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Preselection of lung cancer cases using FGFR1 mRNA and gene copy number for treatment with ponatinib.

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Abstract

Introduction: Pre-clinically, high FGFR1 mRNA (FGFR1-MRNA) and FGFR1 amplification (FGFR1-AMP) predicted sensitivity to FGFR inhibitors in NSCLC and SCLC cell lines. KRAS mutations did not preclude sensitivity.

Patients and Methods: Metastatic EGFR- and ALK-negative lung cancers were screened for FGFR1-MRNA by in-situ hybridization (ISH) and FGFR1-AMP by silver in-situ hybridization (SISH). Positive cases were offered ponatinib, a multi-kinase inhibitor of FGFR1–4. Differences in overall survival (OS) between cohorts were assessed using log-rank test. Association of FGFR1 positivity with clinicopathologic features were assessed using Fisher's exact test and Kruskal-Wallis rank sum test.

Results: 171 cases were prescreened: 9/123 (7.3%) SISH+; 53/126 (42.1%) ISH+; 6 cases concordantly positive for SISH and ISH. SISH+ cases had fewer coincident KRAS mutations ($p=0.03$) than SISH- cases, and ISH+ cases had worse OS ($p=0.020$) than ISH- cases. Data distributions suggested a distinct higher positivity cutpoint for FGFR1 ISH (20%), occurring in 23% [29/126] cases, was associated with SCLC histology ($p=0.022$), soft tissue metastases ($p=0.050$) and shorter OS ($p=0.031$). Four patients received ponatinib on study: All ISH+ by the initial cutpoint, 2/4 by higher cutpoint, 1/4 SISH+. Tolerability was poor. The best response for the two higher ISH cases was SD and PD for the two lower ISH cases.

Conclusions: Elevated FGFR1-MRNA is more common than FGFR1-AMP and associated with worse OS. Higher FGFR1 mRNA expression may be associated with a specific phenotype and is worthy of further exploration. Ponatinib's poor tolerance suggests further FGFR exploration in ISH+ cases should utilize more selective FGFR1 inhibitors.

Micro-Abstract:

A phase II biomarker-driven study using FGFR1 gene copy number by SISH and mRNA expression by ISH in lung cancer patients treated with ponatinib was conducted.

FGFR1 ISH and SISH positivity may be associated with a distinct phenotype. Ponatinib's poor tolerance limited the number of patients treated on study.

Keywords

FGFR1; Biomarker; Ponatinib; Lung cancer

Introduction:

Over the last decade, the paradigm of advanced non-small cell lung cancer (NSCLC) management has been revolutionized by the advent of identifiable, targetable oncogenic

drivers, such as epidermal growth factor receptor (EGFR) activating mutations, anaplastic lymphoma kinase (ALK) and ROS1 gene rearrangement. Treatment of these molecular subsets of lung cancer using specific tyrosine kinase inhibitors (TKI) has led to high objective response rates (ORRs) of 40 to 70%, which are often durable, and generally, the TKIs have had better tolerability compared to cytotoxic drugs.^{1–9} However, success in discovering novel oncogene drivers in lung cancer has been limited to the adenocarcinoma histological subtype of NSCLC. The discovery and exploitation of targetable oncogenic drivers in squamous NSCLC (SQLC) and in small cell lung cancer (SCLC) continues to be explored.

The fibroblast growth factor receptor (FGFR) comprises a family of 4 transmembrane receptor tyrosine kinases (FGFR1–4) that regulate cellular proliferation, differentiation, and survival. Binding of a large range of different mitogenic and hormonal ligands to the relevant receptor leads to FGFR dimerization and downstream signaling via the phosphatidylinositol 3-kinase (PI3K), RAS/RAF/MAPK, and protein kinase C (PKC) pathways.^{10,11} A comprehensive effort to molecularly profile 178 SQLC identified alterations in FGFR1–4 in 27% of cases, with FGFR1 gene amplification being the most common event.¹² Other sequencing efforts of SQLC suggest a similarly high frequency of FGFR1 amplification, and pre-clinical studies demonstrated response to FGFR1 inhibition in FGFR1-amplified SQLC in-vitro and in-vivo.^{13–15} FGFR1 amplification has also been reported in SCLC in a small subset, together with possible dependence on the FGFR signaling pathway.^{16–18} Given the relatively high frequency of FGFR1 amplification, fluorescent in-situ hybridization (FISH) for FGFR1 was used to select SQLC patients for treatment with different FGFR inhibitors in several clinical trials. Unfortunately, ORRs following this approach have been low (8 to 11.5%), with a disease control rate (DCR) of 38.5– 50%.^{19–21}

One possible explanation for this is that gene copy number may not lead to functional mRNA expression and therefore has limited value for predicting FGFR1 pathway addiction. In a cohort of 151 resected primary lung tumors comprising the entire spectrum of NSCLC histology, 6/13 (46%) samples with gene copy number (GCN) ≥ 4 also had high mRNA expression (4+, per RNA scope guidelines). Conversely, 6/44 (13.6%) samples with mRNA 4+ also had GCN ≥ 4 .²²

Regarding the predictive potential of these biomarkers in 58 cell lines across different lung cancer histologies, including SQLC, adenocarcinoma, and SCLC, FGFR1 gene amplification, mRNA transcription and protein expression were quantified prior to FGFR TKI therapy. Following treatment with ponatinib, a multikinase inhibitor of FGFR1, 2, 3, and 4 (IC_{50} = 2, 2, 18, and 8 nmol/L, respectively)²³, as well as PDGFRA, PDGFRB, RET, and VEGFR1–3 (IC_{50} 0.2–8 nmol/L), these cell lines were ranked by their IC_{50} for inhibiting proliferation. When ponatinib sensitivity was correlated with each cell line's corresponding FGFR1 mRNA, protein, and gene copy number score, FGFR1 mRNA (AUC 0.905) and protein (AUC 0.887) were more predictive of ponatinib sensitivity compared to FGFR1 gene copy number (AUC 0.691).²² Based on this pre-clinical data, a phase II trial was designed to determine the prevalence of and factors associated with FGFR 1 biomarker

(gene copy number and mRNA) positivity in NSCLC and SCLC and explore the predictive value of these biomarkers for ponatinib sensitivity.

Methods:

Prescreening for ponatinib biomarkers

Using formalin-fixed paraffin-embedded cell line pellets, clinically translatable mRNA (ISH) and gene copy number (SISH) assays were developed as previously described.²² FGFR1 immunohistochemistry applicable to the same tissues was not available when this protocol was designed.

Unlike oncogenic point mutations or gene rearrangements, gene copy number, mRNA levels and protein expression levels represent continuous variables. This cell line work was then utilized to predict initially clinically relevant cut points for FGFR1 inhibitor sensitivity using a ponatinib IC₅₀ 50 nM based on the predicted serum trough level of 60 nM at the recommended phase 2 dose (RP2D) of 45 mg oral daily in a previous clinical trial.²⁴

Patients 18 years of age with advanced lung cancer of all histologies (NSCLC and SCLC) other than carcinoid were pre-screened for tumoral FGFR1 gene copy number by silver in-situ hybridization (SISH) and for FGFR1 mRNA levels by in-situ hybridization (ISH). If the underlying histology was NSCLC with adenocarcinoma or not otherwise-specified (NOS), the patient had to be negative for both EGFR mutations and ALK rearrangements. Lung cancer specimens with KRAS mutation were not excluded from prescreening because KRAS mutations were present in some FGFR1 positive cell lines that responded to ponatinib.²² If sufficient tissue remained and the RET rearrangement status was not already known, patients with adenocarcinoma or NOS histologies testing negative for both FGFR1 ISH and SISH (and, for the University of Colorado Hospital (UCH) internal cases, who were also 'pan-negative' for other known drivers) were then tested for RET rearrangement, a further known target of ponatinib, by FISH.

FGFR1 mRNA testing (in-situ hybridization [ISH])

mRNA in situ hybridization (ISH) was performed on formalin-fixed paraffin embedded (FFPE) tumor tissue using the RNA scope 2.0 assay system with recommended probes from Advanced Cell Diagnostics, Inc. (Hayward, CA) (FGFR1 Cat. 310071). ISH scores were generated and recorded using the following scoring system at 200 × magnification: 0, no staining; 1, one to 3 dots per tumor cell; 2, 4 to 10 dots per tumor cell; 3, more than 10 dots per cell or presence of dot clusters in 1% and < 10% tumor cells; 4, 10% tumor cells with dot clusters as per the RNA scope system scoring guidelines.¹⁸ The pre-defined cutpoint for FGFR1 ISH positivity was a score of 3 or 4 per this scoring system. FGFR1 SISH has been validated against FGFR1 FISH.²²

FGFR1 gene amplification (silver in-situ hybridization [SISH])

Dual-color silver in situ hybridization (SISH) was performed on FFPE tissue, using a fully automated protocol on the Ventana BenchMark XT instrument (Ventana). All reagents, including FGFR1 2,4-Dinitrophenol (DNP) probe (FGFR1 locus on 8p12, Cat. 760–1217)

and chromosome 8 digoxigenin (DIG) probe (Centromere 8 [CEN8] Cat. 760–1220) cocktails, ultraView SISH, and ultraView Alkaline Phosphatase Red ISH detection kits, were obtained from Ventana Medical Systems Inc. (Tucson, AZ). FGFR1 and CEN8 signals were counted separately in 50 non-overlapping tumor nuclei per core. FGFR1 minor signal clusters and major signal clusters were counted as 6 and 12 signals, respectively (Ventana Interpretation Guide, <http://links.lww.com/JTO/A837>). For each core, the mean copy number per nucleus of each probe (FGFR1 and CEN8), the FGFR1/CEN8 ratio, and the percentage of cells with FGFR1 signal clusters were calculated. The pre-defined cutpoint for FGFR1 amplification (SISH+) was an average of at least four FGFR1 signals per nucleus (gene copy number) or FGFR1/CEP8 ratio ≥ 2.0 .^{22,25} FGFR1 ISH to quantify mRNA expression has been validated against quantitative RT-PCR.²² Two pathologists (HY and TAB) independently scored each core. Any specimens with discrepant results were re-evaluated by both pathologists for a consensus result.

Additional molecular testing including RET rearrangement testing (fluorescent in-situ hybridization [FISH])

Hematoxylin and Eosin-stained paraffin sections were examined by a board-certified anatomic pathologist for testing suitability. Tumor cells were isolated by microscope assisted microdissection followed by tumor cell lysis and DNA extraction.

Mutational analysis of EGFR, KRAS, BRAF and ERBB2 (HER2) were carried out either using real-time polymerase chain reaction (RT-PCR) for individual targets (Quiagen, Inc) or TruSight Tumor sequencing panel (Illumina, Inc.) for multiple gene targets.

Using a manual slide processing fluorescence in situ hybridization (FISH) technique, paraffin sections were hybridized with fluorescent probes. MET probe (Vysis LSI MET SpectrumRed Probe) in combination with CEP7 probe (Vysis CEP7 SpectrumGreen Probe) was utilized to score for MET amplification. 5'ALK probe in combination with 3'ALK probe (Vysis ALK Break Apart FISH Probe kit) was utilized to score for ALK rearrangement. 5'ROS1 probe (SureFISH ROS1 5' from Agilent/DAKO) in combination with 3'ROS1 probe (Vysis LSI ROS1 (Cen) Spectrum Green Probe from Abbott Molecular) was utilized to score for ROS1 rearrangement. 5'RET probe (Vysis LSI RET (Cen) SpectrumGreen Probe) in combination with 3'RET probe (Vysis LSI RET (Tel) SpectrumRed Probe) was utilized score for RET rearrangement. For ALK, ROS1, and RET gene rearrangement, FISH positivity was defined by $> 15\%$ of cells with split signals, with at least 50 cells scored. Clinically significant MET gene amplification was defined by MET to chromosomal centromere (CEP7) ratio ≥ 5.0 .²⁶ The received FFPE specimen was subjected to the standard FISH laboratory protocol and hybridized with these probe sets.²⁷ Analyses were performed in fluorescence microscope with proper interference filters.

Study design and treatment

This was an open-label, phase II, biomarker-driven study of ponatinib monotherapy divided into a prescreening phase and a treatment phase (see below). The study protocol was approved by the Colorado Institutional Review Board (COMIRB) and in accordance with the Declaration of Helsinki. Prescreened patients were categorized into four molecular

cohorts based on ISH and SISH positivity – FGFR1 ISH+/SISH+, FGFR1 ISH+/SISH-, FGFR1 ISH-/SISH+, and FGFR1 ISH-/SISH- (double negative) – with the provision of testing for RET rearrangement in the double negative cohort.

The primary objectives of this study were to determine the prevalence of the defined biomarkers (FGFR1 ISH+, SISH+, or both) in the prescreened population and the objective response rate (ORR, RECIST v1.1) to ponatinib treatment in each molecularly defined cohort. The secondary objectives were to determine the clinical and pathological features associated with biomarker positivity and negativity at the pre-defined cut point and at higher cut points (where applicable), to determine the safety, tolerability, and adverse event profile of ponatinib in patients with advanced lung cancer, to determine the progression free survival (PFS), pattern of failure (CNS vs. extra-CNS) and response duration (in those who achieved an objective response) of ponatinib in the molecularly defined cohorts, and to determine the ORR in RET+ NSCLC and in the FGFR1 double-negative cohort.

The study employed a one-stage design.²⁸ In each molecularly defined cohort, the study required 12 subjects to decide whether ORR is greater than 40% assuming the response rate is 5% without treatment. Twelve subjects per cohort provided over 90% power to detect this rate difference with a targeted type I error rate of 0.05 and an actual error rate of 0.02. If 3 patients responded per molecular cohort, then the hypothesis that $P = 0.05$ (null hypothesis) would be rejected, but if 2 patients responded then the hypothesis that $P = 0.40$ would be rejected.

Toxicity was graded according to the NCI Common Terminology Criteria for Adverse Events (NCI-CTCAE), version 4.0. A dose level was considered tolerable following completion of 1 cycle (28 days) in 9 of 12 patients in the absence of dose limiting toxicity (DLT). Adverse events (AEs) were assessed at screening, on days 1, 8, and 15 of Cycle 1, and day 1 of each further cycle in the first 12 patients accrued in the entire study. All other patients had AEs assessed at screening and on day 1 of each cycle. Disease was assessed by CT or PET/CT (including IV contrast if target lesions include those other than parenchymal lung lesions) +/- brain imaging as indicated per protocol at baseline, and then every 2 cycles (8 weeks) per RECIST v1.1 by local investigators. Median overall survival (OS) was calculated using Kaplan-Meier method.

Patients received ponatinib 45 mg oral daily in the FGFR1 ISH+/SISH+, ISH+/SISH-, ISH-/SISH+, and ISH-/SISH-/RET- cohorts. Exposures at the standard ponatinib dose of 45 mg QD were predicted to be sufficient to affect FGFR1 in vivo (see 'Prescreening for ponatinib biomarkers'). During the course of the trial, a black box warning (October 2013) emerged regarding the potential vascular complications associated with ponatinib use. A series of FDA- mandated protocol changes were then introduced. Due to the potential for long term vascular risks with ponatinib, doses above 45 mg QD were not explored. Subjects with history of vascular complications were excluded. In the absence of contraindications, low dose aspirin 81 mg QD and a statin (e.g., atorvastatin 10 mg QD) were recommended to mitigate the risk of vascular complications. As the IC_{50} for RET is an order of magnitude lower than for FGFR1, the starting dose for the RET cohort was 30 mg QD. Each cycle was 28 days.

Ponatinib treatment eligibility

Key inclusion criteria: All NSCLC and SCLC patients were eligible for prescreening, met biomarker criteria (FGFR1 ISH+/SISH+, ISH+/SISH-, ISH-/SISH+, ISH-/SISH-/RET-, or ISH-/SISH-/RET+) using one of the assays described (see *FGFR1 ISH* and *SISH* and *RET FISH*), had measurable disease per RECIST v1.1, had received prior platinum-based chemotherapy, had life expectancy ≥ 3 months, ECOG performance status 0 to 2, and normal hematologic, renal, and hepatobiliary function. Patients with CNS metastases were eligible if they had stable CNS disease.

Key exclusion criteria: History of clinically significant bleeding disorder, clotting disorder (myocardial infarction, stroke, venous / arterial thromboembolism), acute pancreatitis within 1 year of study or history of chronic pancreatitis, uncontrolled hypertriglyceridemia (triglyceride > 450 mg/dL), and history of ventricular arrhythmia (except premature ventricular complexes).

Clinical and molecular correlation with biomarker positivity

Clinical and pathologic features including age at diagnosis of metastatic lung cancer, sex, histology, sites of metastatic disease at diagnosis, smoking status, other known molecular drivers, including EGFR, KRAS, ALK, ROS1, BRAF V600, MET amplification, MET exon 14 skip lesion, HER2 mutation, HER2 amplification, response to first line platinum doublet chemotherapy for metastatic lung cancer, and overall survival from the date of diagnosis of metastatic disease were collected. Association of these features and treatment response to ponatinib with biomarker positivity at the pre-defined cut point, and at higher cut points (where applicable), were explored. In addition to the pre-defined 4 molecular cohorts (FGFR1 SISH+/ISH+, SISH+/ISH-, SISH-/ISH+, SISH-/ISH-), SISH and ISH were assessed separately to maximize the sample size if each molecular cohort was too small due to slow accrual.

Descriptive information for the molecular subgroups for categorical variables were computed and differences between the molecular cohorts were assessed using Fisher's exact test. For continuous variables, the differences between the molecular cohorts were assessed using the Kruskal-Wallis rank sum test due to their non-normal distribution. Kaplan-Meier curves were used to examine overall survival (from metastatic diagnosis) between the molecular cohorts and differences in survival times was assessed using the log-rank test. A significance level of 0.05 was used for all hypothesis testing and p-values were not corrected for multiple testing due to the exploratory nature of this work.

Results:

In Oct 2013, following the black box warning RE: ponatinib-associated vascular issues, the treatment aspects of the protocol were put on hold, but prescreening continued. From Sept 2013 to November 2017, 171 patient samples were prescreened by FGFR1 ISH and SISH. Forty (23.4%) had insufficient tissue or failed screening; four had incomplete clinical information. As a result, 127 patients were analyzable. Following protocol modifications, the treatment aspect of the protocol was reopened, but the awareness of the risks associated with

ponatinib and the additional inclusion and exclusion criteria severely limited the number of cases eventually treated with the drug.

Initial biomarker-positivity rate

Of the 127 patient samples with analyzable FGFR1 results, four samples did not have reportable SISH scores and one sample did not have a reportable ISH score due to insufficient sample. All four samples missing a SISH score were ISH negative, and the sample missing an ISH score was SISH negative. Therefore, 123 samples had a SISH score, 126 samples had an ISH score, and 122 samples had both SISH and ISH scores reportable. According to the pre-defined FGFR1 molecular subgroups, 6/122 (4.9%) were SISH+/ISH+, 3/122 (2.5%) were SISH+/ISH-, 47/122 (38.5%) were SISH-/ISH+, and 66/122 (54.1%) were SISH-/ISH- ('double negative'). In the FGFR1 SISH/ISH 'double-negative' cohort, 0/33 (0%) patients tested positive for RET gene rearrangement by FISH. Incidentally, 1/3 (33%) patients who had RET FISH performed in the FGFR1 SISH+/ISH+ subgroup had a RET gene rearrangement. Across the four ISH/SISH combination subgroups, there was no significant difference in age, sex, smoking history, sites of metastatic disease at stage IV diagnosis, histology, response to 1st line platinum-double therapy, and concomitant molecular drivers.

Because of the small size of the SISH+ group (N=9), clinical and molecular characteristics associated with FGFR1 biomarker positivity were conducted in ISH and SISH separately. At the pre-defined cut point for SISH positivity, 9/123 (7.3%) patients were SISH positive (Table 1); 6/9 SISH+ patients were concordantly positive for SISH and ISH, all of whom had high percentage (≥ 20%) of tumor cells with dot clusters by ISH (Appendix: Table S1). Stratified by histology, of the SISH-tested cases, 4/81 [4.9%] adenocarcinoma, 2/21 [9.5%] SCLC, and 3/21 [14.2%] SCLC were SISH positive. There was a significant difference in the prevalence of KRAS mutation between the SISH+ (0/6 [0.0%]) and SISH- (45/93 [48.4%]) cohort (p=0.030), even though the patient numbers were small (adenocarcinoma [4/6 SISH+] and squamous [2/6 SISH+]) (Table 1). Otherwise, there was no significant clinical association of age, sex, smoking history, sites of metastases, histology, response to first line platinum-doublet chemotherapy, or non-KRAS mutations with SISH positivity. There was no difference in survival between the SISH+ and SISH- group (median: 417 days [513–870] vs. 649 days [294–NA], p=0.694) (Figure 1).

At the pre-defined cut point for ISH positivity, 53/126 (42.1%) were ISH positive (Table 1). Stratified by histology, of the ISH-tested cases, 30/84 [35.7%] adenocarcinoma, 12/21 [57.1%] SCLC, and 11/21 [52.3%] SCLC were ISH positive. There were no significant differences in age, sex, smoking history, sites of metastases, histology, response to platinum-based chemotherapy, or co-occurring molecular drivers between the ISH+ and ISH- group at the pre-defined positivity criteria. There was a high prevalence of KRAS in both ISH+ and ISH- subgroup, but there was no significant difference in their prevalence (ISH+: 16/41 [39.0%] and ISH-: 32/61 [52.5%]; p=0.226). Importantly, the ISH+ group had a significantly shorter median overall survival than in the ISH- group (median OS: 530 days [394–757] vs. 753 days [625–1247], p = 0.044) (Figure 2).

To summarize important features associated with FGFR1 gene copy number and mRNA level, a heat map diagram showing absolute ISH (RNA scope score 0 to 4 and percentage of tumor cells with dot clusters) and SISH scores (gene copy number and FGFR1/CEP8 ratio) correlated with smoking status, histology, and the most common co-occurring mutations (KRAS, p53, MET exon14, and PIK3CA) is shown in Figure 3. Other notable co-occurring mutations included RET rearrangement (1/3 [33.3%] in SISH+/ISH+, 0% in the other FGFR1 subgroups), HER2 amplification by FISH (1/2 [50%] in SISH-/ISH-, not tested in other FGFR1 subgroups), BRAF G466A (1/46 [2.2%] in SISH-/ISH-, 0% in the other FGFR1 subgroups), which although is associated with kinase impairment, paradoxically may be associated with ERK activation²⁹, and AKT1 mutation (1/32 [3.1%] in SISH-/ISH+ and 0% in the other FGFR1 subgroups).

Ponatinib treatment

A total of 4/56 biomarker positive (6 SISH+/ISH+, 3 SISH+/ISH-, and 47 SISH-/ISH+) patients were treated with ponatinib at a starting dose of 45 mg oral daily, and their biomarker results, treatment response, and on-treatment complications are summarized in Table 2. None of the patients in the double-negative (SISH-/ISH-) cohort were treated with ponatinib. The ORR of the entire treated cohort (N=4) was 0%, but the disease control rate (DCR) was 50% (complete response [CR] + partial response [PR] + stable disease [SD]). The median PFS was 1.8 months (1.8 to 5.1 months). The median duration of treatment was 1.6 months (1.3 to 4.3 months). A detailed account of each patient's treatment course and a history of their post-treatment details and outcomes are provided:

Patient 1 had FGFR1 ISH+ (score 3, 1% tumor cells with dot cluster), SISH and RET negative squamous lung cancer. They were pan-negative for other known molecular oncogene drivers (EGFR, ALK, ROS1, MET amplification, MET exon 14, BRAFV600, HER2). Ponatinib 45 mg oral daily was started on 11/21/2014. They developed disease progression (PD) on the first scan (01/02/2015). The treatment course was complicated by grade 3 hypertension (11/26/14), requiring two anti-hypertensives. Ponatinib was discontinued on 01/02/2015 for Gr. 3 febrile neutropenia and disease progression. The patient went on to receive docetaxel plus ramucirumab, nivolumab, and an antibody-drug conjugate before being admitted for hypoxic respiratory failure, and passing away on 10/17/2015.

Patient 2 had FGFR1 ISH+ (score 4, 80% tumor cells with dot cluster), SISH+ (10.94 gene copy number, FGFR1/CEP8 ratio 3.99), and RET positive squamous lung cancer. They were pan-negative for other known molecular oncogene drivers. Ponatinib 45 mg oral daily was started on 08/21/2014. This was complicated by grade 2 thrombocytopenia and grade 3 abdominal pain due to bowel perforation, which required admission (09/27/2014 to 10/08/2014) for surgery (09/28/2014). CT abdominal imaging (10/02/2014) did not demonstrate PD. Ponatinib was held since day 3 (09/29/2014) of admission due to critical illness and concern that the perforation could be mediated by the study drug. Unfortunately, the patient elected for discharge to hospice, and passed away on 10/10/2014. The duration of stable disease was 41 days.

Patient 3 had FGFR1 ISH+ (score 4, 60% tumor cells with dot cluster), SISH negative and RET negative squamous lung cancer. They were pan-negative for other known molecular oncogene drivers. Ponatinib 45 mg oral daily was started on 08/17/2016 with a best response of stable disease (SD) lasting 4 cycles (153 days). The treatment course was complicated by an admission for atrial fibrillation and hyponatremia (drug held 09/21/2016 to 09/25/2016), edema (drug held 11/22/2016 to 11/28/2016), and persistent cough, low grade fever, and fatigue (drug held 12/23/2016 onwards). Other ponatinib-related adverse events (AE) included grade 1 myalgia, headache, rash, dry skin, alopecia, weakness and somnolence, and grade 2 hypertension. Unfortunately, the patient had a sudden episode of syncope on 01/16/17, 25 days after holding ponatinib, and was pronounced dead after unsuccessful resuscitation in the emergency room. An autopsy was not performed.

Patient 4 had FGFR1 ISH+ (score 3, 1% tumor cells with dot cluster), SISH and RET negative, poorly differentiated NSCLC. They were pan-negative for other known molecular oncogene drivers. Ponatinib 45 mg oral daily was started on 06/16/2015 with a best response of PD (on 08/10/2015) after 2 cycles. The patient developed grade 1 fatigue and grade 2 hypertension, but no SAE or grade 3 to 5 AE were noted. The patient was subsequently treated with an antibody- drug conjugate from 08/25/2015 to 11/17/2015, but discontinued for PD. They passed away on 02/29/2016.

Exploration of alternate FGFR1 positivity cut-points

When the results of all 123 SISH-tested patients were plotted in a continuum from lowest to highest score, the data suggested that the pre-existing definition for SISH positivity, FGFR1/CEP8 2.0 and / or FGFR1 mean GCN 4, was appropriate because this cut point best demarcated the greatest change in the slope of the curve (Appendix: Figure S1). Furthermore, there were only 9 SISH+ patients at the current cutpoint. A higher SISH cutpoint would further reduce the number of SISH+ patients, limiting the ability to detect group differences.

To determine if an alternative FGFR1 ISH positive cut point should be considered, results of all 126 ISH-tested patients were plotted in a continuum from lowest to highest score. ISH 20% tumor cells with dot clusters was selected as an alternative cut point for positivity because this metric clearly separated those with high ISH scores from those with a negative or low-positive ISH scores (Figure 4). Using this new cut-point, all 126 patients with reported ISH scores were then explored to see if the new cut point was associated with distinguishing clinical and molecular features as a means of suggesting that patients with ISH scores above this cut point represented a biologically distinct subset than those below it.

Clinical and molecular characteristics associated with the ISH 20% (N=29) and ISH < 20% (N=97) subgroups are summarized in Table 3. Two of the four patients with a best response of SD from ponatinib were ISH 20%, whereas the two ponatinib-treated patients with a best response of PD were ISH < 20%. Of the 126 patients tested for ISH, 29 (23.0%) patients were ISH 20%. In the ISH 20% group, more patients had soft tissue metastases (17.2% vs 5.0%, p=0.050) and small cell histology (34.5% vs. 11.3%) whereas fewer patients had adenocarcinoma (51.7% vs. 71.1%) and squamous carcinoma (13.8% vs 17.5%) (p=0.022).

Using the new cut point, survival continued to be significantly shorter in the ISH 20% group (median OS 394 days [318-NA] vs. 730 days [548-1046], $p=0.031$) (Appendix: Figure S2). The prevalence of concomitant KRAS mutation in both ISH 20% and ISH < 20% were high (45.0% vs. 47.6%, respectively; $p=1.00$).

Discussion:

Recent efforts have explored FGFR1 gene amplification, as defined by either an increased gene copy number (≥ 4) or an FGFR1/CEP8 ratio ≥ 2.0 , in squamous lung cancer as a predictive biomarker for response to FGFR1 inhibitors, including AZD4547, BGJ398, and Dovitinib, with modest success.¹⁹⁻²¹ The response rate to these agents ranged from 8–11.5%.

In this study, ponatinib, a pan-FGFR inhibitor that was originally developed for treatment-refractory CML, was used in this FGFR1 biomarker-driven study, partly to test the efficacy of ponatinib in 'FGFR1 positive' patients compared to FGFR1 negative patients given the rationale that FGFR1 positivity would more likely identify lung cancers in an FGFR1-addicted state. As demonstrated in our study and in other pre-clinical and clinical studies, FGFR1 ISH and SISH only partially overlap, suggesting that FGFR1 gene amplification does not always lead to downstream transcription and also that elevated transcription can occur in the absence of gene amplification.^{20,22} All of this evidence strongly suggests that FGFR1 gene amplification is insufficient as a predictive biomarker when used in isolation. This study is an early effort to explore the feasibility of using novel biomarkers instead of or in addition to FGFR1 gene amplification to predict response to FGFR1 inhibitors such as ponatinib.

Initial ISH positivity was defined as presence of dot clusters ($\geq 1\%$ of tumor cells) or >10 dots/cell in $\geq 10\%$ tumor cells. Initial SISH positivity was defined as average of at least four FGFR1 signals per nucleus (gene copy number) or FGFR1/CEN8 ratio ≥ 2.0 . Using these criteria, 7.3% patients were SISH+ and 42.1% were ISH+. At the initial ISH and SISH cutpoints, no specific correlation with age, sex, smoking status, or response to platinum therapy were identified. SISH positive disease was less likely to harbor a coincident KRAS mutation than SISH negative disease, and ISH positive cases had worse OS than ISH negative cases.

Exploration of the spread of biomarker positive data did not suggest a more appropriate SISH positivity cutpoint but did suggest a higher biological cutpoint – ISH 20% tumor cells with dot clusters – as a new ISH positive criterion (23% of tested cases) (Figure 4). The objective of exploring FGFR1 ISH at a higher cut point was to see whether a subset of patients with much higher mRNA expression would reveal a biologically distinct subset of patients based on significant differences in clinical and molecular features. Using this higher cutpoint, FGFR1 ISH+ was associated with SCLC histology, soft tissue metastases at diagnosis and remained associated with shorter OS in univariate analysis. Given the small number of patients with small cell and squamous histology, multivariable analysis to control for variables that showed a significant association with FGFR1 positivity in univariate analysis was not feasible.

Unfortunately, due to poor tolerability and safety concerns regarding ponatinib after treating the first few patients, the study did not reach its accrual target (N~70) for the treatment portion of this study and could not confirm or refute the null hypothesis (needed a minimum of 12 patients treated). This study prescreened a more heterogeneous population, including lung adenocarcinoma, which is amenable to a much larger arsenal of treatment options than squamous lung cancer, and SCLC, the latter of which, due to its rapidly progressive nature, often precludes patients from receiving the drug after initial lines of chemotherapy. As a result, all four patients that were enrolled onto the ponatinib treatment arm had either squamous or poorly differentiated lung cancer.

Since the number of patients treated with ponatinib was small (N=4), the ability to deduce the value of these FGFR1 biomarkers for predicting benefit from this drug was limited. Although the four FGFR1 preselected patients treated with ponatinib had a 0% response rate, half of them still had a best response of stable disease.

The apparent lack of response to ponatinib in a FGFR1-preselected population could be due to multiple factors. The treated patients may not have responded because ponatinib was not potent enough to inhibit FGFR1 signaling, and the maximum tolerated dose was limited due to many off-target effects. Our data suggests that FGFR1 biomarker positivity, especially at higher cut points, may enrich for a biologically distinct subset that may be dependent on FGFR1 activation and signaling, which in turn may predict sensitivity to FGFR1 inhibitors such as ponatinib.

However, FGFR1 gene amplification and mRNA expression by SISH and ISH may not be the best biomarker to determine FGFR1 addiction considering FGFR1 signaling may be dependent on autocrine and paracrine ligand binding^{22,30}, FGFR1 gene amplification and mRNA expression may not lead to functional activation, FGFR1 expression is likely heterogeneous within and between tumors²⁰, and neither gene amplification or mRNA overexpression necessarily determine a state of constitutive activation of cell proliferation, differentiation, and survival, unlike gain-of-function mutations such as EGFR activating mutation or KRAS mutation. Finally, a true FGFR1-addicted state may be so rare that the cut point of ISH and SISH needed to be so much higher that our relatively small sample size of prescreened patients was insufficient to differentiate those patients.

This sample of 127 analyzable patients provides a rich data set for biomarker exploration. That FGFR1 mRNA (ISH) did not seem to be very specific for an oncogene-addicted state may reflect a less robust methodology of scoring compared to FGFR1 SISH. Whereas SISH requires the pathologist to average the number of cell signals / nucleus over 50 cells, ISH criteria does not mandate a minimum number of cells examined. Instead, the pathologist scores the tissue sample based on a global impression and provides a best estimate of the number of dots per cell and the percentage of tumor cells with dot clusters. That these two biomarkers are not completely overlapping suggests that neither biomarker is sufficient as a standalone biomarker. Other than suboptimal patient enrollment, another limitation is that a FGFR1 antibody to detect FGFR1 protein expression by immunohistochemistry (IHC) was not implemented into the biomarker set as no validated antibody was available at the time.

Conclusion

High FGFR1 mRNA expression is more common than FGFR1 amplification and was associated with worse OS. Higher levels of FGFR1 mRNA expression and gene amplification may be associated with a specific phenotype and is worthy of further exploration. Ponatinib's poor tolerance suggests further FGFR exploration in ISH+ cases should utilize more selective FGFR1 inhibitors.

Clinical Practice Points:

Recent trials of FGFR1 inhibitors in SCLC with FGFR1 gene amplification showed modest response rates. Pre-clinical experiments on NSCLC and SCLC cancer cell lines and resected tumors demonstrated that FGFR1 mRNA and protein overexpression were more predictive of ponatinib sensitivity than FGFR1 gene amplification. Based on this data, a phase II biomarker- driven study using FGFR1 gene amplification by silver in-situ hybridization (SISH) and mRNA expression by in-situ hybridization (ISH) in NSCLC and SCLC patients treated with ponatinib was conducted. A total of 171 patient samples were prescreened using FGFR1 ISH and SISH, and 127 cases were analyzable. The prevalence of FGFR1 ISH+ and SISH+ cases was 42.1% and 7.3%, respectively. Due to a black box warning regarding potential vascular complications associated with ponatinib, the drug was put on hold. Although the treatment aspect of the protocol was re-opened after protocol modification, due to awareness of ponatinib-associated risks, only 4 patients were treated with ponatinib at 45 mg oral daily. The objective response rate was 0% with a disease control rate of 50% (stable disease in two patients with a high FGFR1 ISH score). Association of clinicopathologic features with FGFR1 ISH and SISH positivity at different cutpoints were explored. FGFR1 ISH positivity at a higher cutpoint may identify a distinct clinical phenotype (soft tissue metastases, small cell histology, worse survival) of lung cancer patients that may benefit from FGFR1 inhibition and is worthy of further exploration.

Ponatinib's poor tolerance suggests further FGFR exploration in ISH+ cases should utilize more selective FGFR1 inhibitors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Overall survival of SISH+ versus SISH- group at pre-defined cut point (GCN ≥ 4.0 GCN or FGFR1/CEP8 ≥ 2.0).

Figure 2.
Overall survival of ISH+ versus ISH- group at pre-defined cutpoint (1% tumor cells with dot clusters)

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Figure 4.
FGFR1 ISH Score by Percentage (%) Cells with Dot Clusters from Low-To-High Score

Table 1.

Summary of relevant clinical and pathologic features associated with FGFR1 ISH and SISH positivity at pre-defined and at exploratory cutpoints.

	SISH (N=123)			ISH (N=126)		
	SISH- (N=114)	SISH+ (N=9)	ISH- (<1%) (N=73)	ISH+ (1%) (N=53)	ISH<20% (N=97)	ISH 20% (N=29)
Age (median [IQR])	64.70 [58.05, 72.45]	68.40 [65.30, 69.50]	63.80 [58.02, 70.15]	65.70 [60.00, 72.60]	64.9 [58.6, 72.5]	64.7 [53.5, 70.5]
Male Sex (%)	55/114 (48.2)	7/9 (77.8)	37/73 (50.7)	26/53 (49.1)	47/97 (48.5)	16/29 (55.2)
Smoking PY (median [IQR])	30.00 [11.3, 45.0]	40.00 [33.0, 57.0]	30.00 [11.3, 45.8]	32.00 [20.0, 50.0]	30.00 [12.9, 48.5]	30.00 [20.0, 50.0]
Sites of metastases						
• Soft tissue (%)	10/114 (8.8)	0/9 (0.0)	4/73 (5.5)	6/53 (11.3)	5 (5.2)	5 (17.2)
Histology (%)						
• Adenoca	77/114 (67.5)	4/9 (44.4)	54/73 (74.0)	30/53 (56.6)	69 (71.1)	15 (51.7)
• Squamous	19/114 (16.7)	2/9 (22.2)	9/73 (12.3)	12/53 (22.6)	17 (17.5)	4 (13.8)
• Small cell	18/114 (15.8)	3/9 (33.3)	10/73 (13.7)	11/53 (20.8)	11 (11.3)	10 (34.5)
•						
KRAS (%)	45/93 (48.4)	0/6 (0.0)	32/61 (52.5)	16/41 (39.0)	39/82 (47.6)	9 /20 (45.0)

Variables of significant difference between biomarker group are highlighted in **bold**

Table 2.

Biomarker Results, and Treatment Outcomes and Complications Related to Ponatinib

Patient	Histology	ISH score	SISH score	RET	Best response	Outcomes /complications
1	Squamous	Positive Score 3 1% dot cluster	Negative	Negative	PD on day 43 (D/C for PD)	AE: Gr. 3 HTN and Gr. 3 FN OS: 330 days
2	Squamous	Positive Score 4 80% dot cluster	Positive GCN 10.94 FGFR1/ CEP8 3.99	Positive	SD for 40 days (D/C for bowel perforation)	AE: Gr. 2 ↓ PLT; Gr. 3 Bowel perforation OS: 51 days
3	Squamous	Positive Score 4 60% dot cluster	Negative	Negative	SD for 153 days (Drug held indefinitely on day 129)	AE: Gr. 3 Atrial fibrillation Gr. 3 Hyponatremia Gr. 2 Edema Gr. 2 Cough OS: 153 days (Syncope, unsuccessful resuscitation)
4	Poorly differentiated	Positive Score 3 1% dot cluster	Negative	Negative	PD on day 56 (D/C for PD)	AE: Gr. 1 fatigue; Gr. 2 HTN No Gr. 3 AE OS: 259 days

All patients were started on Ponatinib 45 mg daily

SD = stable disease; PD = progressive disease; AE = adverse event; OS = overall survival; HTN = hypertension; FN = febrile neutropenia, PLT = platelets

Table 3.

Clinical and Molecular Features of FGFR1 ISH+ vs ISH- subgroup alternate positivity cut point (ISH ≥ 20% tumor cells with dot clusters)

	ISH <20%	ISH ≥ 20%	P-value
N	97	29	
Age at Stage IV Dx (median [IQR])	64.9 [58.6, 72.5]	64.7 [53.5, 70.5]	0.497
Male Sex (%)	47/97 (48.5)	16/29 (55.2)	0.673
Pack Years Smoking (median [IQR])	30.00 [12.88, 48.50]	30.00 [20.00, 50.00]	0.772
Sites of metastases at stage IV			
Adrenal (%)	6 (6.2)	3 (10.3)	0.429
Bone (%)	32 (33.0)	9 (31.0)	1.000
Dx beyond lung			
Brain (%)	16 (16.5)	7 (24.1)	0.412
Liver (%)	12 (12.4)	7 (24.1)	0.142
Soft tissue (%)	5 (5.2)	5 (17.2)	0.050
Pleural /Pericardial (%)	34 (35.1)	7 (24.1)	0.367
Lymph node (%)	73 (75.3)	23 (79.3)	0.805
Histology (%)			
Adenoca /adenosquamous	69 (71.1)	15 (51.7)	0.022
Squamous	17 (17.5)	4 (13.8)	
Small cell /LCNE	11 (11.3)	10 (34.5)	
Platinum Response (%)	PR/CR		
	33 (62.3)	13 (86.7)	0.117
KRAS (%)	39/82 (47.6)	9 /20 (45.0)	1.000
ROS1 (%)	0/68 (0.0)	0/12 (0.0)	---
MET FISH (%)	0/66 (0.0)	0/14 (0.0)	---
METexon14 (%)	3/49 (6.1)	1/13 (7.7)	1.000
BRAF (%)	1/72 (1.4)	0/16 (0.0)	0.761
HER2 (%)	0/72 (0.0)	0/16 (0.0)	N/A
HER2/CEP17 ≥ 2.0 (%)	1/2 (50.0)	N/A1	1.000
RET (%)	0/52 (0.0)	1/13 (7.7)	0.200

¹ N/A: HER2 FISH testing was not performed on any of these patients