

# Inhibitor-Sensitive *FGFR1* Amplification in Human Non-Small Cell Lung Cancer

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## Abstract

**Background:** Squamous cell lung carcinomas account for approximately 25% of new lung carcinoma cases and 40,000 deaths per year in the United States. Although there are multiple genomically targeted therapies for lung adenocarcinoma, none has yet been reported in squamous cell lung carcinoma.

**Methodology/Principal Findings:** Using SNP array analysis, we found that a region of chromosome segment 8p11-12 containing three genes—*WHSC1L1*, *LETM2*, and *FGFR1*—is amplified in 3% of lung adenocarcinomas and 21% of squamous cell lung carcinomas. Furthermore, we demonstrated that a non-small cell lung carcinoma cell line harboring focal amplification of *FGFR1* is dependent on FGFR1 activity for cell growth, as treatment of this cell line either with *FGFR1*-specific shRNAs or with FGFR small molecule enzymatic inhibitors leads to cell growth inhibition.

**Conclusions/Significance:** These studies show that *FGFR1* amplification is common in squamous cell lung cancer, and that FGFR1 may represent a promising therapeutic target in non-small cell lung cancer.

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## Introduction

Lung cancer is the leading cause of cancer-related death in developed countries with deaths in 2009 estimated at approximately 160,000 in the United States, accounting for about 28% of all cancer deaths [1]. Non-small cell lung cancer (NSCLC) accounts for 75% of all lung cancers and includes two predominant subtypes, adenocarcinoma and squamous cell carcinoma (SCC), which comprise 40% and 25% of NSCLCs, respectively [2,3]. Despite clear histologic and biologic distinctions, lung adenocarcinoma and squamous cell carcinoma are largely treated with the same chemotherapeutic agents with the exception of the antifolate agent pemetrexed which is approved for the treatment of non-squamous NSCLC [4].

Significant advances in the treatment of lung adenocarcinoma have stemmed from detailed genomic analyses and the deployment of molecularly targeted agents leading which have led to improvements in patient outcomes. Examples include the use of

epidermal growth factor receptor (EGFR) inhibitors such as gefitinib and erlotinib [5,6,7] for lung adenocarcinomas bearing *EGFR* mutations [8,9,10], and of ALK inhibitors such as crizotinib [11] for lung adenocarcinomas bearing *EML4-ALK* translocations [12,13].

However, little is currently known about the targetable genetic abnormalities underlying squamous cell lung cancer. In addition to *TP53* mutations [14], squamous cell lung carcinomas have been shown to harbor amplifications of *PIK3CA* [15], *SOX2* [16], and *EGFR* [16] as well as *EGFR* variant III mutations [17] *DDR2* mutations [18] and rare amplifications of *PDGFRA/KIT* [19,20] and *BRF2* [21]. A recent study has demonstrated focal amplification of the *FGFR1* locus on chromosome 8p associated with cellular dependency on *FGFR1* and sensitivity to FGFR inhibitors [22]. At this time there are no FDA-approved targeted therapies for squamous cell lung cancer.

Targeting amplified tyrosine kinases with antibodies or with small molecule inhibitors has led to dramatic improvements in

response rates and overall survival of cancer patients whose tumors harbor specific genomic abnormalities. Amplifications of *EGFR* and *ERBB2* have been reported in a variety of malignancies, including head and neck, esophageal, gastric, breast and colon cancers as well as NSCLC [23]. Targeting of these tyrosine kinases, such as the use of cetuximab to target *EGFR* in colorectal and head and neck cancer [24,25] and the use of trastuzumab to target *ERBB2* in breast cancer [26], has resulted in significant improvement in patient outcomes in each of these diseases, though not all patients with these amplifications respond to targeted agents [27,28], likely due to additional genomic alterations within the tumor that result in primary resistance to specific agents [29,30].

The fibroblast growth factor receptor type 1 gene (*FGFR1*) is one of the most commonly amplified genes in human cancer [16]. The fibroblast growth factor receptor (FGFR) tyrosine kinase family is comprised of four kinases, FGFR1, 2, 3, and 4, that play crucial role in development, and have been shown to be targets for deregulation by either amplification, point mutation, or translocation (reviewed in [31]). Translocations involving *FGFR3*, as well as activating somatic mutations in *FGFR3* have been identified in multiple myeloma and bladder cancer [32,33,34]. We and others have identified activating mutations in *FGFR2* in endometrial cancer [35,36]. Amplification or activation of *FGFR1* has been reported in oral squamous carcinoma [37], esophageal squamous cell carcinomas [38], ovarian cancer [39], bladder cancer [40], prostate cancer [41], rhabdomyosarcoma [42], and lung cancer [16,43,44,45,46]. Consistent with this, a pan-FGFR tyrosine kinase inhibitor has been shown to block tumor proliferation in a subset of NSCLC cell lines with activated FGFR signaling but has no effect on cells that do not activate the pathway [47]. *FGFR1* has been identified as the driver event in breast carcinomas and NSCLC, especially squamous cell lung carcinomas, harboring similar amplifications of the 8p11 chromosomal segment [22,48].

Based on SNP array copy number analysis of 732 samples, we report that *FGFR1* is somatically amplified in 21% of lung squamous cell carcinomas as compared to 3.4% of lung adenocarcinomas. We validate FGFR1 as a potential therapeutic target by showing that at least one *FGFR1*-amplified NSCLC tumor cell line is sensitive to FGFR enzymatic inhibition and dependent on *FGFR1* expression for cell viability as evidenced by shRNA treatment. Together with previous reports reviewed above, these results suggest that FGFR1 may be an attractive therapeutic target in NSCLC.

## Materials and Methods

### NSCLC primary samples and cell lines

NSCLC cell lines, NCI-H1703 (squamous), NCI-H2444 (pulmonary), NCI-H520 (squamous), HCC95 (squamous), NCI-1581 (large cell carcinoma), Calu3 (not otherwise specified), NCI-H1734 (not otherwise specified), Colo699 (adenocarcinoma), NCI-H2170 (squamous), NCI-H226 (squamous), A427 (adenocarcinoma), NCI-H1563 (adenocarcinoma), NCI-H1781 (adenocarcinoma) and HCC15 (squamous) were obtained from the collection of A.F. Gazdar, J. Minna, and colleagues [49,50,51], from ATCC (Manassas, Virginia, United States) and/or DSMZ (Braunschweig, Germany). Cells were maintained in RPMI 1640 complete media supplemented with 10% calf serum (Gibco/Invitrogen, Carlsbad, California, United States) and penicillin/streptomycin (Gibco/Invitrogen). The NSCLC tumor/normal pairs analyzed in this study have been described earlier [16,20,45,50,52].

### SNP array data analysis

SNP array experiments were performed on 732 NSCLC tumor and cell line samples and data analyzed as described previously [16,20,45,50,52]. The boundaries of the 8p11 amplicon defined by GISTIC analysis [53] were identified as reported [52](<http://www.broadinstitute.org/tumorscape/pages/portalHome.jsf>). Data display has been performed using the Integrative Genomics Viewer (<http://www.broadinstitute.org/igv>).

### Transfection and infection

Phoenix 293T packaging cell line (Orbigen, San Diego, California, United States) were transfected with pBabe-Puro-based gateway vectors using FuGENE® 6 Transfection Reagent (Roche, Indianapolis, United States) to generate replication incompetent retroviruses. Target cells were infected with these retroviruses in the presence of 8 µg/ml polybrene. Two days post infection, cells were treated with 2 µg/ml puromycin (Sigma, St. Louis, Missouri, United States) for two days. The resulting stable cell lines were used for experimental studies.

### shRNA mediated *FGFR1* knockdown

shRNA vectors were obtained from TRC (The RNAi Consortium). The target sequences of the shRNA constructs are:

*FGFR1* 1 (TRCN 0000121307): 5'- AGTGGCTTATTAA-TTCCGATA-3'.

*FGFR1* 2 (TRCN 0000121308): 5'- GCTTGCCAATGGCG-GACTCAA-3'.

*FGFR1* 3 (TRCN 0000121309): 5'- CTTGTATGTCATC-GTGGAGTA-3'.

*FGFR1* 4 (TRCN 0000121310): 5'- CAAGATGAAGAG-TGGTACCAA-3'.

*FGFR1* 5 (TRCN 0000121311): 5'- GAATGAGTACGGCA-GCATCAA-3'.

*LETM2* 1 (TRCN 0000040243): 5'- CGCACCTTCTACC-TGATAGAT-3'.

*LETM2* 2 (TRCN 0000040244): 5'- CCCAGCACAAAG-GAGATAGTT-3'.

*LETM2* 3 (TRCN 0000040245): 5'- CCAGTTACATCAT-CACCCATA-3'.

*LETM2* 4 (TRCN 0000040246): 5'- CCAGGAAGTAC-TAATACAA-3'.

*LETM2* 4 (TRCN 0000040247): 5'- GCATTGAGTGTAT-CAGAACTA-3'.

*WHSCIL1* 1 (TRCN 0000015613): 5'- CGAGAGTATAA-AGGTCATAAA-3'.

*WHSCIL1* 2 (TRCN 0000015614): 5'- CCATCATCAAT-CAGTGTGTAT-3'.

*WHSCIL1* 3 (TRCN 0000015615): 5'- CGAGAATATC-ATGTCCAGTTT-3'.

*WHSCIL1* 4 (TRCN 0000015616): 5'- GCTTCCATTAC-GATGCACAAA-3'.

*WHSCIL1* 5 (TRCN 0000015617): 5'- GCAGGGAATT-GTTTGAGTCTT-3'.

The sequence targeted by the *GFP* shRNA is 5'-GCAAGCT-GACCCTGAAGTTCAT-3'. Lentiviruses were made by transfection of 293T packaging cells with these constructs using a three-plasmid system as previously described [54]. Target cells were incubated with lentiviruses for 6 hours in the presence of 8 µg/ml polybrene and left in fresh media. Cells were grown for two days. Fifty micrograms of total cell lysates prepared from the infected cell lines was analyzed by Western blotting.



## Growth and Proliferation assays

For survival assays,  $2 \times 10^6$  cells for each tumor cell line expressing shRNAs constructs targeting *FGFR1*, *WHSC1L1*, *LETM2* or *GFP* along with uninfected cells were seeded in 3 replicates on a 6 well plate. Cell viability was determined at 24 hour time points for 4 consecutive days by counting the cells using Beckman Coulter Vi-Cell Automated Cell Viability Analyzer following trypan blue dye staining. The percentage of cell viability was plotted for each cell line of readings obtained on day 4 relative to day 1.

## Soft agar anchorage-independent growth assay

For soft agar assays,  $2 \times 10^4$  NSCLC cells expressing sh*FGFR1* and sh*GFP* were suspended in a top layer of RPMI1640 containing 10% calf serum and 0.4% Select agar (Gibco/Invitrogen, Carlsbad, California, United States) and plated on a bottom layer of RPMI1640 containing 10% calf serum and 0.5% Select agar on a 6 well plate. PD173074 or FIIN-1 [55] was added as described to the top agar. After 3–5 weeks incubation colonies were counted in triplicate. IC50s were determined by nonlinear regression using Prism 5 software (GraphPad Software).

## Cytotoxicity assays

Depending on the growth curves for each cell line, between 800 and 2000 NSCLC cells were seeded in 6 replicates in 96- well plate. One day after plating, increasing doses of FGFR inhibitors PD173074 or FIIN-1 were added and proliferation of cells was assessed 4 days later using the WST-1 assay (Roche Applied Science). Each data point represents the median of six replicate wells for each tumor cell line and inhibitor concentration. IC50s were determined by nonlinear regression using the Prism Graphpad software.

## Western Blots

Total protein was extracted and separated by gel electrophoresis by lysing cells in a buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2.5 mM EDTA, 1% Triton X-100, and 0.25% IGEPAL. Protease inhibitors (Roche Applied Science) and phosphatase inhibitors (Calbiochem) were added prior to use. Before loading to the gel, samples were normalized for total protein content. Total protein lysates were boiled in sample buffer, separated by SDS-PAGE on 8% polyacrylamide gels, transferred to PVDF membrane, and probed overnight using the appropriate primary antibodies. Antibodies used for immunoblotting were: anti- FGFR1 antibody ( 3472, Cell Signaling Technologies, Danvers, MA, United States), anti- phospho FRS2 Y436 ( 3861, Cell Signaling Technologies, Danvers, MA, United States), anti-phospho-FRS2 Y196 ( 3864, Cell Signaling Technologies, Danvers, MA, United States), anti-FRS2 ( sc-17841, Santa Cruz Biotechnology, Santa Cruz, CA, United States). anti-*WHSC1L1* monoclonal antibody ( sc-130009, Santa Cruz

Biotechnology), anti-*LETM2* monoclonal antibody ( ab84626, Abcam), and anti-Actin monoclonal antibody ( sc-1615, Santa Cruz Biotechnology).

## Statistical Analysis

Comparisons between SNP array copy number data for lung adenocarcinoma (AC) and squamous cell carcinoma (SCC) tumors were performed using Fisher's exact T test to calculate two-tailed p-values among samples harboring high level amplification, defined as  $\log_2$  ratio  $>0.7$  or 3.25 normalized DNA copies. P values  $<0.05$  were considered significant.

To determine the IC50 for FGFR inhibitors, the cell viability measurements of six replicates at varying concentrations of inhibitors were normalized to untreated control cells. Sigmoidal dose response curves were fitted to the data by non linear regression using GraphPad Prism software. Standard deviations were determined for the mean of each value using an inbuilt module of the software.

For shRNA experiments, performed in three replicates, the cell number was counted and the mean and standard deviation were determined using functions in Microsoft Excel.

## Results

### *FGFR1* is amplified in non-small cell lung cancer

We examined the 8p11-12 genomic region using Affymetrix 250K SNP array copy number data in a previously reported data set of 732 NSCLC samples, (628 primary tumors and 104 cell lines) (Table S1) [16,20,45,50,52]. We observed high level amplification, defined as  $\log_2$  ratio  $>0.7$  or 3.25 normalized DNA copies, of the 8p11-12 chromosomal segment encompassing the *FGFR1* locus in 44 (6%) of NSCLC samples (Figure 1a; Table S2). The majority (93%; 41/44) of these amplifications were relatively focal events ( $<50\%$  of the length of chromosome 8p) indicating preferential selection of the specific target genes within the region of amplification [52]. The inferred copy number of the amplifications, normalized to a copy number of 2 for each sample, ranged from 3.25 to 25 copies (median = 2.8 copies). The estimated extent of the region of focal amplifications ranged from 0.47 to 112.7 Mb (median = 2.74 Mb).

To identify regions of significant copy-number alteration, we applied GISTIC (Genomic Identification of Significant Targets In Cancer) [53], and identified a 170 Kb region on 8p11 (38.28 to 38.45 Mb) as significantly amplified. While the overall pattern of 8p11 amplification was consistent with the literature on lung cancer as reported, our sample size and resolution provided more power to accurately identify and localize both large-scale and focal chromosomal alterations as compared to earlier reports [45,52]. The sole genes within the region of amplification identified in our analysis across all samples were *FGFR1* and *LETM2*. In our copy number data, *WHSC1L1* was generally amplified with *FGFR1* and *LETM2* (41/44 samples) but the whole *WHSC1L1* gene did not fall

within the GISTIC peak (chr8:38,284,229–38,451,475). Specifically, three primary tumor samples with amplified *FGFR1* and *LETM2* genes had amplicon breakpoints within *WHSC1L1*, explaining the exclusion of *WHSC1L1* from the GISTIC amplification peak (Figure S1; Figure S2). The amplicon breakpoints within *WHSC1L1* are consistent with a lack of amplification

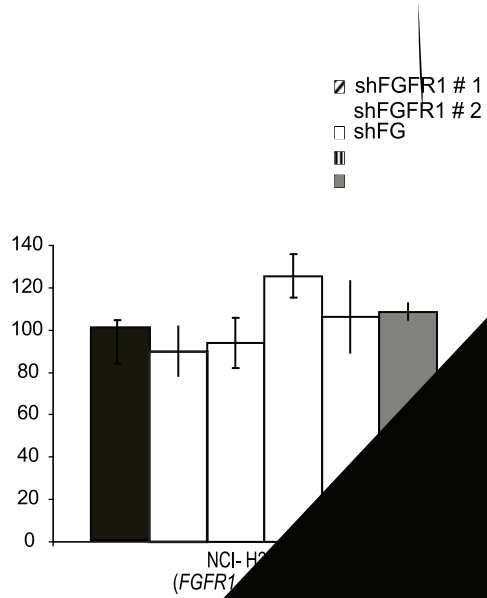
a



b

Percent cell survival

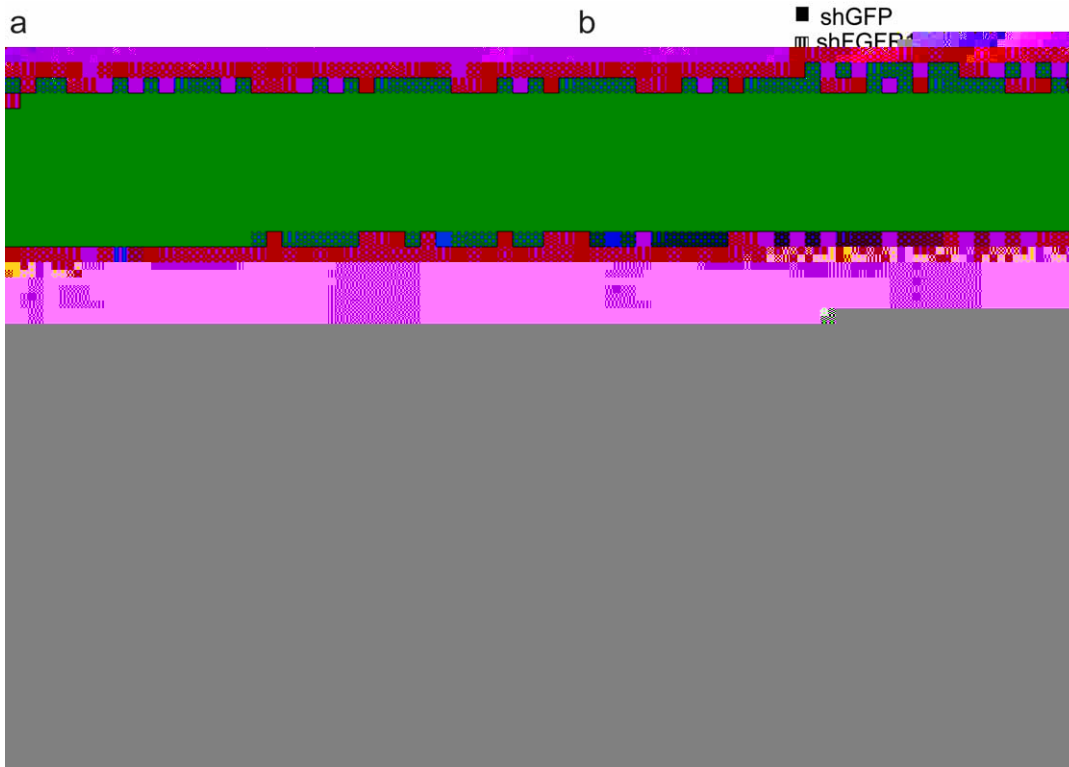
c



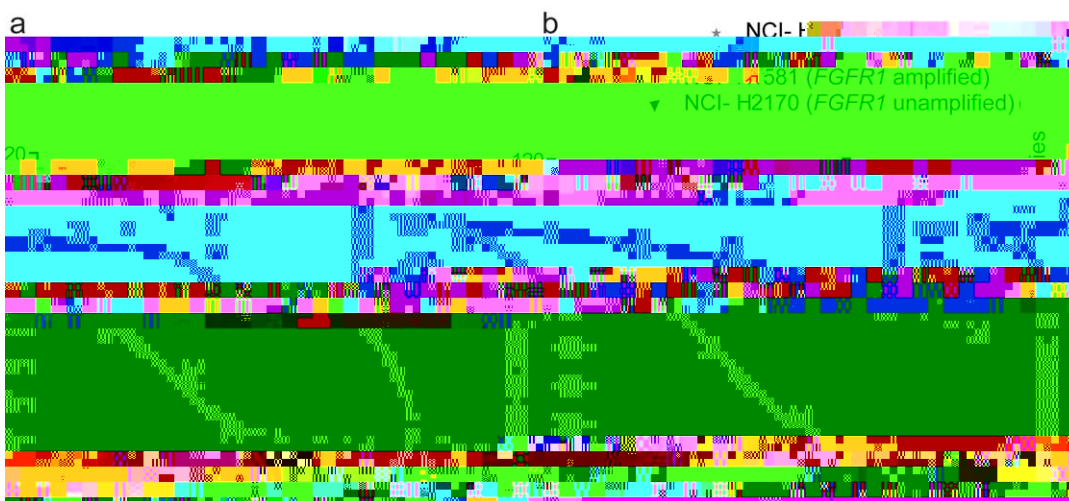
requires the gene as demonstrated by shRNA depletion, and is sensitive to inhibition with FGFR kinase inhibitors.

Genes other than *FGFR1* have been proposed to be a target of amplification on chromosome segment 8, notably *WHSC1L1* [44] and *BRF2* [21]. However, the evidence presented here as well as in a recent study [44] for *FGFR1* as the functional target of amplification in NSCLC cell line. Additionally, in our study, *FGFR1* was amplified in all the *FGFR1* amplified cell lines, which is unlikely to be the only relevant gene in this region. The cell line that was not amplified in our study, NCI-H1703 [44] (Figure 1c), does not show dependence on *FGFR1* for survival, suggesting that its growth is dependent on another gene in this region, possibly *PDGFRA* [19,20]. In contrast, the cell line NCI-H1703 shows a significant impact on *FGFR1* depletion, suggesting that its growth is dependent on *FGFR1* for cellular transformation.

A recent study [44] suggested that the growth of NCI-H1703 cells is dependent on *PDGFRA* for cellular transformation.



**Figure 3. Ectopic expression of *FGFR1* coding region rescues lethality of an shRNA targeting the *FGFR1* 3' UTR.** (A) Bar graph for rescue assay. Lethality due to depleted levels on endogenous *FGFR1* level in NCI-H1581 is rescued by over expression of wild type (Wt) full length *FGFR1* coding sequence. (B) No effect on the survival of NCI-H2170 cells was observed due to over expression of wild type form of *FGFR1*. NCI-H2170 is not dependent on *FGFR1* activity. NI, no infection. shGFP, control hairpin specific for green fluorescent protein used as a negative control. All results are normalized to survival of cells infected with shGFP. Data shown is as mean of three replicates. (C) Validation of *FGFR1* rescue by immunoblotting. Depleted levels of endogenous *FGFR1* level in NCI-H1581 cells infected with *FGFR1* shRNA-expressing lentiviruses targeting the *FGFR1* 3'UTR (lane 1) is rescued by overexpression of wild type form of *FGFR1* cDNA lacking the 3'UTR (lane 2) with concomitant modest rescue in the levels tyrosine residue phosphorylation of the *FGFR1* substrate FRS2 (middle panel). Actin is shown as a loading control (lower panel). doi:10.1371/journal.pone.0020351.g003



**Figure 4. *FGFR1* tyrosine kinase activity is essential in NCI-H1581 cells.** (A) Treatment with the indicated concentrations of pan *FGFR* inhibitor PD173074 inhibited soft agar colony formation by the NCI-H1581 NSCLC cell lines harboring *FGFR1* amplification, as compared with the NCI-H2170 line, which does not harbor *FGFR1* amplification. Colonies were photographed and quantitated after 4 weeks. (B) Treatment with the indicated concentrations of PD173074 inhibited survival of NCI-H1581 cells, but not of NCI-H2170 cells, as determined by WST assay performed after 4 days treatment. IC50s are indicated. doi:10.1371/journal.pone.0020351.g004

that our results suggest that amplification alone will not always predict sensitivity to FGFR1 inhibition, additional work is needed to fully characterize the genetic alterations involved in NSCLC carcinogenesis and dependency on FGFR1.

## Supporting Information

**Figure S1 WHSC1L1 histone methyltransferase activity domain is not likely to be specifically targeted for amplification at 8p11-12q.** Heat map representation of SNP array based segmented copy number on chromosome arm 8p11-12q for 34 NSCLC samples (rows; ordered by amplification of a 170 kb chromosomal segment spanning *WHSC1L1*, *LETM2* and *FGFR1*) having amplification greater than 3.25 copies (log<sub>2</sub> ratio of 0.7) from a collection of 732 NSCLC primary samples and cell lines. Three primary tumor samples marked \* harbor amplicon with breakpoints within *WHSC1L1* and only amplify *FGFR1* and *LETM2* genes. The sample marked \* appears to be a translocation within *FGFR1* removing its first exon. The locations of WHSC1L1 SET domain and FGFR1 kinase domain are indicated. The color scale ranges from blue (deletion) to red (amplification) with estimated copy numbers as shown. Grey denotes a region for which no SNPs are present on the array and therefore represents indeterminate copy number.  
(TIF)

**Figure S2 Exclusion of WHSC1L1 functional domain among primary tumors harboring amplified FGFR1 and LETM2.** Bar graph representation of unsegmented probe-level copy number values for amplicons in 3 primary tumor samples harboring break points within *WHSC1L1*. Estimated copy number values (y axis) are plotted for individual SNPs at 8p11-12 locus (x axis). Copy number of SNPs defining boundary of breakpoint are indicated. Genomic positions of genes in region are shown along the x axis.  
(TIF)

**Figure S3 Elevated FGFR1 gene copy number in NSCLC cell lines.** SNP array based segmented copy number on chromosome arm 8p11-12q for 18 NSCLC cell lines (rows; ordered by amplification) from telomere (left) to centromere (right). The color scale ranges from blue (deletion) to red (amplification) with estimated copy numbers shown.  
(TIF)

**Figure S4 Activation of FGFR1 substrate FRS2 in NCI-H1581 cells.** Western blot analysis of FGFR1 in five different 8p11-12 amplified cells (Colo699, Calu3, NCI-H2077, NCIH1581, NCI-H520 and NCIH1703) indicated by red horizontal bar below and in three NSCLC cell lines harboring deletion of the region (NCI-H1781, NCI-H1563 and HCC15) indicated by blue horizontal bar below. NCI-H1581 cells show increased tyrosine residue phosphorylation of FGFR1 substrate FRS2 as compared to other NSCLC cell lines using actin as a loading control (shown in lower panel).  
(TIF)

**Figure S5 FGFR1 tyrosine kinase activity is essential for NCI-H1581 anchorage independent growth.** Inhibition of soft agar colony formation by the NCI-H1581 NSCLC cell line harboring *FGFR1* amplification, in the presence of increasing concentrations of FGFR inhibitor PD173074, compared with HCC15 and NCI-H2170 cells without *FGFR1* amplification, and NCI-H1703 cells that harbor *FGFR1* amplification but do not over-express FGFR1. Cells were seeded in soft agar and treated with different concentrations of PD173074. Representative plates from two independent experiments are presented. Colonies were photographed and quantitated after 4 weeks.  
(TIF)

**Figure S6 FGFR1 tyrosine kinase activity is essential in proliferation of NCI-H1581 cells.** Treatment with the indicated concentrations of irreversible FGFR inhibitor FIIN-1 inhibited survival of NCI-H1581 cells, but not of NCI-H2170 cells, as determined by WST assay performed after 4 days treatment. IC<sub>50</sub>s are indicated.  
(TIF)

**Figure S7 FGFR1 instead of BRF2 is the more commonly amplified gene at 8p11.**



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