Preselection of Lung Cancer Cases Using *FGFR1* mRNA and Gene Copy Number for Treatment With Ponatinib

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Abstract

A phase 2 biomarker-driven study using epidermal growth factor receptor 1 *FGFR1* gene copy number by silver in-situ hybridization (SISH) and messenger RNA expression by in-situ hybridization (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.

Introduction: Preclinically, high epidermal growth factor receptor 1 (*FGFR1*) messenger RNA (*FGFR1*-MRNA) and *FGFR1* amplification (*FGFR1*-AMP) predicted sensitivity to fibroblast growth factor receptor inhibitors in non–small-cell lung cancer and small-cell lung cancer 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). Patients with positive findings were offered ponatinib, a multi–kinase inhibitor of *FGFR1-4*. Differences in overall survival (OS) between cohorts were assessed by the log-rank test. Association of *FGFR1* positivity with clinicopathologic features were assessed by Fisher exact test and Kruskal-Wallis rank sum test. **Results:** A total of 171 cases were prescreened: 9 (7.3%) of 123 $SISH^+$; 53 (42.1%) of 126 ISH^+; and 6 cases concordantly positive for SISH and ISH. $SISH^+$ cases had fewer coincident *KRAS* mutations ($P = .03$) than $SISH^-$ cases, and ISH^+ cases had worse OS ($P = .020$) than ISH^- cases. Data distributions suggested a distinct higher positivity cut point for *FGFR1* ISH ($\geq 20\%$), occurring in 29 (23%) of 126 cases, was associated with small-cell lung cancer histology ($P = .022$), soft tissue metastases ($P = .050$) and shorter OS ($P = .031$). Four patients received ponatinib on study: All ISH^+ by the initial cut point, 2 of 4 by higher cut point, 1 of 4 $SISH^+$. Tolerability was poor. The best response for the 2 higher ISH cases was stable disease and progressive disease for the 2 lower ISH cases. **Conclusion:** 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 fibroblast growth factor receptor exploration in ISH^+ cases should utilize more selective *FGFR1* inhibitors.

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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 has led to high objective response rates (ORRs) of 40% to 70%, which are often durable, and generally the tyrosine kinase inhibitors 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 histologic 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 PI3K, RAS/RAF/MAPK, and protein kinase C 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 (*FGFR1*-AMP), and preclinical studies demonstrated response to *FGFR1* inhibition in *FGFR1*-amplified SQLC in vitro and in vivo.[13-15] *FGFR1*-AMP 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*-AMP, 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%-11.5%), with a disease control rate of 38.5% to 50%.[19-21]

One possible explanation for this is that gene copy number (GCN) may not lead to functional messenger RNA (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 (46%) of 13 samples with GCN $\geq$ 4 also had high mRNA expression (4+, per RNA scope guidelines). Conversely, 6 (13.6%) of 44 samples with mRNA 4+ also had GCN $\geq$ 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 before FGFR tyrosine kinase inhibitor therapy. After treatment with ponatinib, a multikinase inhibitor of *FGFR1*, -2, -3, and -4 (drug concentration causing 50% inhibition [IC_{50}] = 2, 2, 18, and 8 nmol/L, respectively),[23] as well as platelet-derived growth factor receptors alpha and beta, rearranged during transfection (*RET*), and vascular endothelial growth factor receptors 1 to 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 GCN score, *FGFR1* mRNA (area under the curve [AUC] 0.905) and protein (AUC 0.887) were more predictive of ponatinib sensitivity compared to *FGFR1* GCN (AUC 0.691).[22]

On the basis of this preclinical data, a phase 2 trial was designed to determine the prevalence of and factors associated with FGFR 1 biomarker (GCN and mRNA) positivity in NSCLC and SCLC, and to explore the predictive value of these biomarkers for ponatinib sensitivity.

Patients and Methods

Prescreening for Ponatinib Biomarkers

Using formalin-fixed, paraffin-embedded (FFPE) cell line pellets, clinically translatable mRNA (in-situ hybridization, ISH) and GCN (silver in-situ hybridization, 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, GCN, 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 $IC_{50} \leq 50$ nM based on the predicted serum trough level of 60 nM at the recommended phase 2 dose of 45 mg received orally daily in a previous clinical trial.[24]

Patients $\geq$ 18 years of age with advanced lung cancer of all histologies (NSCLC and SCLC) other than carcinoid were prescreened for tumoral *FGFR1* GCN by SISH and for *FGFR1* mRNA levels by ISH. If the underlying histology was NSCLC with adenocarcinoma or not otherwise specified, 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 not otherwise specified histologies testing negative for both *FGFR1* ISH and SISH (and for the University of Colorado Hospital 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 by ISH*

mRNA ISH was performed on FFPE tumor tissue samples using the RNA scope 2.0 assay system with recommended probes from Advanced Cell Diagnostics (*FGFR1* Cat. 310071). ISH scores were generated and recorded using the following scoring system at 200× magnification: 0, no staining; 1, 1 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 $\geq$ 1% and < 10% tumor cells; and 4, $\geq$ 10% tumor cells with dot clusters as per the RNA scope system scoring guidelines.[18] The predefined cut point 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 by SISH*

Dual-color SISH was performed on FFPE tissue samples using a fully automated protocol on the Ventana BenchMark XT instrument. All reagents, including *FGFR1* 2,4-dinitrophenol probe (*FGFR1* locus on 8p12, Cat. 760-1217) and chromosome 8 digoxigenin probe (centromere 8 [CEN8]; Cat. 760-1220)

cocktails, ultraView SISH, and ultraView Alkaline Phosphatase Red ISH detection kits, were obtained from Ventana. *FGFR1* and CEN8 signals were counted separately in 50 nonoverlapping 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 predefined cut point for *FGFR1*-AMP ($SISH^+$) was an average of at least 4 *FGFR1* signals per nucleus (GCN) or *FGFR1* per centromere of chromosome 8 (CEP8) ratio ≥ 2.0.[22,25] *FGFR1* ISH to quantify mRNA expression has been validated against real-time quantitative PCR.[22] Two pathologists (H.Y. and T.A.B.) independently scored each core. Any specimens with discrepant results were reevaluated by both pathologists for a consensus result.

Additional Molecular Testing

Hematoxylin and eosin–stained, paraffin-embedded 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 by real-time PCR for individual targets (Qiagen) or TruSight Tumor sequencing panel (Illumina) 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. The 5′ *ALK* probe in combination with 3′ *ALK* probe (Vysis *ALK* Break Apart FISH Probe kit) was utilized to score for *ALK* rearrangement. The 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. The 5′ *RET* probe (Vysis LSI *RET* [Cen] SpectrumGreen Probe) in combination with 3′ *RET* probe (Vysis LSI *RET* [Tel] SpectrumRed Probe) was the 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 2, biomarker-driven study of ponatinib monotherapy divided into a prescreening phase and a treatment phase The study protocol was approved by the Colorado institutional review board, and the study was performed in accordance with the Declaration of Helsinki. Prescreened patients were categorized into 4 molecular cohorts on the basis of 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 ORR (Response Evaluation Criteria in Solid Tumors [RECIST] v1.1) to ponatinib treatment in each molecularly defined cohort. The secondary objectives were to determine the clinical and pathologic features associated with biomarker positivity and negativity at the predefined cut point and at higher cut points (where applicable), to determine the safety, tolerability, and adverse event (AE) profile of ponatinib in patients with advanced lung cancer, to determine the progression-free survival, pattern of failure (central nervous system vs. extra–central nervous system) and response duration (in those who experienced 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 used a 1-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 the disease of $\geq$ 3 patients responded per molecular cohort, then the hypothesis that $P \leq .05$ (null hypothesis) would be rejected, but if the disease of $\leq$ 2 patients responded, then the hypothesis that $P \geq .40$ would be rejected.

Toxicity was graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events, version 4.0. A dose level was considered tolerable after completion of 1 cycle (28 days) in 9 of 12 patients in the absence of dose-limiting toxicity. 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 computed tomography or positron emission tomography/computed tomography (including intravenous contrast if target lesions included those other than parenchymal lung lesions) with or without 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 by the Kaplan-Meier method.

Patients received ponatinib 45 mg received orally daily in the *FGFR1* $ISH^+/SISH^+$, $ISH^+/SISH^-$, $ISH^-/SISH^+$, and $ISH^-/SISH^-/RET^-$ cohorts. Exposures at the standard ponatinib dose of 45 mg 4 times a day were predicted to be sufficient to affect *FGFR1* in vivo. 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 US Food and Drug Administration–mandated protocol changes were then introduced. Because of the potential for long-term vascular risks with ponatinib, doses above 45 mg 4 times a day were not explored. Subjects with a history of vascular complications were excluded. In the absence of contraindications, low-dose aspirin provided at 81 mg 4 times a day and a statin (eg, atorvastatin provided at 10 mg 4 times a day) were recommended to mitigate the risk of vascular complications. Because the IC_{50} for *RET* is an order of magnitude lower than for

FGFR1, the starting dose for the *RET* cohort was 30 mg 4 times a day. Each cycle was 28 days.

Ponatinib Treatment Eligibility

Key inclusion criteria were as follows: 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, had measurable disease per RECIST v1.1, had received prior platinum-based chemotherapy, had a life expectancy of $\geq$ 3 months, had Eastern Cooperative Oncology Group performance status 0 to 2, and had normal hematologic, renal, and hepatobiliary function. Patients with central nervous system metastases were eligible if they had stable central nervous system disease.

Key exclusion criteria were as follows: history of clinically significant bleeding disorder, clotting disorder (myocardial infarction, stroke, venous/arterial thromboembolism), acute pancreatitis within 1 year of the 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 were collected, 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 OS from the date of diagnosis of metastatic disease. Association of these features and treatment response to ponatinib with biomarker positivity at the predefined cut point, and at higher cut points (where applicable), were explored. In addition to the predefined 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 as a result of slow accrual.

Descriptive information for the molecular subgroups for categorical variables were computed, and differences between the molecular cohorts were assessed by the Fisher exact test. For continuous variables, the differences between the molecular cohorts were assessed by the Kruskal-Wallis rank sum test because of their nonnormal distribution. Kaplan-Meier curves were used to examine OS (from metastatic diagnosis) between the molecular cohorts, and differences in survival times were assessed by the log-rank test. A significance level of .05 was used for all hypothesis testing, and *P* values were not corrected for multiple testing because of the exploratory nature of this work.

Results

In October 2013, after the black box warning regarding ponatinib-associated vascular issues, the treatment aspects of the protocol were put on hold, but prescreening continued. From September 2013 to November 2017, a total of 171 patient samples were prescreened by *FGFR1* ISH and SISH. Forty (23.4%) had insufficient tissue or failed screening; 4 had incomplete clinical information. As a result, the data of 127 patients were analyzable. After protocol modifications were made, 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, 4 samples did not have reportable SISH scores, and 1 sample did not have a reportable ISH score as a result of insufficient sample. All 4 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 predefined *FGFR1* molecular subgroups, 6 (4.9%) of 122 were $SISH^+/ISH^+$, 3 (2.5%) of 122 were $SISH^+/ISH^-$, 47 (38.5%) of 122 were $SISH^-/ISH^+$, and 66 (54.1%) of 122 were $SISH^-/ISH^-$ (ie, double negative). In the *FGFR1* SISH/ISH double-negative cohort, 0 of 33 patients tested positive for *RET* gene rearrangement by FISH. Incidentally, 1 (33%) of 3 patients who had *RET* FISH performed in the *FGFR1* $SISH^+/ISH^+$ subgroup had a *RET* gene rearrangement. Across the 4 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 first-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 predefined cut point for SISH positivity, 9 (7.3%) of 123 patients were SISH positive (Table 1); 6 of $SISH^+$ patients were concordantly positive for SISH and ISH, all of whom had a high percentage ($\geq$ 20%) of tumor cells with dot clusters by ISH (Supplemental Table 1 in the online version). Stratified by histology, of the SISH-tested cases, 4 (4.9%) of 81 adenocarcinoma, 2 (9.5%) of 21 SQLC, and 3 (14.2%) of 21 SCLC were SISH positive. There was a significant difference in the prevalence of *KRAS* mutation between the $SISH^+$ (0/6) and $SISH^-$ (45/93, 48.4%) cohort ($P = .030$), even though the patient numbers were small (adenocarcinoma, 4/6 $SISH^+$; 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 $SISH^-$ groups (median, 417 days [95% CI: 513-870] vs. 649 days [95% CI: 294-NA], $P = .694$) (Figure 1).

At the predefined cut point for ISH positivity, 53 (42.1%) of 126 were ISH positive (Table 1). Stratified by histology, of the ISH-tested cases, 30 (35.7%) of 84 adenocarcinoma, 12 (57.1%) of 21 SQLC, and 11 (52.3%) of 21 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 predefined 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%;

Table 1 Summary of Relevant Clinical and Pathologic Features Associated With *FGFR1* ISH and SISH Positivity at Predefined and Exploratory Cut Points

Characteristic	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 (y), 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 pack-years, 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)	4/73 (5.5%)	6/53 (11.3%)	5 (5.2%)[a]	5 (17.2%)[a]
Histology						
Adenoma	77/114 (67.5%)	4/9 (44.4%)	54/73 (74.0%)	30/53 (56.6%)	69 (71.1%)[a]	15 (51.7%)[a]
Squamous	19/114 (16.7%)	2/9 (22.2%)	9/73 (12.3%)	12/53 (22.6%)	17 (17.5%)[a]	4 (13.8%)[a]
Small cell	18/114 (15.8%)	3/9 (33.3%)	10/73 (13.7%)	11/53 (20.8%)	11 (11.3%)[a]	10 (34.5%)[a]
KRAS	45/93 (48.4%)[a]	0/6 (0)[a]	32/61 (52.5%)	16/41 (39.0%)	39/82 (47.6%)	9/20 (45.0%)

Abbreviations: FGFR = fibroblast growth factor receptor; IQR = interquartile range; ISH = in-situ hybridization; SISH = silver in-situ hybridization.
[a]Variables with significant difference between biomarker groups.

ISH$^-$, 32/61, 52.5%; $P = .226$). Importantly, the ISH$^+$ group had a significantly shorter median OS than in the ISH$^-$ group (median OS, 530 days [95% CI: 394-757] vs. 753 days [95% CI: 625-1247], $P = .044$) (Figure 2).

To summarize important features associated with *FGFR1* GCN 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 (GCN 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 associated with kinase impairment, paradoxically may be associated with ERK activation[29] and *AKT1* mutation (1/32, 3.1%, in SISH$^-$/ISH$^+$ and 0% in the other *FGFR1* subgroups).

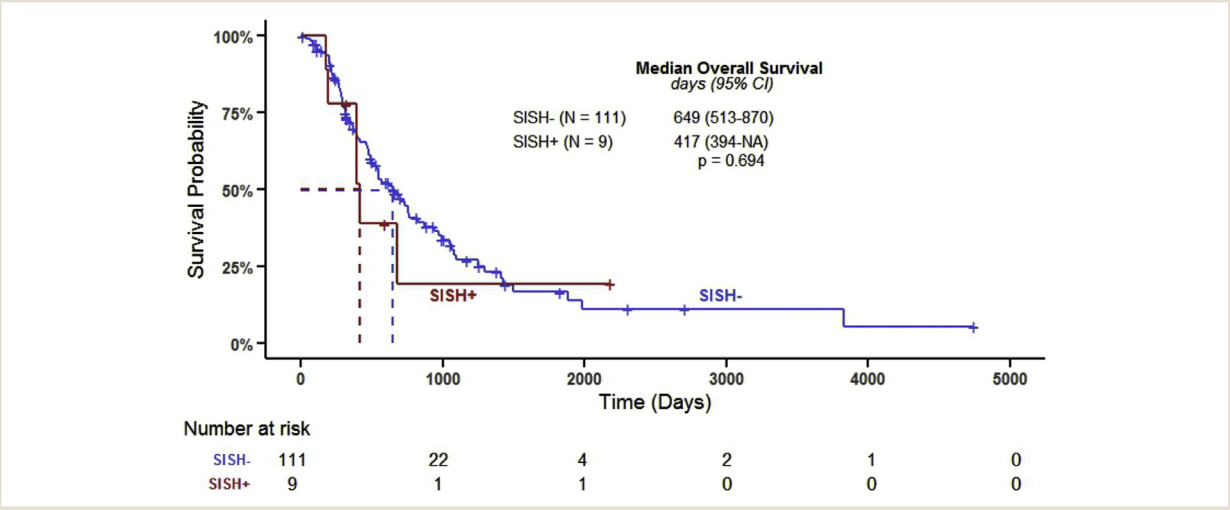
Ponatinib Treatment

A total of 4 of 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 provided orally daily; 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 was treated with ponatinib. The ORR of the entire treated cohort (n = 4) was 0, but the disease control rate was 50% (complete response + partial response + stable disease). The median progression-free survival was 1.8 months (range: 1.8-5.1 months). The median duration of treatment was 1.6 months (1.3-4.3 months). A detailed account of each patient's treatment course and a history of their posttreatment details and outcomes are provided in what follows.

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, *BRAF* V600, *HER2*). Ponatinib 45 mg provided orally daily was initiated on November 21, 2014. The patient had disease progression (PD) on the first scan (January 2, 2015). The treatment course was complicated by grade 3 hypertension (November 26, 2014), requiring 2 antihypertensives. Ponatinib was discontinued on January 2, 2015, for grade 3 febrile neutropenia and PD. The patient went on to receive docetaxel plus ramucirumab, nivolumab, and an antibody–drug conjugate before being admitted for hypoxic respiratory failure and dying on October 17, 2015.

Patient 2 had *FGFR1* ISH$^+$ (score 4, 80% tumor cells with dot cluster), SISH$^+$ (10.94 GCN, *FGFR1*/CEP8 ratio 3.99), and *RET*-positive squamous lung cancer. They were pan-negative for other known molecular oncogene drivers. Ponatinib 45 mg provided orally daily was initiated on August 21, 2014. This was complicated by grade 2 thrombocytopenia and grade 3 abdominal pain due to bowel perforation, which required admission (September 27, 2014, to October 8, 2014) for surgery (September 28, 2014). Computed tomographic abdominal imaging (October 2, 2014) did not demonstrate PD. Ponatinib was held since day 3 (September 29, 2014) of admission because of critical illness and concern that the

Figure 1 Overall Survival of SISH$^+$ Versus SISH$^-$ Group at Predefined Cut Point (GCN ≥ 4.0 GCN or *FGFR1*/CEP8 Ratio ≥ 2.0)



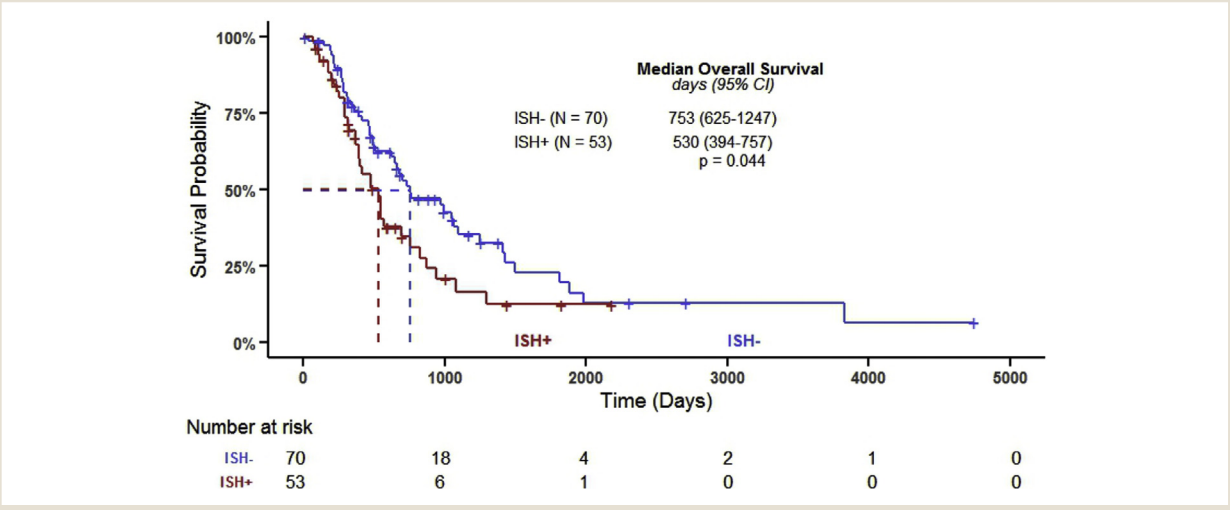
Abbreviations: CEP8 = centromere of chromosome 8; FGFR = fibroblast growth factor receptor; GCN = gene copy number; SISH = silver in-situ hybridization.

perforation could be mediated by the study drug. The patient elected for discharge to hospice and died on October 10, 2014. The duration of stable disease was 41 days.

Patient 3 had *FGFR1* ISH$^+$ (score 4, 60% tumor cells with dot cluster), SISH$^-$, and *RET*-negative squamous lung cancer. They were pan-negative for other known molecular oncogene drivers. Ponatinib 45 mg provided orally daily was initiated on August 17, 2016, with a best response of stable disease lasting 4 cycles (153 days). The treatment course was complicated by an admission for atrial fibrillation and hyponatremia (drug held September 21-25, 2016), edema (drug held November 22-28, 2016), and persistent cough, low-grade fever, and fatigue (drug held December 23, 2016, onward). Other ponatinib-related AEs included grade 1 myalgia, headache, rash, dry skin, alopecia, weakness and somnolence, and grade 2 hypertension. Unfortunately, the patient experienced a sudden episode of syncope on January 16, 2017, 25 days after holding ponatinib, and was pronounced dead after unsuccessful resuscitation in the emergency room. An autopsy was not performed.

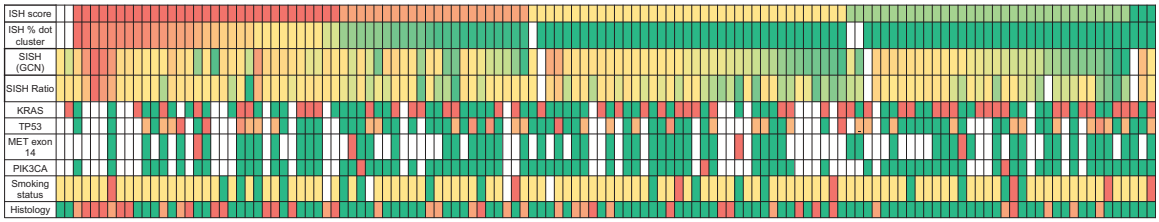
Patient 4 had *FGFR1* ISH$^+$ (score 3, 1% tumor cells with dot cluster), SISH$^-$, and *RET*-negative poorly differentiated NSCLC. They were pan-negative for other known molecular oncogene drivers. Ponatinib 45 mg provided orally daily was initiated on June 16, 2015, with a best response of PD (on August 10, 2015) after 2

Figure 2 Overall Survival of ISH$^+$ Versus ISH$^-$ Group at Predefined Cut Point (≥ 1% Tumor Cells With Dot Clusters)



Abbreviation: ISH = in-situ hybridization.

Figure 3 Heat Map of *FGFR1* ISH and SISH Score Correlation With Smoking, Histology, and Co-occurring Mutations. Mutations Include *KRAS*, *TP53*, *MET* Exon 14, and *PIK3CA*



ISH score (1-4)	4	3	2	1	0													
ISH % dot cluster (0-100%)	100	80	50	40	30	20	10	7.5	5	2.5	1	0						
SISH (GCN) (0.00-20.00)	18	16	12	8	4	3	2.5	2	1.8	1.6	1.4	1.2	1	0.8	0.6	0.4	0.2	0
SISH Ratio (0.00-10.00)	8	7	5	4	3	2.8	2.5	2.25	2	1.75	1.5	1	0.5	0				
KRAS (0=no, 1=yes)				1	0													
TP53 (0=no, 1=missense, 2=nonsense, 3=frameshift)	3	2	1	0														
MET exon 14 (0=no, 1=yes)				1	0													
PIK3CA (0=no, 1=yes)				1	0													
Smoking status (0=never, 1=former, 2=current)		2	1	0														
Histology (1=adeno, 2=squamous, 3=SCLC)		3	2	1														

Abbreviations: FGFR = fibroblast growth factor receptor; ISH = in-situ hybridization; SISH = silver in-situ hybridization.

cycles. The patient developed grade 1 fatigue and grade 2 hypertension, but no serious AEs or grade 3 to 5 AEs were noted. The patient was subsequently treated with an antibody–drug conjugate from August 25 to November 17, 2015, but discontinued for PD. The patient died on February 29, 2016.

Exploration of Alternative 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 preexisting definition for SISH positivity, FGFR1/CEP8 $\geq$ 2.0, and/or *FGFR1* mean GCN $\geq$ 4 was appropriate because this cut point best demarcated the greatest change in the slope of the curve (Supplemental Figure 1 in the online version). Furthermore, there were only 9 $SISH^+$ patients at the current cut point. A higher SISH cut point 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

Table 2 Biomarker Results, and Treatment Outcomes and Complications Related to Ponatinib

Patient No.	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: Grade 3 HTN and Grade 3 FN • OS: 330 d
2	Squamous	• Positive • Score 4 • 80% dot cluster	Positive GCN 10.94 *FGFR1*/CEP8 3.99	Positive	SD for 40 d (D/C for bowel perforation)	• AE: Grade 2 ↓ PLT; grade 3 bowel perforation • OS: 51 d
3	Squamous	• Positive • Score 4 • 60% dot cluster	Negative	Negative	SD for 153 d (drug held indefinitely on day 129)	• AE: Grade 3 atrial fibrillation • Grade 3 hyponatremia • Grade 2 edema • Grade 2 cough • OS: 153 d (syncope, unsuccessful resuscitation)
4	Poorly differentiated	• Positive • Score 3 • 1% dot cluster	Negative	Negative	PD on day 56 (D/C for PD)	• AE: Grade 1 fatigue; grade 2 HTN • No grade 3 or higher adverse events • OS: 259 d

All patients initiated therapy with ponatinib 45 mg provided orally daily.
Abbreviations: AE = adverse event; CEP8 = centromere of chromosome 8; D/C = discontinued; FGFR = fibroblast growth factor receptor; FN = febrile neutropenia; GCN = gene copy number; HTN = hypertension; ISH = in-situ hybridization; OS = overall survival; PD = progressive disease; PLT = platelets; *RET* = rearranged during transfection; SD = stable disease; SISH = silver in-situ hybridization.

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, the data of 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 4 patients with a best response of stable disease from ponatinib were ISH ≥ 20%, whereas the 2 ponatinib-treated patients with a best response of PD were ISH < 20%. Of the 126 patients tested for ISH, 29 patients (23.0%) were ISH ≥ 20%. In the ISH ≥ 20% group, more patients had soft tissue metastases (17.2% vs. 5.0%, $P = .050$) 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%) ($P = .022$). Using the new cut point, survival continued to be significantly shorter in the ISH ≥ 20% group (median OS, 394 days [95% CI: 318-NA] vs. 730 days [95% CI: 548-1046], $P = .031$) (Supplemental Figure 2 in the online version). The prevalence of concomitant *KRAS* mutation in both ISH ≥ 20% and ISH < 20% was high (45.0% vs. 47.6%, respectively; $P = 1.00$).

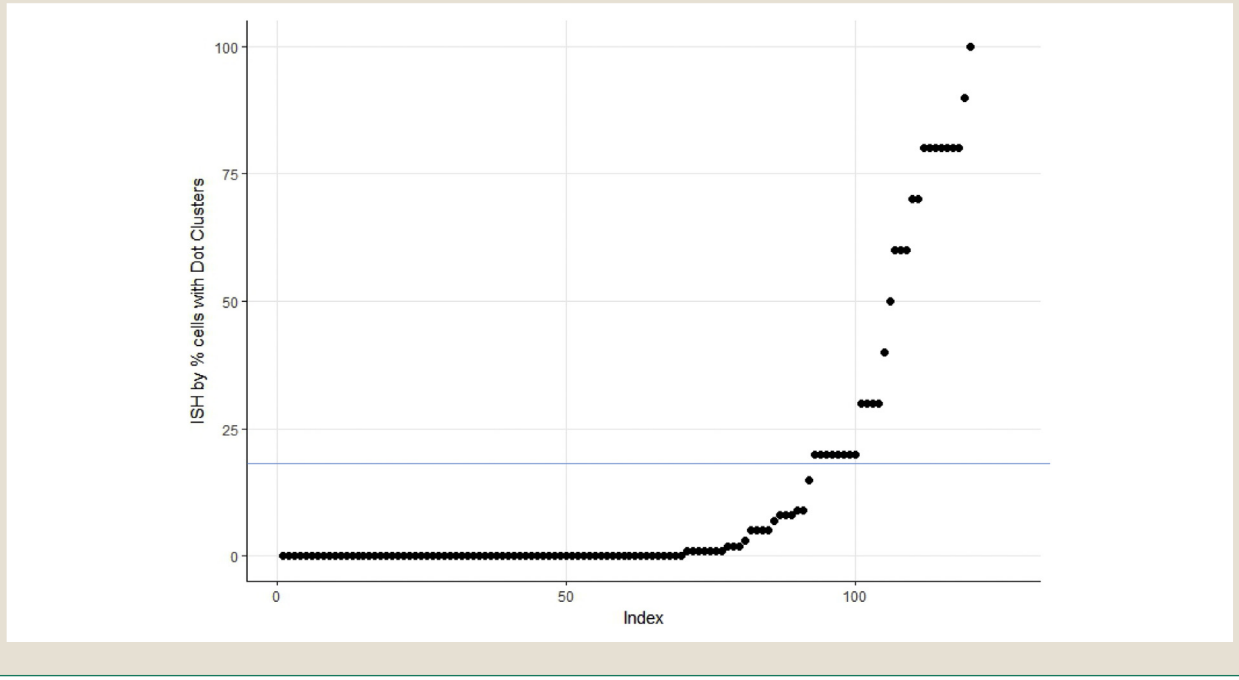
Discussion

Recent efforts have explored *FGFR1* gene amplification, as defined by either an increased GCN (≥ 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 ranges 8% to 11.5%.

In this study, ponatinib, a pan-FGFR inhibitor that was originally developed for treatment-refractory chronic myelogenous leukemia, 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 preclinical 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 (≥ 1% of tumor cells) or > 10 dots per cell in ≥ 10% tumor cells. Initial SISH positivity was defined as average of at least 4 *FGFR1* signals per nucleus (GCN) 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 cut points, 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

Figure 4 ***FGFR1*** **ISH Score by Percentage of Cells With Dot Clusters From Low to High Score**



Abbreviations: FGFR = fibroblast growth factor receptor; ISH = in-situ hybridization.

Table 3 Clinical and Molecular Features of *FGFR1* ISH$^+$ Versus ISH$^-$ Subgroup Alternate Positivity Cut Points (ISH ≥ 20% Tumor Cells With Dot Clusters)

Characteristic	ISH <20%	ISH ≥ 20%	*P*
No. of subjects	97	29	
Age at diagnosis of stage IV disease (y), median [IQR]	64.9 [58.6, 72.5]	64.7 [53.5, 70.5]	.497
Male sex	47/97 (48.5)	16/29 (55.2)	.673
Smoking pack-years, median [IQR]	30.00 [12.88, 48.50]	30.00 [20.00, 50.00]	.772
Site of Metastases at Diagnosis of Stage IV Disease Beyond Lung			
Adrenal	6 (6.2%)	3 (1.3%)	.429
Bone	32 (33.0%)	9 (31.0%)	1.000
Brain	16 (16.5%)	7 (24.1%)	.412
Liver	12 (12.4%)	7 (24.1%)	.142
Soft tissue	5 (5.2%)	5 (17.2%)	.050[a]
Pleural/pericardial	34 (35.1%)	7 (24.1%)	.367
Lymph node	73 (75.3%)	23 (79.3%)	.805
Histology			.022[a]
Adenocarcinoma/adenosquamous	69 (71.1%)	15 (51.7%)	
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%)	.117
KRAS	39/82 (47.6%)	9/20 (45.0%)	1.000
ROS1	0/68 (0)	0/12 (0)	—
MET FISH	0/66 (0)	0/14 (0)	—
MET exon 14	3/49 (6.1%)	1/13 (7.7%)	1.000
BRAF	1/72 (1.4%)	0/16 (0)	.761
HER2	0/72 (0)	0/16 (0)	NA
HER2/CEP17 ≥ 2.0	1/2 (50.0%)	NA	1.000
RET	0/52 (0)	1/13 (7.7)	.200

Abbreviations: CR = complete response; FGFR = fibroblast growth factor receptor; FISH = fluorescence in-situ hybridization; IQR = interquartile range; ISH = in-situ hybridization; LCNE = large-cell neuroendocrine carcinoma; NA = not applicable (HER2 FISH testing was not performed on any of these patients); PR = partial response; *RET* = rearranged during transfection.
[a]Statistically significant (≤ 0.05). Fisher's exact test for categorical variables. Kruskal-Wallis rank sum test for continuous vairables.

biomarker-positive data did not suggest a more appropriate SISH positivity cut point but did suggest a higher biologic cut point—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 that is based on significant differences in clinical and molecular features. Using this higher cut point, *FGFR1* ISH positivity was associated with SCLC histology and 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, as a result of 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, so we could not confirm or refute the null hypothesis, as we 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, which, because of its rapidly progressive nature, often precludes patients from receiving the drug after initial lines of chemotherapy. As a result, all 4 patients who were enrolled onto the ponatinib treatment arm had either squamous or poorly differentiated lung cancer.

Because 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 4 *FGFR1* preselected patients treated with ponatinib had a response rate of 0, 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 disease of the treated patients may not have responded because ponatinib was not potent enough to inhibit *FGFR1* signaling, and the maximum tolerated dose was limited because of its many off-target effects. Our data suggest 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 tumors,[20] and neither gene amplification nor mRNA overexpression necessarily determines 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 patients with analyzable data 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 per nucleus over 50 cells, ISH criteria does not mandate a minimum number of cells examined. Instead, the pathologist scores the tissue sample on the basis of 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 2 biomarkers are not completely overlapping suggests that neither biomarker is sufficient as a stand-alone biomarker. Other than suboptimal patient enrollment, another limitation is that a *FGFR1* antibody to detect *FGFR1* protein expression by immunohistochemistry was not implemented into the biomarker, set no validated antibody was available at the time.

Conclusion

High *FGFR1* mRNA expression is more common than *FGFR1*-AMP 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 SQLC with *FGFR1* gene amplification showed modest response rates. Preclinical 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.
- On the basis of these data, a phase 2 biomarker-driven study using *FGFR1* gene amplification by SISH and mRNA expression by 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. Because of 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 reopened after protocol modification, as a result of awareness of ponatinib-associated risks, only 4 patients were treated with ponatinib 45 mg provided orally daily.
- The ORR was 0 with a disease control rate of 50% (stable disease in 2 patients with a high *FGFR1* ISH score).
- Association of clinicopathologic features with *FGFR1* ISH and SISH positivity at different cut points were explored.
- *FGFR1* ISH positivity at a higher cut point may identify a distinct clinical phenotype (soft tissue metastases, small-cell histology, worse survival) of lung cancer patients who 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.

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Disclosure

The authors have stated that they have no conflict of interest.

Supplemental Data

Supplemental figures and table accompanying this article can be found in the online version https://doi.org/10.1016/j.cllc.2018.09.001.

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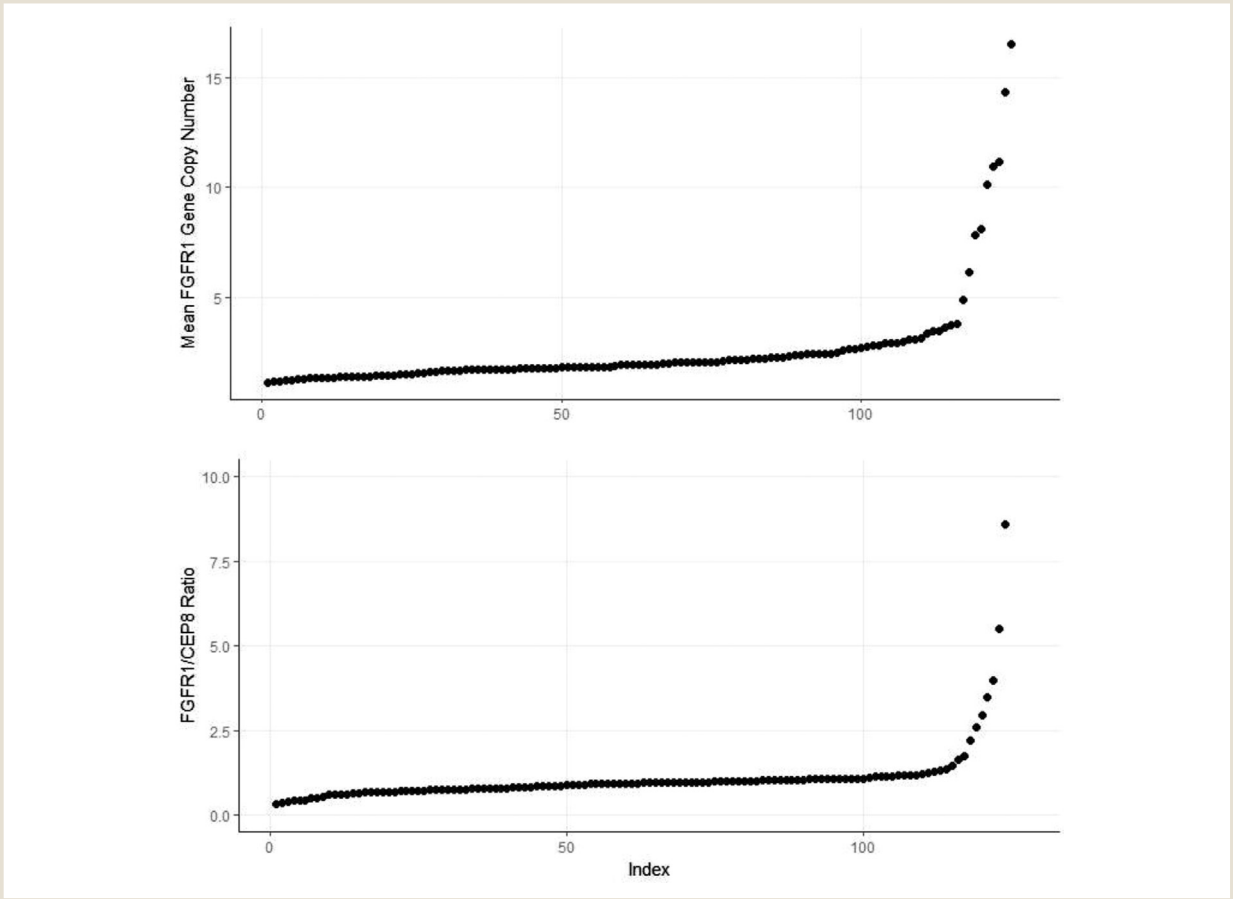
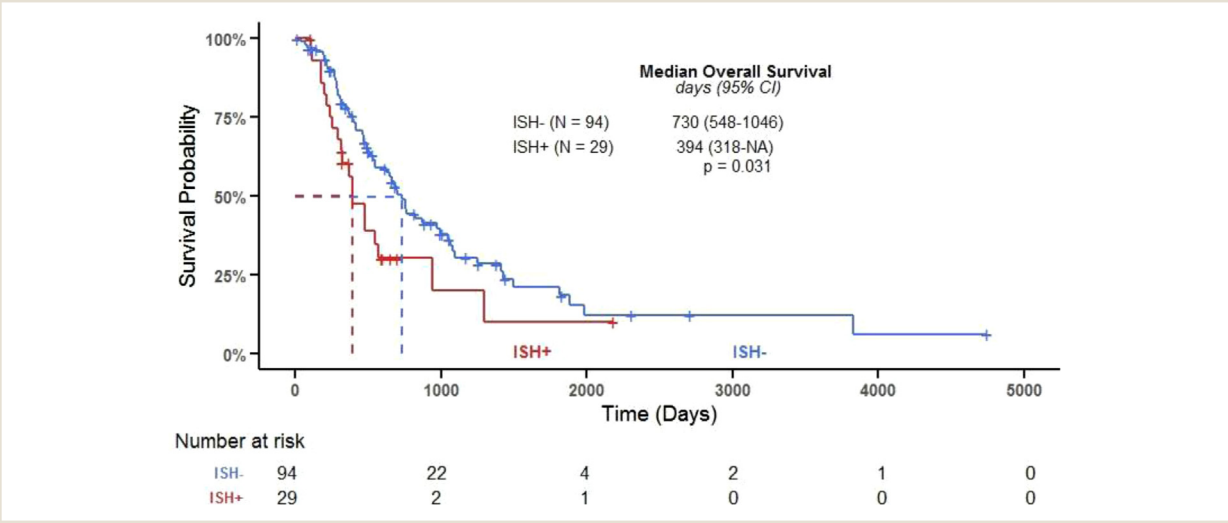
Supplemental Data

Supplemental Figure 1 Mean *FGFR1* Gene Copy Number and FGFR1/CEP8 Ratio From Low to High Score



Abbreviations: CEP8 = centromere of chromosome 8; FGFR = fibroblast growth factor receptor.

Supplemental Figure 2 Overall Survival of ISH$^+$ Versus ISH$^-$ Group Shown Using Predefined 20% Cut Point



Abbreviation: ISH = in-situ hybridization.

Supplemental Table 1 Comparison of Concordant and Discordant ISH Score by Percentage Dot Cluster With All 9 SISH$^+$ Cases

SISH$^+$ Case No.	ISH Score	ISH Cells With Dot Clusters (%)	SISH GCN	SISH FGFR1/CEP8 Ratio
1	0	0	6.14	2.21
2	2	0	8.11	1.63
3	2	0	7.84	2.61
4	4	20	10.12	3.47
5	4	60	4.86	1.48
6	4	80	10.94	3.99
7	4	80	14.34	5.52
8	4	80	16.54	8.61
9	4	90	11.18	2.95

ISH$^+$ if score 3 or 4 (3 = % dot clusters $\geq$ 1% to 10%, 4 = % dot clusters $>$ 10%); SISH$^+$ if GCN $\geq$ 4.0 or FGFR1/CEP8 $\geq$ 2.0.
Abbreviations: CEP8 = centromere of chromosome 8; FGFR = fibroblast growth factor receptor; GCN = gene copy number; ISH = in-situ hybridization; SISH = silver in-situ hybridization.