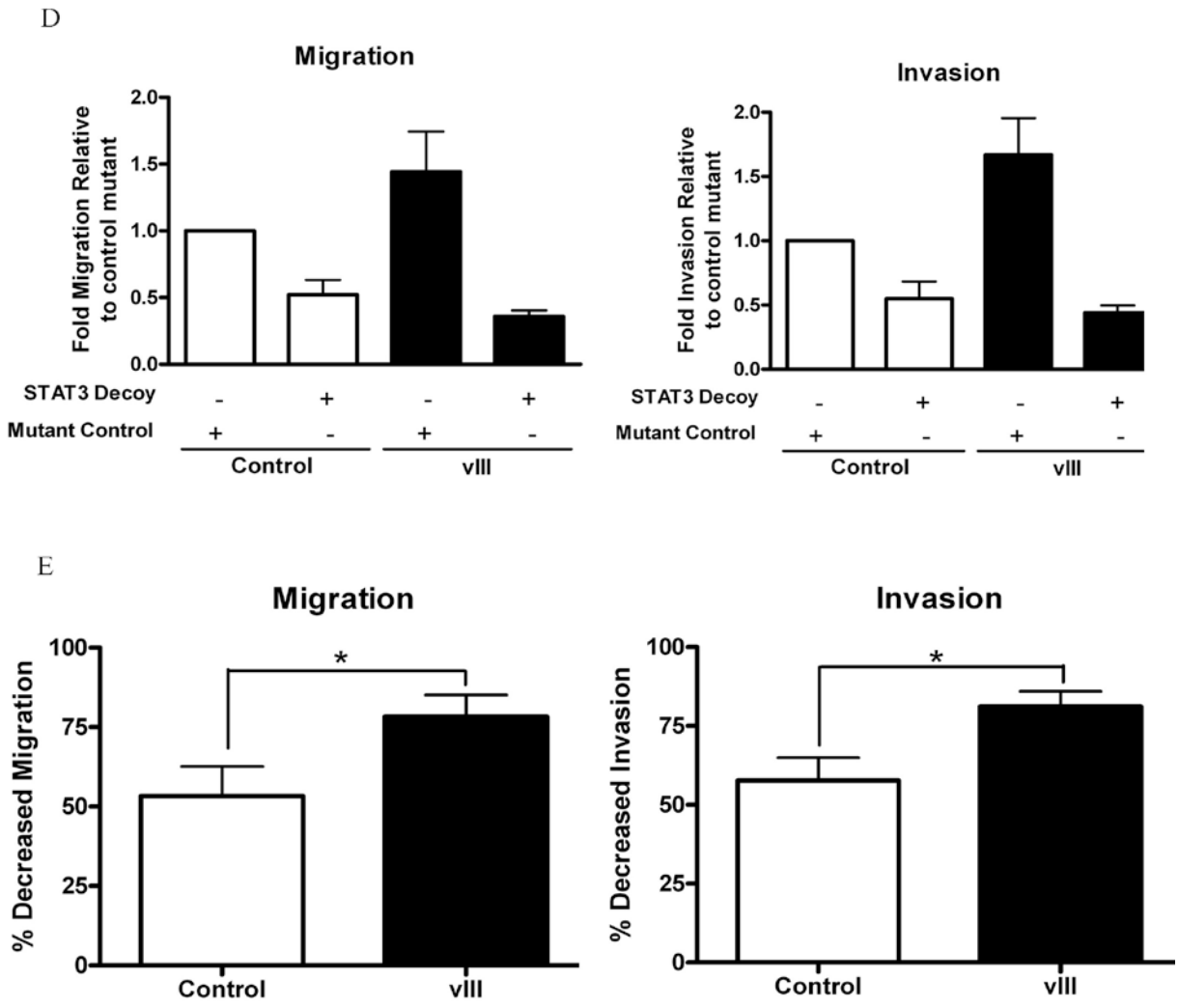


Figure 4.4 STAT3 plays a critical role in EGFRvIII-mediated migration and invasion. (A) EGFRvIII-transfected 686LN cells (vIII) and vector transfected control cells (control) were subsequently transfected with non-targeting siRNA or STAT3 siRNA for 6 h. Cells were collected at hours 48 for the analysis of total STAT3 protein levels by immunoblotting. β-tubulin levels demonstrate equal loading of protein in all lanes. The experiment was repeated 4 times with similar results. (B) STAT3 siRNA decreases HNSCC migration and invasion. Forty eight hours after non-targeting or STAT3 siRNA transfection, EGFRvIII-transfected 686LN HNSCC cells (vIII) and vector-transfected controls (control) were subjected to migration (left panel) and invasion assays (right panel) with the same methods described in materials and methods. Both control cells and EGFRvIII transfected cells decreased their migration and invasion when cells are treated with STAT3 siRNA compared to non-targeting siRNA treated condition. (C) Percent reduction of migration and invasion with STAT3 siRNA in both control and vIII was

calculated from the results shown in panel B. The degree of both migration (left panel) and invasion (right panel) with STAT3 siRNA was greater in EGFRvIII expressing 686LN cells than in control cells (*p=0.03 and 0.016 respectively). (D) STAT3 decoy decreases HNSCC migration and invasion. After mutant or STAT3 decoy treatment, EGFRvIII-expressing HNSCC 686LN cells (vIII) and vector-transfected controls (control) were subjected to migration (left panel) and invasion assays (right panel). Both control cells and EGFRvIII-expressing cells demonstrated decreased migration and invasion when treated with STAT3 decoy compared to mutant control decoy treatment. (E) Percent reduction of migration and invasion with STAT3 decoy in both control and vIII was calculated from the results shown in Figure 4D. HNSCC cells expressing EGFRvIII had significantly reduced migration (*p=0.05) and invasion (*p=0.05) compared to control cells in the presence of STAT3 decoy.

Others have reported that PI3K/AKT is activated downstream of EGFRvIII in glioma [223, 224]. To determine whether motility and invasion are also mediated by this pathway, in addition to STAT3, we examined the expression of AKT phosphorylation in EGFRvIII and vector-transfected control HNSCC cells and found that similar to results in previous reports, EGFRvIII-expressing HNSCC cells expressed modestly increased levels of pAKT (Figure 4.5A). However, in contrast to our observations with STAT3 targeting, blockade of PI3K/AKT using the pharmacologic inhibitor NVP-BEZ235-AN-4 (BEZ) abrogated cell growth but not invasion or metastasis, in HNSCC cells expressing EGFRvIII (Figures 4.5B-C). Similar results were also obtained using siRNA directed against the p85 subunit of PI3K in a urothelial cancer model expressing EGFRvIII. These results suggest that STAT3 is specifically required for the EGFRvIII-mediated enhancement of HNSCC cell motility and invasion.

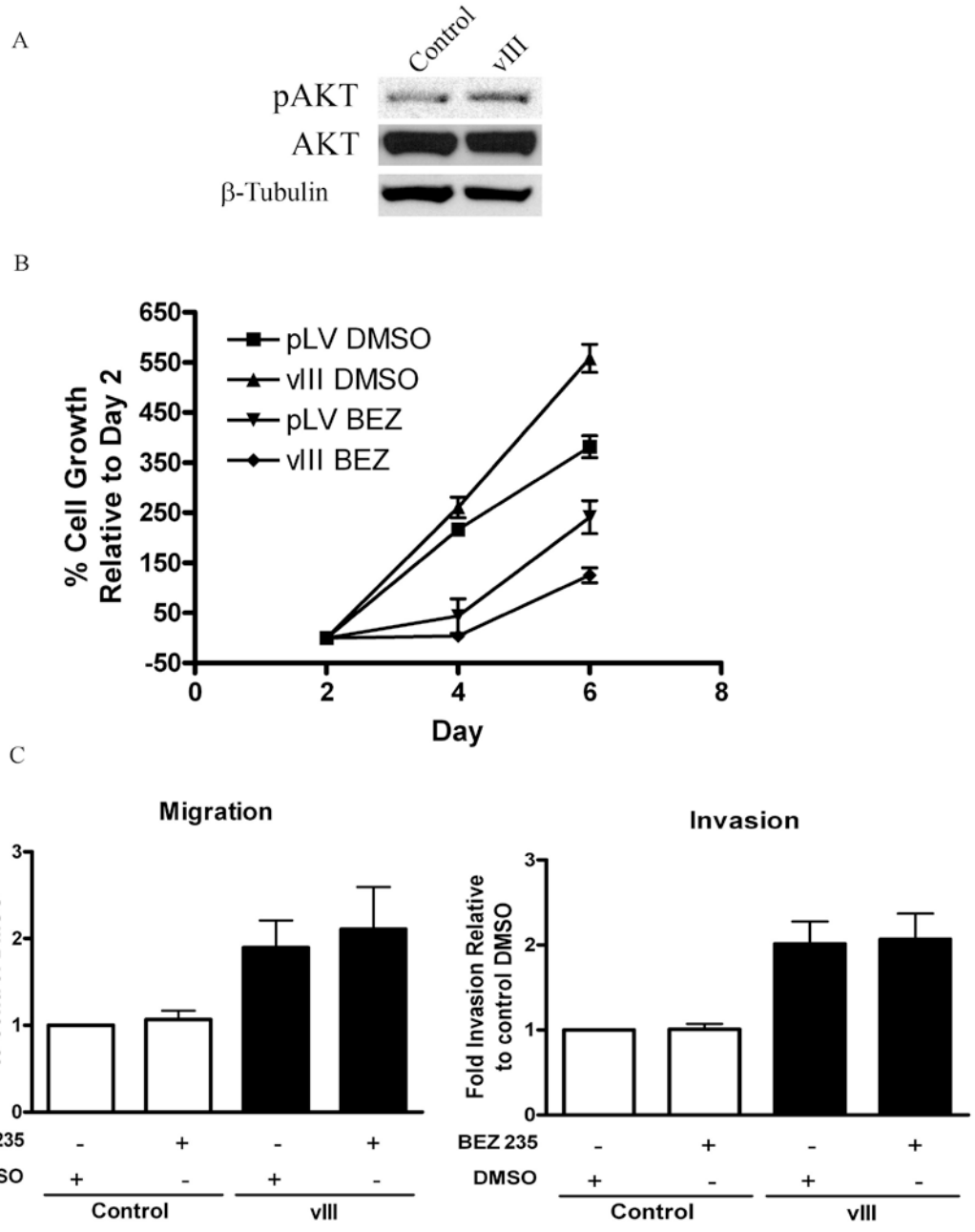


Figure 4.5 Increased PI3K/AKT signaling in EGFRvIII-expressing HNSCC cells mediates proliferation, but not invasion. (A) EGFRvIII-expressing HNSCC 686LN cells express modestly increased levels of phosporylated AKT. After serum starvation for 24 hours, cell extracts from vector-transfected control cells (control) and EGFRvIII-transfected cells (vIII) were analyzed by Western blot analysis. The blot was incubated with phosphoserine-AKT antibody (Ser473), stripped and immunoblotted for total AKT and β-tubulin to ensure equivalent loading. The experiment was performed 3 times with similar results. (B) Blockade of PI3K/AKT

abrogates cell growth in HNSCC 686LN cells expressing EGFRvIII. HNSCC cells expressing EGFRvIII and vector-transfected control cells were plated onto 6 well plates with presence (◆, vIII), (▼, control) or absence (▲, vIII), (■, control) of 100nM of NVP-BEZ235-AN-4. Growth curve of the transfected cell lines were obtained by MTT at several time points for 6 days. HNSCC cells expressing EGFRvIII showed increased growth rates compared with the vector- transfected control cells where cell growth was abrogated by PI3K/AKT blockade in both cell lines. Experiment was repeated 3 times with similar results. (C) Cell migration (left panel) and invasion (right panel) was not abrogated by PI3K/AKT blockade. Cell invasion assay was performed with HNSCC 686LN cells transfected with EGFRvIII or vector-transfected control with or without PI3K/AKT inhibition using 100nM of NVP-BEZ235-AN-4. HNSCC cells expressing EGFRvIII show increased cell invasion compared with the vector-transfected control cells but cell invasive capacity was not decreased by PI3K/AKT blockade in either cell line.

4.3.5 Cetuximab does not abrogate EGFRvIII-induced HIF-1α expression under hypoxic conditions.

Solid tumors, including HNSCC, contain large regions of low oxygen concentrations (hypoxic) regions, which contribute to resistance to treatment with standard approaches including chemotherapy and radiation. Hypoxia potently induces expression of hypoxia inducible factor (HIF-1α), which has been shown to be a STAT3 target gene [225]. EGFRvIII has been reported to contribute to hypoxia-mediated tumor growth in conjunction with radiation therapy but has not been previously linked to HIF-1α expression [226]. We therefore examined the expression of HIF-1α following treatment of HNSCC cells expressing EGFRvIII (or vector-transfected controls) with EGF and/or cetuximab. As shown in Figure 4.6, hypoxia-induced expression of HIF-1α was reduced by cetuximab in vector-transfected control cells but not in HNSCC cells expressing EGFRvIII. This experiment was also validated in urothelial cancer T-24 cells stably

transfected with EGFRvIII. These results suggest that STAT3 signaling via HIF-1α may contribute to cetuximab resistance in EGFRvIII-expressing HNSCC tumors under hypoxia.

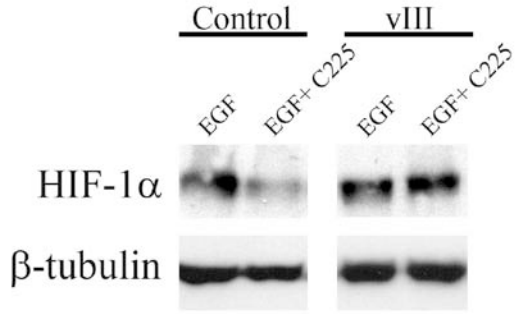


Figure 4.6 EGFRvIII induces HIF-1alpha expression under hypoxic conditions, which is not abrogated by ceutximab. EGFRvIII-expressing 686LN cells (vIII) and vector transfected control cells (control) were treated with (or without) EGF (10 ng/ml) and/or cetuximab (C225, 7 μg/ml) in hypoxic conditions for 24 h. Under hypoxic conditions and EGF treatment, EGFRvIII-transfected cells maintained expression of HIF-1α with cetuximab treatment whereas the expression of HIF-1α in vector-transfected control cells was significantly decreased by cetuximab. The experiment was performed 4 times with similar results.

4.4 DISCUSSION

We previously reported that EGFRvIII is expressed in up to 40% of HNSCC tumors where expression of this altered receptor mediates growth and resistance to chemotherapy or EGFR targeting using cetuximab [65]. Patients with HNSCC succumb to their disease due to invasion into surrounding tissues and regional and distant metastasis. Although the addition of cetuximab to radiation was shown to improve survival, it did not decrease metastasis [14]. The present study was undertaken to determine the effects of EGFRvIII on the migration and invasion of

HNSCC cells and the signaling pathways that mediate these properties. Our results suggest that EGFRvIII increases HNSCC motility and invasion, at least in part, through activation of STAT3.

EGFRvIII is the most common EGFR alteration in human cancers. Deletion of exons 2-7 gives rise to a receptor that lacks a ligand binding site and is constitutively activated in a ligand-independent manner. EGFRvIII has not been observed in normal tissue, but it has been detected in carcinomas of the brain, breast, ovary [196], lung [227], prostate [197] and head and neck [65]. Expression of EGFRvIII has been correlated with poor prognosis in brain tumors [228]. Further, EGFRvIII can to transform fibroblasts *in vitro* [221] and enhance the tumorigenicity of cancer cells *in vivo,* supporting its oncogenic function [229].

The effect of EGFRvIII on tumor cell behavior is incompletely understood. Nagane *et al.* reported that EGFRvIII-expressing cells demonstrated less apoptosis in response to cisplatin treatment [230]. Others have reported that EGFRvIII induced glioma cell migration and invasion via induction of metalloproteases and extracellular matrix components [103]; [231]. The precise signaling events that mediate EGFRvIII-induced migration and invasion need further investigation. Moscatello *et al.* reported that EGFRvIII activates PI3-K pathway instead of the Ras-Raf-MEK pathway, which is preferentially activated by wild-type EGFR [120]. Further investigation suggested that constitutive PI3-K/AKT activation by EGFRvIII may contribute to chemoresistance and radioresistance in these cells [224, 232]. Antonyak *et al.* showed that c-Jun N-terminal Kinase (JNK) was constitutively activated by EGFRvIII and was down-regulated by PI3-K inhibition [223]. To date, signaling through PI3-K/AKT has not been correlated with tumor cell migration or invasion mediated by EGFRvIII. We found that although EGFRvIII-

expressing HNSCC cells expressed increased levels of phosphorylated AKT, abrogation of PI3-K/AKT using either NVP-BEZ235-AN-4 or p85 siRNA, did not abrogate invasion in EGFRvIII-expressing HNSCC cells. NVP-BEZ235-AN-4 treatment decreased the proliferation of EGFRvIII-expressing HNSCC cells, suggesting that activated PI3K/AKT by EGFRvIII contributes to EGFRvIII-induced HNSCC cell proliferation, but not migration or invasion (Figure 5).

There are few, but conflicting, reports linking EGFRvIII to STAT3 signaling. While Mizoguchi *et al.* reported a correlation of expression levels of EGFRvIII and phosphotyrosine STAT3 in glioblastoma [132], Andersen *et al.* recently reported that glioma cells that express EGFRvIII fail to induce IRF-1 via STAT3 phosphorylation [233]. Cumulative evidence has implicated STAT3 as an critical oncogene where elevated expression levels of tyrosine phosphorylated STAT3 are detected in numerous human cancers [73]. Studies in HNSCC demonstrate that STAT3 is activated downstream of receptor and non-receptor tyrosine kinases including EGFR and Src family kinases as well as IL-6/gp130 [234], [84], [235]. Targeting STAT3 in HNSCC preclinical HNSCC models inhibited tumor growth but not the growth of normal epithelial cells [219]. Expression of tyrosine phosphorylated STAT3 in the primary HNSCC tumor has been correlated with nodal metastasis, advanced tumor stage and decreased survival [236]. The results of the present study suggest that activation of STAT3 downstream of EGFRvIII in HNSCC contributes to the increased migration and invasion.

STAT3 target genes include cell cycle regulators [237], anti-apoptotic genes [238] and pro-angiogenic factors [239], each of which has been implicated in tumorigenic processes

including invasion and metastasis. STAT3 has been shown to contribute to cancer migration and invasion thorough regulation of genes that stimulate these processes including matrix metalloproteinases (e.g. MMP-2 and MMP-9), VEGF and/or bFGF [240]; [241]. In addition to the transcriptionally mediated effects of STAT3 on cell migration and invasion, transcription-independent pathways have also been described for the effects of STAT3 on cell motility. Specifically, STAT3 has been found to directly interact with cell motility components such as focal adhesion components, FAK, paxillin [242], $p130^{CAS}$ [243], or cytoskeltal microtubles [244]. We did not detect increased expression of MMP-2, MMP-9 or VEGF in association with the EGFRvIII-mediated migration and invasion observed in these cells, suggesting that other STAT3 target genes or STAT3 interacting proteins may be playing a role. Additionally, the lack of HIF-1α decrease in EGFRvIII cells treated with cetuximab under hypoxic conditions suggests a more complex regulatory balance between oxygen-dependent and –independent factors that influence HIF-1α.

Aberrant activation of STAT3 has been shown to contribute to tumor progression making STAT3 an attractive therapeutic target. To date, no STAT3 targeting strategies have undergone clinical evaluation. We have developed a highly specific transcription factor decoy approach to block STAT3 signaling and demonstrated that it inhibits tumor growth *in vitro* and *in vivo* in HNSCC preclinical models [219]. Using the same STAT3 decoy, others have reported antitumor effects in a murine model of cutaneous squamous cell carcinoma [245]. Toxicology studies in non-human primates were recently completed and demonstrated no evidence of toxicity [246]. In the present study, treatment of EGFRvIII-expressing HNSCC cells with the STAT3 decoy abrogated cell motility and invasion. These results suggest that selective activation by EGFRvIII

in HNSCC contributes to the invasive phenotype, which can potentially be targeted with therapeutic strategies that inhibit STAT3. In addition to the decoy, others have reported the use of siRNA, peptidomimetic strategies, and G-quartet oligonucleotides to inhibit STAT3 in cancer models [19].

These cumulative results suggest that STAT3 activation is critical for cancer progression mediated by both wild-type EGFR and EGFRvIII, which are often co-expressed in HNSCC tumors [65]. Knockdown or blockade of STAT3 preferentially abrogated the migration and invasion of HNSCC cells that expressed EGFRvIII implicating STAT3 as a critical pathway in mediating HNSCC invasion in tumors that express this altered receptor. We have previously demonstrated that both PLCγ-1 and c-Src mediate HNSCC invasion downstream of EGFR [88, 95]. Thus EGF stimulation in cells expressing both EGFR and EGFRvIII likely results in invasion via multiple downstream signaling molecules including STAT3.

Therapeutic agents with selective activity against EGFRvIII are presently under clinical investigation including the immunotoxin MR1-1, the chimeric antibody 806 [247] and irreversible HER1/HER2 inhibitors [129] that appear to have selective activity against EGFRvIII. The EGFR monoclonal antibody cetuximab is the only FDA-approved EGFR targeting strategy for HNSCC. We previously reported that HNSCC xenografts expressing EGFRvIII were resistant to the growth inhibitory effects of cetuximab [65]. Here we demonstrate that EGFRvIII cells are resistant to anti-invasive effects of cetuximab in HNSCC. Further, EGFRvIII expression results in an increase in phosphorylation of STAT3 in HNSCC cells. These results suggest that HNSCC tumors that express EGFRvIII may be best treated with strategies

that selectively block EGFRvIII, or its downstream signaling pathways, in addition to targeting wild-type EGFR.

5.0 THE ROLE OF SRC FAMILY KINASES IN EGFRVIII EXPRESSING HNSCC

5.1 INTRODUCTION

SRC is one of the earliest known oncogenes, originally described by Peyton Rous that could induce solid tumors in avians [248]. However, it wasn't until the development of molecular biology that this discovery could be fully appreciated and utilized. Studies showed that the viral oncogene responsible for the cellular transformation was *v-SRC* which also had a cellular counterpart *c-SRC* [249]. More recently the discovery of highly selective and specific Src kinase inhibitors has brought this laboratory finding into the clinic.

5.1.1 Src Structure and function

There are nine members of the Src family of tyrosine kinases (SFKs): LYN, FYN, LCK, HCK, FGR, BLK, YRK, YES, and c-Src (Src hereafter). Src is the best studied of these family members and is most often implicated in malignancy [250]. In HNSCC Src, Lyn, Fyn and Yes are expressed at detectable levels [84].

SFKs are a family of homologous non-receptor tyrosine kinases with extensive sequence homology. SFKs contain four Src-homology domains (SH1-4), a c-terminal regulatory domain and a unique region. SH1 is the catalytic domain and contains the autophosphorylation site

necessary for full kinase activity. The SH2 and 3 domains are modular domains present in various cellular proteins and are involved in protein binding. The SH4 domain contains signals for lipid modifications that aid in membrane association and targeting. The c-terminal regulator domain contains a negative regulatory tyrosine residue (Y530) and the unique region, as the name suggests, is unique to each SFK [251].

Src has active (open) and inactive (closed) conformations that are involved in molecular regulation. Phosphorylation of Y530 causes binding to the SH2 domain and Src folds into an inactive conformation [252-255], which contributes to blocking substrates from binding to the kinase domain. Dephosphorylation of Y530 causes Src to resume an open conformation but full activity requires autophosphorylation of the Y419 residue within the catalytic domain [256].

Src activity is regulated by both kinases and phosphatases that act at Y530. C-terminal Src Kinase (CSK) maintains Src in an inactive conformation by mediating phosphorylation of tyrosine 530 on Src (negative-regulatory tyrosine) [256]. CSK may have a tumor suppressor role as overexpression of CSK in an *in vivo* metastatic mouse colon carcinoma model showed significant suppression of tumor metastasis [257, 258]. Correlative studies in colon cancer and hepatocellular carcinoma report that downregulation of CSK may be a mechanism of enhanced Src activity in cancer [259, 260].

Y530 can be dephosphorylated by various protein phosphatases allowing access of substrates to the kinase domain. Protein tyrosine phosphatase-alpha [261] and SHP1/SHP2 [250] are the best studied examples of Src specific dephosphorylation. Additionally, in breast cancer

protein-tyrosine phosphatase 1B has been shown to be involved in Src-specific dephosphorylation [262]. Src can also be activated by direct binding of the SH2 and SH3 domains to intracellular proteins or growth factor receptors, which disrupts the inhibitory conformation of Src allowing for activation [250, 263-265].

5.1.2 Src in cancer

Src is involved in many normal cellular functions such as cell adhesion, migration, proliferation, survival, angiogenesis and differentiation and deregulation of these pathways contributes to tumorigenesis, tumor progression and metastasis [251, 266]. The role of Src in these cellular functions is to mediate cell signaling between many cell surface proteins including integrins [267], g-coupled protein receptors [88, 268] and growth factor receptors [269].

Src is rarely mutated in cancer [270, 271]. Activated Src is common in colorectal and breast cancers and elevated levels of Src protein have been reported in several cancers including colon, breast, lung, endometrial, ovarian, pancreatic and head and neck [266, 272]. In the absence of Src mutations or amplification increased Src activity is likely due to increased activation of intracellular molecules that activate Src family kinases. Src is activated in response to stimulation of several RTKs including PDGFR, EGFR, IGF-1R, GPCRs, cytokines, integrins, cell adhesion complexes and others [273].

Src has been reported to be activated in HNSCC compared to normal and pSrc correlates with invasiveness and lymph node metastasis [274]. Aberrant Src activation has been shown to contribute to HNSCC progression and metastasis [90, 91].

5.1.3 Src in the presence of EGFR blockade

There are four primary signaling pathways involved in mediation of EGFR signaling. Ras-MAPK, PI3K-Akt, PLCgamma-PKC, STAT pathways [275]. Src kinases have been reported to be activated in numerous solid tumors, including HNSCC, that have high EGFR expression levels and can function to potentiate EGFR signaling [269, 276]. FAK (involved in the regulation of cell adhesion and migration), PI3K and STAT3 are known substrates for Src [17]. The multiple upstream activators for Src indicate that even in the presence of EGFR blockade Akt-PI3K and STAT pathways may be activated in a Src-dependent manner. In HNSCC SFKs are activated following EGFR stimulation and physically associated with EGFR [84]. SFKs have reduced activity following EGFR inhibition [277].

First evidence that STATs were activated by Src came from studies into v-Src mediated transformation of fibroblasts and hematopoietic cells [278, 279]. Many human cancers have shown a role for Src in constitutive STAT activation [280-283] and in HNSCC STAT3 is a Src-dependent mediator of EGFR-stimulated growth *in vitro* [284] and decreased apoptosis [80] and increased tumor growth *in vivo* [81].

5.1.4 Src and EGFRvIII

Epidermal growth factor receptor variant 3 (EGFRvIII) lacks exons 2-7, is constitutively active and is absent in normal tissue [67, 109]. HNSCC cells expressing EGFRvIII have been

shown to be resistant to apoptosis by cisplatin *in vitro* [65]and other *in vitro* models of EGFRvIII have shown decreased sensitivity to tyrosine kinase inhibitors (TKIs) compared to wtEGFR only cells [285, 286].

EGFRvIII expression has been shown to influence cell survival, proliferation, motility, invasiveness and treatment resistance [102-106]. In glioblastoma, breast cancer and HNSCC cell lines EGFRvIII enhances *in vivo* tumorigenicity [65, 107, 108], within the tumors proliferation is increased and apoptosis is decreased [102, 107].

Differential activation of EGFRvIII signaling pathways compared to EGFRwt has been reported [112-114] and recent work in glioblastoma indicates that Src is an appropriate therapeutic target in combination with EGFR monoclonal antibodies [83, 287].

5.1.5 Src inhibitors (Dasatinib)

Src blockade has inhibited proliferation in several tumor models including breast cancer, head and neck cancer, prostate cancer and GBM models [91, 288-291] Many tumor cell models have shown that treatment with a Src or SFK inhibitor or siRNA blocked tumor cell invasion and migration [291-294].

Dasatinib is a potent SFK inhibitor with activity against BCR-ABL, KIT, PDGFRalpha/beta and EPHA2 [295, 296] that has been FDA approved for use in chronic myeloid leukemia. In GBM dasatinib has been shown to decrease cell viability and induce apoptosis through its SFK activity [297]. In several solid tumor cell line models *in vitro* and *in*

vivo reduced expression of vascular endothelial growth factor (VEGF) has been reported following Src inhibition or down-regulation of Src expression [298-300]. In HNSCC dasatinib has been reported to reduce invasion and migration in vitro and inhibit Src and downstream mediators of cell adhesion including focal adhesion kinase (FAK) [90].

In glioblastoma several studies have looked at the effects of altered src activity on EGFRvIII expressing tumors. Genetic disruption of Src (DNSrc) in EGFRvIII expressing glioblastoma xenografts decreases tumor growth rate and significantly increases the efficacy of EGFRvIII specific Ab treatment [83]. Dasatinib has also been used with success in *in vivo* glioblastoma model systems. In an endogenously expressing EGFRvIII model dasatinib has been shown to inhibit growth and increase survival and apoptosis [287]. In a GBM cell line model engineered to express EGFRvIII and DASrc dasatinib alone had no effect on tumor volume but it significantly increased the xenograft sensitivity to EGFR mAb treatment (mAb 806) in a combination treatment group [287].

5.1.6 Purpose of this Study

The role of Src activity in EGFRvIII expressing HNSCC has not been defined. Given the constitutive activity of EGFRvIII it is possible that SFKs are key downstream mediators of the increased oncogenic phenotype observed in EGFRvIII expressing tumor cells. We undertook this study to determine if SFKs were differentially activated in EGFRvIII expressing HNSCC compared to EGFRwt and if inhibition of SFK signaling could abrogate EGFRvIII mediated invasion, migration and tumor growth.

5.2 MATERIALS AND METHODS

5.2.1 Cell lines, reagents and cell culture

Cal33 and UMSCC1 (SCC1) cells were a kind gift from Dr Gerard Milano (Centre Antoine-Lacassagne, Nice, France) and Dr. Thomas E. Carey (University of Michigan, Michigan, USA), respectively. FaDu and 293T cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA). All cells were maintained in Dulbecco's modified Eagle's medium (Mediatech Inc., Herndon, VA, USA) with 10% heat-inactivated fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA). SCC1 cells were supplemented with 0.4 ug/ml hydrocortisone (Invitrogen) and FaDu cells were supplemented with 1% NEAA (Invitrogen). Cells were incubated at 37 °C in the presence of 5% CO_2.

EGFRvIII-transfected HNSCC cells (Cal33vIII) and vector control-transfected HNSCC cells (Cal33control) have been previously described [301]. EGFRvIII was subcloned in to the pMSCV Neo plasmid (Clontech, Mountain View, CA). EGFRvIII plasmid DNAs were a kind gift from Dr Frank Furnari (Ludwig Institute for Cancer Research, La Jolla, CA). SCC1 and FaDu cells were infected with vector alone (MSCV) or EGFRvIII vector (vIII). Briefly, 293T cells were plated at 80-90% confluency in a 10 cm dish and reverse transfected using lipofectamine 2000 (Invitrogen), manufacturer’s plasmids and parent vector or EGFRvIII plasmid overnight. Fresh media was placed on the cells after 16 hours and virus produced over 48 hours. Target cells were plated in 10 cm dishes at 25% confluency 16 hours prior to treatment to allow cells to adhere. Viral supernatant was collected, centrifuged, filtered,

supplemented with polybrene and placed on target cells for 72 hours. Viral supernatant was replaced with complete media for 24 hours and cells were selected with 0.5 mg/ml G418 (Invitrogen) for 72 hours. Resulting cells were tested for EGFRvIII expression via RT-PCR as described previously [65, 301] and maintained under selection pressure.

Recombinant human EGF was purchased from Sigma-Aldrich Corp. (St Louis, MO, USA). BCR-Abl/Src inhibitor dasatinib (Das) was a kind gift from Bristol-Myers Squibb (New York, NY, USA) for *in vitro* experiments and was purchased from Chemie Tek (Indianapolis, IN, USA) for *in vivo* experiments.

5.2.2 Immunoprecipitation and Western Blotting

HNSCC cells were plated at 50% confluency in a 10cm dish for 48 hours and harvested as indicated in western blotting. For immunoprecipitation cells were serum starved for 24 hours following cell adhesion. 1 mg of whole cell lysate, 2-4ug antibody and 40 ul Protein G beads (Millipore, Temecula, CA) were combined and allowed to rotate overnight. Beads were pelleted the following day by centrifugation and washed 3 times with fresh lysis buffer. Beads were resuspended in 1x western blot loading dye and boiled for 8 min before loading onto a 10% SDS-page gel.

Cell lines and tumor pieces were lysed in detergent containing 1% NP-40, 0.1 mM phenylmethylsulfonyl fluoride, 1 mg/ml leupeptin and 1 mg/ml aprotinin, and protein levels were

determined using the Bio-Rad protein assay method (Bio-Rad Laboratories, Hercules, CA, USA). Total protein (40μg unless otherwise noted) were separated on 8%SDS–page gel and transferred to nitrocellulose membranes using a semidry transfer machine (Bio-Rad Laboratories). Membranes were blocked with Odyssey blocking buffer (Li-Cor Biosciences; Lincoln, NE), probed with primary and subsequently secondary antibodies and visualized using Odyssey Infrared Imaging System (Li-Cor Biosciences) according to manufacturer's instructions. Primary antibodies used for blotting included β-actin, phospho-AKT (Ser473), AKT, phospho-STAT3 (Tyr705) and STAT3, phospho-Src (Y416), phospho-STAT3 (Y705XP), STAT3, Lyn, Fyn, Src, Yes, phospho-EGFR Y992, and phospho-EGFR Y1068 from Cell Signaling Technology, Beverly, MA, Src B-12 (Santa Cruz Biotechnology; Santa Cruz, CA), and EGFR (BD Transduction Laboratories; San Jose, CA), anti-phospho-Src Y416 clone 9A6 (Millipore, Temecula, CA). The EGFRvIII 4-5H antibody was a kind gift from Dr. Careen Tang (Georgetown University Medical Center; Washington D.C.). Secondary antibodies used for blotting included goat anti rabbit IRDye 680 or goat anti mouse IRDye 800CW (Li Cor Biosciences).

5.2.3 Animal Experiment

Nu/nu athymic nude mice (Harlan Sprague-Dawley, Indianapolis, IN, USA) were injected subcutaneously with $5x10^5$ cells per flank suspended in 100 ul serum free media with Cal33control and Cal33vIII in opposing flanks. Tumor volumes were measured in two dimensions with vernier calipers and calculated using the formula: (length × width^2) × 0.52. At the end of the study, mice were killed by cervical dislocation under anesthesia; the tumors

surgically excised and snap frozen in dry ice. Tumors were allowed to develop and 10 days after inoculation tumors were measured and stratified randomization performed dividing the mice into 2 groups of 8. Mice were treated with either 80mM citric acid in PBS by oral gavage or dasatinib (50mg/kg daily) by oral gavage.

5.2.4 Statistical analysis

For migration and invasion studies, the statistical significance of differences in the number of invading cells or migrated area was assessed using Wilcoxon–Mann–Whitney two-tailed exact test. Statistical analysis and graphs were created in GraphPad Prism (version 4.03; GraphPad Prism Software, Inc.; La Jolla, CA).

5.2.5 Proliferation Assay

To assay proliferation 5000 cells per well were plated in triplicate in a black-walled 96 well plate and allowed to adhere overnight. Following adhesion cells were treated with DMSO (control) or 100nM dasatinib for 72 hours and subsequently assayed with CellTiter-Glo Luminescent Cell Viability Assay (Promega; Madison WI) according to manufacturer's instructions. Breifly, 100 ul Cell Titer Glo reagent was added to each well and the plate was rocked gently for 2 minutes and the luminescent signal allowed to stabilize for 10 minutes before the plate was read on a Victor^{3}V 1420 mulitlabel counter with Wallac 1420 software (Perkin Elmer; Waltham, MA). Values were normalized to DMSO vector control cells and plotted in GraphPad Prism (version 4.03; GraphPad Prism Software, Inc.; La Jolla, CA).

5.2.6 Matrigel invasion assay and cell migration assay

Cell invasion was evaluated *in vitro* using Matrigel-coated semipermeable modified Boyden inserts with a pore size of 8 μm (Becton Dickinson/Biocoat, Bedford, MA, USA). Cell migration was evaluated *in vitro* using semipermeable modified Boyden inserts with a pore size of 8 μm (Becton Dickinson/Biocoat). For both assays, cells were plated in duplicate at a density of 1.3×10^4 cells per well in serum-free media in the insert. At the same time, cells were plated in 24-well plates to serve as proliferation controls, these cells were subjected to the same medium composition as the holding well. Both the insert and the holding well were subjected to the same medium composition with the exception of serum. The insert contained no serum, whereas the lower well contained 10% FBS that served as a chemoattractant. After 24 h of treatment at 37 °C in a 5% CO_2 incubator, the cells in the insert were removed by wiping gently with a cotton swab. Cells on the reverse side of the insert were fixed and stained with Hema 3 (Fisher Scientific, Hampton, NH, USA) according to the manufacturer's instructions. Cells plated in 24-well plates were subjected to 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays and the cell numbers across the groups were normalized. The number of invading or migrating cells was adjusted accordingly.

5.2.7 siRNA transfections

The siRNA sequences targeting Lyn human mRNA (sense: AAUGGUGGAAAGCAAAGUCCCUU, antisense: GGGACUUUGCUUUCCACCAUUUU;

Sigma-Aldrich, St. Louis, MO, USA) were transfected into HNSCC cells for target silencing. The nontargeting siRNA (D-001210-01, sense 5′-UAGCGACUAAACACAUCAAUU-3′ and antisense 5-UUGAUGUGUUUAGUCGCUAUU-3′; Thermo Scientific Dharmacon) was used as a control. The siRNA or transfections were performed using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. HNSCC cells were transfected with 800 pmol of siRNA or nontargeting control siRNA per 10cm dish. The transfection medium was replaced with complete media after 4-6 h of transfection and cells incubated for 48 hours before plating for invasion and migration assays or harvested for western blotting.

5.3 RESULTS

5.3.1 EGFRvIII expression in engineered HNSCC cell lines

It has been previously reported that EGFRvIII is present in 40% of HNSCC [65, 131]. EGFRvIII is generally lost *in vitro* for unknown reasons, consequently EGFRvIII studies are generally performed on exongenously transfected model systems [68]. We previously reported on the Cal33 cell line stably expressing EGFRvIII [301]. We stably expressed EGFRvIII constructs in 2 other HNSCC cell lines as described in materials and methods (Figure 5.1). To confirm EGFRvIII expression we immunoblotted whole cell lysates with an EGFRvIII antibody and found that EGFRvIII was expressed in each of the two cell lines at distinct levels (Figure 5.1A). We confirmed EGFRvIII expression via RT-PCR as well (Figure 5.1B) and found that both the FaDu and UMSCC1 infected cell lines expressed EGFRvIII mRNA.

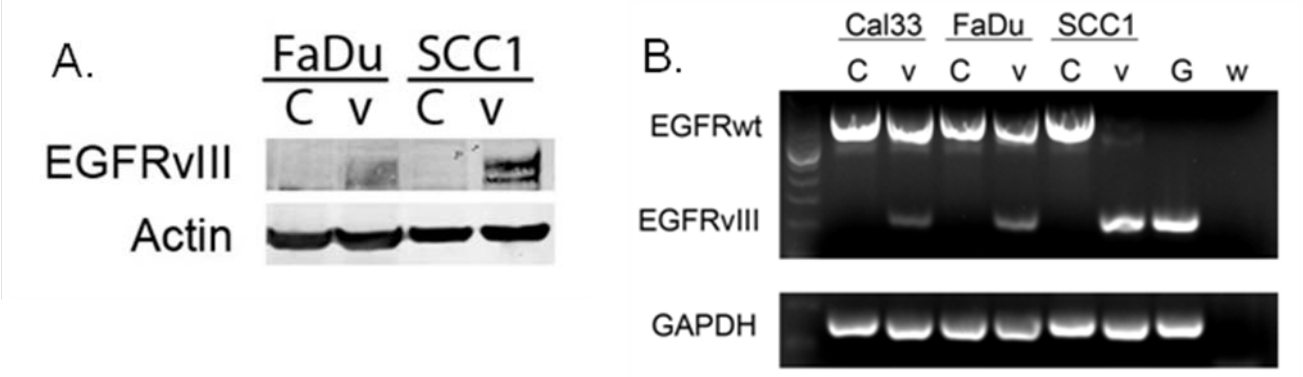


Figure 5.1 EGFRvIII expression in HNSCC cell lines. EGFRvIII was stably introduced into the HNSCC cell lines FaDu and UMSCC1. A) Cells expressing the vector alone (C) or EGFRvIII (v) were probed for EGFRvIII using the 4-5H antibody to demonstrate protein expression of EGFRvIII. Due to the difference in protein expression 80ug of FaDu lysate was loaded, while 40ug of UMSCC1 lysate was loaded. B) HNSCC cells expressing vector alone (C) or EGFRvIII (v) were assayed for EGFRvIII expression via RT-PCR. The glioma cell line U87MG EGFRvIII was used as a positive control (G). A control lane is included to ensure no contamination of reagents (w) which has all reagents added with water instead of transcript.

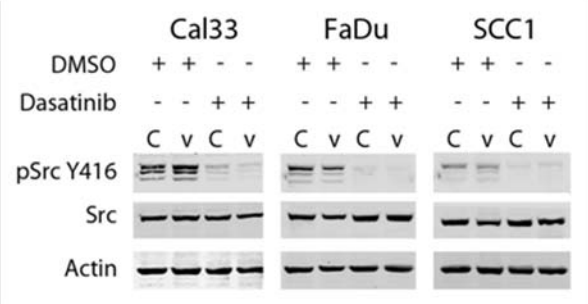
5.3.2 Src family kinases mediate proliferation, invasion and migration in EGFRvIII expressing HNSCC cells

We previously reported that expression of EGFRvIII enhances proliferation *in vivo* and cell motility and invasion *in vitro* [65, 173]. EGFRvIII has been best studied in glioma where SFKs have been shown to be key mediators in EGFRvIII related cell motility and tumor growth [287]. We assessed SFK activation by immunoblotting for phosphorylation at the activation site (Y416). All three EGFRvIII expressing cell lines had phosphorylation at Y416 (Figure 5.2A) which could be inhibited by treatment with the src inihibitor dasatinib (Figure 5.2A). To assess the role of SFKs in EGFRvIII expressing HNSCC cell proliferation we treated cells with dasatinib and assayed for proliferation (Figure 5.2B). We found that dasatinib significantly

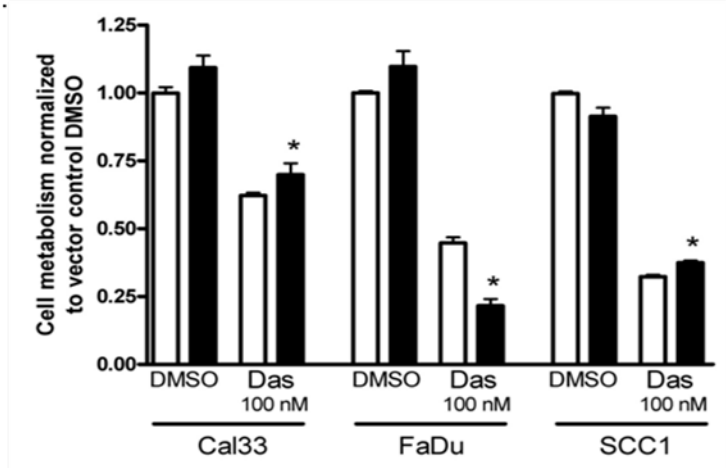
inhibits cell proliferation in vector control and EGFRvIII expressing cells compared to vehicle treated vector control cells.

We have previously demonstrated that EGFRvIII increases cell motility *in vitro* in HNSCC [301]. To determine if SFKs were mediators of cell migration and invasion we treated vector control and EGFRvIII expressing HNSCC cells with dasatinib and assayed for changes in migration or invasion. We found that dasatinib significantly inhibited EGFRvIII mediated HNSCC cell migration (Figure 5.2C) and invasion (Figure 5.2D). Both migration and invasion were also significantly inhibited by dasatinib in vector control HNSCC cells indicating that SFKs are a plausible target in both EGFRvIII-associated HNSCC as well as HNSCC that only expresses wtEGFR.

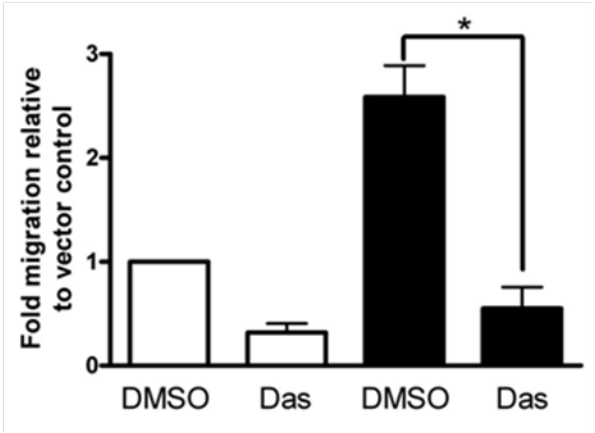
A.


Cal33
FaDu
SCC1
DMSO
Dasatinib
C v C v C v C v C v C v
pSrc Y416
Src
Actin

B.


Cell metabolism normalized to vector control DMSO
1.25
1.00
0.75
0.50
0.25
0.00
DMSO
Das
100 nM
Cal33
FaDu
SCC1

C.


Fold migration relative to vector control
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2
1
0
DMSO
Das
DMSO
Das

D.

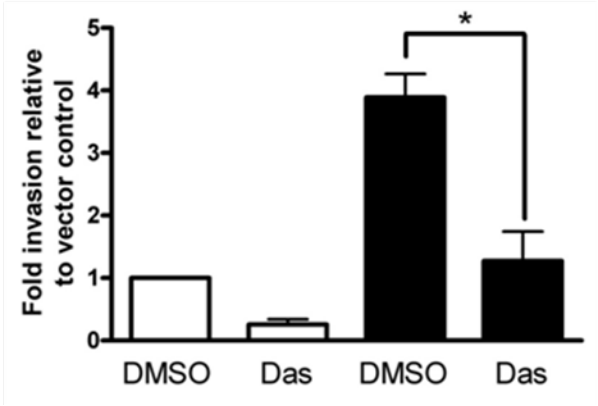

Fold invasion relative to vector control
5
4
3
2
1
0
DMSO
Das
DMSO
Das

Figure 5.2 Src inhibition decreases proliferation, migration and invasion in cells expressing EGFRvIII and EGFRwt. A) Vector control (C) and EGFRvIII (v) expressing clones were cultured for 24 hours in the presence of DMSO or 100nM dasatinib and immunoblotted for src activation via phosphorylation at Y416. B) Cell proliferation was assayed in each HNSCC vector (open bar) or EGFRvIII (closed bar) expressing clone following 72 hours of treatment with DMSO or 100nM dasatinib. We used the Mann-Whitney test to determine statistically significant differences between EGFRvIII expressing HNSCC DMSO and dasatinib treatments; *$p<0.0001$. C) UMSCC1 vector control (open bars) and EGFRvIII expressing (closed bars) cells were assayed for cell motility in the presence of DMSO or 100nM dasatinib (Das). Mann-Whitney test $p<0.001$. D) UMSCC1 vector control (open bars) and EGFRvIII expressing (closed bars) cells were assayed for cell invasion in the presence of DMSO or 100nM dasatinib (Das). Mann-Whitney test $p=0.003$.

Dasatinib is known to inhibit several other kinases in addition to the SFKs [296]. To confirm that SFK inhibition by dasatinib was inhibiting the downstream pathways of SFK signaling involved in cell proliferation and motility we performed immunoblots of dasatinib treated vector control and EGFRvIII expressing HNSCC cells to evaluate phosphorylation of the active sites of MAPK, Akt and FAK. We found that MAPK and AKT were phosphorylated in control treated cells and phosphorylation was significantly inhibited by treatment with dasatinib (Figure 5.3A). With dasatinib treatment FAK had reduced levels of phosphorylation at tyrosine 576/577 and tyrosine 861 which are phosphorylated by active Src. Phosphorylation at the autophosphorylation site tyrosine 397 was unchanged or slightly increased following dasatinib treatment. This site, when authophosphorylated creates a binding site for Src via the SH2 domain of Src which activates src by displacing the inhibitory phosphorylation at Y527 [302] (Figure 5.3B).

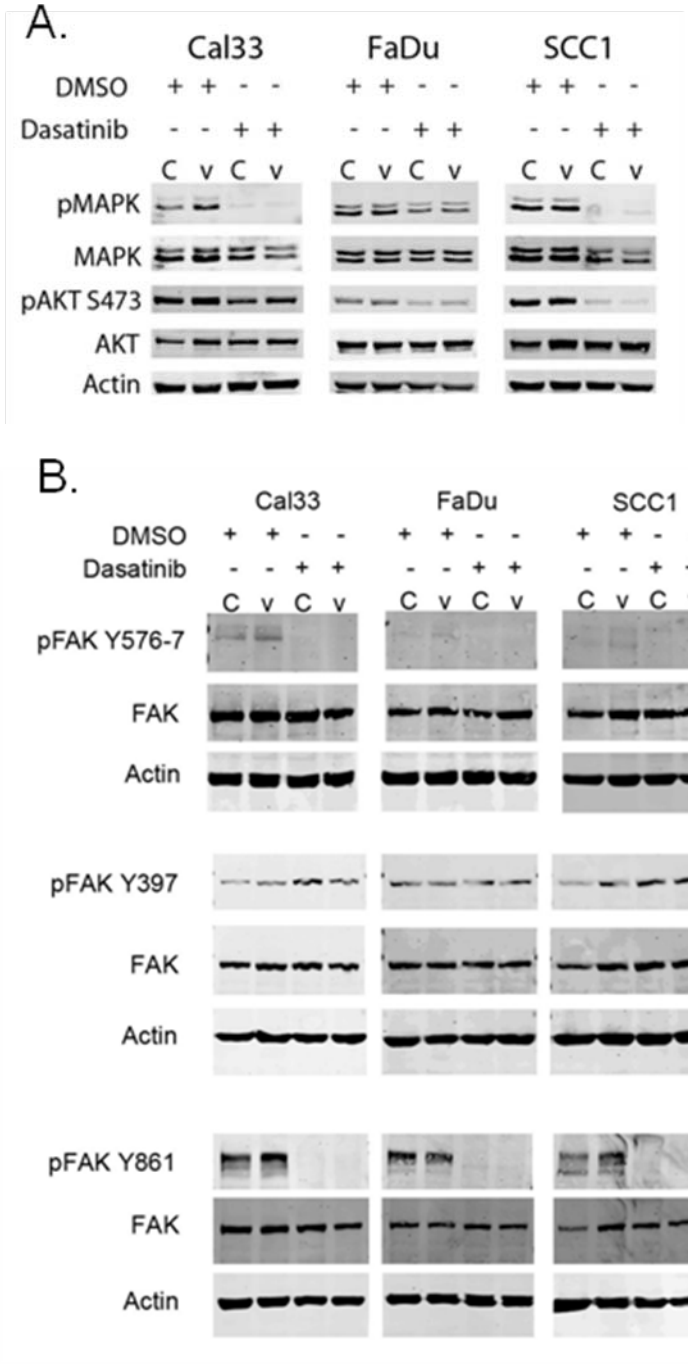


Figure 5.3 Dasatinib inhibits common signaling pathways. Vector (C) and EGFRvIII (v) expressing cells were cultured for 24 hours in the presence of DMSO or 100nM dasatinib and immunoblotted for: A. MAPK and AKT activation via phosphorylation or B. FAK activation via phosphorylation at the Src activating sites Y576/7 and Y861 and the autophosphorylation site Y397.

5.3.3 SFKs mediate tumor growth in EGFRvIII expressing HNSCC xenografts

We previously reported that EGFRvIII expressing HNSCC xenografts proliferate more rapidly than vector control xenografts and show relative insensitivity to cetuximab, the only FDA approved molecular targeted therapy for HNSCC [65]. We hypothesized that SFKs are effectors of EGFRvIII mediated tumor growth in EGFRvIII expressing HNSCC and that inhibition of SFKs would reduce tumor growth. To test this we innoculated nude mice with vector control or EGFRvIII expressing HNSCC and initiated treatment when tumors were visible and of equal volume. Mice were treated with vehicle control or dasatinib to assess the effects of SFK inhibition. Control treated EGFRvIII expressing HNSCC xenografts had significantly higher final tumor volumes than vector control xenografts (Figure 5.4A). In vector control xenografts SFK inhibition failed to significantly reduce tumor volume compared to vehicle control treatment (Figure 5.4B, D). In EGFRvIII expressing xenografts SFK inhibition significantly reduced tumor volume (Figure 5.4C, D), possibly indicating that EGFRvIII is more reliant on SFKs for tumor growth than EGFRwt. To verify that SFKs were durably inhibited by dasatinib treatment we immunoblotted lysates prepared from control and treated xenografts and probed for phosphorylated SFK. We found that phosphorylated SFK at the activation site was significantly decreased in dasatinib treated xenografts expressing EGFRvIII or vector control (Figure 5.4E).

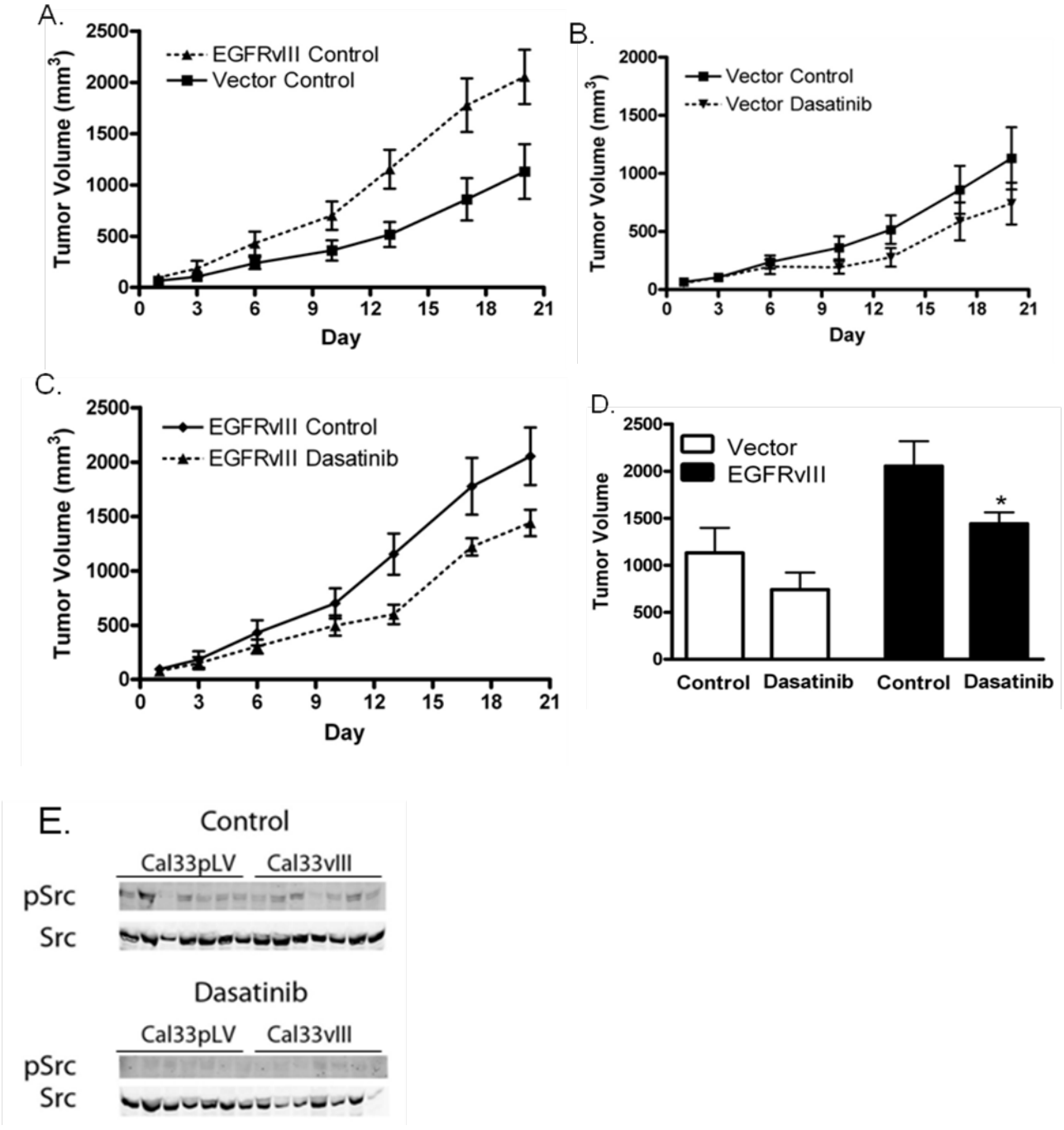


Figure 5.4 Dasatinib inhibits tumor growth in EGFRviII expressing cells *in vivo*. Mice were inoculated with Cal33 cells expressing vector alone (Vector) or EGFRvIII constructs and treated daily with vehicle control (Control) or dasatinib. A) Tumor volumes of Cal33 vector and EGFRvIII expressing cells under control treatment plotted by day (Day 1 = treatment initiation). Final tumor volumes (Day 20) were analyzed for statistical

significance using the Mann-Whitney test; p=0.019. B) Tumor volumes of Cal33 vector expressing cells treated with vehicle control or dasatinib and plotted by day. Final tumor volumes (Day 20) were analyzed for statistical significance using the Mann-Whitney test; p=0.19. C) Tumor volumes of Cal33 EGFRvIII expressing cells treated with vehicle control or dasatinib and plotted by day. Final tumor volumes (Day 20) were analyzed for statistical significance using the Mann-Whitney test; p=0.047. D) Final tumor volumes of vector and EGFRvIII expressing cells (Day 20). Mann-Whitney test EGFRvIII expressing xenografts control treatment compared to dasatinib treatment; p=0.047. E) Immunoblot of xenografts probed for pan-src phosphorylation at Y416.

5.3.4 pSTAT3 is not involved in *in vivo* resistance to SFK inhibition

In HNSCC it has been demonstrated that EGFR, Src and STAT3 physically associate and that Src inhibition leads to a reduction in STAT3 phosphorylation [84]. More recently JAK dependent phosphorylation of STAT3 has been reported to be a feedback mechanism involved in resistance to dasatinib treatment in HNSCC model systems *in vitro* and *in vivo* [303]. We assessed vector control and EGFRvIII expressing xenografts for STAT3 phosphorylation at Y705 via immunoblot and found that dasatinib treated xenografts had reduced levels of pSTAT3 compared to control treated xenografts (Figure 5.5A). We found no significant difference between the levels of STAT3 inhibition in EGFRvIII expressing xenografts compared to vector control xenografts.

To confirm this *in vivo* finding in other model systems we tested *in vitro* phosphorylation of STAT3 following 24 hour SFK inhibition by dasatinib. Dasatinib treated EGFRvIII and vector control expressing cells showed no significant difference in pSTAT3 levels in the 3 cell

lines (Figure 5.5B). Vector control cells showed a more marked trend towards an increase in pSTAT3 levels in 2 of the cells lines (Figure 5.5B: FaDu, SCC1) and no change in one of the cell lines (Figure 5.5B: Cal33). The Cal33 cell line was used for the xenograft experiments that showed inhibition of pSTAT3 with chronic SFK inhibition. These results indicate that the reported pSTAT3 feedback mechanism is not a widespread in HNSCC and that pSTAT3 inhibition may be variable upon treatment with an SFK inhibitor.

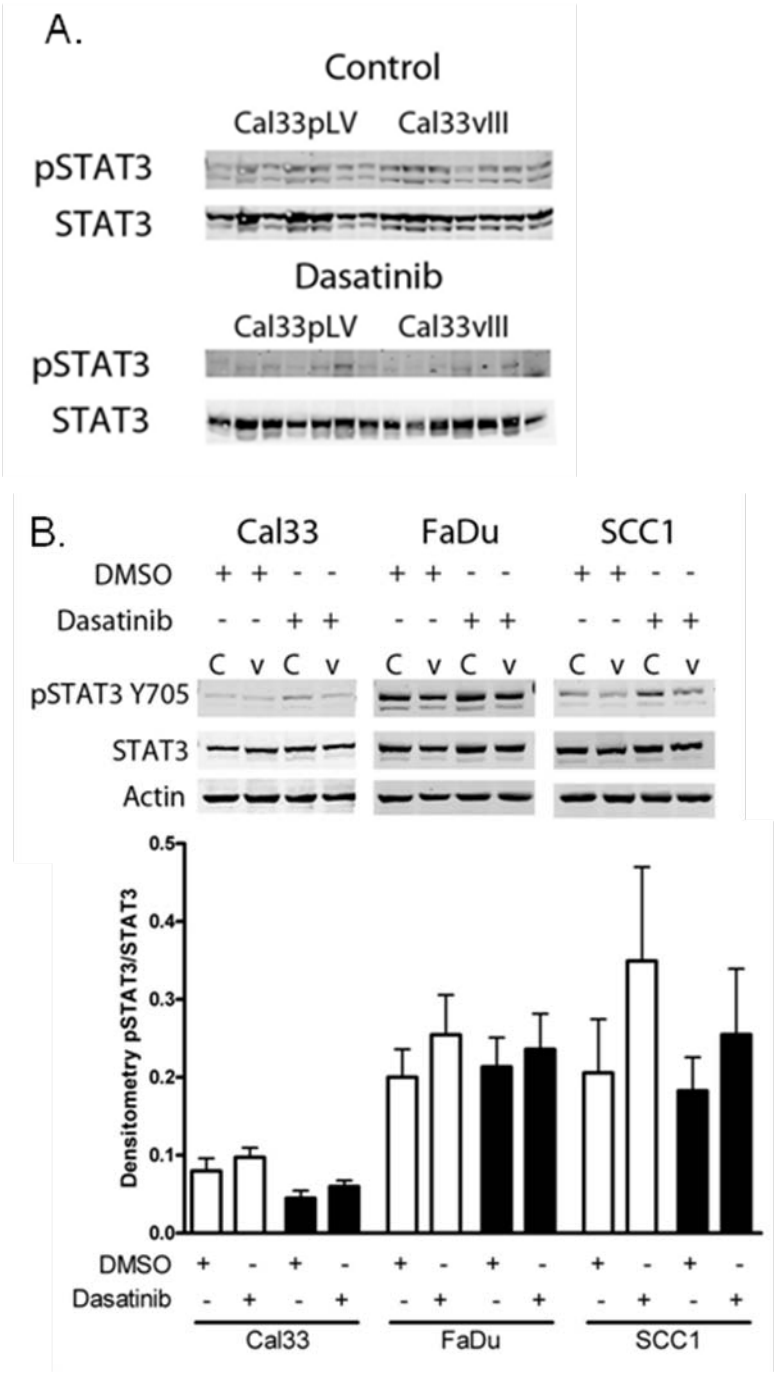


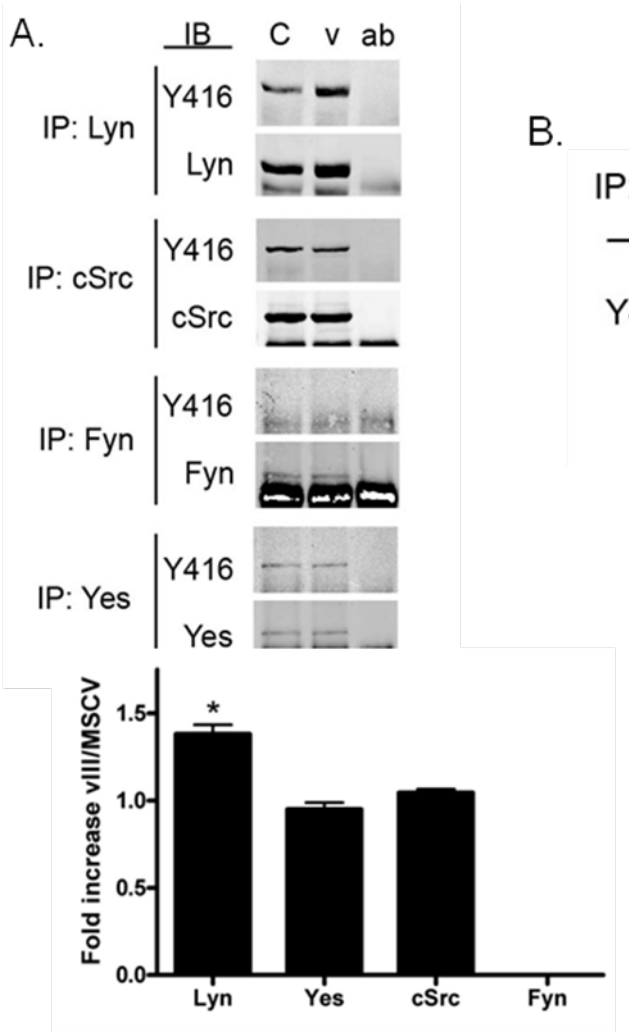
Figure 5.5 pSTAT3 does not appear to be a mechanism of resistance to dasatinib in EGFRvIII expressing cells *in vitro* or *in vivo*. A) HNSCC vector or EGFRvIII expressing xenografts treated with control or dasatinib were immunoblotted for phosphorylated STAT3 (Y705). B) Vector control (C, open bar) and EGFRvIII (v, closed bar) expressing cells were cultured for 24 hours in the presence of DMSO or 100nM dasatinib and

immunoblotted for STAT3 activation via phosphorylation at Y705, densitometry was performed and the results plotted as a ratio of pSTAT3/STAT3 levels . n=3

5.3.5 Lyn may be a key mediator in EGFRvIII expressing HNSCC migration and invasion

Reports in glioma expressing EGFRvIII indicate that Fyn and Src are key effectors of EGFRvIII signaling [287]. We sought to determine which, if any, SFKs were specifically activated in EGFRvIII expressing HNSCC. We immunoprecipitated Lyn, Fyn, Src and Yes and immunoblotted with SFK Y416 and total protein antibodies. We found that Lyn was the only SFK with increased phosphorylation in EGFRvIII expressing cells (Figure 5.6A). Fyn, while expressed, was not phosphorylated and Src and Yes were not differentially phosphorylated. Increased phosphorylation of Lyn in EGFRvIII expressing cell lines was confirmed in two other head and neck cell lines expressing EGFRvIII as well (Figure 5.6B).

We examined the effects of Lyn knockdown on the EGFRvIII phenotype to evaluate the hypothesis that phosphorylation of Lyn is a key signaling intermediate for EGFRvIII mediated invasion and migration. On treating cells with Lyn siRNA we found that total levels of both Lyn and Fyn were reduced. We treated cells for 48 hours with Lyn (Fyn) siRNA (Figure 5.6C) and subjected them to migration and invasion assays *in vitro*. We found that Lyn (Fyn) siRNA significantly reduced invasion and migration in EGFRvIII expressing cells (Figure 5.6D,E) indicating that Lyn (and Fyn) are likely intermediates of EGFRvIII mediated cell motility.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cSrc
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0.0
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Lyn
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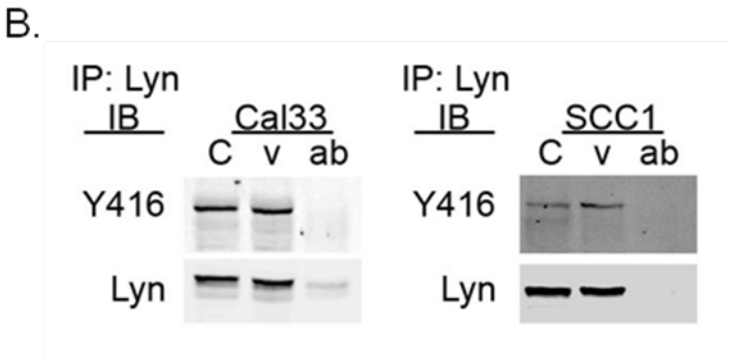
cSrc
Fyn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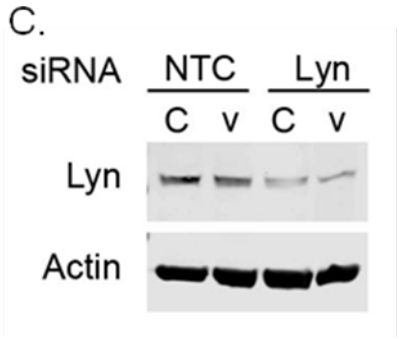
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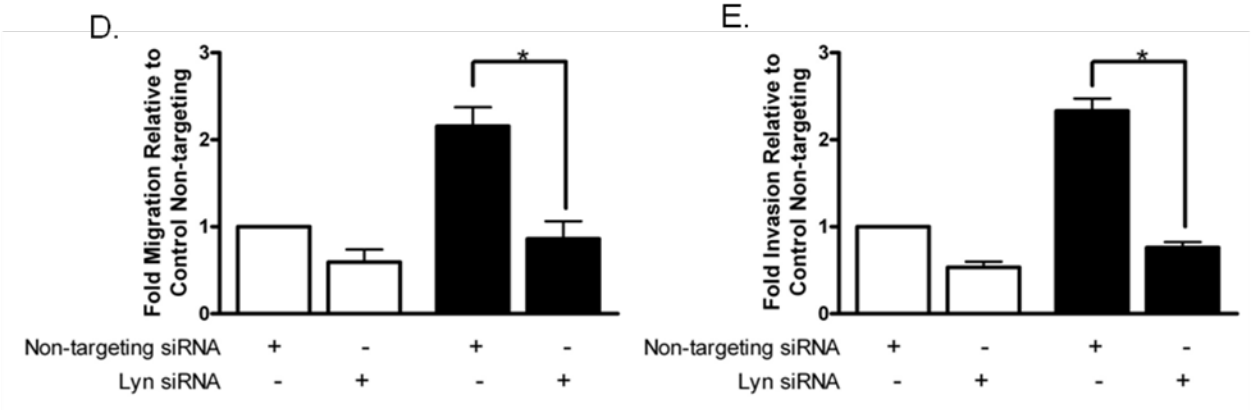
D.
Fold Migration Relative to Control Non-targeting
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Non-targeting siRNA + - + -
Lyn siRNA - + - +

Figure 5.6 Lyn has increased phosphorylation at Y416 in EGFRvIII expressing cells compared to vector control cells. A) FaDu vector or EGFRvIII cells were serum started for 24 hours and harvested for immunoprecipitation (IP) with an SFK antibody as noted. Following IP proteins were immunoblotted for phosphorylation at Y416 and subsequently for total protein. Densitometry was performed. pSFK was normalized to total SFK and graphed as a fold increase in EGFRvIII expressing cells compared to vector only cells. B) Increased phosphorylation of Lyn Y416 was confirmed in Cal33 and UMSCC1 vector and EGFRvIII expressing cells as noted in (A). C) UMSCC1 vector and EGFRvIII expressing cells were treated with Lyn siRNA for 48 hours and immunoblotted to confirm Lyn knockdown. D) UMSCC1 vector control (open bars) and EGFRvIII expressing (closed bars) cells were assayed for cell motility in the presence of non-targeting siRNA or Lyn siRNA. Mann-Whitney test p=0.029. D) UMSCC1 vector control (open bars) and EGFRvIII expressing (closed bars) cells were assayed for cell invasion in the presence of non-targeting siRNA or Lyn siRNA. Mann-Whitney test p=0.029.

5.4 DISCUSSION

EGFRvIII is more tumorigenic than EGFRwt [102, 107, 109-111] despite the fact that there is no difference in the cytoplasmic signaling domain of EGFRwt and EGFRvIII. Differential activation of EGFRvIII signaling pathways compared to EGFRwt has been reported [112-114]. Altered oncogenic phenotypes may be attributed to altered signaling kinetics [116, 117]. Ligand binding to wtEGFR results in rapid receptor internalization, dephosphorylation and degradation or recycling of the receptor [114]. EGFRvIII is constitutively tyrosine phosphorylated at lower levels than ligand stimulated wtEGFR [112]. The lower levels of phosphorylation prevent interaction with the Cbl-SETA-endophilin complex that enables

receptor internalization of the wtEGFR after ligand binding. EGFRvIII is therefore retained at the plasma membrane and continues to participate in oncogenic signaling pathways [116].

EGFRvIII expression is found in 40% of HNSCC [65, 131] and in many other tumor types including glioma, breast, lung [195, 196] and prostate [197]. EGFRvIII has been shown to contribute to increased oncogenicity through various signaling pathways [100, 101]. The mechanism through which EGFRvIII expression increases oncogenicity is incompletely understood.

EGFRvIII has been shown *in vitro* and in patient samples to induce constitutive activation of the PI3K/Akt pathway [119-121]. Blockade of this pathway has been shown to reduce the EGFRvIII enhanced oncogenic phenotype [120, 121]. We have previously demonstrated that in EGFRvIII expressing HNSCC inhibition of the PI3K/Akt pathway reduces cell proliferation but has no effect on cell motility or invasion [301]. MAPK has been reported to be involved/activated in EGFRvIII expressing models [110, 122, 123] as well as independent/not activated in EGFRvIII expressing models [112, 124, 125]. These differences may indicate that MAPK plays a minor and secondary role in the oncogenic phenotype of EGFRvIII in cancer biology.

Both STAT3 and SFKs have been implicated as key mediators in the EGFRvIII oncogenic phenotype. Mellinghoff et al. [126] found significant correlation between activated STAT3 levels and EGFRvIII (but not wtEGFR) in 82 malignant astrocytic gliomas using immunohistochemistry. We have previously shown that STAT3 is required for EGFRvIII

expressing HNSCC motility and invasion [301]. Many human cancers have shown a role for Src in constitutive STAT activation [280-283] and in HNSCC STAT3 is a Src-dependent mediator of EGFR-stimulated growth *in vitro* [284] and decreased apoptosis [77] and increased tumor growth *in vivo*[81].

SFKs have been shown to contribute to invasion in EGFRvIII expressing gliomas [287]. Genetic and molecular inhibition of SFKs reduced invasion of EGFRvIII expressing gliomas [287]. In HNSCC, it has been reported that SFK inhibition through dasatinib treatment reduces cell motility and invasion by regulating downstream cell adhesion molecules such as FAK [90]. We have demonstrated that abrogation of SFK activity through dasatinib significantly decreases proliferation, invasion and cell motility of EGFRvIII and vector control expressing HNSCC cells. We found that AKT and MAPK were down regulated by SFK inhibition as were phosphorylation sites on FAK phosphorylated by SFKs. pFAK Y397, which is the FAK autophosphorylation site and is a docking site for Src appeared to be unchanged or slightly increased with SFK inhibition. Dasatinib treatment of HNSCC cells by Johnson et al. also showed no change in pFAK Y397 levels [90]. This is not suprising as integrins and several RTKs activate this site (Y397) which can then recruit Src [302]. While invasion and cell motility were significantly inhibited by SFK blockade, it appears that there are other mechanisms that contribute to EGFRvIII expressing HNSCC mediated invasion and cell motility as these phenotypes were not completely abrogated. Continued phosphorylation of FAK at Y397 may be one cause of the remaining invasion and migration noted after dasatinib treatment.

In glioblastoma several studies have addressed the effects of altered src activity on EGFRvIII expressing tumors. Genetic disruption of Src (DNSrc) in EGFRvIII expressing glioblastoma xenografts decreased tumor growth rates and significantly increases the efficacy of EGFRvIII specific Ab treatment [83]. Dasatinib has also been used with success in *in vivo* glioblastoma model systems. In an endogenously expressing EGFRvIII *in vivo* model dasatinib has been shown to inhibit growth and increase survival and apoptosis [287].

In HNSCC dasatinib treatment of xenografts has only been reported in the context of downstream signaling markers [303] and has not been evaluated for tumor growth inhibition or abrogation of metastasis [303]. We found that *in vivo* inhibition of SFK signaling by treatment with dasatinib significantly reduced tumor volumes in EGFRvIII expressing cells, but not in vector control cells expressing only EGFRwt . This may indicate that EGFRvIII expressing cells are more reliant on the SFK signaling pathway for proliferation than EGFRwt cells.

In glioma, Fyn and Src have been reported to be preferentially activated over other SFKs and may mediate SFK signaling in this cancer type [287]. Lyn has been implicated as a key signaling mediator in several cancers including prostate [304] , glioma [305], leukemia [306, 307] and breast [308, 309]. To the best of our knowledge a specific role for Lyn in HNSCC has not been investigated. We sought to determine if a specific SFK was the key signaling intermediate for the SFK dependent effects we observed *in vitro*. We found that Lyn had elevated phosphorylation levels in EGFRvIII expressing HNSCC cells compared to vector control cells in three model systems. Unfortunately, the high sequence homology between SFKs can often cause cross reaction of siRNA targeting with other family members. We found that

Lyn specific siRNA crossreacted with Fyn. We used this Lyn (Fyn) siRNA to knockdown Lyn expression and found reduced cell motility and invasion in EGFRvIII expressing HNSCC. This finding may be important in view of the Lyn/BCR-ABL therapeutics in development. Due to the side-effects of SFK/BCR-ABL inhibitors, more specific inhibitors have been under development [310]. The Lyn/BCR-ABL inhibitor bafetinib is being developed in two phase II clinical trials for patients with prostate cancer and B-cell chronic lymphocytic leukemia, and a trial is in progress for patients with brain tumors [311].

We have shown that SFKs are important in EGFRvIII mediated cell proliferation, motility and invasion in HNSCC. We have also shown that SFK blockade is effective at inhibiting tumor growth in EGFRvIII expressing HNSCC but not vector control HNSCC. Lyn may be the primary SFK involved in the increased cell motility and invasion observed in EGFRvIII expressing cells. It appears that SFKs are involved preferentially in EGFRvIII mediated signaling, however, there are also other mechanisms associated with EGFRvIII signaling in HNSCC that remain to be studied. In the future testing of SFK inhibition in an *in vivo* model of EGFRvIII expressing HNSCC metastasis may show greater effects as we show *in vitro* that SFK inhibition has effects on both proliferation and cell motility/invasion.

6.0 GENERAL DISCUSSION

6.1 TREATMENT OF HNSCC

A population-based estimate of 36,540 cases of oral cavity and pharynx cancers and 12,720 laryngeal cancers will occur in the U.S. in 2010 [3]. Standard of care for locally advanced HNSCC has included surgery with curative intent followed by radiation therapy (RT) with or without concurrent chemotherapy (CT) [312]. Despite these efforts, five-year survival rates for cancer of the larynx have remained unchanged from the 1970s and as of 2006 were at 63%. Five-year survival rates for oral cavity cancer increased from 54% to 63% during this time period [3]. Molecular studies to clarify HNSCC progression found EGFR overexpression in up to 90% of cases as a result of gene amplification and transcriptional activation [8, 9]. EGFR overexpression contributes to tumor progression and is an independent predictor of poor prognosis [12]. This dramatic overexpression has led to the development of EGFR-targeted therapies including monoclonal antibodies, tyrosine kinase-specific inhibitors, ligand-linked immunotoxins, and antisense approaches [13]. More recently, the addition of the FDA-approved, EGFR-targeted chimeric antibody therapeutic, Erbitux (cetuximab, C225; Imclone Systems), has been included in regimens for HNSCC treatment following a phase III clinical trial demonstrating improved survival of cetuximab plus RT compared to RT alone [14]. Tyrosine kinase inhibitors of EGFR are also under clinical investigation for HNSCC [313].

While HPV, tobacco use and alcohol consumption are significant risk factors in HNSCC carcinogenesis [314-316] not all people exposed to these risk factors will develop this disease, indicating the presence of underlying genetic predispositions and environmental factors that remain to be elucidated. In the face of the unchanged mortality rates reported, targeted therapies such as cetuximab seemed promising in treating this EGFR overexpressing cancer. Unfortunately, despite the majority of HNSCC overexpressing EGFR, cetuximab combined with radiotherapy (RT) only has a 10% increase in response rate compared to RT alone [14]. Studies are underway to discover the population that will respond best to cetuximab treatment and mechanisms of resistance to cetuximab.

6.2 EGFR IN HNSCC PROGNOSIS

To better determine which tumors will respond to EGFR targeted therapies it is important to better understand EGFR biology and the possible roles for EGFR in patient prognosis. Prior to this work it had been well reported that high EGFR protein levels as determined by IHC were associated with poorer patient prognosis [63, 133, 135, 137, 146]. EGFR gene amplification as detected by FISH had in some studies shown a correlation with worse prognosis [64, 144-146]. Overall, the connection between EGFR gene amplification, mRNA levels and protein levels was minimally explored and poorly understood [64, 144-146]. Additionally, the role of site specific EGFR phosphorylation was relatively unexplored but promised to be important as it would likely be a better indication of the reliance of the cancer on EGFR signaling. We have shown that while EGFR gene amplification may be indicative of patient prognosis, all cases with EGFR

gene amplification also have protein overexpression detected by IHC, and several cases with high EGFR by IHC did not have gene amplification. This is of clinical import for patients as testing for EGFR gene amplification by FISH is expensive and rarely covered by insurance companies, while IHC is routine and generally the costs are covered. We have also shown that EGFR mRNA levels are a poor test for patient prognosis and do not correlate with protein or gene amplification. This discordance is likely due to the labile nature of mRNA. HNSCC surgeries are extensive and often take a full day to perform, following this the tumors must be analyzed by pathology before processing for mRNA, it is therefore not surprising that EGFR mRNA levels may not be as reliable as DNA and protein measures.

Levels of EGFR expression do not correlate to EGFR targeted therapy response [14]. This may not be unexpected as EGFR expression does not necessarily correlate to EGFR dependence in a tumor. It is more likely that elevated signaling through EGFR is indicative of EGFR dependence. In non-small cell lung cancer (NSCLC) activating EGFR mutations have been found to be reliable markers of EGFR targeted therapy sensitivity [317, 318]. These activating kinase domain mutations may be related to cancer cell dependence on the EGFR signaling pathway. While such mutations are rare in HNSCC, it is possible that activity measured via phosphorylation of key sites on EGFR would also be related to efficacy of EGFR targeted therapies. We determined that site specific phosphorylation of EGFR at tyrosine 1068 (Y1068) was correlated with poorer patient survival. Future studies will be needed to evaluate the efficacy of pEGFR Y1068 in EGFR targeted therapy response.

6.3 EGFRVIII EXPRESSION

The kinase domain mutations found in NSCLC patients are not common in HNSCC but the EGFR variant 3 that lacks exons 2-7 has been reported to be present in 40% of HNSCC [65, 131]. EGFRvIII lacks a large portion of the extracellular binding domain and is constitutively active at low levels [116]. EGFRvIII has been shown to confer increased oncogenicity in several tumor types [102, 107, 109-111], but the mechanism of expression is still not clear. One previous study indicated that Alu sequences may be involved in loss of these exons through non-allelic homologous recombination in gliomas with EGFR gene amplification [202]. Analysis of TCGA gene expression and SNP data for 116 glioblastomas revealed that while 9/14 EGFRvIII positive tumors had loss of exons 2-7 at the DNA and RNA levels, 4/14 showed EGFRvIII expression only at the RNA level. In PCR based studies in our laboratory I found that in EGFR gene amplified glioma there was a joining of exons 1 and 8 in pre-mRNA but not in genomic DNA. In HNSCC I did not detect exon 1-8 joining in DNA or pre-mRNA, only at the mRNA level. We additionally screened glioma and HNSCC EGFRvIII positive tumors for alterations in the splice acceptor and donor sites that may indicate causes of alternative splicing. No alterations were found in either tumor type. Together these data may indicate that the mechanisms of EGFRvIII expression are different in GBM and HNSCC. The data in glioma support a role for genomic instability and transcriptional alterations, while the evidence in HNSCC is more suggestive of an alternative splicing mechanism. This is in concordance with IHC evidence from University of Pittsburgh pathologists that indicates that EGFRvIII when expressed in glioma produces strong and homogenous signal in the tumor sections, while in HNSCC tumor sections have heterogenous expression of EGFRvIII and it is present at a lower level (personal communications).

These findings require further investigation to elucidate the mechanisms of expression and support or invalidate these theories with more rigorous methodologies. The determination of these mechanisms is important in the treatment and screening of these tumors. EGFRvIII confers a more oncogenic phenotype *in vitro* and *in vivo* and in further studies we show that it responds differentially to therapeutic agents, consequently EGFRvIII will be an important screen for GBM and HNSCC tumors. Understanding the mechanism of EGFRvIII expression will allow us to develop the most effective and reliable screening assays to better aid in patient treatment regimens.

6.4 TARGETING SIGNALING PATHWAYS IN EGFRVIII EXPRESSING CANCER

Cancer treatments are moving towards personalized therapies that screen patients for molecular markers and treat according to these markers. The most notable of these types of personalized therapies are NSCLC and breast cancer. In NSCLC activating EGFR kinase domain mutations predispose patients to EGFR inhibitor sensitivity [317, 318] and allow physicians to select patients that will benefit most from these treatment regimens. In breast cancer HER2, PR and ER screening are now standard practice and aid in selecting patients for endocrine therapy, trastuzumab or chemotherapy [319]. Unfortunately, cancers are more complex than just one oncogene that can be targeted and acquired resistance to targeted therapies is common. More therapeutic targets are needed to treat cancer effectively.

EGFRvIII is an ideal target as it is not present in normal tissue [320]. Clinical trials testing EGFRvIII vaccines have met with some success and work is underway to improve on the efficacy of these agents [321, 322]. The EGFRvIII specific immunotoxin MR1-1 is currently undergoing phase 1 clinical trial at Duke University in glioblastoma and we are awaiting the results of this pre-clinically promising therapeutic (NCT01009866).

While targeting of EGFRvIII itself is underway, current literature indicates that dual targeting of EGFR and a downstream signaling molecule may be more effective in EGFRvIII expressing cells [83, 287]. EGFRvIII is constitutively active and has been reported to have differential downstream signaling in GBM [112-115, 323]. In GBM targeting SFKs with dasatinib led to increased tumor sensitivity to EGFR monoclonal antibody treatment in a xenograft model. In GBM PI3K is a key effector of EGFRvIII downstream signaling that is differentially regulated compared to EGFRwt only cells and constitutively activated [112, 115, 232]. In HNSCC we noted only a modest increase in pAKT levels and found that they mediated proliferation but not invasion or migration. It appears that the downstream signaling effects of EGFRvIII may vary in different cancer types, underscoring the need for characterization of EGFRvIII differential signaling in each cancer type to understand the therapeutics that may be most effective.

6.4.1 STAT3 in EGFRvIII Expressing HNSCC

STAT3 is constitutively activated in many cancers in addition to HNSCC, including ovarian, breast, prostate, leukemia and lymphoma [324]. Previous studies in GBM implicated STAT3 activation in EGFRvIII signaling but failed to elucidate specific functional pathways for

STAT3 involvement [132, 325]. We are the first group to explore the role of STAT3 in EGFRvIII invasion and migration. We showed that STAT3 is necessary for EGFRvIII mediated HNSCC invasion. This work has potential for clinical application as there are several STAT3/JAK inhibitors currently under clinical development including: the STAT3 specific inhibitor OPB-31121 currently recruiting for a Phase 1 clinical trial in patients with advanced solid tumors at MD Anderson Cancer Center (NCT00955812) and the STAT3 Decoy undergoing a Phase 0 clinical trial in head and neck cancer patients at the University of Pittsburgh (NCT00696176) [326-331]. General STAT3 targeting strategies under development include: peptidomimetics, small molecule inhibitors (against the SH2 and DNA binding domains), antisense RNA, siRNA, and indirect inhibition by inhibiting upstream targets [332]. Our laboratory is actively developing the STAT3 transcriptional decoy that has passed toxicity tests. We showed that the transcriptional STAT3 decoy was effective in abrogating HNSCC invasion in EGFRvIII expressing HNSCC as well as vector control cells and further work should be done in *in vivo* preclinical models to determine the efficacy of STAT3 inhibitors on tumor volume and metastasis in EGFRvIII expressing HNSCC.

6.4.2 Src Family Kinases in EGFRvIII Expressing HNSCC

In addition to STAT3 SFKs are common therapeutic targets. Src is one of the oldest oncogenes and has had several generations of inhibitors developed to target it [250]. GBM has already shown efficacy for SFK targeting alone and in combination with EGFR [83, 287]. We showed that SFKs are an effective target in EGFRvIII expressing xenografts, but not EGFRwt only HNSCC. We also demonstrated that SFKs, in part, mediate EGFRvIII expressing HNSCC invasion and cell motility which may be specifically mediated by Lyn.

For both STAT3 and SFK inhibition tumor volume is likely a poor measure of efficacy as we have shown that *in vitro* both markedly inhibit invasion. Orthotopic xenograft models of metastasis are available in HNSCC and are the next step in testing the efficacy of these treatments alone or in combination with the FDA approved EGFR targeting monoclonal antibody cetuximab.

6.5 AREAS FOR FUTURE STUDY

EGFRvIII was discovered in the late 1980's [333] and much remains to be explored with respect to the biology of EGFRvIII expression, signaling and cancer treatment. Most studies that will need to be performed are a complex combination of patient analysis, in vitro support and preclinical model testing of new therapies. Below are a few areas that need to be further explored in the context of the work we have performed to allow for a more efficacious understanding of EGFRvIII biology and treatment choice.

6.5.1 EGFRvIII and Metastasis

We have described a striking increase in cell invasion in EGFRvIII expressing HNSCC compared to vector control *in vitro* which indicates that EGFRvIII expressing HNSCC in patients may be more metastatic. Few studies have been done on *in vivo* metastasis of EGFRvIII expressing xenografts or patient tumors. A preclinical mouse model of metastasis that utilized renal carcinoma cells engineered to express EGFRvIII or overexpress EGFR found that EGFR overexpressing cells formed more lung metastasis at a faster rate, while EGFRvIII expressing

cells formed larger lung metastasis [334]. In this study no metastasis were found in other sites. In a breast cancer study EGFRvIII appeared to contribute to metastasis as 40% of Her2 positive breast cancers coexpressed EGFRvIII while 75% of Her2 positive lymph node metastases coexpressed EGFRvIII [335]. This limited work on EGFRvIII mediated metastasis *in vivo* is inconclusive and requires validation in other cancer types as well as larger sample sizes. With advances in imaging, xenograft metastasis experiments could be performed utilizing live fluorescence imaging. Established orthotopic xenograft models are available in HNSCC, making this cancer a natural choice for study of these metastasis problems. In HNSCC patients, design of a tissue microarray with metastatic and non-metastatic tumors subsequently probed with EGFRvIII antibodies would provide evidence for the hypothesis that EGFRvIII increases metastasis in patient tumors. An important limitation of this study is that patients that present for HNSCC resection generally have late stage disease and this TMA may be biased if EGFRvIII is a late event in HNSCC progression. A study of EGFRvIII incidence in the setting of HNSCC metastasis would be novel and timely as EGFRvIII vaccines are beginning to expand into cancer types beyond GBM.

Should the hypothesis that EGFRvIII promotes metastasis *in vivo* prove to be correct there are several *in vitro* studies that would naturally result. Some work has been done *in vitro* to characterize potential metastasis mechanisms despite the lack of *in vivo* evidence. In breast cancer EGFRvIII expressing cells exhibit increased CXCR4 expression and enhanced CXCR4 mediated invasion *in vitro* [336]. CXCR4 has previously been implicated in breast cancer metastasis where it directs CXCR4 expressing cells to regions rich in the CXCR4 ligand

(CXCL12) which include lung, liver, bone marrow and lymph nodes [337]. This chemotactic mechanism would need further validation in *in vivo* model systems of HNSCC.

In vitro evidence for a role for EGFRvIII in epithelial to mesenchymal transition came from work in ovarian cancer. This study demonstrated that cells exongenously expressing EGFRvIII had increased N-cadherin and vimentin with decreased E-cadherin, keratins and mucins compared to vector control cells [338]. Other molecules possibly associated with metastasis and shown to be upregulated in EGFRvIII expressing cells include tissue factor [339] and EphA2 [340].

6.5.2 EGFRvIII Cross-talk

EGFRvIII cross-talk with other receptors has been minimally explored in the setting of EGFRvIII and has not been addressed in EGFRvIII expressing HNSCC. In GBM the activating phosphorylation site of c-Met was found to be phosphorylated in correlation with increasing levels of EGFRvIII receptor. *In vitro* cell viability assays found that the combination of a c-Met inhibitor and an EGFR inhibitor or cisplatin resulted in enhanced cytotoxicity in EGFRvIII expressing cells compared with either compound alone [115].

In breast cancer indirect evidence for heterodimerization with Her2 has been demonstrated. Coexpression of EGFRvIII and Her2 has been identified in a subset of tumors [108, 335]. *In vitro* coexpression of EGFRvIII and Her2 enhances Her2 activation and increases signaling through downstream pathways [335].

6.6 CONCLUSIONS

HNSCC is a disease currently characterized by high mortality and morbidity rates that remain unchanged. Overexpression of EGFR appeared promising for EGFR targeted therapies such as cetuximab but unexpected mechanisms of resistance have emerged making patient selection for these inhibitors important. We have shown that in HNSCC EGFR levels as detected by IHC are currently the best prognostic indicator and pEGFR Y1068 levels may be of importance in determining sensitivity to EGFR targeted therapies. EGFRvIII is a mechanism of EGFR targeted therapy resistance in HNSCC and is likely a splice variant in HNSCC but not in GBM. These expression differences will be of importance in determining the best clinical screens for the presence of EGFRvIII expression as EGFRvIII targeted therapies and appropriate combination therapies are determined. In EGFRvIII expressing HNSCC both STAT3 and SFKs (specifically Lyn) are appropriate therapeutic targets that are involved in EGFRvIII mediated invasion. While these are important therapeutic targets, it appears that there are also other pathways involved in EGFRvIII mediated oncogenic phenotypes and further investigation of EGFRvIII signaling and crosstalk is needed. *In vivo* validation via preclinical and clinical models is needed for STAT3 and SFKs with emphasis on metastatic outcomes.

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