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# The activity of the Nrf2/Keap1 pathway regulates A549 Non-small-cell Lung Cancer (NSCLC) cells motility through RhoA–ROCK1 pathway

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**Abstract. Purpose:** It is observed that Nrf2 transcription factors initiate the production of proteins that play a role in regulating tumor growth, the study aims to discover whether Nrf2 will regulate cell motility in A549 NSCLC cells. The ROCK-RhoA pathway is a significant contributor to cell growth and migration, which can relate to cell motility. There might be a connection between Nrf2 and ROCK-RhoA pathway, the study aims to see whether Nrf2 regulates A549 NSCLC cell motility through ROCK-RhoA pathway. **Method:** The study will use wound healing assay to visualize cell motility, comparison is made between positive control group(A549 cells treated with brusatol) and negative control group(A549 cells not treated with brusatol), respectively representing the expression level of Nrf2 where it is inhibited or normal. The pathway by which Nrf2 regulates cell motility is studied by western blotting and chemiluminescence. **Possible result:** There are four main possible results: (1), Nrf2 regulates cell motility in A549 NSCLC cells, the pathway involved in this regulation is ROCK-RhoA pathway. (2), Nrf2 regulates cell motility in A549 cells, however, the pathway involved in this regulation requires further studies to decide. (3), There is no obvious correlation between Nrf2 expression level and cell motility, the inhibition of Nrf2 results in change of expression level of the ROCK-RhoA pathway, however. (4), There is no obvious correlation between Nrf2 expression level and cell motility, ROCK-RhoA pathway is not significantly affected by inhibition of Nrf2 expression, further studies are needed to decide pathways involved. **Conclusion:** The result of this study provides valuable insights for studying the role of Nrf2 in regulating cell motility and the pathway in the process. It would improve understanding of Nrf2 transcription factor, and how it interacts with pathways to carry out regulation of cell motility. The study also provides future possibilities in terms of cancer treatment and development of gene-based medicine.

**Keywords:** Cancer, Cell Migration, Cell Signaling, Lung Cancer, Non-small-cell lung cancer.

## 1. Background

NSCLC is any type of epithelial lung cancer other than small cell lung cancer (SCLC). Squamous cell carcinoma, large cell carcinoma, and adenocarcinoma are the most common types of NSCLC, several other types can occur but less frequently, while all types can occur in unusual histological variants[1]. Most lung cancer statistics include both small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). In general, about 10% to 15% of all lung cancers are SCLC, and about 80% to 85% are

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NSCLC. Lung cancer is the second most common type of cancer in the United States (excluding skin cancer). Lung cancer is most prevalent among elderly people, most patients are 65 years and above, with a small number of patients between 45 and 65 years old. The risk of a man developing lung cancer is around 6.25%, and 5.88% for women. The survival rate of lung cancer is mainly related to the type of lung cancer and its stage of development. [2]

Organisms receive oxidants from both interior metabolism and exterior environment. Oxidants are commonly reactive oxygen series (ROS) that cause oxidative stress if oxidants and antioxidants levels are not balanced[3]oxidative stress may result in damage to cell structures and tissues, disrupting the body from functioning normally[4]. Organisms also receive electrophilic stress, which is caused by electrophilic compounds that have electron-defiant regions and are hence attracted to electron-rich regions on other molecules, which causes the formation of new chemical bonds and lead to modification and conformational change to specific structures in the body. [5]

Nuclear factor-erythroid 2-related factor 2 (Nrf2) is a transcription factor and a regulator of the expression of several genes that contribute to cellular resistance to oxidative stress. [3]The KEAP1 gene encodes a protein containing KELCH-1 like domains, as well as a BTB/POZ domain[6]. Under homeostasis, Keap1 forms part of an E3 ubiquitin ligase, which regulates the quantity of Nrf2 transcription factor[7] by targeting it for ubiquitination and proteasome-dependent degradation[8]. Nrf2 binds to its endogenous inhibitor Kelch-like ECH-associated protein 1 (Keap1) in the cytoplasm under normal conditions, which is an adaptor subunit of Cullin 3-based E3 ubiquitin ligase[8,9]. This binding is controlled by redox, when under oxidative stress, the protein will dissociate from Nrf2, which will then be transported to the nucleus[6]. Inside the nucleus, Nrf2 will activate the expression of various genes that have antioxidant response elements (AREs) in their promoters, hence producing proteins and enzymes that increase the cell's resistance to oxidants. [3]

RhoA–ROCK1 pathway is a signal transduction pathway that plays an important role in cell growth, differentiation, migration and development[10]The Keap1-Nrf2 pathway responds to oxidative and electrophilic stresses to maintain the balance between oxidative stress and antioxidantsUnder homeostatic conditions[11,12].

Brusatol is a triterpene lactone compound mainly from Brucea javanica[13]. It has been proved to provoke a rapid and transient inhibition of Nrf2 signaling[14]. It can be used to modify the activity of Nrf2. Dimethyl Sulfoxide(DMSO), with formula (CH3)2SO is a common medium used in cell culture. Keap1-Flag is Keap1 specifically for experimental purposes, it forms part of an E3 ubiquitin ligase and serves as an inhibitor of Nrf2[7]. siNrf2 is an inhibitor of Nrf2 while exogenously introducing Nrf2-EGFP will result in the overexpression of Nrf2[15]. Horseradish peroxidase can catalyze the reaction between RhoA–ROCK1 proteins and secondary antibodies.

I predict that the positive control group, consisting of A549 cells that have transcription factor Nrf2 inhibited by brusatol will show decreased cell motility compared to the control group consisting of A549 cells cultured with DMSO only. This is tested by wound healing assay within 24 and 48 hours by calculating area of migrated cells using processing software Image-J. Additionally, it is predicted that the inhibition of Nrf2 will result in a reduced level of expression of ROCK and RhoA proteins in A549 cells in the positive control group. This can be observed by using western blotting followed by chemiAs fewer RhoA–ROCK1 proteins are produced and binded to secondary antibodies conjugated with chemiluminescence by incubating proteins with antibodies and observing the chemiluminescence signal. This is detected by an enhanced chemiluminescence (ECL) system.

## 2. Method

### *2.1. Cell Culture and Reagents*

The human NSCLC cells line A549 were cultured using RPMI1640 supplemented with 10% foetal bovine serum (FBS), 1% penicillin/streptomycin at 37 ℃ in a humidified atmosphere that contains 5% CO2. Brusatol was dissolved in dimethyl sulfoxide (DMSO). Before wound healing assay (group 1) starts, A549 NSCLC cells in the positive control group are treated with brusatol (10 nM) that's dissolved

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in DMSO(0.1%) for 24 hours, and A549 cells in the control group are treated with DMSO for 24 hours. Before wound healing assay(group 2) starts, Keap1-Flag is transfected into A549 cells in the positive control group and Mock-Flag is transfected into A549 cells. [15]

### *2.2. Cell Migration Assay*

Cell migration was measured by wound healing assay. Cells were arranged into 6-well plates and cultured until they form a single layer, this may last for 24 hours. The single layer of cells were then collected using the tip of 200-μl sterile pipette and washed using phosphate buffered saline (PBS) for 2 times to remove any cells that were not on the layer. Afterwards, culture cells in RMPI-1640 medium with 1% serum to reduce cell proliferation during assay. At time zero, obtain initial images from four independent areas after each collection of cells. Each collection was examined, photos of the same location were taken, and measures of the healed area needed to be done after the fixed time. Use Image J to calculate the area of migrated cells to obtain the degree of migration. [15]

### *2.3. Western Blotting*

Western blotting is used to detect the presence of RhoA–ROCK pathway. Harvest A549 NSCLC cells from the migration assay and wash with PBS, obtain cell lysate by lysing cells in ice-cold cell lysis buffer containing 1× protease Inhibitor Cocktail. SDS-PAGE is used to separate proteins in cell lysate based on their size, transfer protein onto a PVDF membrane for binding with antibodies later. Incubate proteins with primary antibodies(ROCK1, RhoA), and incubate with corresponding goat anti-mouse Ig G or goat anti-rabbit Ig G conjugated with horseradish peroxidase (HRP). Enhanced Chemiluminescence System(ECL) is used to detect whether there is a decrease in expression level of ROCK1 and RhoA proteins in the positive control group, where Nrf2 activity is inhibited with brusatol.

### *2.4. Statistical Analysis*

Results were obtained from at least three independent experiments, indicated as the average ± standard deviations (SDs). Comparisons between groups were performed using an ANOVA test. p-values $\leq 0.05$ were considered statistically significant.

## 3. Results

### *3.1. A549 NSCLC cells motility*

**Possible result 1:** A decrease in cell motility is observed in A549 NSCLC cells whose Nrf2 is inhibited by brusatol. This indicates that the inhibition of Nrf2 does result in a decrease in cell motility in A549 cells, the activity of Nrf2 transcription factor and cell motility of A549 cells forms a positive relationship. This result aligns with the hypothesis.

**Possible result 2:** There is no significant change observed in A549 NSCLC cells whose Nrf2 is inhibited by brusatol. This indicates that the inhibition of Nrf2 does not cause cell motility to decrease, this result possibly suggests that Nrf2 transcription factor does not play a major role in regulating cell motility in A549 cells.

**Possible result 3:** An increase in cell motility is observed in A549 NSCLC cells whose Nrf2 is inhibited by brusatol. This indicates that the inhibition of Nrf2 results in an increase in cell motility in A549 cells, the activity of Nrf2 transcription factor and cell motility of A549 cells forms a negative relationship.

### *3.2. Expression of ROCK protein*

**Possible result 1:** A decrease in expression level of ROCK protein is observed by western blotting and chemiluminescence, when Nrf2 is inhibited, this observation suggest that activity of Nrf2 transcription factor forms positive relationship with ROCK protein expression, and the pathway in which Nrf2 regulates cellular response through a pathway that involves ROCK protein.

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**Possible result 2:** There is no significant decrease of ROCK protein expression level observed by western blotting and chemiluminescence, while this result might indicate that there is no obvious association between Nrf2 activity and ROCK protein, it is also possible that Nrf2 inhibition results in deactivation of ROCK protein through, for example, changing its phosphorylation status, so that certain regulation involving ROCK is inhibited while concentration of ROCK protein is not decreased.

**Possible result 3:** An increase in expression level of ROCK protein is observed by western blotting and chemiluminescence, when Nrf2 is inhibited, this observation suggests that the activity of Nrf2 forms negative correlation with ROCK protein expression, hence inhibiting Nrf2 will result in increased cell motility in A549 cells.

### *3.3. Expression of RhoA protein*

**Possible result 1:** A decrease in expression level of RhoA protein is observed by western blotting and chemiluminescence, this result indicate that activity of Nrf2 forms a positive correlation with expression level of RhoA, and Nrf2 regulates certain cellular response through pathway that involves Nrf2

**Possible result 2:** There is no significant changes observed for RhoA proteins expression level when Nrf2 is inhibited, while this result might suggest that Nrf2 does not have an obvious association with the expression of RhoA protein, it is also possible that the inhibition of Nrf2 results in deactivation of RhoA protein, which inhibited the pathway by which Nrf2 regulates certain cellular response without RhoA protein expression level changing.

**Possible result 3:** RhoA protein expression level was observed to increase by western blotting and chemiluminescence, this result indicates that activity of Nrf2 and expression of RhoA proteins forms a negative correlation.

### *3.4. Combination of Possible Results Table(CR)*

**Table 1.** Possible Results Table of Cell Motility and Protein Presence

| Possible Observations | Cr1 | Cr2 | Cr3 | Cr4 | Cr5 | Cr6 | Cr7 | Cr8 |
|---|---|---|---|---|---|---|---|---|
| A549 cell motility decreases when Nrf2 is inhibited? | + | + | + | + | - | - | - | - |
| Chemilumines-cence detects less ROCK1 protein? | + | + | - | - | + | + | - | - |
| Chemilumines-cence detects less RhoA protein? | + | - | + | - | + | - | + | - |
| Supporting Hypothesis? | Yes | Partially | Partially | Partially | Partially | Partially | Partially | No |

+ Represents that a decrease of cell motility in A549 NSCLC cells is observed in the positive control group, where Nrf2 transcription factor is inhibited by brusatol (row 1). In row 2 and 3, it represents that decrease of expression level of ROCK1 and RhoA proteins are observed in the positive control group, where Nrf2 is inhibited by brusatol.

- Represents that no decrease of cell motility in A549 NSCLC cells is observed in the negative control group, where Nrf2 transcription factor is not affected/not inhibited(row 1). In row 2 and 3, it represents that decrease of expression level of ROCK1 and RhoA proteins are not observed in the positive control group, where Nrf2 is inhibited by brusatol.

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## 4. Discussion

Cell migration is an important factor of the development of various diseases, including cancer. By studying genes and pathways that regulate cell migration, understanding of this process will be improved, this might help discover new treatments of cancer as well as enhancing treatments that already exist to make them more effective. Nrf2 is a transcription factor known to be regulating the cell's response to oxidative stress. In homeostasis, the activity of Nrf2 is regulated strictly by E3 ubiquitin ligase that has KEAP1 binded to it as this complex is in charge of the degradation of Nrf2. Under oxidative stress, however, Nrf2 will be translocated to the nucleus, it will then bind to DNA promoters and transcribe genes that code for antioxidative proteins, thus protecting the cell from the harm of oxidative stress. On another hand, there is much evidence showing that the consistent activation of Nrf2 activities in cancer is associated with progression and metastasis of cancerous cells, as well as the development of resistance to chemotherapy and radiotherapy. As the Nrf2-Keap1 pathway can regulate cancer in many ways, it is considered a potential field of research for cancer treatment. Although the effect that Nrf2 asserts on cancer is clear, its role in the regulation of cell motility and the cell signaling pathway involved in the process is due to be known.

In Cr1, the result indicates that A549 NSCLC cell's motility decreases when Nrf2 transcription factor is inhibited, and reduced level of ROCK1, RhoA proteins is also observed as a result of Nrf2 inhibition. This observation perfectly aligns with the hypothesis that inhibition of Nrf2 will result in decreased cell motility and this is regulated through ROCK-RhoA pathway.

In Cr2, the result indicates that A549 NSCLC cell's motility decreases when Nrf2 transcription factor is inhibited. Reduced level of ROCK1 protein is observed, but a decrease in the expression level of RhoA protein is not observed. This result may suggest that although Nrf2 does play a role in regulating cell motility, the pathway in which it achieves this is not ROCK-RhoA as RhoA does not seem to be affected, instead, it might be a pathway that involves ROCK and other proteins but not RhoA. This result can partially support the hypothesis as it shows Nrf2 does play a role in regulating cell motility, while the hypothesized ROCK-RhoA pathway is not proved. However, this can also probably be because ROCK1 is a serine kinase[16] and its activity is largely regulated by ligands, phosphorylation, and mutation[17]while RhoA is a small GTPase[18] and is regulated by GTPase-activating proteins[19]. The different regulation mechanism might be the cause of different responses from ROCK and RhoA proteins. What might be the pathway that involves ROCK proteins in which Nrf2 transcription factors regulate cell motility in A549 NSCLC cells?

In Cr3, the result indicates that A549 NSCLC cells' motility decreases when Nrf2 transcription factor is inhibited. Reduced level of ROCK1 protein is not observed, but a decrease in the expression level of RhoA protein is observed. This result may suggest that although Nrf2 does play a role in regulating cell motility, the pathway in which it achieves is probably not ROCK-RhoA as ROCK does not seem to be affected, hence it can only partially support the hypothesis. Instead, it might be a pathway that involves RhoA and other proteins but not ROCK. What is the pathway involving RhoA that is involved in Nrf2 regulating cell motility?

In Cr4, it is observed that A549 NSCLC cells' motility decreases when Nrf2 is inhibited by brusatol, this result suggest that Nrf2 does play a role in regulating cell motility at least in A549 NSCLC cells. However, the expression level of ROCK1 and RhoA proteins are not decreased, which suggests that ROCK-RhoA pathway is probably not involved in Nrf2 signaling. However, it is also possible that Nrf2 affects the activity of ROCK and RhoA without changing their expression level. For example, the inhibition of Nrf2 may result in the deactivation of ROCK and RhoA proteins by causing change in their phosphorylation status. The hypothesis can hence be generated: Does inhibition of Nrf2 decrease motility of A549 NSCLC cells through deactivation of ROCK-RhoA proteins? Or is the regulation of cell motility of Nrf2 achieved through other signaling pathways? There is also the possibility that brusatol affects the expression of other components of A549 NSCLC cells, for example, other transcription factors, and decreased cell motility is a result of regulation from other signaling pathways. Overall, this observation can partially support the hypothesis as Nrf2 might regulate the motility of A549 NSCLC cells.

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In Cr5, it is observed that A549 NSCLC cells' motility does not decrease when Nrf2 is inhibited by brusatol. But a decrease in expression level of ROCK and RhoA proteins is observed. This result might suggest that the Nrf2 transcription factor does not play a major role in the regulation of motility of at least A549 NSCLC cells, while the inhibition of Nrf2 does result in a decreased level of activity of ROCK-RhoA pathway, which suggest that the inhibition of Nrf2 does result in certain cellular response, although it is remains due to be discovered. There is also the possibility that certain key proteins involved in the process, such as Nrf2, ROCK or RhoA are mutated in A549 NSCLC cells, hence the whole regulation pathway is disrupted. For instance, there is a mutation that causes Keap1, which regulates Nrf2 expression, to dysfunction in A549 cells[20]. Can the mutation of Keap1 in A549 NSCLC cells, which causes dysfunction of Keap1, inhibit Nrf2's ability to regulate motility of A549 NSCLC cells' motility through ROCK-RhoA pathway? This observation may partially support the hypothesis as the inhibition of Nrf2 does seem to result in decreased levels of ROCK and RhoA protein expression.

In Cr6, it is observed that the motility of A549 NSCLC cells does not decrease when Nrf2 is inhibited, and while a decreased level of expression is observed on ROCK proteins, the expression level of RhoA protein is not decreased. This result suggest that Nrf2 does not play a major role in the regulation of cell motility in at least A549 NSCLC cells, but the inhibition of Nrf2 does result in a change in expression level of ROCK proteins, which indicates that certain cellular activity is regulated by Nrf2 and involves ROCK protein, and the specific target of this regulation is due to be discovered. This observation can partially support the hypothesis as it shows inhibition of Nrf2 results in a decreased level of activity of a pathway that involves RhoA protein.

In Cr7, the motility of A549 NSCLC cells does not decrease when Nrf2 is inhibited by brusatol, and ROCK protein expression level is not decreased, while RhoA protein expression level decreases. This result indicates that probably Nrf2 does not play a significant role in regulation of cell motility at least in A549 NSCLC cells, however, the inhibition of Nrf2 does result in regulation of certain cellular activity as there is a decrease in RhoA proteins. This pathway involving RhoA is due to be discovered.

In Cr8, a decrease in motility of A549 NSCLC cells is not observed, and a decrease in the level of expression of ROCK and RhoA proteins is not observed as well. This result suggests that 1. Nrf2 does not play a primary role in regulating cell motility in at least A549 NSCLC cells, which can be further verified as inhibition of Nrf2 does not result in a decreased expression level of ROCK-RhoA proteins. 2. Nrf2 inhibition results in deactivation of Rock-RhoA proteins, hence the ROCK-RhoA pathway is inhibited while protein level does not decrease, but this regulation pathway of Nrf2 does not result in decreased motility in at least A549 NSCLC cells, but perhaps something else. Further research may be needed to discover the effector of this regulation. 3.Mutated proteins in A549 NSCLC cells may cause the dysfunction of the pathway, and hence result in dysregulation of cell motility. For example, KEAP1 is mutated and not functioning in A549 cells. Can the mutated KEAP1 in A549 NSCLC cells cause the Nrf2 transcription factor to dysfunction and fail to regulate cell motility through the ROCK-RhoA pathway? This observation involves various possibilities that make it inappropriate for supporting the hypothesis.

## 5. Concentration and Treatment Discussion

Brusatol is proved to be an inhibitor of the Nrf2 transcription factor. As the concentration of brusatol increases, more Nrf2 will be inhibited, which might result in a more significant decrease in cell motility in at least A549 NSCLC cells. However, there might be a negative feedback loop that tightly controls the expression of Nrf2, if a large number of Nrf2 is inhibited, it might trigger the loop and cause the expression of Nrf2 to rise up again.

## 6. Conclusion

In conclusion, the study explores whether Nrf2 regulates the motility of A549 NSCLC cells, and whether this process is carried out via RhoA–ROCK1 pathway. The result of this study may indicate if Nrf2 plays a role in regulating the motility of some NSCLC cells, and the importance of RhoA–ROCK1 pathway for this function of Nrf2 to be carried out. The study may also provide ideas for treatment of

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specific NSCLCs from the perspective of metastasis by controlling the activity of Nrf2, which can either be accomplished by applying medication that inhibit Nrf2, or, according to Nrf2's role of countering oxidative stress, may be accomplished by reducing oxidative stress that patient is exposed to. Further research might still be needed to prove the feasibility and effectiveness of such methods, however.

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