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A miRNA panel predicts sensitivity of FGFR inhibitor in lung cancer cell lines

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Abstract

Purpose: To test whether a miRNA panel may serve as an alternative biomarker of FGFR TKI sensitivity in lung cancer.

Methods: Histologically diverse lung cancer cell lines were submitted to assays for ponatinib and AZD4547 sensitivity. miRNAs, FGFR1 mRNA, gene copy number and protein expression were detected using the method of RT-PCR, FISH and immunoblotting in 34 lung cancer cell lines respectively.

Results: Among 34 cell lines, 14 exhibited ponatinib sensitivity and 20 exhibited AZD4547 sensitivity (IC_{50} values < 100 nmol/L). 39 out of 377 miRNAs set were initially identified from the 4 paired ponatinib sensitive or insensitive cell lines to have at least an 8-fold differential expression, and then were detected in the whole of 34 cell lines. A predictive panel of three miRNAs (let-7c, miRNA155 and miRNA218) was developed that had an area under the curve (AUC) of 0.886 with sensitivity of 71.4% and specificity of 77.3% to predict response to ponatinib. The miRNA panel performed similar to FGFR1 protein expression (AUC = 0.864) and mRNA expression (AUC = 0.939), and superior to FGFR1 amplification (AUC = 0.696).

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Disclosure

None

Furthermore, we validated this panel using data for sensitivity to AZD4547 in the cell line cohort with an AUC of 0.931 with sensitivity of 73.3 % and specificity of 76.2% respectively.

Conclusions: The developed miRNA panel (let-7c, miRNA155 and miRNA218) may be useful in predicting response to FGFR TKIs, either ponatinib or AZD4547 in lung cancer cell lines, and warrants further validation in the clinical setting.

Micro-abstract:

We investigate whether miRNAs may serve as alternative biomarker of FGFR TKI sensitivity in lung cancer cell lines, and found a miRNA panel (let-7c, miRNA155 and miRNA218) could be useful in predicting response to FGFR TKIs, either ponatinib or AZD4547.

Keywords

miRNA; FGFR inhibitor; lung cancer; cell lines

INTRODUCTION

The fibroblast growth factor receptor (FGFR) pathway is an important oncogenic driver in malignant cancer (1). It controls cellular processes such as cell proliferation, differentiation, migration, cycle progression, metabolism, and survival. In NSCLC, the most frequent alteration of the FGFR pathway is represented by FGFR1 amplification, which is reported as occurring in up to 20% of squamous NSCLC; other, less frequent alterations include point mutations or translocations of the genes encoding for FGFR1–4 (2).

Currently, several FGFR inhibitors are being investigated in phase 1–3 clinical trial in solid tumors with FGFR amplification or protein over-expression (2), such as nintedanib (Boehringer Ingelheim; Ingelheim am Rhein, Germany) (3), Ponatinib (Ariad Pharmaceuticals; Cambridge, MA) (4), AZD4547 (AstraZeneca; London, UK) (5), and BJG398(Novartis, Switzerland) (6). In these studies, preliminary results showed that only a subset of patients with FGFR amplification or protein over-expression responded to the FGFR TKIs AZD4547 and BJG003(5–6). The response rates did not reach those observed for other lung cancer driver mutation genes such as mutant EGFR or ALK/ROS1 fusion, suggesting that the biomarkers used for enrolling into the FGFR TKI trials were inaccurate (7). In a set of 58 lung cancer cell lines, sensitivity to ponatinib was correlated with FGFR1 amplification, mRNA and protein expression, as well as mRNA expression of FGF2 and FGF9 (8). This study reported better correlation of FGFR1 TKI sensitivity with FGFR1 mRNA or protein expression as compared to FGFR1 amplification. These data clearly identify the need for further investigation to find additional biomarkers which may be better able to predict response to FGFR inhibitors in the clinical setting.

MicroRNAs (miRNAs) are small, non-coding, stable sequences of RNA with regulatory functions exerted through inhibition of crucial mRNA (9). Recent studies demonstrated that pathologic conditions, such as solid tumors, are associated with specific intracellular miRNA patterns and are also able to affect circulating miRNA. Based on this assumption, several studies have identified specific miRNAs or groups of miRNAs (miRNA signatures) with a potential diagnostic or prognostic role in solid tumors (10–11). Some miRNA, such as

miR-34bc, are currently considered promising predictors of poor outcome for early-stage lung cancer, apparently due to a correlation between their target genes inactivation and an aggressive phenotype (12). Another study suggested the existence of a circulating miRNA signature able to detect lung cancer (13). Furthermore, our previous study also found miRNA signatures were also reported as able to predict the sensitivity of lung cancer to EGFR-TKIs (14–15). In this present study, we performed a comprehensive analysis of miRNAs in a panel of human lung cancer cell lines that were previously characterized for sensitivity to two FGFR1 TKIs (8). We developed a 3-miRNA panel that accurately predicts the sensitivity to ponatinib in 34 cell lines and the chemically-distinct TKI, AZD4547.

METHODS

Cell culture

All cell lines were cultured in RPMI-1640 growth medium supplemented with 10% FBS at 37°C in a humidified 5% CO_2 incubator. The following cell lines were available in our laboratories and submitted to DNA fingerprint analysis for authentication: H1703, HCC95, NE-18, DMS-114, SKMES-1, H460, SW1573, H520, H661, H125, HCC44, H1299, H157, Colo699, H1581, HCC15, H2126, H1869, H1435, and H441. The remaining 14 cell lines were obtained directly from the University of Colorado Cancer Center Tissue Culture Core (Aurora, CO). The core laboratory routinely performs DNA fingerprint analyses on all banked cell lines to ensure their authenticity.

IC_{50} analysis to ponatinib and AZD4547 in cell lines

Sensitivity to ponatinib was defined as an $IC_{50} \leq 100$ nM/L in a cohort of cell lines that were previously characterized for FGF ligand and FGFR receptor-mediated autocrine signaling status (8,16). The IC_{50} for AZD4547 was also determined in the lung cancer cell lines, including 13 ponatinib-sensitive cell lines and 44 ponatinib-resistant cell lines by using the IC_{50} cutoff value of 100 nM/L. Cell line information is presented in Table S1.

RT-qPCR for miRNA analysis

We used a comprehensive, commonly employed commercial panel (Life Technologies, TaqMan® Array Human MicroRNA A Card v2.0) to perform the miRNA expression level detection; this panel utilizes a 384-well microfluidic card, enabling to test 377 miRNA plus three provided endogenous controls and one non-human negative control.

Initially, 4 lung cancer cell lines determined to be sensitive to ponatinib (COLO699, H1581, SW1573, and H520) were selected as well as 4 insensitive cell lines (H125, NE-18, H2126, and SK-MES-1) and were analyzed for miRNA expression. RNA was extracted from treatment-naïve cell cultures, and real-time quantitative polymerase chain reaction (RT-qPCR) was achieved using a 7900HT Fast Real-Time cycler (Applied Biosystems). Cycle thresholds (ΔCt) were normalized using U6 small nuclear RNA, as per the manufacturer's protocol. Each cell line was analyzed in triplicate and averaged data generated. In order to determine the ΔCt, the threshold was set at 0.2, and baseline data collected at cycles 3–15. Based on the results of the RT-qPCR, a statistical analysis of microarrays (SAM) plot analysis was performed (Stanford University; http://statweb.stanford.edu/~tibs/SAM/) which

identified the 39 most differentially expressed miRNAs from the original set of 377. Here miRNA which exhibited an 8-fold expression difference between sensitive and insensitive cell lines were included. Following selection, the panel of 39 miRNAs were then assayed across the 34 lung cancer cell lines (8).

FGFR1 protein, mRNA expression and gene copy number detection

Selected lung cancer cell lines were submitted to the University of Colorado Cancer Center Molecular Pathology shared resource for evaluation of FGFR1 **gene copy number** by FISH analysis. FGFR1 protein and mRNA levels were measured by immunoblotting with an antibody against the carboxyl-terminus of FGFR1 and quantitative PCR with primers annealing to sequences within the invariant second immunoglobulin domain. The methods for FISH, protein immunoblotting and QPCR are described previously (8).

Transfection of let-7c inhibitor

Based on the observed data, the interaction between the most relevant miRNA, let-7c and the status of FGFR1 mRNA expression were further explored through an additional experiment. We included 11 ponatinib-sensitive cell lines, treated with let-7c inhibitor and its negative control oligonucleotide (Invitrogen; Carlsbad, CA). Cells were added into 6-well plates (2×10^5 cells/well) and transfected with miRNA inhibitor using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's protocol. After 72 hours, cells were harvested and tested for alternations in the levels of specific miRNA and FGFR1 mRNA by qRT-PCR.

Statistical analysis

We assessed the expression of miRNAs for classification of lung cancer cells as sensitive or insensitive to ponatinib using logistic regression by computing the receiver operating characteristic (ROC) curve and used the area under the ROC curve (AUC) as an accuracy index. Among potential biomarkers, miRNAs characterized by a statistical $p< 0.1$ in the univariate analysis were selected to enter into multivariable logistic regression, which were selected in a combination of biomarkers potentially able to predict sensitivity to ponatinib. We further validated the biomarker panel for a distinct FGFR inhibitor, AZD4547, in the same cell line cohort. All P values were two-sided, with values < 0.05 considered statistically significant. SAS 9.3 (SAS Institute Inc., Cary, NC, USA) and SPSS (Version 21.0, IBM, Chicago, IL) were used for the statistical analyses.

RESULTS

Cell lines and their sensitivity to ponatinib and AZD4547

The IC_{50} for ponatinib and AZD4547 are listed in Table S1. We used our previous criteria to distinguish ponatinib sensitive cell lines ($IC_{50}\leq 100$ nM) from resistant cell lines and identified 14 ponatinib-sensitive cell lines and 20 ponatinib-resistant cell lines [11]. As for AZD4547, the IC_{50} was not available for the H1048 cell line. Thus, when we use the IC_{50} cutoff of 100 nM, we identified 13 AZD4547-sensitive cell lines and 20 AZD4547 insensitive cell lines. Among the 33 cell lines, there were 4 cell lines, H211, H1703, H1993, H125 that showed an opposite sensitivity to ponatinib and AZD4547.

Developing a miRNA panel to predict sensitivity to ponatinib

The RT-qPCR analysis enabled us to identify the median ΔCt for each miRNA and to compare them between 4 paired ponatinib-sensitive and ponatinib-resistant cell lines. Notably, a lower ΔCt indicates a higher concentration of each specific miRNA, as the amplification takes place earlier in presence of increased nucleic acid. Full data regarding the initial 377 miRNAs set, including ΔCt for sensitive and resistant cell lines are reported in table S2 and table S3 respectively. SAM analysis identified 39 miRNAs that have more than an 8-fold difference in expression which were used in subsequent investigations in the 34 cell lines (see Table 1).

Univariate analysis identified 8 distinct miRNAs whose concentration was significantly or marginally significantly associated with sensitivity to ponatinib, including Let-7c, miR155, miR218, miR3383p, miR200b, miR379, miR200c, miR429(see Table 1). Due to the limited cell lines included in this study, we performed the ROC curve analysis with the above 8 individual miRNAs and found that miR200c or miR429 alone was not significantly predictive of the sensitivity to ponatinib in cell lines. Finally, Let-7c, miR155, miR218, miR3383p, miR200c, miR379 and miR200b were evaluated by multivariate analysis and allowed the identification of a miRNA panel associated with sensitivity to ponatinib, including increasing let-7c, increasing miR-218 and decreasing miR-155. This 3-miRNA panel was associated with a model AUC= 0.886 with a sensitivity of 71.4% and specificity of 77.3% to predict the response to ponatinib. The ROCs are depicted in Figure 1.

Comparing the developed miRNA panel with other biomarkers including FGFR mRNA, protein expression and amplification

In a previous study, we found that FGFR mRNA or FGFR protein expression, but not FGFR amplification were associated with sensitivity to ponatinib in lung cancer cell lines (8). These biomarkers were studied in all 34 cell lines deployed in the present study. Similar to our previous reports, we found that FGFR1 mRNA or FGFR1 protein expression could predict sensitivity to ponatinib with an AUC of 0.939 and 0.864 respectively. We determined that FGFR amplification predicts sensitivity to ponatinib with an AUC of 0.696. The 3-miRNA panel developed in this study has an AUC of 0.886, which is similar to the sensitive biomarkers of FGFR1 mRNA or FGFR1 protein expression in the study (see Figure 1).

Validating the 3-miRNA panel and evaluating other biomarkers for sensitivity to AZD4547 in the cell line cohort

Data for sensitivity to AZD4547 were established for 33 out of 34 study cell lines. Here we chose to evaluate the same concentration for AZD4547 that was used for ponatinib (100 nM), resulting in the identification of 13 sensitive and 20 resistant cell lines for the validation cohort. The 3-miRNA panel developed for ponatinib predicted cell line sensitivity to AZD4547 with an AUC of 0.931 and a sensitivity of 73.3 % and specificity of 76.2%. We further investigated the biomarkers of FGFR1 mRNA, protein expression and amplification in the setting of the 34 cell lines cohort. Analysis revealed that FGFR1 mRNA or FGFR1 protein expression could predict the sensitivity of AZD4547 with an AUC of 0.871 and 0.735 respectively, while FGFR amplification was less able to predict the sensitivity of ponatinib with a AUC of 0.585 (see Figure 1).

Comparing the distinguished yield of the miRNA panel using different IC50 cutoff

We further investigated whether the miRNA panel exhibits a consistent distinguished yield by using a different IC_{50} cutoff. When 50 nM was used as the IC_{50} cutoff value for both ponatinib and AZD4547, 13 ponatinib sensitive and 21 resistant cell lines were identified, while 8 sensitive and 25 resistant cell lines were identified for AZD4547. This 3-miRNA panel was associated with a model AUC= 0.853 to predict the response to ponatinib. The 3-miRNA panel developed for ponatinib predicted cell line sensitivity to AZD4547 with an AUC of 0.865. These results using the cutoff value of 100 nM were consistent to those for both ponatinib and AZD4547 (see Figure 2).

The relationship of let-7c with FGFR1 mRNA expression

Since let-7c was the most robust miRNA in the univariate analysis (in terms of p value), it was considered the best candidate for studying interactions with FGFR1 mRNA. Experiments employed transient transfection with a let-7c silencing RNA (Life Technologies) to explore effects of let-7c levels on FGFR1 mRNA expression. In these experiments, the high mobility group AT-hook 2 (HMGA2) gene served as a surrogate measurable target of repression exerted by let-7c (17–18). After 72 hours, 6 out of 11 cell lines showed an increase in HMGA2 levels (reflecting a decrease in let-7c levels); of these 6 cell lines, 5 also showed a reduction in FGFR1 mRNA levels from baseline (Table 2).

DISCUSSION

Precision therapy, guided by biomarkers of response, has dramatically improved the prognosis of patients with advanced lung cancer (19). Examples include mutant EGFR that can be effectively inhibited by EGFR TKIs and deficient mismatch repair (MMR), high microsatellite instability (MSI), are susceptible to anti-PD-1 immunotherapy respectively (20–22). In this study, we investigated the role of miRNAs as a biomarker to predict sensitivity to FGFR inhibitors. Analysis revealed differences in the miRNA expression between cell lines determined to be sensitive and resistant to ponatinib with identification of a predictive miRNA panel including let-7c, miRNA155 and miRNA218. Analysis of this panel identified an AUC of 0.886 to predict sensitivity to ponatinib, which is as good as other predictive biomarkers including FGFR1 mRNA and protein expression as previously reported. Furthermore, the predictive role of this miRNA panel was validated when comparing sensitivity of AZD4547 in the cell line cohort. Moreover, we found that the mechanism of let-7c to predict the sensitivity of ponatinib may involve regulating the mRNA expression of FGFR1.

After the successful development of molecular targeted drugs in lung adenocarcinoma, substantial efforts have been made to provide similar targeted drugs in lung squamous carcinoma (23). Since FGFR gene alteration is the most frequent occurrence in squamous carcinoma, targeted therapies for FGFR including ponatinib, AZD4547 and BJG003 are being evaluated (4–6). Preliminary clinical data found that only a small subset of enrolled patients respond to these FGFR1 TKIs. One potential explanation may be due to the improper use of FGFR amplification as a patient selection biomarker (7). As identified in our previous study, FGFR1 mRNA or protein expression, but not gene copy number better

predicts FGFR TKI sensitivity across all lung cancer cell lines studies (8). This previous data suggested the need for better biomarkers to predict tumor sensitivity to targeted drugs.

Currently, efforts to identify patients who are likely to experience anti-cancer treatment failure are ongoing, and evaluations of miRNA dysregulation to support this endeavor have been reported (24–25). Lim EL et al further used RNA sequencing to comprehensively analysis the miRNA difference expression between primary and refractory pediatric AML samples and found three candidate miRNAs, indicating that they may be associated with treatment resistance (26). In this study, we firstly developed a miRNA panel to predict sensitivity to FGFR inhibitors through comprehensive analysis of miRNA expression in 34 thoracic cancer cell lines, which showed consistent results both in ponatinib and AZD4547 cell line cohorts. While this work was performed exclusively in cell lines, further studies on tumor tissue specimens from patients must be performed to validate the potential role for this miRNA panel. However, liquid biopsy showed obvious advantage comparing with tumor biopsy in the areas of efficacy surveillance and relapse monitoring because liquid biopsy is nearly non-invasive (27). Thus, our miRNA panel might show superiority than FGFR1 mRNA or protein expression if this miRNA panel in circulating miRNA was identify as a reliable predictive factor for screening patients for FGFR1 inhibitor, which is also our next step work in the near future. Besides that, 34 cell lines included into this study contained different histological subtypes such as adenocarcinoma, squamous, large cell and small cell cell lines without known oncogenic mutations, which might not represent the whole lung cancer population. However, since FGFR1 inhibitor are rarely effective in patients with EGFR/ALK/ROS1 mutation, investigation the biomarker for FGFR1 inhibitor in oncogenic driver pan-negative cell lines will be helpful to identify the potential one for clinical implication.

Additionally, since let-7c was the most robust miRNA in the univariate analysis to predict the sensitivity to ponatinib, we further investigated the correlation between let-7c and FGFR1 mRNA. We found that let-7c silencing was significantly associated with decreased expression of FGFR1, suggesting that let-7c predicts, but also participates in regulation of FGFR1 mRNA levels and thereby sensitivity to ponatinib. It has been reported that Let-7 has been demonstrated to be a direct regulator of RAS (28) and high mobility group A2 (HMGA2) expression (29) in human cells through binding sequences in their 3’UTRs. However, the detailed mechanism of let-7c and correlated changes in FGFR1 mRNA is still unknown and warrants further investigation.

Conclusion

This study comprehensively evaluated the predictive role of miRNA to FGFR inhibitors in 34 cell lines and developed a miRNA panel (let-7c, miRNA155 and miRNA218), which was validated with sensitivity to AZD4547 in the cell line cohort. Similar to FGFR1 mRNA or protein expression, the miRNA panel predicted the sensitivity of ponatinib and AZD4547. Since miRNA panel could also be detected by a non-invasive liquid biopsy, we will further explore its role for predicting sensitivity to FGFR-TKIs in the clinical setting.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Clinical Practice Points

- The improper use of FGFR amplification as a patient selection biomarker might be the potential explanation that preliminary clinical data found that only a small subset of enrolled patients respond to these FGFR1 TKIs.
- Our previous study, FGFR1 mRNA or protein expression, but not gene copy number better predicts FGFR TKI sensitivity across all lung cancer cell lines studies.
- This study found that a miRNA panel (let-7c, miRNA155 and miRNA218) could predict the sensitivity of FGFR-TKIs, not only ponatinib but also AZD4547.

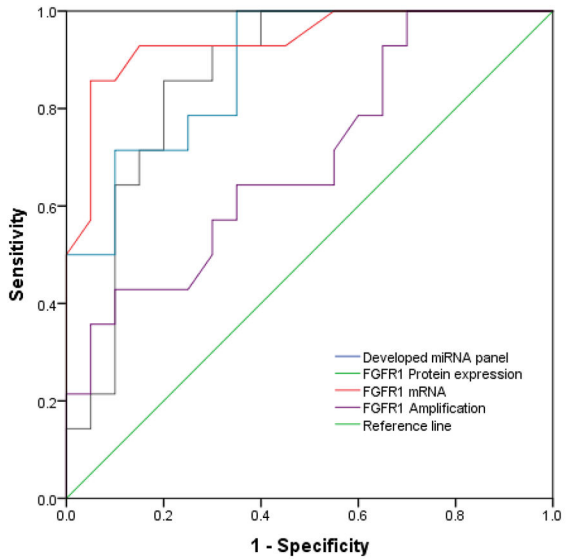

Figure 1A

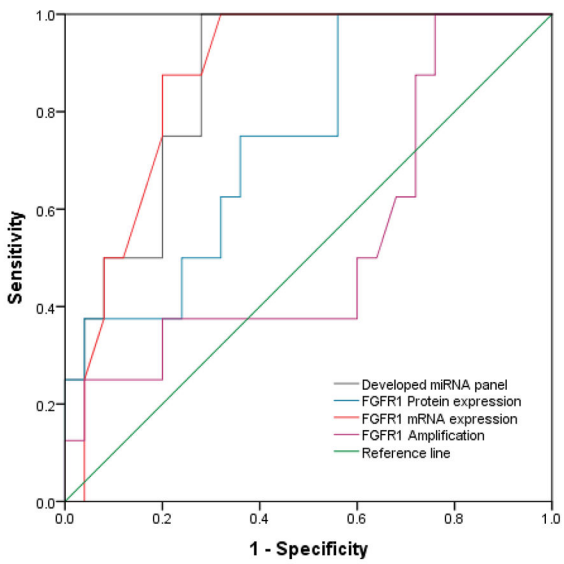

Figure 1B

Figure 1.

Receiver operating curves (ROCs) for the developed miRNA (let-7c, miR-155, and miR-218) together with FGFR1 mRNA, protein expression and amplification to predict the sensitivity of ponatinib (Figure 1A, on the left) and AZD4547 (Figure 1B, on the right) by using the IC50 cutoff values 100 nmol/l.

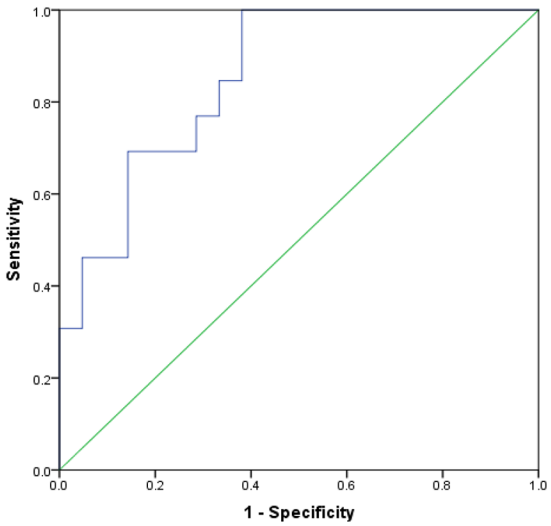

Figure 2A

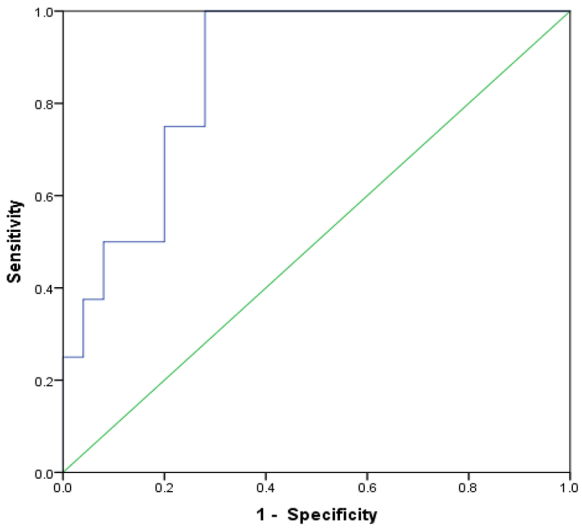

Figure 2B

Figure 2.

Receiver operating curves (ROCs) for the developed miRNA (let-7c, miR-155, and miR-218 to predict the sensitivity of ponatinib (Figure 2A, on the left, AUC=0.853) and AZD4547 (Figure 2B, on the right, AUC=0.865) by using the IC50 cutoff values 50 nmol/l.

Table 1.

Logistic regression analysis of sensitivity to ponatinib according to the 39 miRNAs selected through SAM analysis.

N#	VARIABLE	ESTIMATE	P
1	let7c	7.073	0.008
2	miR22	0.836	0.360
3	miR23b	0.804	0.370
4	miR34c	2.033	0.154
5	miR99a	0.172	0.678
6	miR100	0.451	0.502
7	miR105	0.543	0.461
8	miR127	0.158	0.691
9	miR134	1.188	0.276
10	miR141	2.547	0.110
11	miR146a	1.560	0.212
12	miR155	3.602	0.058
13	miR200a	1.245	0.264
14	miR200b	2.769	0.096
15	miR200c	3.576	0.059
16	miR203	1.778	0.182
17	miR204	0.396	0.529
18	miR205	1.188	0.276
19	miR218	4.060	0.044
20	miR221	0.984	0.321
21	miR224	1.513	0.219
22	miR335	0.869	0.351
23	miR3375p	0.844	0.358
24	miR3383p	4.514	0.034
25	miR376a	0.438	0.508
26	miR376c	1.153	0.283
27	miR379	3.169	0.075
28	miR382	1.708	0.191
29	miR411	2.094	0.148
30	miR429	2.840	0.092
31	miR452	2.434	0.119
32	miR487b	1.146	0.284
33	miR492	0.054	0.817
34	miR495	1.624	0.203
35	miR539	1.325	0.250
36	miR5425p	0.004	0.949
37	miR655	0.431	0.511
38	miR8863p	0.674	0.412

N#	VARIABLE	ESTIMATE	P
39	miR8865p	1.148	0.284

Table 2.

modifications in FGFR1 mRNA after silencing let-7c in ponatinib-sensitive cell lines.

CELL LINE	Base-line LET-7C	Base-line FGFR1 mRNA (qPCR)	HGMA2			FGFR1 mRNA		
			Change trend	Response	% change	Change trend	Response	% change
H1581	6.7	0.6	None	N/A	N/A	None	N/A	N/A
H226	12.8	0.26	Increase	sat	395.00%	None	N/A	N/A
H2066	11.6	0.49	None	N/A	N/A	None	N/A	N/A
Colo699	12.1	1	Increase	dose	575.00%	Decrease	dose	36.10%
H1563	11	0.28	Increase	sat	542.00%	Decrease	dose	40.80%
H661	8.3	0.13	None	N/A	N/A	None	N/A	N/A
H522	7.9	1.1	Increase	sat	436.00%	Decrease	dose	50.10%
H1734	8.5	0.26	None	N/A	N/A	None	N/A	N/A
SW1573	13.7	0.24	Increase	sat	182.00%	Decrease	dose	33.40%
DMS-114	10.8	0.4	None	N/A	N/A	None	N/A	N/A
H520	10	0.49	Increase	dose	170.00%	Decrease	dose	29.40%