Mechanisms of Resistance for Cancers with Fibroblast Growth Factor Receptor Gene Fusions

Research Thesis

Presented in partial fulfillment of the requirement for graduation with research distinction in the undergraduate colleges of The Ohio State University

By

Hannah Parks

The Ohio State University
April 2015

Project Advisor: Dr. Sameek Roychowdhury, College of Medicine
Abstract

Background: Alterations in the fibroblast growth factor receptor (FGFR) tyrosine kinase have been found in cancers such as glioblastoma multiforme, breast cancer, bladder cancer, cholangiocarcinoma, prostate cancer, thyroid cancer, oral cancer, and lung cancer. Advances in sequencing technology and small molecule inhibitors allow for matching patients with targeted therapies of tyrosine kinases. However, despite initial response to therapy, patients’ cancers soon become resistant to inhibitors over time and develop acquired resistance. We will be using protein arrays and Western blot analysis to understand acquired secondary resistance mechanisms to tyrosine kinase inhibitors used in cancer treatments. The RT-4, a bladder cancer cell line with a FGFR3-TACC3 fusion will be used as model for resistance to the FGFR tyrosine kinase inhibitors Ponatinib and BGJ398.

Methods: Over a period of one year the cell line RT-4 was exposed to increasing dosages of BGJ398 and Ponatinib. RT-4 cell lines became less sensitive to the drug, and they were determined to be resistant while demonstrating a two-log fold difference in cell viability compared to the parental RT-4 cell line. Reverse Phase Protein Array (RPPA) provides a broad assessment of proteins and signaling pathways. Further confirmation of the changes in protein expression between the parent and resistant cell lines was done by Western blot analysis in an attempt to identify a possible resistance mechanism.

Results: After resistant RT-4 cell lines were created, we identified the Akt pathway as a possible resistance mechanism by RPPA and confirmed by Western blot analysis. Combined treatment in resistant cell lines of FGFR inhibitor, BGJ398, and Akt inhibitor, GSK2141795, was more effective in reducing cell viability than either inhibitor alone. The
identification of the Akt signaling pathway as a secondary resistance mechanism can be a potential secondary inhibitor target.

Discussion: FGFR acquired resistance has not been studied and we identified the Akt pathway as one mechanism for resistance in cancers with FGFR fusions. RPPA analysis can be a useful tool for identifying candidate resistance pathways and targets. Combination treatment with FGFR and AKT inhibitors may be a more effective therapy than individual inhibitor treatment.

Introduction

Genetic alterations in fibroblast growth factor receptors (FGFR) can occur through point mutations, gene fusions, or amplifications, leading to activation of FGFR signaling, which can result in diseases such as cancer. Thus, FGFR has recently become a novel target for new small molecule inhibitors that turn FGFR signaling off in cancer cells. However, despite initial responses to therapy, patients in clinical trials receiving novel FGFR inhibitors are expected to develop acquired resistance over time. Therefore, it is imperative that we understand acquired resistance to FGFR inhibitors so that we can develop rational combination treatments to overcome impending resistance.

The predominant FGFR inhibitors are tyrosine kinase inhibitors. Tyrosine kinases (TK) are enzymes that transfer a phosphate to a tyrosine residue in polypeptides. They regulate a wide variety of cell functions including proliferation, survival, differentiation, function and motility. TK can be receptor TKs, such as FGFRs, which are found in the cell membrane and bind ligands, or nonreceptor TKs, which are found in the cytosol, nucleus, and the inner surface of the plasma membrane. A receptor TK contains three parts: a N-terminal lobe to bind ATP and magnesium, a C-terminal lobe containing an activation loop,
and a cleft to bind a polypeptide substrate. ¹ When a receptor TK binds a ligand, it dimerizes and autophosphorylates. This reaction creates a binding site for signaling proteins downstream of the TK. Tyrosine phosphatases and inhibitory molecules often terminate the reaction.¹

FGFR is a classic example of a receptor TK and consists of a family including FGFR1, FGFR2, FGFR3, and FGFR4, and 18 FGF ligands that bind to the four receptors. ² FGFR signals to a variety of downstream pathways, such as MAPK, STAT, and PI3K pathway, and helps to regulate cell growth. When FGFR is de-regulated, through a variety of methods, cells can proliferate at an abnormal rate, which leads to diseases, such as cancer. Genetic alterations that de-regulate FGFR include gene amplifications, translocations, and point mutations. ²

Gene translocations can result in TK protein fusions. The fusion partner of the TK has several important functions that include stabilized structure, increased expression, loss of wild-type expression, oligomerization activation, loss of inhibitory domains, and avoidance of degradation.³ Many fusion partners can result in a direct or indirect oligomerization and constitutently activated the TK, which results in the activation of proteins downstream and excessive cell proliferation.³ Previous FGFR studies show that FGFR inhibitors have maximum potency on cell lines with high levels of wild-type or mutant FGFR3 expression. All three inhibitors, PD173074, TKI-258, and SU5402, decreased downstream signaling, but had no effect in normal human urothelial cells.⁴ Later studies have attributed high levels of sensitivities not only to the over-expressed FGFR3 protein, but to cell lines with FGFR fusions.⁵
To study acquired resistance to FGFR inhibitors, we have used a simplified cell line model. The cell line we will be using is RT-4, which is a bladder cancer that has an FGFR3-TACC3 fusion. We decided to study acquired resistance in the RT-4 cell line with two FGFR3 inhibitors, BGJ398 and Ponatinib. Both BGJ398 and Ponatinib are currently being used in clinical trials at OSU for patients with cancers that have FGFR alterations. BGJ398 binds directly to the ATP binding site in FGFR, while Ponatinib binds to a site adjacent to the ATP pocket and causes a conformation change in FGFR.\textsuperscript{2,6} After it was determined that we had established a RT-4 BGJ398 resistant cell line and a RT-4 Ponatinib resistant cell line, we began to look at proteins that were upregulated and downregulated that may result in secondary resistance. These secondary resistance mechanisms are also possible therapeutic targets and could eventually be administered directly after resistance develops in patients. Using a variety of assays we will find a secondary resistant mechanism pathway, which will allow for a secondary inhibitor target and a decrease in cell viability in the resistant cell lines.

**Materials and Methods**

*Cell Culture*

RT-4 cells were obtained from American Type Culture Collection (ATCC) and maintained in McCoy media supplemented with 10% Fetal Bovine Serum and 1% penicillin-streptomycin. Ponatinib, BGJ398, and GSK2141795 were purchased from Selleck Chemicals. Drug-resistant RT-4 cells were generated by exposing the parental cell line to increasing concentrations of the Ponatinib and BGJ398 until there was an approximate two logarithmic fold difference in the viability of the parental and resistant cell lines.
**Viability Assay**

Both parental and resistant cell lines were plated in 96-well plates, with 3x10³ cells per well, and 24 hours later exposed to a variety of drug concentrations ranging from 50nM to 15uM for 72 hours. Cell viability was determined by CellTiter-Glo assay following the manufacturers protocol (Promega). Cell viability is calculated by setting the vehicle control at 100% viability and calculating the viability of other concentrations as a ratio compared to the vehicle control.

**Reverse phase protein array (RPPA)**

To extract protein, cells were lysed using Lysis Buffer (1% Triton X-100, 50mM HEPES pH 7.4, 150nM NaCl, 1.5mM MgCl₂, 1mM EGTA, 100mM Ma pyrophosphopate, 1mM Na₃VO₄, 10% glycerol, and phosphatase and protease inhibitors) and after sonication, were centrifuged at 4°C for 10 minutes at 12,700×g. After protein concentrations were determined using Bio-Rad Protein Assay Dye Reagent Concentrate and Nanodrop technology, the lysate was mixed with 4× SDS sample buffer (40% glycerol, 8% SDS, .25M Tris-HCl pH 6.8) and boiled for 5 minutes before being stored at -80°C. Samples were sent to MD Anderson RPPA Core Facility, where the samples were diluted and added to nitrocellulose-coated slides. Antibodies, using the tyramide amplification method, tag proteins on the slide and then the slide is scanned on a flatbed scanner. Detailed protocol and analysis completed by MD Anderson can be found on

Western blotting and antibodies

Cells were lysed using RIPA Buffer (25mM Tris×HCl pH 7.6, 150mM NaCl, 1% sodium deoxycholate, .1% SDS, and phosphatase and protease inhibitors) followed by centrifugation at 4°C for 10 minutes at 12,700×g. Protein concentrations were quantified using Bio-Rad Protein Assay Dye Reagent Concentrate and Nanodrop technology. Proteins were separated on SDS-Polyacrylamide gels (either made in house 10% gel or 4-15% Mini-Proteam TGX Gel from Bio-Rad). Antibodies used for Western blotting were pFGFR3 (Abcam), total FGFR3 (Santa Cruz Biotechnology, INC), pFRS2 (Cell Signaling), total FRS2 (Santa Cruz Biotechnology, INC), GAPDH (Santa Cruz Biotechnology, INC), pAkt S473 (Cell Signaling), pAkt T308 (Cell Signaling), total Akt (Cell Signaling), α/β Tubulin (Cell Signaling), p4E-BP1 (Cell Signaling), total 4E-BP1 (Cell Signaling), total eEF2k (Cell Signaling), p GSK-3β (Cell Signaling), pGSK-3α/β (Cell Signaling), total GSK-3β (Cell Signaling), and total GSK-3α/β(Cell Signaling).

Results

Generating RT-4 cell lines that are resistant to FGFR inhibitors BGJ398 and Ponatinib

To generate RT-4 cell lines resistant to FGFR inhibitors, we first had to determine at what drug concentrations the parental cell line was sensitive to BGJ398 and Ponatinib. The parental cell lines were plated in three 100mm dishes and each was treated with DMSO (control), 5μM BGJ398 or 1.5μM Ponatinib for 24 hours. Western blot analysis showed a decrease of pFGFR3 and pFRS2 in the parental RT-4 cell lines that were treated with the inhibitors. α/β Tubulin was used to verify that the proteins were loaded in equal amounts (Figure 1).
After determining that BGJ398 and Ponatinib inhibited the phosphorylation of FGFR3, we next sought to generate and prove resistance to inhibitors. Two sets of parental RT-4 cell lines were treated with either 50nM BGJ398 or 25nM Ponatinib based on published IC50 values and the IC50 values of our initial cell viability curves of the parental cells lines treated with the inhibitors. The concentrations of the inhibitors were continually increased over an eight month period until the concentrations reached 5uM BGJ398 and 1.5uM Ponatinib. Cell viability curves showed a separation between the parental cell lines and treated cell lines indicating a decrease in sensitivity to the inhibitors, which provided evidence for resistance (Figure 2). For example, the IC50 of the parental cell line is approximately 150nM Ponatinib, but in the 1.5uM Ponatinib resistant cell line the IC50 is approximately 2uM Ponatinib. Similarly, the IC50 of the parental cell line is approximately 100nM BGJ398, but in the 5uM BGJ398 resistant cell line the IC50 is 7uM BGJ398 (Figure 2).

In addition to increased cell viability, the resistant cell lines showed a change in cellular morphology compared to the parent. The parental cells have a clustered, colony morphology, while both resistant cell lines appear to have a more spindle-shaped morphology and expand across the plate more uniformly compare to the parental cell line (Figure 3). Changes in cellular morphology could be representing an underlying change in protein expression either at the DNA, RNA, or protein level.

Using RPPA analysis to determine candidate resistance mechanisms

To evaluate potential mechanisms for the acquired resistance, we used RPPA analysis to look at a broad picture of protein expression in both the parental and resistant cell lines. After it was determined that the separation on the viability curve between the
parent and the resistant cell lines was two logarithmic fold difference, the parental and two resistant cell lines were treated with either DMSO or FGFR inhibitors for 24 hours and triplicate samples were harvested and proteins were isolated and sent to MD Anderson for RPPA testing and analysis of over 200 proteins (Figure 4). We then narrowed the list of candidate proteins by filtering out those with less than a 20% upregulation or downregulation fold change. From that list we considered only those proteins with a q-value of less than .05, giving us 96 significant proteins from the Ponatinib resistant cell line and 88 significant proteins from the BGJ398 resistant cell line. Both the Ponatinib and BGJ398 resistant cell lines showed an upregulation in the Akt, MAPKinase, and PI3-Kinase pathways (Figure 5).

In an attempt to find a possible resistant mechanism to FGFR inhibitors, we analyzed RPPA data from other cancer cell lines with resistance to FGFR inhibitors. Simultaneously, in our lab two additional cancer cell lines, DMS114 (lung cancer with FGFR1 amplification) and RT-112 (bladder cancer with FGFR3 amplification and fusion), became resistant to BGJ398 and their proteins were analyzed using RPPA testing and analysis. All significant proteins, based on q-values and fold changes, were cross-referenced between the four resistant cell lines. 8 of the significant proteins found in the resistant cell lines interact with the PI3K and STAT pathways, which are downstream targets of FGFR signaling (Figure 6).

*Using Western blot analysis to verify candidates from RPPA data*

We used Western blot analysis in addition to RPPA analysis to verify upregulated pathways found in RPPA and to assess changes in specific proteins relevant to FGFR signaling that could not be studied by RPPA analysis, such as FGFR3 and FRS2. There is
little difference in phosphorylation of FGFR3 between the parental cell line and the resistant cell line, even though the resistant cell lines are being treated with FGFR inhibitors. While the parental and 5uM BGJ398 resistant cell line have similar pFRS2 expression, the pFRS2 is downregulated in the 1.5uM Ponatinib resistant cell line (Figure 7).

Next, we performed Western blot analysis to verify the results observed from the RPPA analysis of certain upregulated pathways. The PI3K pathway is part of FGFR signaling, and the RPPA revealed many upregulated proteins across the four resistant cell lines within this pathway. These proteins include, but are not limited to pAkt S473, pAkt T308, pGSK 3β, and pGSK 3α/β. The most significant changes in phosphorylation of the resistant cell lines when compared to the parental can be seen in pAkt S473, pGSK 3β, and pGSK 3α/β. pAkt S473, pGSK 3β, and pGSK 3α/β appears to be more phosphorylated in 5uM BGJ398 resistant cell line when compared to the 1.5uM Ponatinib resistant cell line (Figure 8). With RPPA analysis showing upregulated proteins and supporting Western blot analysis, we decided to assess the effects of inhibiting Akt activity with GSK2141795, a clinical grade Akt inhibitor, in the 5uM BGJ398 resistant cell line.

*Use of a GSK2141795 to inhibit Akt activity in RT-4 5uM BGJ398 resistant cell lines*

We designed an experiment that not only compared the effect of GSK2141795 on the parental cell line versus the resistant cell line, but also the effect of GSK2141795 when used in combination with BGJ398 on the resistant cell line. We treated the parental and the 5uM BGJ398 resistant cell lines with a variety of BGJ398 and a variety of GSK2141795 concentrations ranging from 1nM to 10uM. The 5uM BGJ398 resistant cell line was also
treated with a combination of 500nM GSK2141795 with various concentrations of BGJ398 and 1.5uM GSK2141795 with various concentrations of BGJ398. There was little effect on the parental cell line when GSK2141795 was administered in various concentrations, which is consistent with what we expected to see, because FGFR can use a variety of pathways other than Akt. The parental cell line treated with GSK2141795 had an IC₅₀ of 10uM. We saw a decrease in cell viability in the 5uM BGJ398 resistant cell line when GSK2141795 is administered, with an IC₅₀ of approximately 1.5uM. However we saw an even larger decrease in cell viability when BGJ398 and GSK2141795 are used in combination, with an IC₅₀ of 10nM BGJ398 when treated in combination with 500nM GSK2141795 and an IC₅₀ of 1nM BGJ398 when treated in combination with 1.5uM GSK2141795 (Figure 9). The difference between cell viability of the parental cell line and resistant cell line when exposed to GSK2141795 provides evidence of changes in protein dependence after chronic exposure to an inhibitor. The effectiveness of GSK2141795 on the resistant cell line verifies the use of RPPA to suggest resistant mechanisms and the ability to use a second inhibitor to reduce cell viability on a resistant cell line.

**Discussion**

Although tyrosine kinase inhibitors may represent a new focused treatment for cancer, many patients develop resistance to the inhibitors over time despite initial responses or benefits. Some inhibitors’ resistance patterns have been studied heavily, such as ABL1, EGFR, BRAF, and ALK, but there has been little research done on acquired drug resistance for FGFR since it is a novel target. We have demonstrated the ability to produce FGFR resistant cell lines over a period of months and utilized RPPA analysis to study a variety of possible resistance mechanisms. After cross-referencing RPPA data from four different
resistant cell lines, we were able to narrow our focus to resistance mechanisms within the FGFR pathway. Using western blot analysis, we hypothesized that multiple proteins within Akt pathway were upregulated. With this information, we inhibited the Akt pathway and showed that a combination therapy was more effective than the individual inhibitors.

There are many pathways, such as the Akt pathway, within a cell that could lead to a resistance mechanism. RPPA analysis could be a useful tool for identifying mechanisms of resistance after treatment with an inhibitor. It’s low cost, quick turnaround, and high output are ideal to analysis a wide range of protein pathways in the cell. Suggestion of a resistance pathway and not a specific protein allows for the opportunity to use a variety of inhibitors found within a pathway. The analysis can be easily compared over multiple samples. Although RPPA is a valuable tool to narrow down the path of resistance, it has its limitations. Some proteins that are known to drive cancer are found in the extracellular membrane, which are not easily extracted from the cell membrane and are not tested for in RPPA analysis. It is important to verify the results found and study missing components in RPPA analysis with some other form of analysis, such as Western blot, DNA sequencing, or RNA sequencing.

Although RPPA analysis and Western blot analysis has provided the theory of Akt resistance, there are more experiments that need to be completed to convince us of Akt’s involvement in resistance. We will be completing cell viability curves using Akt siRNA to analyze the effect of a specific Akt knockdown. In addition to cell viability curves, we will also be completing Western blot analysis using Akt siRNA and the Akt inhibitor GSK2141795 to verify that both are inhibiting Akt and not causing a decrease in cell viability simply through toxicity. Using the resistant cell lines, we will also perform wound
healing assays to identify changes in cell migration between the parental cell line, resistant cell line, and resistant cell lines treated with Akt siRNA. In addition to these experiments, we will be studying other researchers work on the Akt pathway.

Several papers have demonstrated that proteins within the Akt pathway contribute to resistance and changes in cellular morphology. In Grygielewicz et al.’s paper, “Epithelial-mesenchymal transition confers resistance to selective FGFR inhibitors in SNU-16 gastric cancer cells,” described that resistance using FGFR inhibitors has a common pathway of resistance that is independent of the inhibitor itself. They discovered upregulation in the Akt, ERK, STAT3, and HER2 pathways. Thorne et al. describes the effect that GSK-3 can have on a variety of kinase inhibitors. Because of the prevalence of proteins in the Akt pathway’s contribution to resistance, many inhibitors have been designed to target this pathway. This could be important for potential combination therapy for patients in the future.

This study highlights the importance of understanding secondary resistance mechanisms. While suggesting a technique to find resistance patterns within patients, we were also able to identify a common secondary resistance pathway with FGFR dependent cancers. In the future, this could narrow the search for resistance patterns if we find a common pathway within FGFR dependent cell lines and administer combination therapies earlier in treatment plans.
Figures and Tables

Figure 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>RT-4 Parental Cell line</th>
<th>RT-4 Parental Cell line</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5uM Ponatinib</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>5uM BGJ398</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Western Blot analysis showing inhibition of pFGFR3 and pFRS2 in parental RT-4 cell line after 24 hours of exposure to 1.5uM Ponatinib and 5uM BGJ398.

Figure 2

(a)

Cell Viability Curve of RT-4 Parent and 1.5uM Ponatinib Resistant Cell Lines with Varing Concentration of Ponatinib
Figure 2: (a) The parental and 1.5uM Ponatinib-resistant RT-4 cell lines were plated with different dosages of Ponatinib, incubated for 72 hours, viability was assessed using CellTiterGlo Assay, and graphed on a log scale. (b) Similarly, the parental and 5uM BGJ398-resistant RT-4 cell lines were plated with different dosages of BGJ398 and graphed on a log scale.
Figure 3: (a) Morphology of parental cell line. (b) Morphology of 1.5µM Ponatinib resistant cell line. (c) Morphology of 5µM BGJ398 resistant cell line.

Figure 4
(a)

(b)

Figure 4: (a) Heat map of RPPA analysis of proteins from control and 1.5µM Ponatinib resistant RT-4 cell line. (b) Heat map of RPPA analysis of proteins from control and 5µM BGJ398 resistant RT-4 cell line.
Figure 5
(a)
Figure 5(a) Heat map with proteins of 20% fold change upregulated and downregulated proteins, according to RPPA analysis, in the 1.5uM Ponatinib resistant cell line. (b) Heat map with top 20% fold change upregulated and downregulated proteins, according to RPPA analysis, in the 5uM BGJ398 resistant cell line.
Figure 6 (a)

Significant Protein Changes Found in 3 Cell Lines
- Akt_pS473
- Akt_pT308
- Cyclin-B1
- eEF2K
- GSK-3ab_pS21_S9
- GSK-3b_pS9
- Stat3_pY705
- Stat5a
- TSC1
- YAP_pS127
Figure 6: (a) Number of proteins, with at least 20% fold change and sorted by q values, in common between three FGFR resistant cell lines, RT-112 5uM BGJ398, DMS114 3uM BGJ398 and RT-4 5uM BGJ398 according to RPPA analysis. List of proteins with significant
fold changes and sorted by q values found in the 3 resistant cell lines. (b) Number of proteins, with at least 20% fold change and sorted by q values, in common between three FGFR resistant cell lines, RT-112 5uM BGJ398, DMS114 3uM BGJ398 and RT-4 1.5uM Ponatinib according to RPPA analysis. List of proteins with significant fold changes and sorted by q values found in the 3 resistant cell lines.

Figure 7

Figure 7: The parental cell line was treated with DMSO, the 5uM BGJ398 resistant cell line was treated with 5uM BGJ398, and the 1.5uM Ponatinib resistant cell line was treated with 1.5uM Ponatinib for 24 hours. The first Western blot on the left presents minimal reactivation of the pFGFR3 pathway in resistant cell lines. The first Western blot on the right demonstrates no reactivation within the Ponatinib resistant cell line and minimal reactivation within the BGJ398 resistant cell line. GAPDH is a protein loading control.
Figure 8: Western blot analysis showing changes in protein concentration between resistant cell lines and parental cell. Proteins were chosen based on RPPA analysis that suggested changes in PI3K and STAT pathway. The parental cell line was treated with DMSO, the 5uM BGJ398 resistant cell line was treated with 5uM BGJ398, and the 1.5uM Ponatinib resistant cell line was treated with 1.5uM Ponatinib for 24 hours. Both STAT3 and STAT5, proteins in the STAT pathway, are downstream targets of FGFR3 and are upregulated in the BGJ398 resistant cell line when compared to the parental cell line. pAkt S473, pAkt T308, pGSK-3α/β, pGSK-3β, proteins in the PI3K, are downstream targets of FGFR3 and are upregulated in both resistant cell lines when compared to the parental cell line.
Figure 9: Parental cell line was treated with Akt inhibitor (GSK2141795) and BGJ398. The 5uM BGJ398 resistant cell line was treated with GSK2141795, BGJ398, and 2 concentrations of GSK2141795 and varying dosages of BGJ398. The parental cell line treated with GSK2141795, the dark green line, indicates minimal effect of the inhibitor compared to the resistant cell line treated GSK2141795, the light purple line. However, when the resistant cell line is treated with both GSK2141795 and BGJ398 there is a greater decrease in cell viability compared to the resistant cell line that is treated only with GSK2141795.
References


**Acknowledgements:**

I would like to thank the members of the Roychowdhury lab for their help with the project, Datta J, Damodaran S, Miya J, Ocrainiciuc C, Samorodnitsky E, Bhatt D, and Roychowdhury S. I would also like to thank the Pelotonia Fellowship Program, the Mayers Summer Research Scholarship, and the Undergraduate Research Scholarship.