# Effect of the Knockout of FGL1 by CRISPR on the Gefitinib Sensitivity of PC9/GR Cells

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## Abstract

This paper will investigate the effect of the Knockout of FGL1 by CRISPR on the EGFR signaling analyzed phosphor ERK western blot and how it may control the gefitinib sensitivity of PC9/GR cells measured by the MTT Assay. This experiment will knock out FGL1 using CRISPR. The Knockout will then be verified through Phospho-ERK western blot. Moreover, the sensitivity of the gefitinib will be measured by the MTT Assay. The possible results generated from the reports' experiments may provide insight into future clinical trials of FGL1 Knockout consequences on gefitinib sensitivity. Gefitinib is a potential drug for lung cancer. Therefore, investigating this drug may help scientists better understand how the FGL1 may increase the sensitivity of Gefitinib cells and potentially discover a breakthrough to cure cancer..

**Keywords:** ***FGL1, CRISPR, PC9/GR, Gefitinib Sensitivity***

## 1. Introduction

In 2021, an estimated 1.9 million lung cancer cases were diagnosed, and 608,570 deaths were caused by lung cancer alone in the United States [1]. Among all lung cancer cases, 13% are small cell lung cancer (SCLC ), and 84% are non-small cell lung cancer (NSCLC) [2]. Of the 84% of NSCLC patients, approximately 80% miss the best opportunity for treatment by the time of diagnosis; for this reason, the five-year survival rate of NSCLC patients maintains a low percentage, only 15% [3].

In this report, FGL1 will be an essential chemical for the experiment. Fibrinogen-like protein 1 (FGL1) is a ligand of lymphocyte-activation gene 3, and it is found in the cytoplasm of NSCLC cells and on the surface of breast cancer cells [4]. It can regulate proliferation factor expression and repair liver damage and regeneration or the liver [5]. Essentially, FGL1 also regulates the growth and proliferation of tumor cells as it has a role in the cell proliferation pathway. In recent studies, FGL 1 overexpression has been discovered in solid tumors, especially those with NSCLC [6]. It has been reported that the patient generally with overexpression only had a shorter survival time of 5 years [5].

PC9/GR is a lung adenocarcinoma cell line with a deletion in exon 19 of the EGFR gene, which exhibits a high sensitivity to Tyrosine kinase inhibitors (TKIs) [7]. Several tyrosine residues are trans-auto phosphorylated when EGFR is activated by its ligands. On top of this, EGFR stimulates EGFR intracellular signaling cascades, such as the RAS/RAF/ERK (MAPK) pathway [8]. The two principal downstream effectors of EGFR activation are the RAS-MAP pathway and the PI3K-AKT-mTOR pathway, controlling cell growth and proliferation [9].

In the RAS-MAP pathway, the RAS activates MARP/ERK kinase (MEK) 1/2 dual-specificity protein kinases that activate ERK1/2 [7]. ERK activation will promote the upregulated expression of EGFR ligands. It will then activate BAX, a member of the Bcl-2 family that acts as a core regulator of the intrinsic apoptosis pathway [10]. The Caspases, including caspase-3, are known to act downstream of BAX/BCL-2 control and are essential in executing apoptosis [10]. Because the activated caspase-3 cleaves proteins, including poly(ADP-ribose) polymerase-1 (PARP-1), which is essential in DNA repair, thus promoting apoptosis [10]. The role of FGL1 in these pathways is to block the activation of RAS and the phosphorylation of EGFR and ERK. (See figure 1)

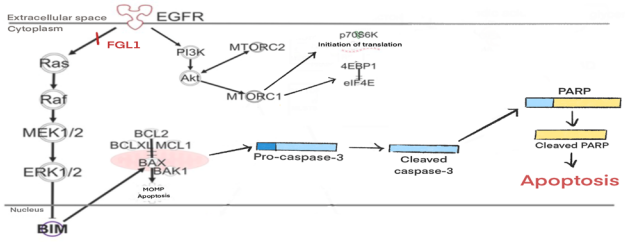


**Figure 1: EGFR and RAS-MAP Pathway. This figure shows how the EGFR and RAS-MAP signalling pathways can lead to apoptosis and the position and function of FGL1 within the pathway.**

The EGFR tyrosine kinase can be inhibited by an inhibitor – Gefitinib. Gefitinib is a drug that has been demonstrated to have an anti-tumor in NSCLC, and it is a drug that is

legally approved in over 20 countries across the globe. Scientific evidence has proved that NSCLC expresses high levels of EGFR and that Gefitinib was more effective against adenocarcinoma than squamous cell carcinoma, and the latter expresses more EGFR [11]. Not only so, but previous experiments were also conducted to analyze the expression of phosphorylated ERK1/2 and compare the results with EGFR (HER1/ErbB1) expression levels [11]. The results of the experiments suggest that Gefitinib is potentially effective against cancers with lower EGFR expressions. In addition, other experiments prove that FGL1 regulates acquired resistance to Gefitinib by inhibiting apoptosis in non-small cell lung cancer by investigating the FGL1 expression in the lung tumor cell lines [12].

While the previous research suggests that FGL1 regulates resistance to Gefitinib, there is necessary to find out how exactly FGL1 regulates the resistance. Since FGL1 blocks the activation of RAS, it should also block the phosphorylation of EGFR and ERK. Thus, this essay will investigate the Knockout of FGL1 by CRISPR, resulting in decreased EGFR signaling and affecting the gefitinib sensitivity of PC9/GR cells.

***Research question***

Can the Knockout of FGL1 by CRISPR result in decreased EGFR signalling and affect the gefitinib sensitivity of PC9/GR cells?

***Hypothesis***

This research tries to predict the Knockout of FGL1 by CRISPR results in decreased EGFR phosphorylation and p-Erk and increased gefitinib sensitivity of PC9/GR cells. Measure EGFR signaling by Phospho-Erk/Erk and phosphoEGFR/EGFRwestern blot and gefitinib sensitivity by MTT Assay. The negative control is PC9/GR without the Knockout of FGL1, and no positive control.

# 2. Methods

## 2.1 *Gene Knockout of FGL1 by CRISPR*

A guide strand is engineered to target the FGL1 locus and used CRISPR to introduce mutation, which is then used to test whether the FGL1 protein is successfully knocked out.

## 2.2 *Western blot*

The FGL1 cells are plated in 6-well plates and cultured for 24 hours. The growth medium is added to the medium containing 0.5% serum and kept overnight [13]. The cells are treated with PEITC for different periods and harvested by MAPK lysis buffer (10 mmol/L Tris-HCl, 50 mmol/L sodium chloride, 30 mmol/L sodium PPi, 50 mmol/L sodium fluoride, 100 μmol/L sodium orthovanadate, 2 mmol/L iodoacetic acids, 5 mmol/L ZnCl2, 1 mmol/L phenylmethyl sulfonyl fluoride, and 0.5% Triton X-100) [14]. Then, prepare protein extracts with the concentration determined by Bio-Rad micrograms protein assay according to the manufacturer's instruction [14]. Twenty micrograms of protein from each sample will be separated by SDS-PAGE and transferred onto polyvinylidene fluoride membranes by the semidry transfer system [14]. The membranes are then blocked with 5% bovine serum albumin or 5% nonfat milk in TBST buffer [2.42 g/L Tris-HCl, 8 g/L NaCl, 1 mL/L Tween 20 (pH 7.6)], and it is kept overnight at 4 degrees celsius with primary antibody conjugated with horseradish peroxidase [14]. The detection will be done with enhanced chemiluminescence Western blotting reagents [13]. The different antibody binds to erk and also binds to phosphorylated erk. And then there should be no changes with total erk, but something changing with phosphorylation.

## 2.3 *Gefitinib sensitivity testing*

The FGL1 cultured with EGFR is seeded at 2.5 104 cells per well onto 96-well plates and cultivated for 24 hours. The Gefitinib is added to the sample and cultured for 24 hours [11]. An MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium assay is used to examine the gefitinib sensitivity against the FGL1 cultured EGFR. The Gefitinib cells with the FGL1 cultured EGFR will be seeded into 96-well microtiter plates, and 10 μL of the Gefitinib will be added at various concentrations (0.25 μL, 0.5 μL, 1.0 μL, 2.0 μL). [11] After incubation for 72 hours at 37 degrees, 20 μL of the MTT solution (5 mg/ml in phosphate-buffered saline [PBS]) will be added to the well, and incubation will continue for 4 more hours at 37 degrees. In the cell growth curves, the IC50 value is the amount of Gefitinib required to reduce absorbance by 50% (560 nm) [15]. The statistical significance of all numerical data gathered through the Western Blot MTT Assay will be analyzed using a T-Test ($p < 0.05$). Each experiment will be repeated five times.

# 3. Results

**Table 1: Possible results**

| Measured parameter | Result 1 | Result 2 | Result 3 | Result 4 | Result 5 | Result 6 | Result 7 | Result 8 |
|---|---|---|---|---|---|---|---|---|
| FGL1 KO in PC9/GR decreases p-EGFR WB | + | + | - | - | + | + | - | - |
| FGL1 KO in PC9/GR decreases p-ERK WB | + | - | - | + | - | + | + | - |
| FGL1 KO in PC9/GR increases MTT killing | + | + | + | + | - | - | - | - |
| Support the hypothesis? | Yes | Partially | Partially | Partially | Partially | Partially | Partially | No |

*Note*. "+" represents a result different from the negative control group "-" represents a result close to the negative control group, which is PC9/GR cells without FGL1 knockout.

**Possible result 1:** All of the results indicate a result is different from the negative control group.
The knockout of FGL1 in PC9/GR cells successfully increased the phosphorylated EGFR and ERK as well as the MTT killing.
**Possible result 2:** Indicates that the result of the phosphorylated EGFR Western blot and sensitivity of gefitinib by MTT assay is different to the negative control group. The result of the phosphorylated ERK Western blot is close to the result from the negative control.
The knockout of FGL1 in PC9/GR cells decreased the Phosphorylated EGFR and increased MTT killing but did not decrease the Phosphorylated ERK.
**Possible result 3:** Indicates that the result of the sensitivity of gefitinib by MTT Assay is different from the negative control group. The result of the phosphorylated EGFR Western blot and phosphorylated ERK Western blot is close to the result from negative control.
The knockout of FGL1 in PC9/GR cells only increased the MTT killing and did not decrease phosphorylated ERK and Phosphorylated EGFR.
**Possible result 4:** Indicates that the result of phosphorylated ERK Western blot and sensitivity of gefitinib by MTT Assay is different from the negative control group. The result of the phosphorylated EGFR Western blot is close to the result from the negative control.
The knockout of FGL1 in PC9/GR cells decreased phosphorylated ERK and the increased MTT killing but did not decrease the Phosphorylated EGFR.
**Possible result 5:** Indicates that the result of the Phosphorylated EGFR Western blot is different from the negative control group. The result of the phosphorylated ERK Western blot and gefitinib sensitivity by MTT Assay is close to the result from the negative control.
The knockout of FGL1 in PC9/GR cells only decreased the phosphorylated EGFR measured and did not decrease the Phosphorylated ERK and increase MTT Killing.
**Possible result 6:** Indicates that the result of the Phosphorylated EGFR Western blot and phosphorylated ERK western blot is different from the negative control group. The result of the gefitinib sensitivity by MTT Assay is close to the result from the negative control.
The knockout of FGL1 in PC9/GR cells decreased the phosphorylated EGFR and ERK but did not increase MTT killing.
**Possible result 7:** Indicates that the result of the Phosphorylated ERK Western blot is different from the negative control group. The result of the Phosphorylated EGFR Western blot gefitinib sensitivity by MTT assay is close to the result from the negative control.
The knockout of FGL1 in PC9/GR cells only decreased the phosphorylated ERK and did not decrease the Phosphorylated EGFR and increase MTT killing.
**Possible result 8:** All three results indicate a result that is similar to the result of the negative control.
The knockout of FGL1 in PC9/GR cells failed to decrease the EGFR and ERK phosphorylation and increase MTT killing.

## 4. Discussions

Possible results 1: indicate that the knockout of FGL1 led to decreased phosphorylated EGFR and phosphorylated ERK measured by western blot. It will lead to an increase in sensitivity measured by MTT. Thus it fails to reject the hypothesis. Scientists can conduct further investigations on how to use this concept of knocking out FGL1 to increase the sensitivity of cancer drugs and potentially develop a drug that manipulates the knockout and slow down the development of tumor more efficiently.
**Possible result 2:** indicates that the FGL1 knockout decreased the phosphorylated EGFR without decreasing the phosphorylated ERK and increasing the sensitivity of Gefitinib. It only partially supports the hypothesis. A possible reason that it only decreased the phosphorylated EGFR might be because an interruption occurred between RAS, RAF, and MEK1/2. Therefore the phosphorylation

continued during the process yet. It still managed to create apoptosis. Further investigation is required on whether RAS, RAF, and MEK1/2 were activated in the first place; if so, then the result must be resulting because the inhibition of phosphorylation of ERK was not in the EGFR signaling pathway. Nevertheless, if they were not activated in the first place, then an interruption must have occurred. Further experiments should involve researching the interruptions and alternative pathways that lead to apoptosis without phosphorylating ERK.

Possible results 3 and 4: indicate that even though the phosphorylation of EGFR did not decrease, the Gefitinib sensitivity nevertheless increased. The evidence partially supports this research hypothesis. A possible explanation may be that the knockout of FGL1 inhibited the bind of ligands to EGFR, failing to decrease the EGFR phosphorylation. However, for possible result 3, the RAS was somehow inhibited by another pathway leading to the decrease in phosphorylation of ERK and not the EGFR, thus leading to apoptosis. In possible result 4, another pathway must have activated BAX and led to apoptosis. Experiments should be done to investigate how the knockout might have affected the binding of ligands to EGFR and any alternative pathways that can cause ERK and BAX, which then leads to changes in the sensitivity of Gefitinib.

Possible results 5 and 6: indicates that the decrease in phosphorylation of EGFR does not increase the sensitivity of Gefitinib. This only partially supports the hypothesis. A possible reason for this is that there was an interruption within the pathway after the phosphorylation of EGFR, stopping the activation of BAX and apoptosis. It may also mean that the EGFR signaling pathway is not correlated with the sensitivity of Gefitinib. Therefore, further experiments are needed to prove the correlation or discover new pathways that may have been activating apoptosis.

**Possible result 7:** indicates that the knockout of FGL1 in PC9/GR cells does not decrease the phosphorylation of EGFR and does not affect the sensitivity of Gefitinib measured by MTT. It partially supports this research hypothesis. A possible reason for this may be that the knockout of FGL1 did not inhibit the binding of Ligand to EGFR, so the EGFR was still phosphorylated. However, the phosphorylation of ERK was somehow decreased by another pathway. However, the decrease in phosphorylated ERK either does not activate BAX, or there was an interruption between the RAS/MAP signaling pathway, which both results may lead to no apoptosis. Further investigations are needed on alternative pathways that may activate ERK or interruptions that may have stopped ERK from activating other chemicals and check for ligand binding to EGFR.

**Possible result 8:** indicates that the knockout of FGL1 in PC9/GR cells does not decrease the phosphorylation of the EGFR and does not increase the sensitivity of Gefitinib measured by MTT. It completely rejects this research hypothesis. A possible reason for this is that the knockout of FGL1 does not decrease the ligand binding of EGFR and therefore is not correlated to the EGFR signaling it. Experiments in the future should be based on pathways or mechanisms likely to cause an effect or investigate more deeply to see the consequences of FGL1 knockout..

## 5. Conclusion

In summary, this study investigates the effect of the Knockout of FGL1 by CRISPR on the EGFR signaling analyzed phosphor ERK western blot and how it may control the gefitinib sensitivity of PC9/GR cells measured by the MTT Assay. The possible results generated from the experiments in the report may provide some possible insight into the correlations and mechanisms between FGL1, EGFR, and Gefitinib. Further investigations include experimenting with factors that may affect the FGL1 knockout process, interruptions within the EGFR and RAS/MAP pathway with phosphorylation, and further development into how FGL1 knockout can be further developed Gefitinib as a cure for cancer. Cancer has long been an incurable disease. Cancer is causing millions of human deaths worldwide, and developing a permanent cure for cancer is vital. Gefitinib is a potential drug for lung cancer. It still needs to be investigated in more evident detail for scientists to understand better how the FGL1 may increase the sensitivity of Gefitinib cells and potentially discover a breakthrough to cure cancer.

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