FGFR3 REGULATES EGFR MEDIATED LIGAND INDEPENDENT ACTIVATION OF c-MET IN NON SMALL CELL LUNG CANCER.

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ABSTRACT:

Lung cancer is the leading cause of cancer deaths in the world. More than 85% of these cases are classified as Non small cell lung cancer (NSCLC) which has a dismal 5-year survival rate of 15% in addition to which most patients are diagnosed at an advance stage. Receptor tyrosine kinases are high affinity cell surface receptors that are often deregulated in a host of cancers including NSCLC thereby making them attractive targets. RTK inhibitors in the market however are facing the growing problem of resistance owing to compensatory kinases that compensate for the loss of the original oncogenic driver receptor. While attempting to identify compensatory kinases in NSCLC cell lines we observed a unique interaction between three RTKs- the Fibroblast growth factor receptor 3 (FGFR3), the Epidermal growth factor (EGFR) and mesenchymal epidermal transition (c-Met). We were able to show that upon inhibition of FGFR3 by a small molecule pan FGFR inhibitor LY2874455 there was complete attenuation of c-Met phosphorylation. Previous studies and our own observations led us to believe that c-Met activation occurred in a ligand independent manner which has previously been shown to be mediated by EGFR. EGFR phosphorylation was decreased on treatment with LY 2874455 along with a subsequent decrease in the levels of the EGFR ligand-Amphiregulin. The combined effect of LY2874455 with a c-Met inhibitor, Crizotinib, showed increased cell death as compared to either drug alone. This interaction was seen in the FGFR1 non amplified cell lines thereby suggesting that the use of this FGFR based therapy can be used in patients lacking the said amplification. We therefore have substantial evidence linking the FGFR3, EGFR and c-Met pathways suggesting that inhibition of FGFR3 inhibits the ligand independent activation of c-Met as regulated by EGFR.

TABLE OF CONTENTS:

TABLE OF FIGURES:

Chapter 1: Introduction

Chapter 2: Results

TABLE OF FIGURES

INTRODUCTION

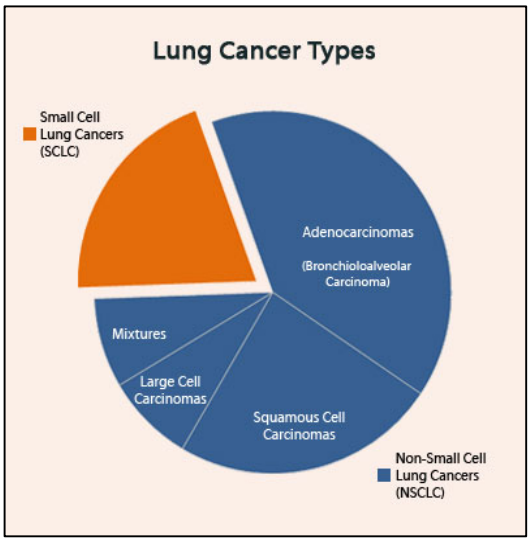
LUNG CANCER: EPIDEMIOLOGY AND RISKS

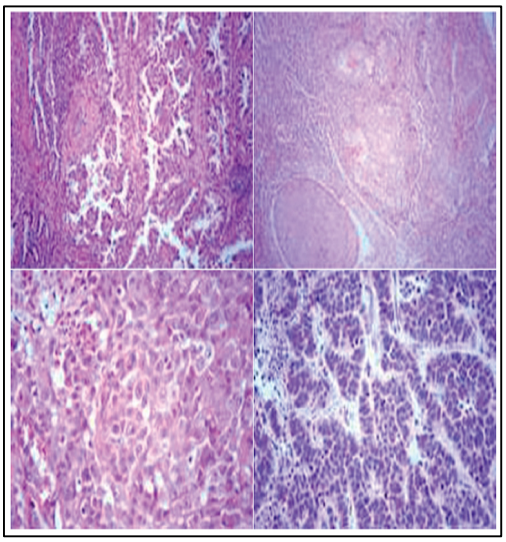
According to the World Cancer Report 2014, Cancer is one of the foremost causes of mortality in the world that accounted for 8.2 million deaths in 2012 out of which lung cancer caused a staggering 1.59 million deaths thereby making it the leading cause of cancer related deaths worldwide. Lung cancers are carcinomas- malignancies that arise from epithelial cells and are classified by the size and appearance of the malignant cells. Risk factors include cigarette smoking, passive smoking, alcohol, air pollution and exposure to carcinogens like crystalline silica [1]. Lung carcinomas are histologically divided into two major subtypes: small cell lung cancer (SCLC) and non small cell lung cancer (NSCLC), the latter being relatively chemo-resistant.

20% of lung cancer cases are classified as SCLC which are more aggressive and are generally treated non surgically [2]. Greater than 85% of lung cancer cases, as of now, are classified as NSCLC which has a poor predicted five-year survival rate of 15.9%. The three main histological sub types of NSCLC are- adenocarcinomas (ADC; ~50%), squamous cell carcinoma (SCC; ~40%) and large cell carcinoma (LCC); **(Fig 1 A and B)**. ADC arise in the distal airways and are less likely to be associated with smoking and chronic inflammation while SCC arise in proximal airways and are more associated with smoking and chronic inflammation. SCC appear to be more aggressive as compared to ADC, the latter being a slower-growing tumor although capable of metastasizing early. The third subtype, LCC, is

identified if the tumor cells appear to be neither glandular nor squamous or do not express the biomarkers for ADC and SCC [3].

A





B

FIG 1: (A) Lung cancer subtypes. (B) Histological subtypes of Lung cancer**: Top left**: Adenocarcinoma; **Top right:** Squamous cell carcinoma; **Bottom left:** Large cell carcinoma; **Bottom right:** Small cell carcinoma. *(Lung Cancer: Peter J. Mazzone, Humberrto K. Choi, Duc Ha; Published: March 2014; Cleveland Clinic.)*

Surgical intervention remains the foremost option in patients diagnosed with lung cancer. However, this line of treatment remains limited only to patients who can tolerate the surgery and whose tumor is completely resectable. Chemotherapy is the treatment of choice for about 70% of lung cancer patients with locally advanced or metastatic disease at the time of diagnosis. The widely accepted standard of care for metastatic lung cancer is Platinum-based chemotherapy [1].

Major signaling and regulatory pathways undergo alterations owing to overexpression or gene sequence variation. Such alterations are observed quite frequently in lung cancer. During tumor pathogenesis receptor tyrosine kinases (RTKs) that transduce growth stimulating signals into the interiors of the cell are also deregulated. Receptor over expression makes the cell hyper responsive to normal levels of growth factors that would otherwise not trigger cell proliferation or in other words, render the kinases oncogenic. Overexpression of these receptors can further contribute to ligand independent signaling. The cells therefore start relying on these abnormally activated proteins for survival and growth; a phenomenon called oncogene addiction [4]. Targeted therapy against such aberrant RTKs such as epidermal growth factor receptor (EGFR), fibroblast growth factor receptor (FGFR), the mesenchymal epidermal transition (c-MET) etc. results in an improved response rate in selected group of patients in NSCLC that harbor specific mutations [1]. For example- Exon 19 and exon 21 substitution accounts for 90% of EGFR+ NSCLC [5], ROS1 rearrangements occur in 1-2% NSCLC patients [6], MET amplification is oncogenic in 3-7% NSCLC patients [7] etc **(Fig 2)**.

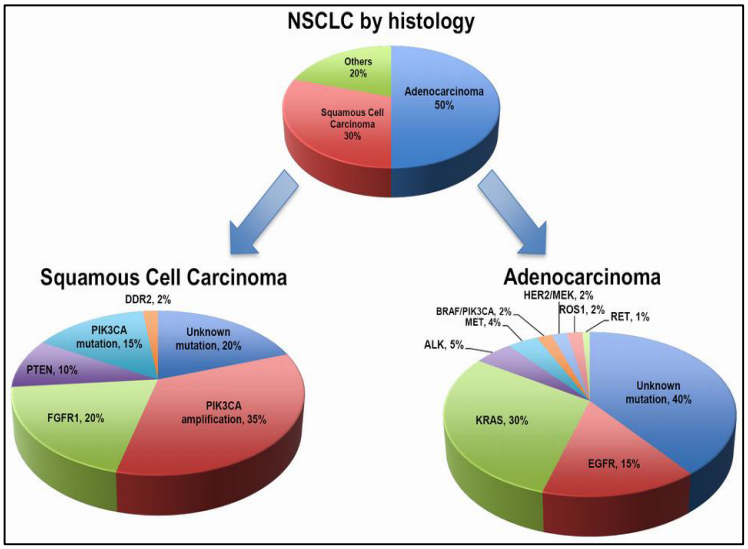


FIG 2: Oncogenic driver mutations in NSCLC subtypes.

COMPENSATORY KINASES AS A MEANS TO OVERCOME TKI RESISTANCE:

In spite of showing high clinical activity initially the use of RTK inhibitors in the treatment of cancer is being hindered due to the growing prevalence of resistance. An accepted theory of resistance is the role of compensatory kinases **(Fig 3)** where in the dependence on either one is compensated by the activation of the other. For example, acquired resistance to EGFR inhibitors gefitinib and erlotinib in non small cell lung cancers carrying activating EGFR mutations due to loss of EGFR dependence through activation of the RTK MET [8], [9]. Met inhibitor-driven resistance can be rescued by inactivating FGFR by small molecules [10], [11]. Enhanced expression of FGFR1 and FGF2 plays an escape mechanism for cell survival of EGFR inhibitor resistant cell lines [12].

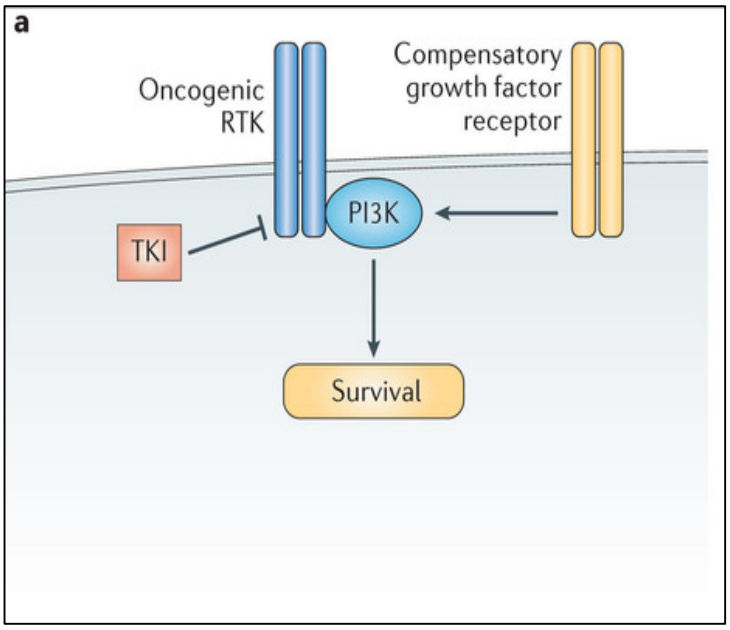


FIG 3: General mechanism of how compensatory kinases function wherein one RTK compensates for the loss or inhibition of the other thereby driving tumor growth.

FGFR SIGNALING PATHWAY:

FGFRs are transmembrane RTKs involved in proliferation, differentiation, survival, migration and angiogenesis. There has been several compelling evidence suggesting the oncogenic potential of abnormal FGFR signaling thereby making it an attractive target for cancer treatment and mitigation. Structurally, FGFRs have three extracellular immunoglobulin like domains and one intracellular tyrosine kinase domain **(Fig 4)**. While only four FGFRs are known (FGFR 1-4), there exist multiple FGF genes that encode 22 functionally distinct ligands. FGFS are secreted glycoproteins that are easily sequestered by heparin sulfate proteoglycans (HPSG) that stabilize the FGF-FGFR interaction by protecting the complex from protease mediated degradation. Binding of an FGF to an FGFR results in receptor dimerization and transphosphorylation of the tyrosine kinase domain. Activation of downstream signaling pathway occurs via intracellular receptor substrates such as FGFR substrate 2 (FRS2) and phospholipase Cγ (PLCγ) rsulting in an upregulation of RAS/mitogen activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K)/AKT signaling pathways [13].

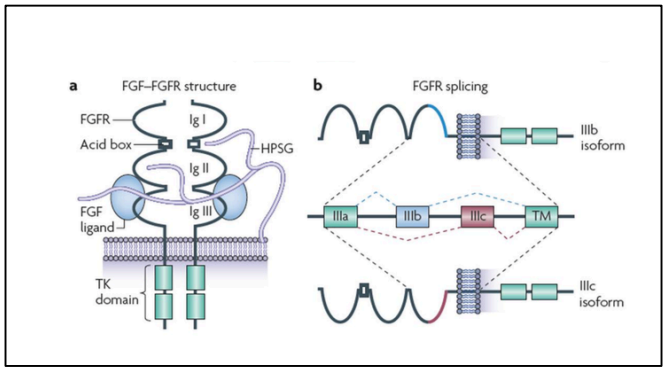


FIG 4: FGFR structure and alternative splicing. **a.** Basic structure of an FGFR-FGF complex consisting of an FGFR dimer, two FGFs and one heparan sulphate proteoglycan(HSPG) chain. **b.** Ligand binding specificity is determined by alternative splicing of the of the Ig III domain.

Alternative splicing of the extracellular domains of RTKs determines ligand binding specificity **(Fig 5).**

Receptor	ligands
FGFR1 IIIb	FGF1, FGF2, FGF3, FGF10, FGF22
FGFR1 IIIc	FGF1, FGF2, FGF4, FGF5, FGF6, FGF8, FGF9, FGF16, FGF17, FGF18, FGF20, FGF21, FGF23
FGFR2 IIIb	FGF1, FGF3, FGF7, FGF10, FGF22
FGFR2 IIIc	FGF1, FGF2, FGF4, FGF5, FGF6, FGF8, FGF9, FGF16, FGF17, FGF18, FGF20, FGF21, FGF23
FGFR3 IIIb	FGF1, FGF9, FGF16
FGFR3 IIIc	FGF1, FGF2, FGF4, FGF5, FGF6, FGF8, FGF9, FGF16, FGF17, FGF18, FGF20, FGF21
FGFR4	FGF1, FGF2, FGF4, FGF5, FGF6, FGF8, FGF9, FGF16, FGF17, FGF18, FGF19, FGF20, FGF21, FGF23

Table 1.1 FGF-FGFR binding specificity.

FIG 5: Ligand binding specificity of the FGFR family. (*Mechanisms underlying distinct EGFR versus FGFR3 and -1 dependency in human bladder cancer cells; Tiewei Cheng)*

Abnormal FGFR signaling contributes to carcinogenesis in three ways:

1) **<u>Molecular aberrations in the FGFR signaling pathway that contribute as driver mutations for oncogenesis:</u>**
    - **FGFR fusion genes:** Several fusion genes play a vital role in driving cancer and thus make attractive targets for treatment. Examples include EML4-ALK with crizotinib in lung

adenocarcinoma and BCR-ABL1 with imatinib in chronic myeloid leukemia.

- **FGFR-activating mutations:** Such mutations cause abnormal FGFR signaling either by increased activation of the kinase domain, ligand independent dimerization of the receptors or altered affinity of the FGF ligands.
- **FGFR overexpression:** Overexpression of FGFRs may cause ligand independent signaling and is caused due to focal amplifications. FGFR1 amplification occurs in about 7%-20% of squamous non small cell lung carcinomas and has been associated with sensitivity to FGFR inhibitors in preclinical *in vivo* models.
- **Aberrant autocrine/paracrine signaling loops:** Essentially these autocrine and paracrine signaling loops result in increased release of FGFs thereby contributing to cell proliferation, survival and angiogenesis.

2) **FGFR signaling pathway and tumor angiogenesis:** Activation of FGFR1 and FGFR2 plays a critical role in the development and maintenance of tumor vasculature. Indirect proangiogenic effects of FGFR signaling involve the activation of vascular endothelial growth factor receptor (VEGFR) thereby promoting neoangiogenesis.

3) **FGFR signaling pathway contributes to resistance against anti cancer therapy:** This signaling pathway contributes to resistance against anti cancer regimen by crosstalk with other oncogenic pathways [13].

There is compelling evidence that suggests the role of abnormal FGFR signaling in the initiation and progression of cancer. The emergence of resistance against small molecule FGFR inhibitors owing to maintenance of downstream signaling molecules by alternative kinases in spite of sustained inhibition of the original drug target **(Fig 6)** highlights the need for the development of multi drug regimens to address the compensatory kinase mechanisms as one of the main drivers for resistance.

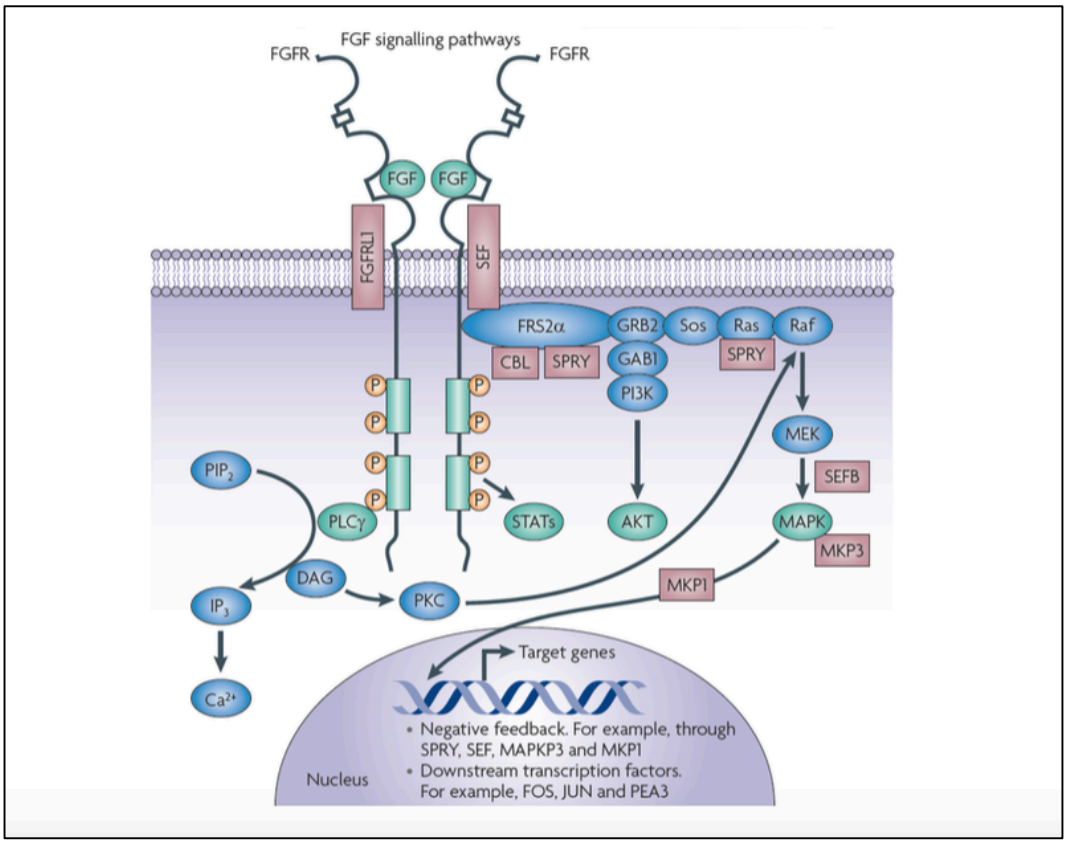


FIG 6: FGFR signaling

C-MET SIGNALING PATHWAY:

FGFRs have been shown to compensate for MET inhibition thereby acting as rescuers for MET inhibitor driven resistance[10], [11]. The c-MET receptor is a cell surface RTK involved in embryonic development, organogenesis and wound healing. Upon activation by it's ligand the hepatocyte growth factor (HGF) it activates a wide range of signaling pathways involving those responsible for proliferation, migration, motility and invasion. Structurally c-MET has three extracellular domains and one intracellular tyrosine kinase catalytic domain flanked by a distinct juxtamembrane and carboxy terminal sequence. Activation of c-Met activates MAPK through the activation of RAS and RAF. MAPK then translocates to the nucleus and activates a host of transcription factors responsible for regulating a large number of genes. Chromosomal rearrangement that causes the tyrosine kinase domain to fuse with an upstream translocating promoting region (TPR) causes constitutive dimerization and hence activation of the encoded protein. The expression of TPR-MET in transgenic mice resulted in the development of multiple tumors [14]. Amplification of the c-MET gene has been reported in a number of human primary tumors. Non small cell lung carcinomas with an acquired resistance to EGFR inhibitors tend to show amplifications in MET [15],[16].

EGFR SIGNALING PATHWAY:

The human EGF receptor family encompasses four members which belong to the ErbB lineage of proteins and have the following designations: 1) EGFR/HER1/ERBB1 2) HER2/ERBB2/NEU 3) ERBB3/HER3 4) ERBB4/HER4

[17]. According to cDNA analysis the the primary structure of EGFR consists of an extracellular domain that is divided into four parts. Domains 1 and 3 participate in ligand binding and domains 2 and 4 participate in disulphide bond formation. In addition to the extracellular domain the receptor also contains a single hydrophobic transmembrane segment and an intracellular domain that has protein kinase activity [18] **(Fig 7)**. ErbB activating ligands or growth factors include- Amphiregulin, Betacellulin, EGF, Epigen, Epiregulin, HB-EGF, TGF α and NRG 1-4. Out of these, seven bind to EGFR, none bind to HER2, two bind to HER3 and seven bind to HER4. HER3 is kinase impaired. The ErbB family functions as dimers or higher oligomers. HER2 fails to bind to any ligand and thus does not form a functional homodimer [17]. However, unphysiological overexpression of HER2 leads to the formation of a functional homodimer [19]. Once the activated dimerization state has been achieved due to ligand binding, the kinase domain catalyzes the phosphorylation of additional tyrosine residues resulting in the activation of protein kinase and in addition to this creates docking sites for adaptor proteins or enzymes that result in downstream signaling [17]. The ErbB/HER signaling involves a host of interconnected and overlapping modules [20] including the PI3K/AKT pathway which is involved in mediating cell survival, the RAS/Raf/MEK/ERK1/2 pathway and PLC γ pathway, both of which participate in cell survival [21].

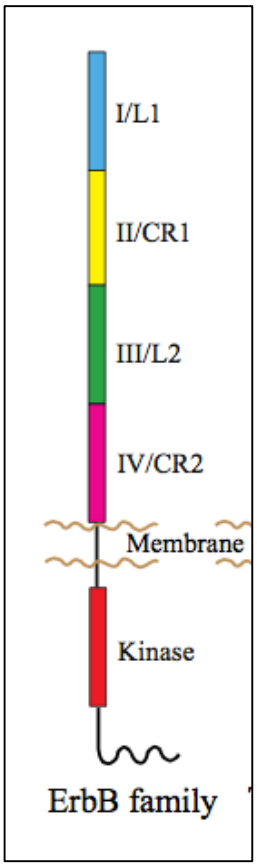


FIG 7: EGFR structure.

There has been significant evidence linking EGFR activation to c-Met [16]. Deregulated HGF/c-Met signaling has been implicated in a host of cancers including NSCLC [22],[23],[24]. Studies have indicated that delayed activation of c-Met is initiated by wild type EGFR. EGFR ligands increased c-Met expression and phosphorylation of vital c-Met residues. This activation requires gene transcription and phosphorylation of c-Met by EGFR occurs without the secretion of HGF or any other secreted factor suggesting that this is a ligand independent mechanism. Kinase activity of both the receptors is required and c-Src pathway is essential for c-Met EGFR communication. Inhibition of EGFR-c-Src-c-Met axis might be effective in the treatment of NSCLC [25].

Overexpression of EGFR occurs in about 60% of NSCLCs [26]. The high percentage of increased EGFR levels in NSCLC cases led to the development of EGFR inhibitors like gefitinib, erlotinib and afatinib all of which form a complex with the EGFR's ATP binding site. While gefitinib and erlotinib are reversible inhibitors of EGFR, afatinib is an irreversible inhibitor of the same [17]. Afatinib is an irreversible covalent inhibitor of EGFR and ErbB2 (HER2) and is the approved first line of treatment for NSCLC patients positive for EGFR mutations. Such multi kinase inhibitors have been developed for overcoming the growing problem of resistance to RTK inhibitors. Afatinib resistant cells showed- down regulation of EGFR proteins and their phosphorylated molecules, up regulation of FGFR1 and its ligand FGF2 and sensitivity to a small molecule FGFR-TKI. Thus

proving that FGFR1 activation acts as a compensatory mechanism in PC9 cells which are a NSCLC adenocarcinoma cell line resistant to Afatinib [12].

AMPHIREGULIN:

Amphiregulin (AREG) is a ligand for EGFR **(Fig 8)** and modulates a host of cell functions like proliferation, apoptosis and migration. The ligand has been found to be over expressed in a host of cancers and has been identified as a pro oncogenic factor. Owing to it's participation in cell migration and invasion it can be used as a predictive marker for metastasis [27]. AREG expression has been linked to the development of resistance to conventional chemotherapeutic agents such as doxorubicin as well as multikinase inhibitors such as sorafenib [28].

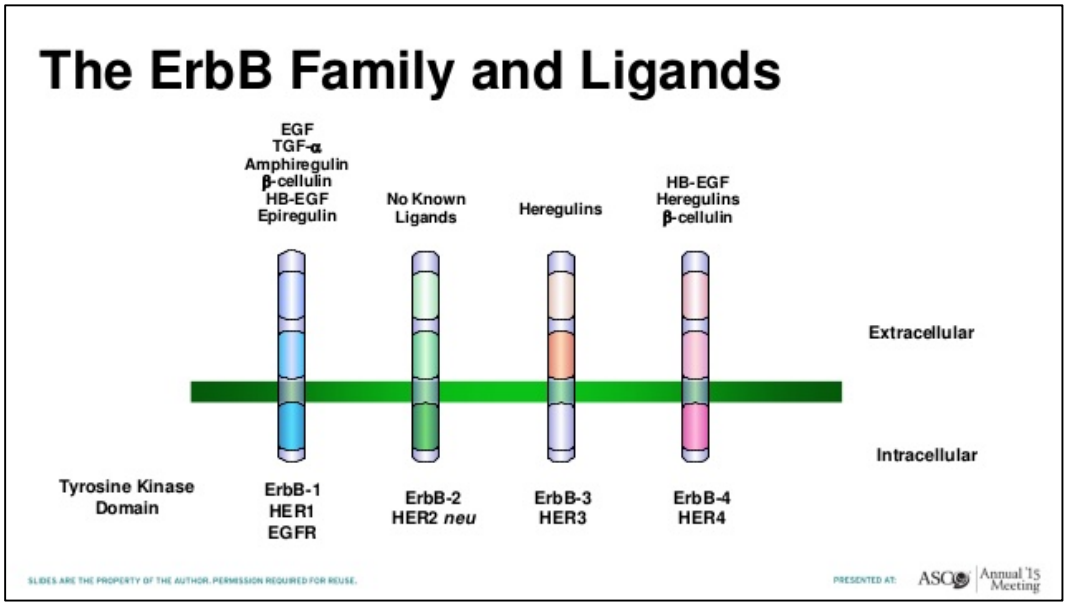


FIG 8: EGFR family and their respective ligands.

LY2874455 :

LY 2874455 **(Fig 9)** is a pan FGFR inhibitor with a significantly lower activity against the Vascular Endothelial Growth Factor receptor (VEGFR)-2. It is more efficacious against cancer cell lines that have increased FGFR or FGF expression as opposed to those with little or no FGFR expression. It has been shown to be an extremely potent FGFR inhibitor *in vivo* and this inhibition has been correlated with its inhibition of tumor growth. The molecule further lacks side effects like hypertension at it's efficacious dose. LY2874455 therefore is a good alternative for patients in whom anti-VEGF/VEGFR- based treatment is contraindicated [29].

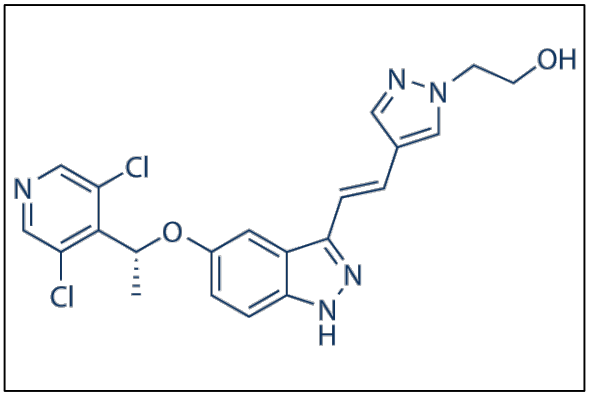


FIG 9: LY 2874455; a pan FGFR inhibitor

CRIZOTINIB:

Crizotinib **(Fig 10)** is a small molecule and a highly potent competitive inhibitor of ALK and c-Met. It inhibits cell proliferation associated with G1-S-phase cell cycle arrest [30]. The drug is generally well tolerated and the adverse effects are generally moderate. The visual disorders generally disappear after discontinuation of

Crizotinib therapy [31],[32]. However, a majority of patients relapse during the first year of treatment and develop resistance to Crizotinib therapy [33].

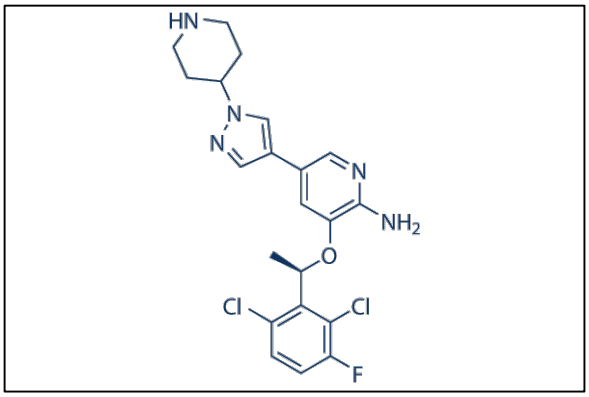


FIG 10: Crizotinib; a c-Met (IC50: 8 nM) and ALK inhibitor (IC50: 20 nM)

Aberrant FGFR, EGFR and c-Met signaling have been shown to promote cancer progression. Resistance to Met inhibitors can be rescued by inactivating FGFR[34], resistance to EGFR inhibitors like afatinib can be rescued by inactivating FGFR1 and Met [12], [35]. This suggests that there is significant cross talk between the three receptors.

Our first goal was to examine possible compensatory kinases that are activated upon FGFR inhibition. We used two wild type NSCLC cell lines- 201T (Adenocarcinoma) and 273T (Squamous cell carcinoma) and two FGFR1 amplified cell lines- H1703 (Squamous cell carcinoma) and H520 (Squamous cell carcinoma). Western blots showed that all four cell lines had the presence of FGFR3, FGFR4, EGFR and c-Met. We were unable to demonstrate that neither

EGFR nor c-Met were activated after FGFR inhibition with the LY 2874455 contrary to our expectations. We observed complete inhibition of c-Met phosphorylation relative to the control in the FGFR non amplified cell lines. We were able to prove that the LY2874455 itself does not directly block the c-Met receptor. Lack of HGF production by 201T and 273T cell lines lead us to believe that c-Met activation is occurring in a ligand independent manner. The possibility of HGF independent phosphorylation of c-Met by EGFR led us to study the effects of LY2874455 on the levels of phosphorylated molecules of the EGFR family. In both the 201T and 273T cell lines we observed that on treatment with LY2874455 there was a decrease in phosphorylated molecule of EGFR. We have been able to show that LY2874455 by itself does not inhibit the phosphorylation of EGFR. ELISA performed post treatment with LY2874455 showed a decrease in the levels of Amphiregulin, a prominent ligand of EGFR, in both cell lines at 4 hours post treatment. LY2874455 was also observed to inhibit phosphorylation of FGFR3. Combination of LY287445 with a c-Met inhibitor, Crizotinib, showed decreased cell growth as compared to the individual drug treatments.

RESULTS:

To measure basal RTK expression levels in both FGFR1 amplified and non amplified cell lines

In order to characterize the basal expression of nine RTKs we chose to draw a comparison between two FGFR1 amplified cell lines (H520 and H1703) and two FGFR1 non amplified cell lines (201T and 273T). The nine RTKs characterized included- FGFRs 1-4, EGFR, HER2, HER3, HER4 and c-MET; all RTKs that are commonly involved in the progression of cancer **(Fig 11 and 12)**. FGFR2 was not expressed in any of the cell lines used. We then quantified the expression levels in all four cell lines while using MCF-7 (a human breast adenocarcinoma cell line) as a control for most of the RTKs, using densitometry **(Fig 13)**.

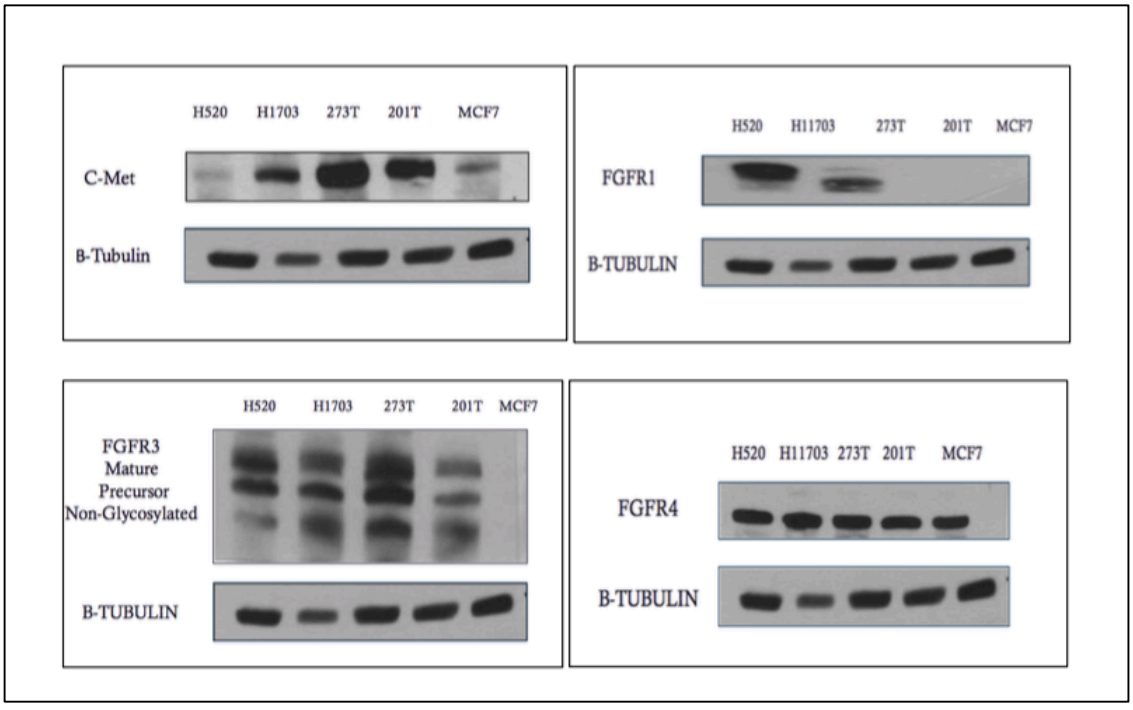


FIG 11: A. Basal expression of RTKs- c-Met, FGFR1, FGFR2, FGFR3 and FGFR4.

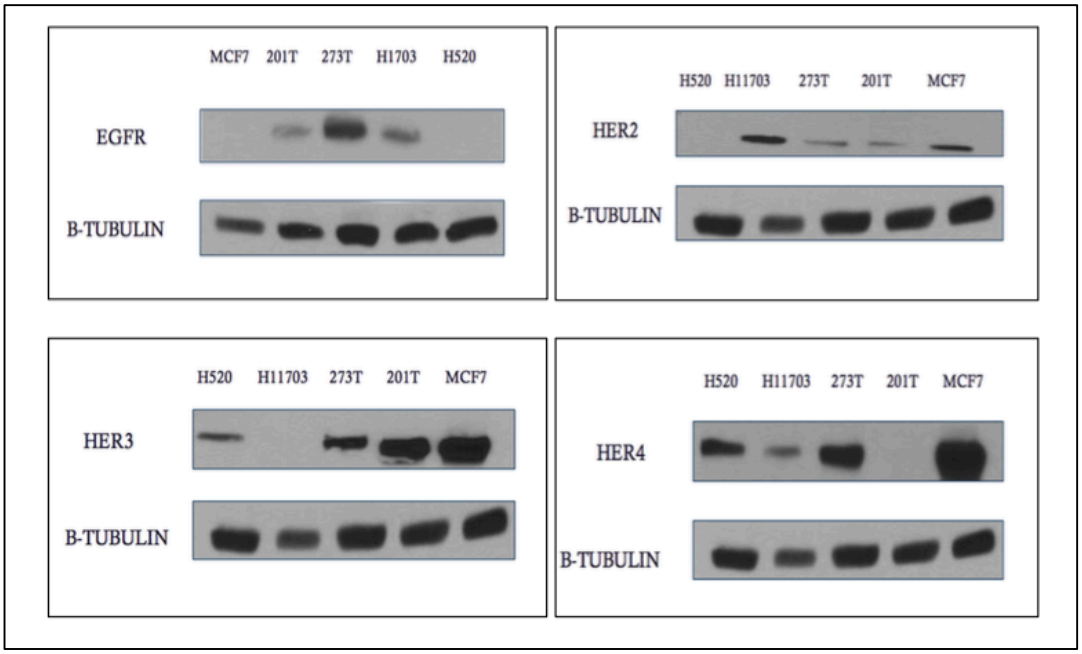


FIG 12: Basal expression of EGFR, HER2,HER3 and HER4

CELL LINES	CMET	FGFR1	FGFR2	FGFR4	EGFR	HER2	HER3	HER4
H520	0.19	1.01	_	0.73	_	_	0.18	0.35
H1703	2.11	0.91	_	1.3	0.24	0.86	_	0.19
273T	2.02	_	_	0.73	0.82	0.13	0.41	0.56
201T	1.8	_	_	0.73	0.35	0.12	0.86	0.004

CELL LINES	FGFR3 NON GLYCOSYLATED	FGFR3 PRECURSOR	FGFR3 MATURE
H520	0.5	0.9	1.04
H1703	1.45	1.4	1.34
273T	0.96	1.1	1.13
201T	0.82	0.6	0.6

FIG 13: Densitometric evaluation of nine receptor tyrosine kinases.

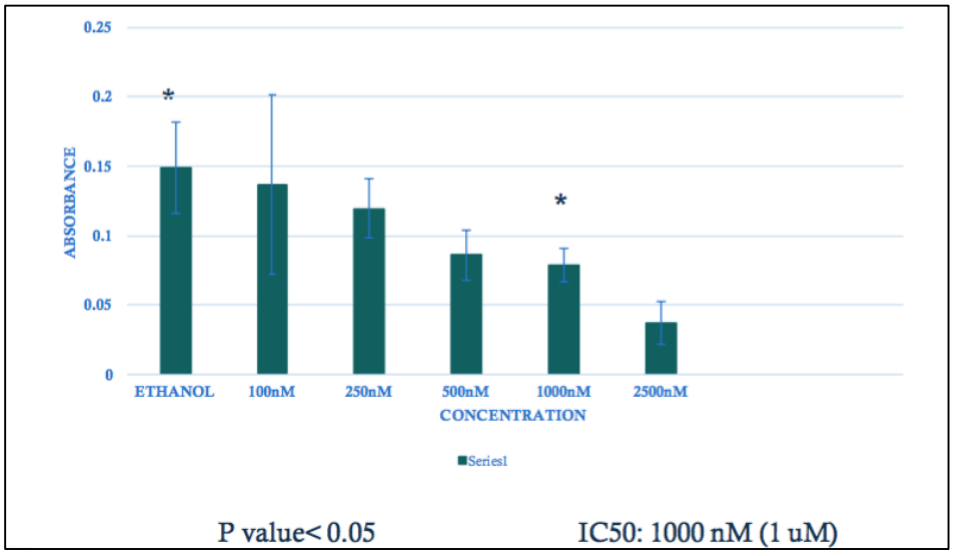
FGFR 4 was consistently expressed in all four cell lines where as FGFR1 was seen only in the FGFR1 amplified cell lines. FGFR2 was found to be absent in all four cell lines. H520 was found to lack EGFR and HER2 while H1703 was found to lack HER3. 201T does not express HER4 while 273T expressed all four of the HER family receptors. Thus we concluded that in order to inhibit the FGFR pathway we would preferably want a pan inhibitor acting on FGFR3 and FGFR4 which were both expressed in all four cell lines.

Selection of an appropriate small molecule FGFR inhibitor and determining the IC50 for each cell line

In order to identify the compensatory kinase mechanisms driving resistance to FGFR inhibitors in NSCLC it was vital to select an appropriate small molecule FGFR inhibitor and to determine the appropriate dose that would cause 50% inhibition *in vitro*. It was necessary to pick a drug that would be effective in both the FGFR amplified as well as in the FGFR non amplified cell lines. After careful screening we concluded that LY 2874455, a pan FGFR inhibitor, effectively inhibited 50% of the cell growth in all four cell lines. Using **GraphPad Prism** we determined the IC50 for 273T and 201T **(Fig 14 A and B);** H520 and H1703 **(15 A and B).** The FGFR1 non-amplified cell lines have an IC50 of 1uM. H520 has an IC50 of 0.7uM while H1703 being the most sensitive cell line has an IC50 of

0.09uM. This high sensitivity is attributed to the fact that H1703 expressed the highest levels of the FGFR family.

A



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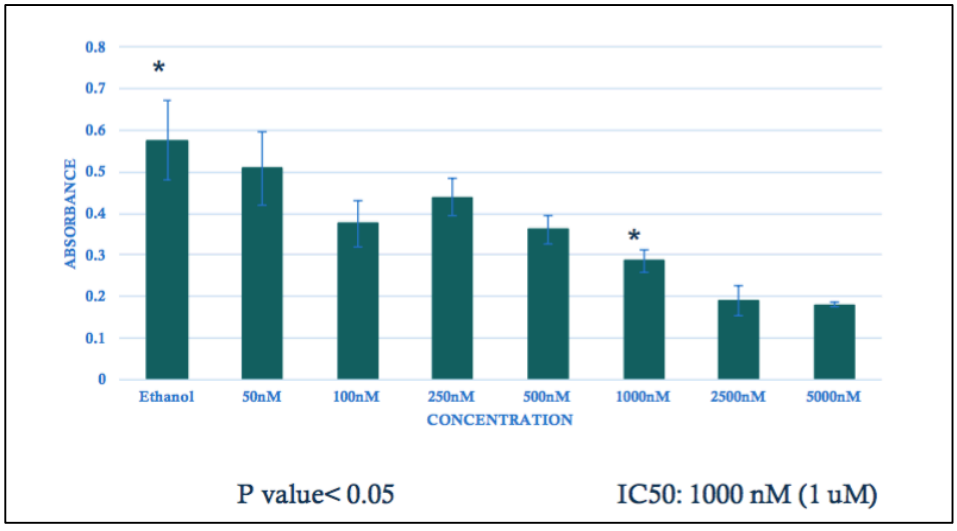
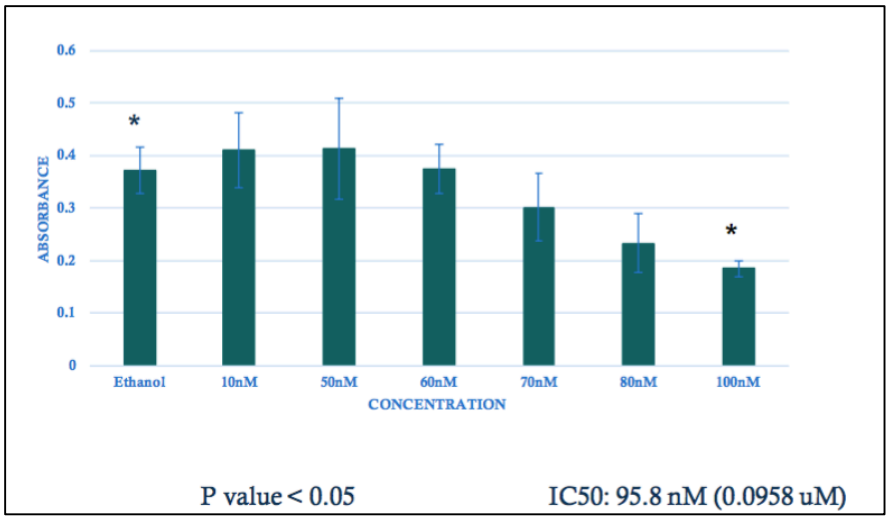


FIG 14: IC50 for LY2874455 for **(A) 273T and (B) 201T** measured at 48 hours following drug treatment.

A



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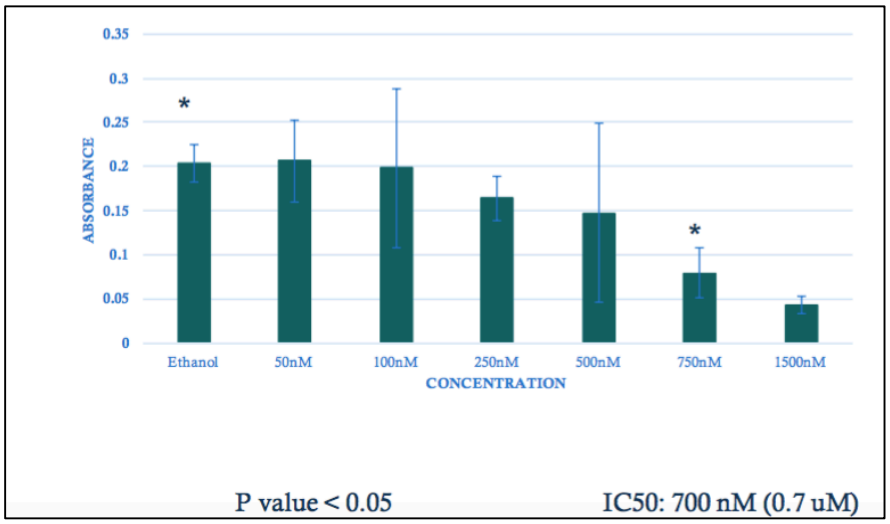
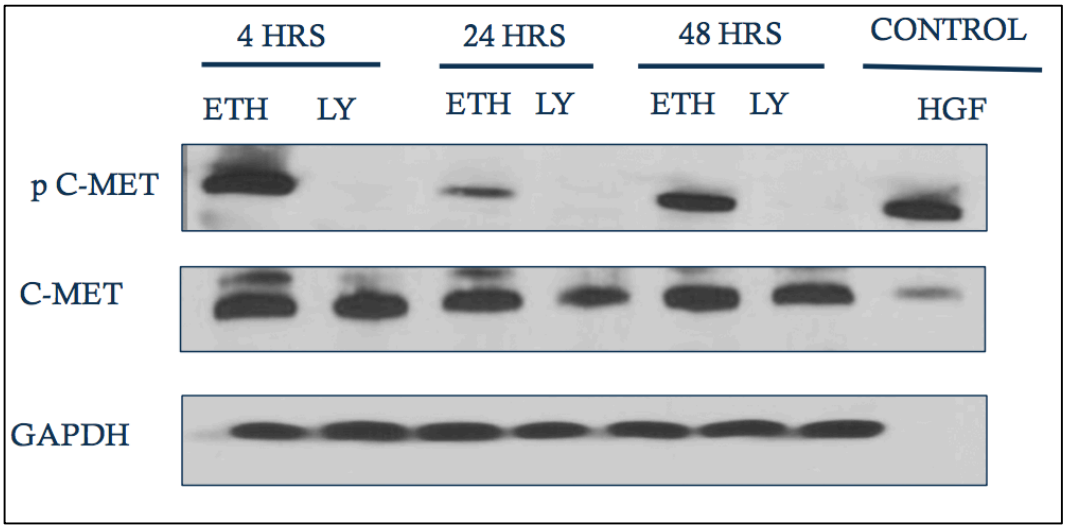


FIG 15: IC50 for LY2874455 for **(A) H1703 and (B) H520** measured at 48 hours following drug treatment.

LY2874455, a pan FGFR inhibitor, completely inhibits c-MET phosphorylation:

To identify the possible compensatory kinases activated on inhibition of FGFR by LY2874455, we treated the 201T, 273T, H520 and H1703 cell lines with their respective IC50 doses of the drug and probed for phosphorylated molecules of various RTKs. Its been previously shown that resistance to Met inhibitors can be overcome by inactivating FGFR by small molecules **(Ref: Synthetic lethal screening)** i.e. FGFR compensates for the loss of the c-Met receptor. However, what we observed upon inhibition of FGFR using LY2874455 was that instead of the reverse of the above interaction (i.e. increased phosphorylation of c-Met upon FGFR inhibition) we saw complete inhibition of c-Met phosphorylation in 201T and 273T **(Fig: 16 A and B).** This suggests that there exists some interaction between the FGFR and c-Met pathways which drives c-Met activation dependent on FGFR phosphorylation such that inhibition of FGFR phosphorylation eliminated c-Met phosphorylation. This effect was not seen in the H520 and H1703 cell lines **(17 A and B)**. The control used in the following experiment is just the vehicle with exogenous HGF (50 ng/ml) with the purpose of showing that c-Met is functional in all four cell lines. To focus on the interaction between FGFR3 and c-Met we continued experiments only in the 201T and 273T cell lines.

A



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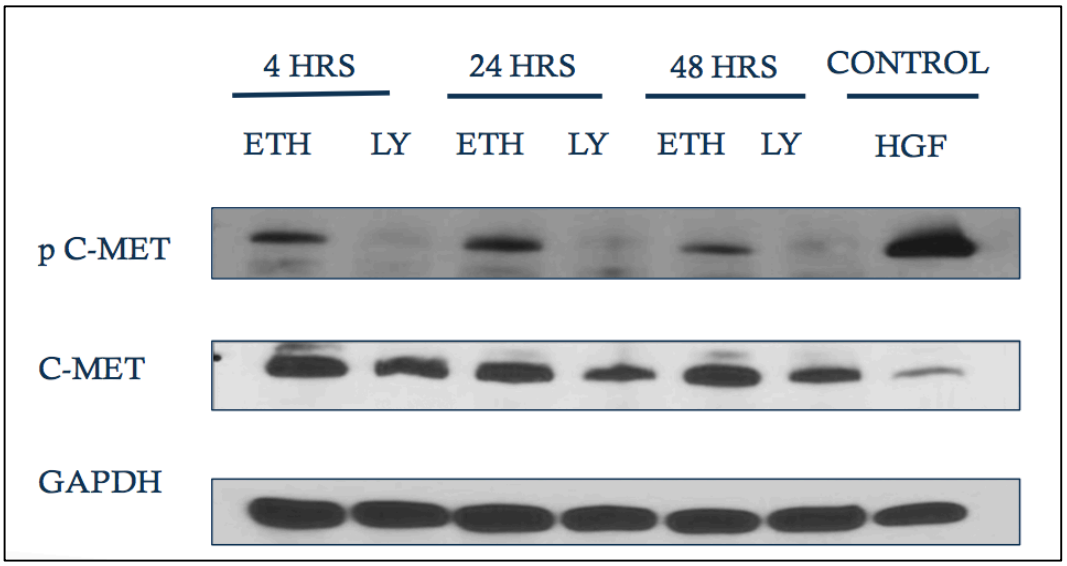
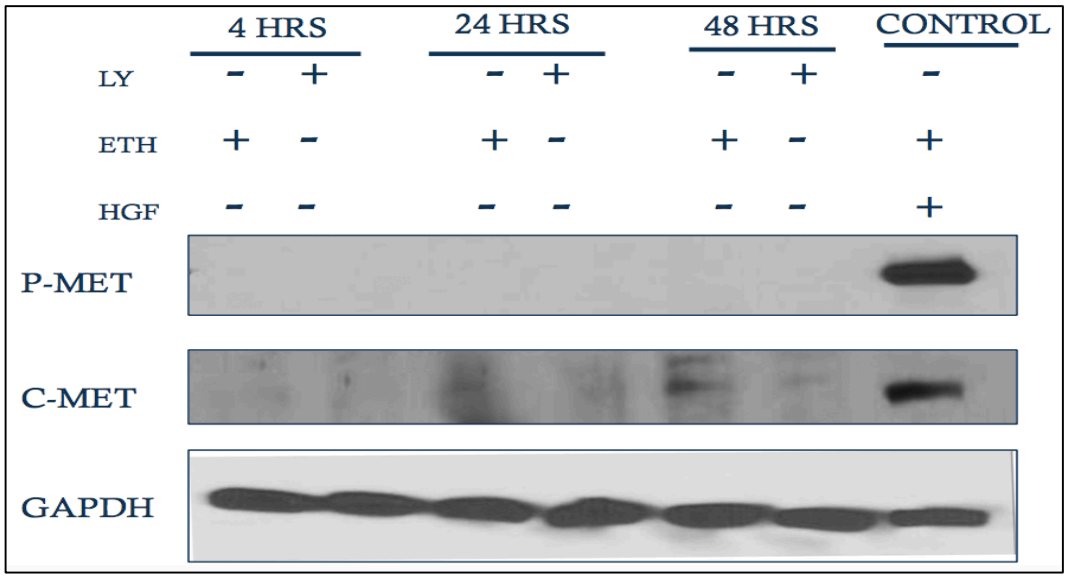


FIG 16: Status of c-Met phosphorylation of **(A) 201T** and **(B) 273T** following drug treatment with pan FGFR inhibitor LY2874455.

A



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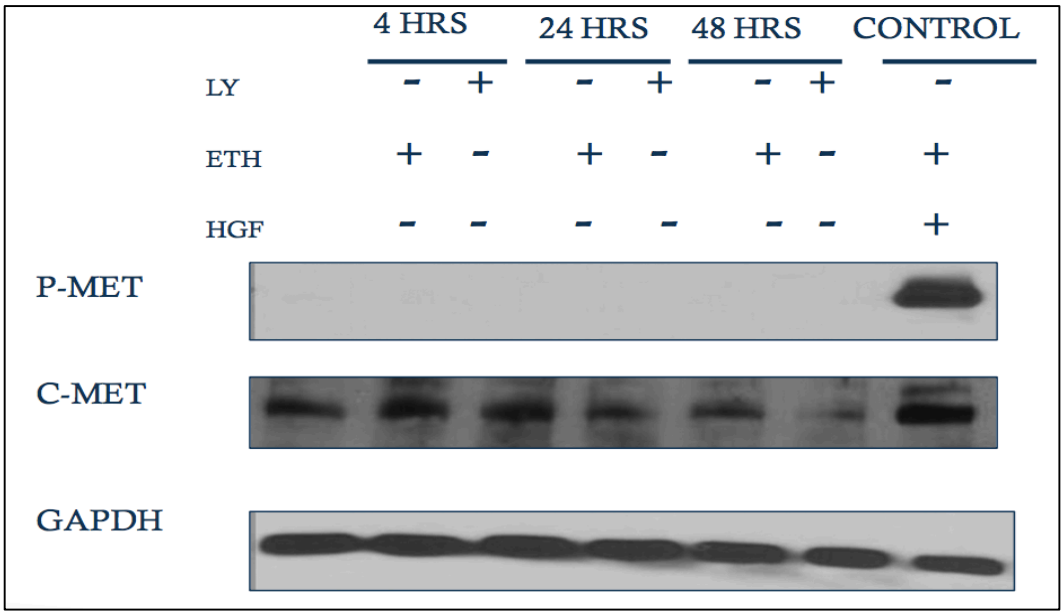


FIG 17: Status of c-Met phosphorylation of **(A) H520** and **(B) H1703** following drug treatment with pan FGFR inhibitor LY2874455.

Pan FGFR inhibitor, LY2874455, does not inhibit c-Met phosphorylation directly:

To eliminate the possibility of LY2874455 directly blocking the phosphorylation of c-Met, we pre-treated the cells with the IC50 doses of LY 2874455 and Ethanol control for two hours in serum free media. The media was then spiked with 10 ng/ml of HGF. The lysates, collected at 10 and 30 minutes' post HGF addition, were then probed for phosphorylated molecules of c-Met **(Fig: 18 and 19)**. Reappearance of phosphorylation of c-Met upon addition of HGF as compared to absence of phosphorylation in the absence of exogenous HGF proved that LY2874455 does not directly block the phosphorylation of c-Met.

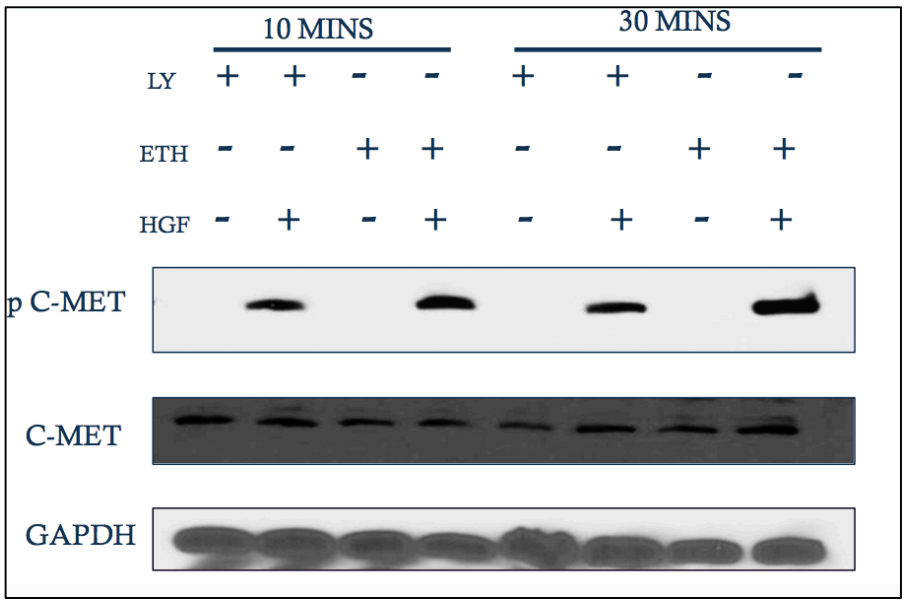


FIG 18: LY2874455 does not directly block c-Met phosphorylation in 273T as seen by the rescue of phosphorylation by exogenous HGF.

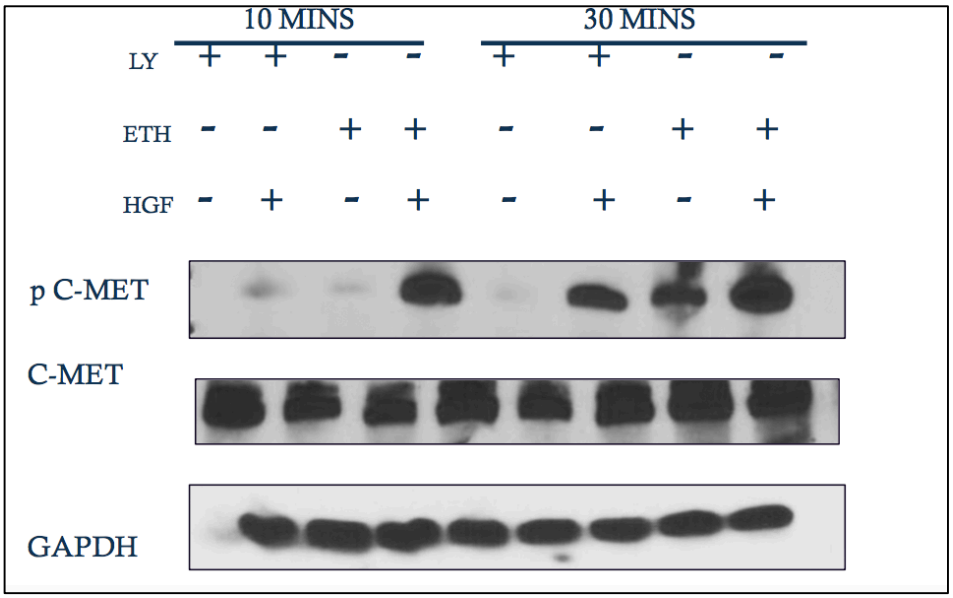


FIG 19: LY2874455 does not directly block c-Met phosphorylation in 201T cells as seen by the rescue pf phosphorylation by exogenous HGF.

Treatment with LY2874455 inhibits phosphorylation of pFGFR3

Both the 273T and the 201T lack FGFR1 and FGFR2. We observed that LY2874455, a pan inhibitor of the FGFR family, inhibited the phosphorylation of FGFR3 at 30 minutes after treatment with the said drug **(Fig 20 A and B)**. We observed that there was a high level of basal phosphorylation of FGFR3 in the untreated cells. Considering that both 201T and 273T cells expressed high levels of FGFR4 in addition to FGFR3 we probed for FGFR4 as well. Unfortunately, the quality of the pFGFR4 antibody was poor and we could not verify if LY2874455 inhibited FGFR4 phosphorylation or not.

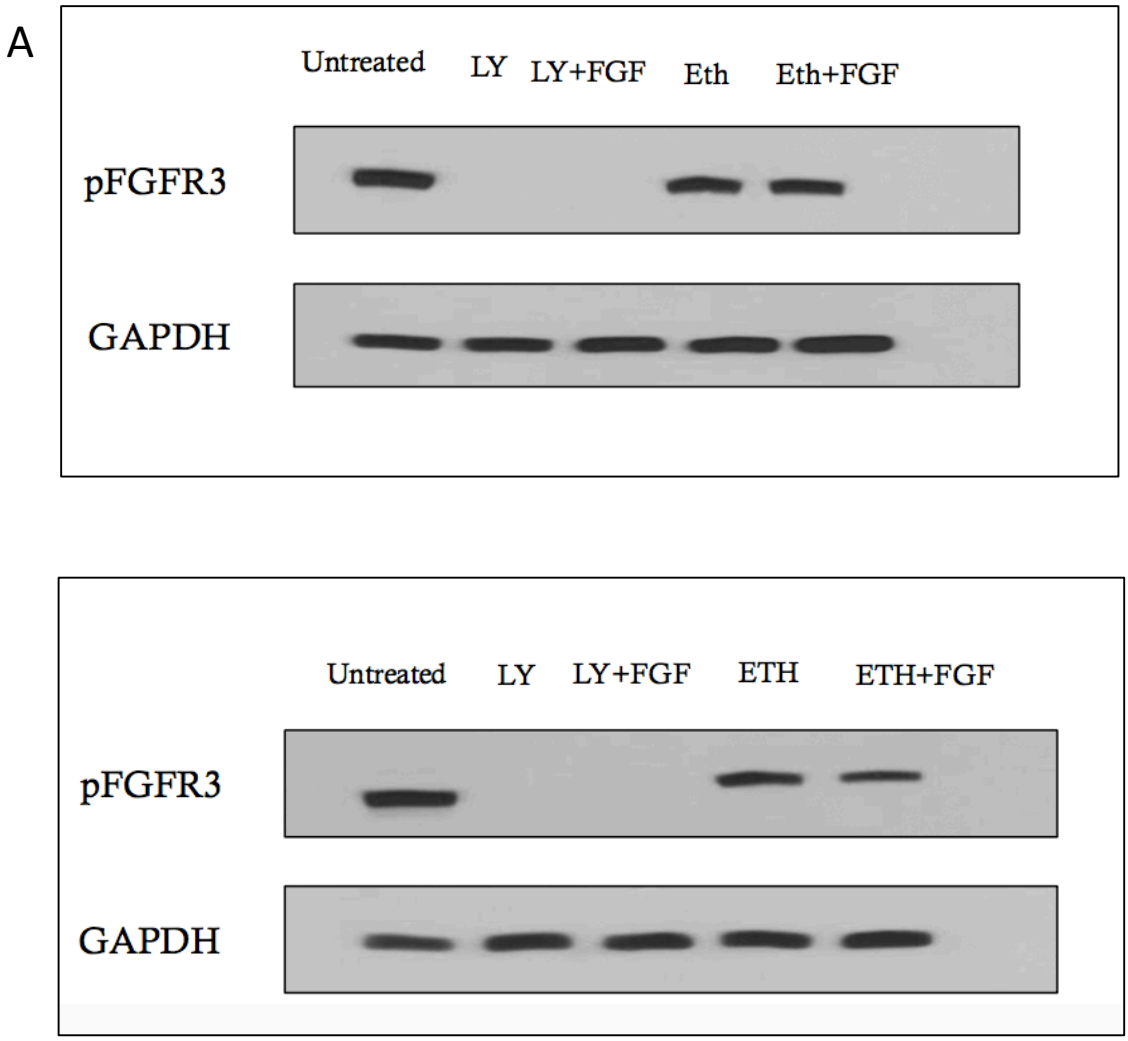


FIG 20: (A) 201T and **(B)** 273T pre-treated with LY2874455 for 2 hours followed by addition of 50 ng/ml each of FGF 8,17 and 18 in serum free media. Lysates collected at 30 minutes after addition of FGF cocktail. Inhibition of pFGFR3 by LY2874455 was observed in both cell lines.

Treatment with LY2874455 decreases phosphorylation of EGFR in the FGFR1 non amplified cells.

Evidence suggests that 201T and 273T do not secrete HGF and for this reason we expected the activation of c-Met, in the absence of LY2874455, to be occurring in a ligand independent manner. Based on previous studies, such a ligand independent activation of c-Met has been shown to be regulated by EGFR. It is for that reason that we decided to look at the phosphorylation status of EGFR following treatment with LY2874455. Treatment with the pan FGFR inhibitor, led to a decrease in the phosphorylation status of EGFR in both cell lines. This decrease was observed at 2 in the 273 T **(Fig 21)** cells and at 4 hours in the 201T cells **(Fig 22)**. This further adds evidence to the presence of a downstream interaction between FGFR and EGFR.

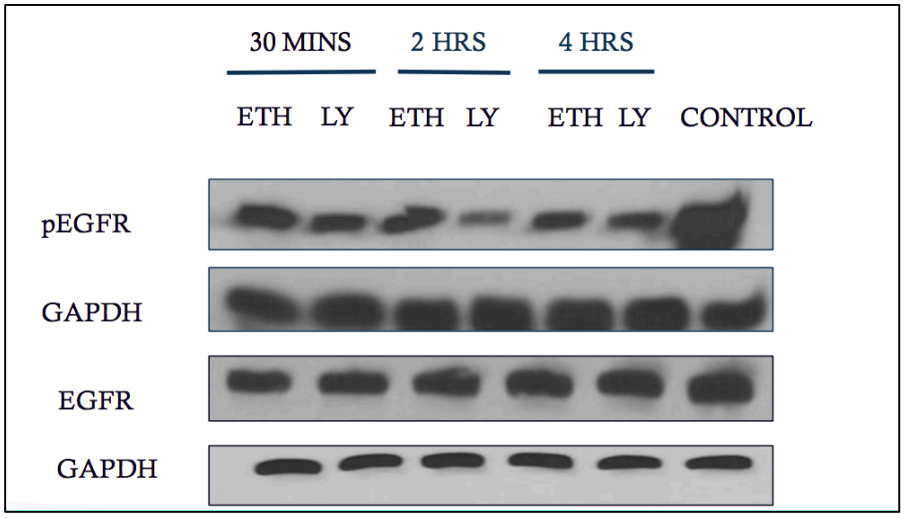


FIG 21: 273T cells show a decrease in the phosphorylation of EGFR at 2 hours post treatment with LY2874455. (ETH= Ethanol; vehicle)

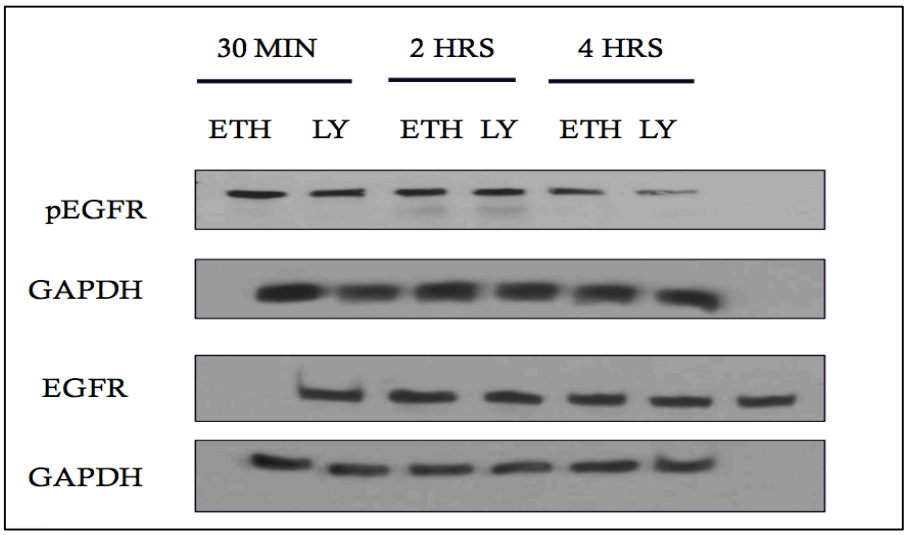
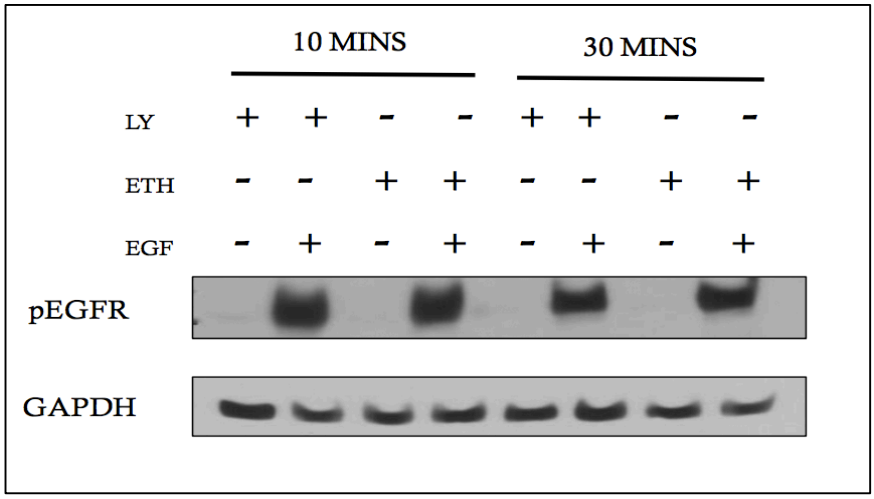


FIG 22: 201T cells show a similar decrease in pEGFR at 4 hours post treatment with LY2874455

LY2874455 itself does not directly inhibit phosphorylation of EGFR in both the FGFR1 non amplified cell lines

Treatment with LY28974455 itself did not directly block EGFR phosphorylation as seen by the EGFR phosphorylation rescue by exogenous EGF following pre-treatment with LY2874455 **(Fig 23 A and B)**.



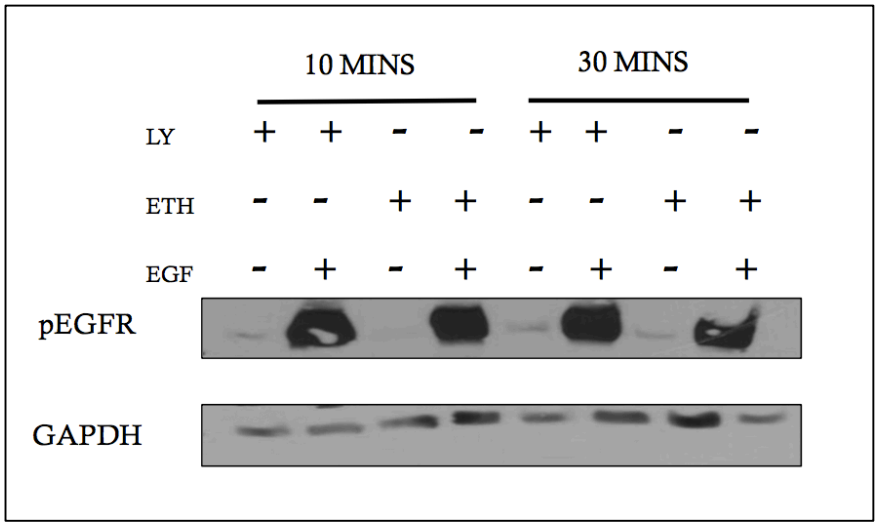


FIG 23: (A) 273T and **(B)** 201T: Treatment with exogenous EGF rescues inhibition of EGFR phosphorylation by pan FGFR inhibitor LY2874455 in both cells lines, thereby showing that LY2874455 does not directly block EGFR phosphorylation.

Amphiregulin levels decrease with FGFR inhibition.

Observing a fall in EGFR phosphorylation status on treatment with LY2874455, we next looked at whether the same decrease was observed in prominent EGFR ligands- Amphiregulin, EGF, Hb EGF and TGF α. There was a significant fall in the levels Amphiregulin, at 4 hours after treatment with LY2874455 in both 273T **(Fig 24)** and 201T **(Fig 25).** We did not detect any of the other prominent ligands.

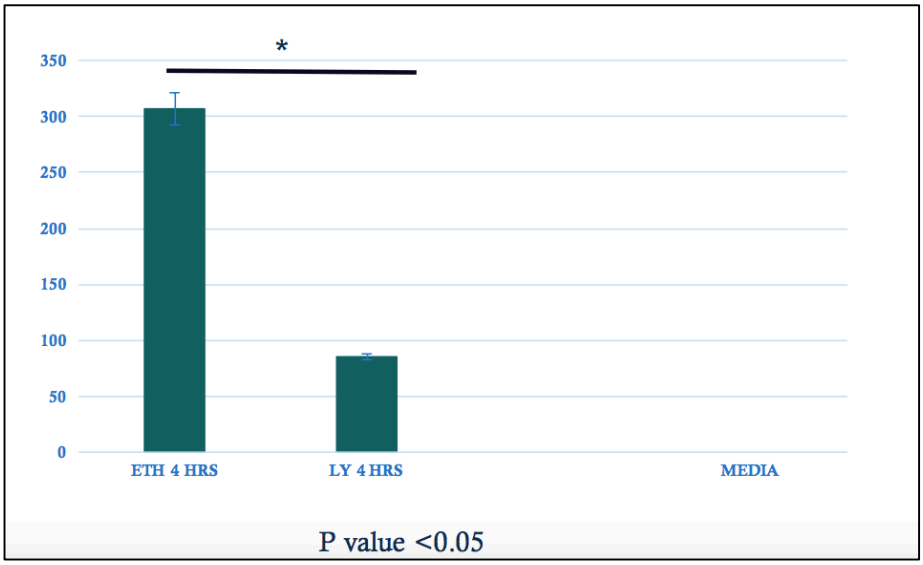


FIG 24: 273T- Significant decrease in the level of Amphiregulin at 4 hours following treatment with pan FGFR inhibitor, LY2874455 in 273T cells.

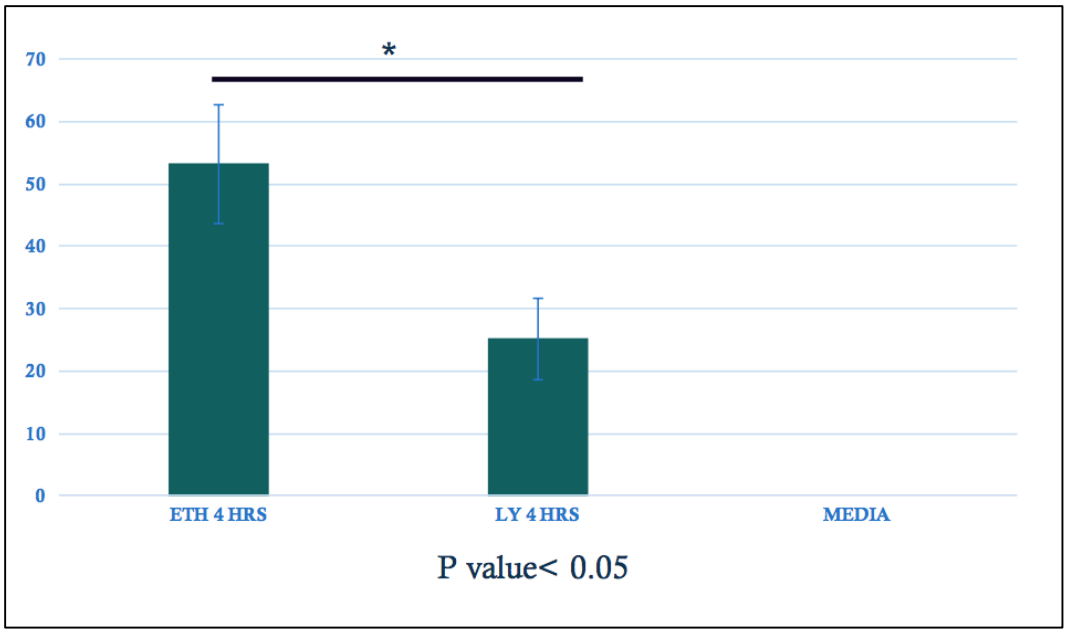


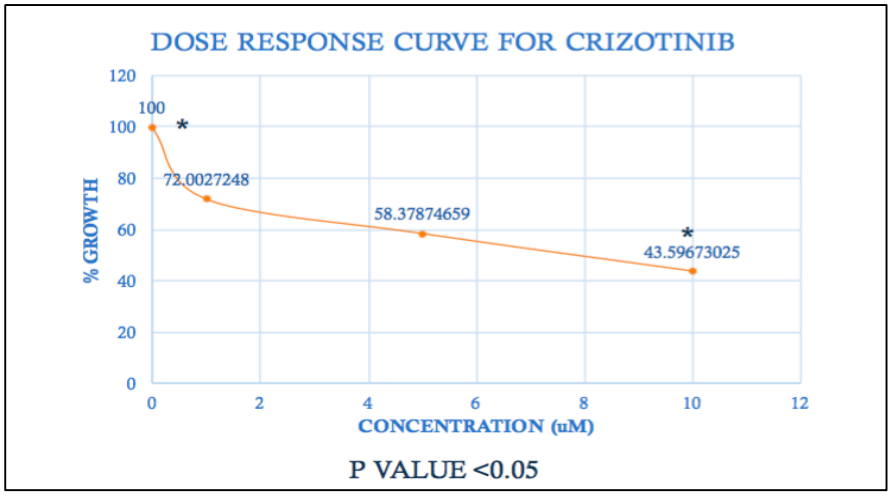
FIG 25: 201T- Significant decrease in the level of Amphiregulin at 4 hours following treatment with pan FGFR inhibitor, LY2874455 in 201T cells.

Combination treatment of LY2874455 and Crizotinib (a c-Met and ALK inhibitor) has a better inhibitory effect on cell growth.

Having established such a correlation between FGFR and c-Met it became evident that LY 2874455 was inhibiting EGFR mediated ligand independent activation of c-Met. However, HGF is secreted by the stromal cells in lung tumors and is present in the serum. Therefore, we decided to combine LY2874455 with a c-Met inhibitor to take care of the ligand dependent activation of c-Met by HGFthat could occur under these experimental conditions with 1% serum. Keeping the dose of LY2874455 constant (IC50) in combination with three varying doses of Crizotinib.

As compared to Crizotinib alone, the combination yielded increased cell death in both cell lines **(Fig 26 and 27).**

A



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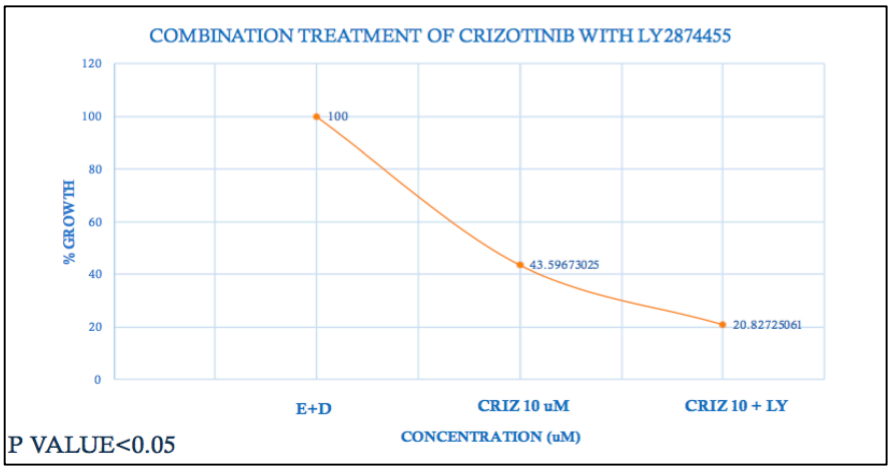
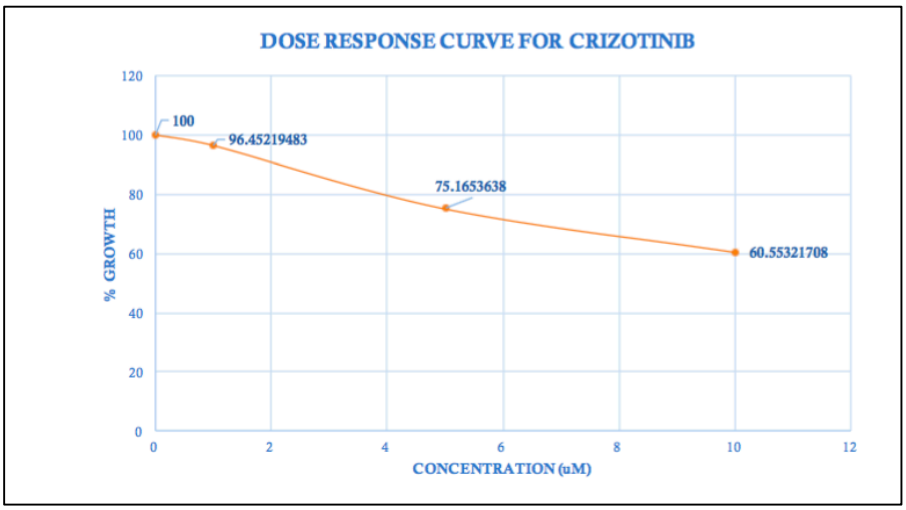


FIG 26: (A) 201T- Dose response for c-Met inhibitor Crizotinib measured at 48 hours following drug treatment using MTS assay. **(B) 201T-** Combination treatment of pan FGFR inhibitor with Crizotinib measured at 48 hours following drug treatment using MTS assay. (Dose- LY28974455 IC50- 1uM, Crizotinib- 1,5 and 10 uM). Abbreviations: E+D= Ethanol+DMSO, Criz= Crizotinib, LY= LY2874455).

A



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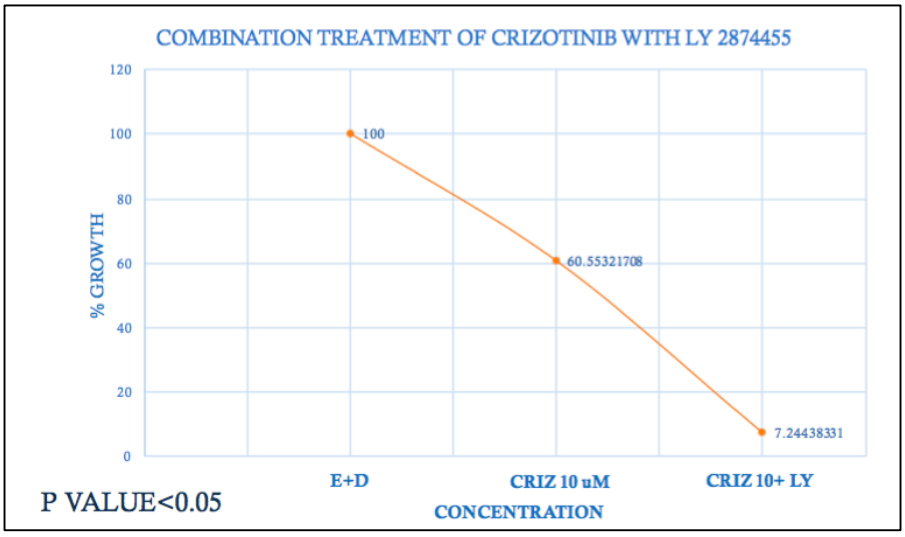

FIG 27: (A) 273T- Dose response for c-Met inhibitor Crizotinib measured at 48 hours following drug treatment using MTS assay. **(B) 273T-** Combination treatment of pan FGFR inhibitor with Crizotinib measured at 48 hours following drug treatment using MTS assay. (Dose- LY28974455 IC50- 1uM, Crizotinib- 1,5 and 10 uM). Abbreviations: E+D= Ethanol+DMSO, Criz= Crizotinib, LY= LY2874455).

DISCUSSION:

Here we have aimed at studying the compensatory kinase mechanisms that come into play in the event of FGFR inhibition. FGFRs are overexpressed in several cancer sub types including NSCLC. There is compelling evidence that certain FGFs and FGFRs are components of an autocrine signaling pathway in NSCL [36]. We used a small molecule pan FGFR inhibitor called LY2874455 to study the possible kinase interactions upon FGFR inhibition. Recent studies have suggested that resistance to Met inhibitors can be rescued by inactivation of FGFR by small molecules [34]. However, we observed that on the inhibition of FGFR by LY2874455, there was complete inhibition of the Met receptor in our FGFR non amplified cell lines. This brought us to a conclusion that while FGFR functioned as a compensatory kinase in the event of Met inhibition, FGFR inhibition was likely to affect some secondary event that was inhibiting the phosphorylation of Met. We were able to validate that LY2874455 inhibits phosphorylation of FGFR3 in the FGFR1 non amplified cell lines. However, owing to poor quality of the antibody we could not validate the same for FGFR4. This validates the idea that in the FGFR1 non amplified cells the said interaction is taking place through FGFR3 and possibly through FGFR4. We also observed a decrease in the phosphorylation the Epidermal Growth Factor Receptor (EGFR) in both the FGFR non amplified cell lines in the presence of LY2874455. We did not detect any secreted HGF upon treatment with LY2874455 leading us to believe that the activation of c-Met in the two cell lines was occurring in a ligand independent manner. Previous studies have shown that EGFR ligands result in the accumulation of activated c-Met and that the

phosphorylation of c-Met by EGFR occurs independent of HGF or any other secreted factor [25]. In the 201T cell line we observed an increase in HER3 phosphorylation upon FGFR inhibition thereby suggesting that the RTK functions as a possible compensatory kinase for the latter. In the 273T cell line however, there was a decrease in the phosphorylation of HER3. Seeing as there was a decrease in the phosphorylation of EGFR on treatment with LY2874455, we looked at the levels of Amphiregulin (AREG) - the ligand for EGFR upon treatment with the said drug. At 4 hours post treatment we observed a significant fall in the levels of AREG in both 273T and 201T cell lines. Expression of AREG has been strongly associated with the development of resistance to conventional chemotherapeutic agents including a multi kinase inhibitor called sorafenib [27]. Also, high level of AREG expression has been correlated with resistance to EGFR and ErbB2 targeted therapies in breast cancer and in NSCLC patients [27]. Previous work in our lab has shown that 201T cells produced FGFs 2,3 and 19 while 273T cells produced FGFs 2,3,9 and 19. There is a possibility that these FGFs may be driving Amphiregulin release in the absence of LY2874455.

FUTURE DIRECTIONS:

Owing to the high basal phosphorylation of FGFR3 using a cocktail of FGFs to stimulate and thereby verify this pathway is not possible. However, using RNA interference with FGFR3 and FGFR4 to check for c-Met and EGFR inhibition is an alternative. Using immunoprecipitation for FGFR4 may improve performance of

the antibody so pFGFR4 status can be examined. We also suspect that c-Met may be forming a complex with EGFR and therefore a co-immunoprecipitation with c-Met and EGFR is what we intend to do. Src has been suggested as a mediator of ligand independent phosphorylation of c-Met, so we can use Src inhibitors to investigate this. It is also worth observing if recombinant Amphiregulin can activate c-Met and stimulate cell proliferation. Cleavage of pro-AREG followed by release of Amphiregulin could be mediated by Matrix Metalloproteinases (MMPs). Thus in order to verify this hypothesis treatment with an MMP inhibitor to observe the effects on c-Met and EGFR phosphorylation followed by its effect on Amphiregulin release is the next experiment worth considering. Lastly an i*n vivo* tumor model to test the combination of LY2874455 and Crizotinib.

CONCLUSION

In summary, we have established that inhibition of FGFR3 phosphorylation results in a significant fall in Amphiregulin thereby preventing the phosphorylation of EGFR which can then no longer activate c-Met in an HGF independent manner **(Fig 28).** This has been further bolstered by the fact that we were able to show increased cell death by combining an FGFR inhibitor with a c-Met inhibitor.

This interaction has been solely found thus far in FGFR non amplified cell lines meaning that FGFR inhibitors can be used in combination with a c-Met inhibitor in patients lacking the said amplification, if the tumor expresses FGFR3.

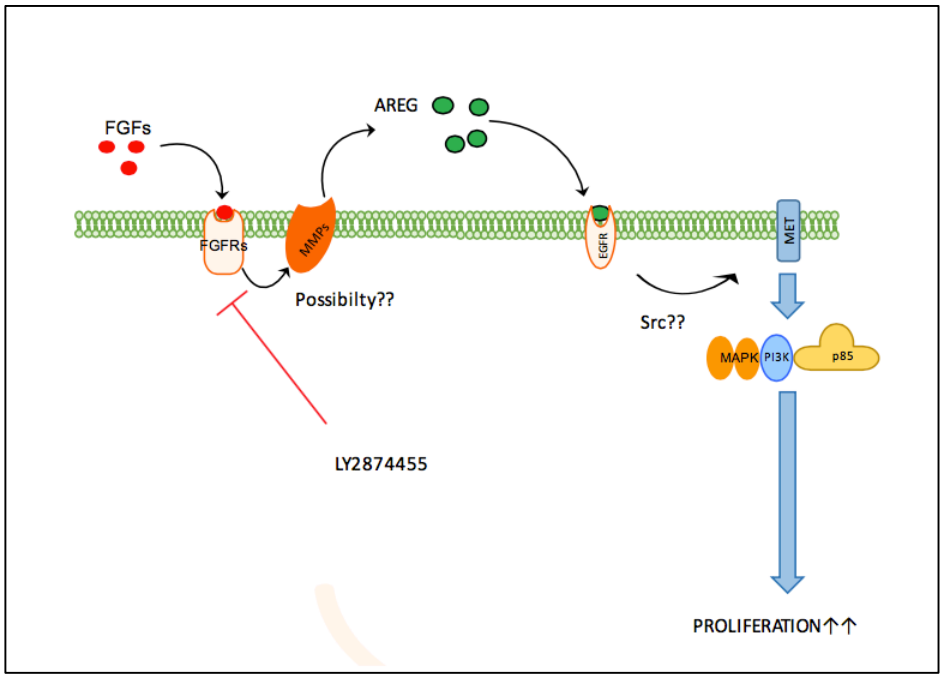

FIG 28: Inhibition of FGFR3 phosphorylation by LY2874455 results in a fall in Amphiregulin levels possibly through the activation of matrix metalloproteinases thereby preventing the phosphorylation of EGFR which can then no longer activate c-Met in an HGF independent manner thereby decreasing cell proliferation.

MATERIALS AND METHODS:

Reagents and cell culture

NSCLC cell lines 201T and 273T were established in our laboratory from primary tissue and were maintained at 37° Celsius in 5% CO2 (Siegfried *et al.,* 1999). Both the cell lines were grown in basal medium eagle (BME) with 10% fetal bovine serum, 2mM L-Glutamine and 50X Penicillin Streptomycin all purchased from GIBCO by Life technologies. NSCLC cell lines H520 and H1703 were obtained from American Type Culture Collection (Manassas, VA, USA). Both cell lines

were grown in Roswell Park Memorial Institute Medium (RPMI) with 10% fetal bovine serum and 50X Penicillin Streptomycin. Rabbit monoclonal c-Met (SC-10; 1:500), FGFR3 (SC-123; 1:500) and FGFR4 (SC-9006; 1:500) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit monoclonal FGFR2 (#11835; 1:1000), FGFR1(#9740; 1:1000), EGFR (#4267;1;1000), HER2 (#42901:1000), HER3 (#12708; 1:1000), HER4 (#4795; 1:1000), β-Tubulin (#2146; 1:2000) antibodies were obtained from Cell Signaling (Beverly, MA, USA). The pan FGFR inhibitor- LY2874455 (S7057) was purchased from Selleckchem.com (Selleckchem, TX, USA). Rabbit monoclonal phospho-Met (Tyr 1234/1235 #3077; 1:1000), phospho-EGFR (Tyr1068; 1:1000), phospho-HER3 (Tyr1289; 1:1000) GAPDH (1:5000) and anti-Rabbit Igg HRP linked (#7074; 1:2000) antibodies were purchased from Cell Signaling (Beverly, MA, USA). Human Amphiregulin ELISA kit (DY262) was purchased from R&D Systems (Minneapolis, MN, USA). Recombinant human HGF protein was purchased from R&D Systems (Minneapolis, MN, USA). Cell Titre Aqueous One Solution Cell Proliferation Assay MTS was obtained from Promega (Madison, WI, USA). SuperSignal West Pico Chemiluminescent Substrate (1:1) was purchased from Thermo Scientific (Waltham, MA, USA) and Luminata Forte Western HRP Substrate was obtained from Millipore Corporation (Billerica, MA, USA). Immobilon-P transfer membrane was obtained from Millipore Corporation (Billerica, MA, USA). The selective FGFR inhibitor LY2874455 (50 mM stock in Ethanol) and the c-Met inhibitor Crizotinib (20 mM stock in DMSO) were

purchased from Selleckchem (Selleckchem, TX, USA). FGFs 8,17 and 18 were obtained from PeproTech (Rocky Hill, NJ, USA).

Cell treatments and Immunoblotting

For measuring the basal expression of RTKs, 201T and 273T cells were grown in BME medium while H520 and H1703 were grown in RPMI medium, both containing 10% fetal bovine serum. Cells were washed with phosphate-buffered saline and lysed with RIPA whole lysis buffer. The insoluble fraction was cleared by centrifugation at 12,000 x g for 30 minutes at 4°C. Protein concentrations were measured by Bradford assay (BIO-RAD). Samples were prepared containing 30 μg of proteins, 4X NuPAGE LDS Sample buffer with BME and RIPA buffer. Prepared samples were then subjected to denaturation by heating at 100°C for 5 minutes. 50ul of the samples were loaded in each well of the gel. Gels were prepared using NEXT gel 7.5% Acrylamide solution, 10% APS and TMED. Gels were run at 100 volts for one hour. Proteins were then transferred to a PVDF membrane (Immobilon-P) over a period of one and a half hours. Membranes were then blocked with 5% milk for one hour followed by overnight incubation with the primary antibodies at 4°C. The membranes were then subjected to four washes of ten minutes each in TBST. Following this they were incubated with the secondary antibody at room temperature for one hour. Membranes were then washed four times with TBST for ten minutes each. Developing solution used was SuperSignal

West Pico Chemiluminescent Substrate (1:1). Developed films were obtained from the X-ray film developer.

In order to calculate the IC50 of the selective FGFR inhibitor LY2874455- 10,000 cells of each of the four cell lines were plated per well in a 96 well plate in BME medium containing 1% FBS. The cells were then treated with LY2874455 48 hours from the date of plating. Absorbance was measured using the Cell Titre Aqueous One Solution Cell Proliferation Assay MTS, 48 hours from the time of treatment at 490 nm. IC50 for each cell line was calculate using GraphPad.

Effect of LY2874455 on the phosphorylation of c-Met, in all four cell lines, was measured by plating 450,000 cells per well in BME medium containing 10% FBS in a 6 well plate. The cells were then allowed to grow to the desired confluence. Cells were treated with LY2874455 and the vehicle Ethanol in BME medium containing 1% FBS using the IC50 measured for each cell line. Lysates were collected at 30 minutes, 2 hours and 4 hours from the time of treatment. Lysates were then subjected to immunoblotting as described above and the membranes were subsequently probed for phospho-c-Met and GAPDH.

To show that LY2874455 was indeed inhibiting phosphorylation of FGFR3, 450,000 cells of each 201T and 273T per well were plated in 10% FBS containing BME medium in a 6 well plate. Once the cells had reached the desired confluence

they were serum starved in serum free medium for 24 hours. The cells were then pre treated with LY2874455 and Ethanol for two hours following which 50 ng/ml of FGF cocktail containing FGF 8,17 and 18 were added. Lysates were collected 30 minutes following the addition of FGFs. Immunoblotting was conducted as mentioned above and the blots were probed for pFGFR3.

In order to reaffirm that LY2874455 was a selective FGFR inhibitor and was not directly blocking c-Met phosphorylation- 450,000 cells of 201T, 273T, H520 and H1703 each were plated per well in BME medium containing 10% FBS, in a 6 well plate, followed by starvation in medium containing 1% FBS for 24 hours. Cells were pre-incubated with LY2874455 and Ethanol (in serum free media) for 2 hours following which 10ng/ml of recombinant HGF protein was added to the wells. Lysates were collected 10 and 30 minutes after HGF addition and were subjected to immunoblotting as described previously. The membranes were then probed for phospho-c-Met, total c-Met and GAPDH.

To study the effects of LY2874455 on the phosphorylation status of phospho-EGFR- 450,000 cells of 201T and 273T per well were plated in BME medium containing 10% FBS in a 6 well plate. The cells were allowed to grow to the desired confluence following which they were treated with Ethanol and the IC50 dose of LY2874455 in BME medium containing 1% FBS. Cell lysates were collected at 30 minutes, 2 hours and 4 hours from the time of treatment. Immunoblotting was

carried out as mentioned above and the membranes were probed for phospho-EGFR and GAPDH.

In order to validate that LY2874455 did not directly inhibit phosphorylation of EGFR- 450,000 cells of both 201T and 273T were plated in BME medium containing 10% FBS in a 6 well plate. The cells were then allowed to grow to the desired confluence following which they were starved in BME medium containing 1% FBS for 24 hours. The cells were then pre-treated with the IC50 dose of LY2874455 and Ethanol in serum free medium for 2 hours after which 10 ng/ml of EGF was added into the treatment wells. Lysates were collected at 10 and 30 minutes following EGF addition. Immunoblotting was carried out as mentioned above and the membranes were probed for pEGFR and GAPDH.

To determine the levels of Amphiregulin secreted by the cells- 450,000 cells of 201T and 273T per well were plated in a 6 well plate in BME medium containing 10% FBS. Cells were then starved in Minimal Essential Medium (MEM) for 24 hours. Cells were treated with Ethanol and the IC50 dose of LY 2874455 in BME medium containing 1% FBS. Media was collected for Amphiregulin ELISA at 4 hours from the time of treatment.

To show that LY287455 was indeed inhibiting the phosphorylation of FGFR3- 201T and 273T were plated at a density of 450,000 in BME media containing 10% FBS. The cells, once at desired confluence, were serum starved for 24 hours. The

cells were then pre-incubated with LY2874455 and Ethanol for 2 hours, following which 50 ng/ml of FGF cocktail was added to the treatment wells. Lysates were collected at 30 minutes following the addition of the FGF cocktail. Immunoblotting was carried out as mentioned above. Blots were probed for pFGFR3 and GAPDH.

In order to measure the combination effect of LY 2874455 and the c-Met inhibitor- Crizotinib- 10,000 cells of 201T and 273T cell lines were plated in a 96 well plate in BME medium containing 1% FBS. Cells were grown to the desired confluence. Cells were then treated with Ethanol, LY2874455 and a combination of LY2874455 and Crizotinib; keeping the IC50 of LY 2874455 as the constant dose and varying doses of Crizotinib. Absorbance was measured at 48 hours after treatment using Cell Titre Aqueous One Solution Cell Proliferation Assay MTS at 490 nm.

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