Silencing ERK Signaling Pathway in TNBC by Silencing BAG3

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Abstract

TNBC(Triple negative breast cancer) is cancer with a high mortality rate and recurrence rate. Other studies have shown that overexpression of EGFR in TNBC is also associated with the overexpression of BAG3 and that silencing of BAG3 will downregulate the expression of EGFR. This study investigates the possible molecular function of BAG3 in treating TNBC by silencing BAG3, weakening the efficiency of the ERK signaling pathway in vivo and in vitro. The study will use BT-549 human breast cancer cell lines, with negative control, a wild-type cancer cell, and positive control treated with YM-1. CRISPR knockout BAG3 will change the expression of BAG3 in the sample, while U0126 treatment maintains the silencing of the ERK signaling pathway. Using MTT assay and xenograft metastasis, the growth and migration of cancer cells will be tested; the western blot result will show the molecular process after the inhibition of BAG3. These results, collectively, can show whether or not a knockout of BAG3 can downregulate EGFR by weakening the ERK signaling pathway.

Keywords: TNBC, ERK Pathway, BAG3, EGFR, U0126, Proliferation

1. Introduction

One of the most common causes of death, cancer, has taken countless number of human beings lives. Among them, one is especially noticeable, whose name is Triple-negative breast cancer. Even after chemotherapy treatment, patients at an early stage will still receive a poor prognosis and a high relapse rate [1]. This disease is resistant as a result of its characteristics: the mutated cells show no estrogen and progesterone receptor. Current studies show that EGFR is over-expressed in TNBC, but silencing EGFR itself is not efficient in treating the disease, while targeting chaperones which plays a key role in cell signaling in treatment, has become the new hope. P53, PI3K/AKT, and MAPK pathways were all shown to have an immune association with TNBC [2].

BAG is a family of co-chaperone proteins that regulate cell functions such as growth and reproduction. BCL2-Associated Athanogene 3 (BAG3) is the essential protein that affects cancer cell proliferation and migration by binding to a motif in Hsp70 proteins' ATP domain, as well as WW domain and SH3 domain. BAG3 has a special two Ile-Pro-Val motif which allows itself to bind to some small heat shock proteins. BAG3 is often found overexpressed in cancer cells, and studies have shown that depletion of BAG3 is useful in the acceleration of cancer cell death [3]. On the other hand, BAG3 is the important key bridge in the activation of ERK signaling pathway, which contains FAK and AKT phosphorylation, which in turn affects the expression of EGFR [3, 4]. Since there are a great number of people who are suffering from this condition, it is necessary to investigate the molecular mechanism in how BAG3 is able to affect cell adhesion, proliferation, and metastasis.

U0126, which is a MEK1/2 inhibitor, is used as a ERK signaling pathway inhibitor. In this investigation, it is used to ensure that ERK signaling pathway is the major pathway that activates EGFR [5].

According to those recent findings about the role of BAG3 in TNBC cells and ERK signaling pathway, this paper hypothesizes that if BAG3 is silenced in TNBC cells, because the absence of BAG3 will cause AKT and FAK efficiency to decrease, lowering the expression of EGFR, then TNBC cells will be less effective in cell growth and migration.

2. Methods

2.1. Materials

This experiment will use human BT-549 cell lines, which show high expression of BAG3 and MDA-MB-231 cell lines in mice (ATCC, United States). All mice will be held under pathogen-free conditions. Any animal experiment will follow AAAALAC guidelines.

2.2 In Vitro Cell Culture

MDA-MB-231 cells will be cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% Fetal Bovine Serum and 5mM glutamine. BT-549 cells were cultured in Iscove-modified Dulbecco's medium (IMDM) supplemented with 10% Fetal Calf Serum, 4 mM L-glutamine, 10*2 IU/ml penicillin and 100 μg/ml streptomycin. All cultures were maintained at humidified 37°C and 5% CO2 incubator before analyzation [1, 3].

2.3 Reagent

U0126 was dispersed in ultrapure water or DMSO as a stock solution. Stock solutions will be used with isotonic saline before use [6,7].

2.4 MTT Assay

Cell viability will be determined by MTT assay. All cells will be seeded on 96-well plates, each cultured for 24 hours. After this period, 20 ul of MTT at 5mg/ml solution will be added in each well, incubated in the humidified environment at 37 °C and 5% CO2. After the incubation, wildtype cells will receive docetaxel and no drug(two groups). The BAG3 knockout cells will receive no drug. The absorbance will be tested after 24 hour period [3, 8].

2.5 Xenograft Metastasis

Three test groups will be transplanted on mice: BT-549 wildtype cells, BAG3 KO BT549 cells, and BT-549 wildtype cells given docetaxel. For a continuous of 21 days, 50 ul of PBS saline will be provided for all three groups, except the positive control, which will be given docetaxel. Tumors will be excised after the tumor reached 2-3 square centimeters in volume, and later saved for further examination [9].

2.6 CRISPR Knockout BAG3

Mix 150 µl 3.3mM BG-GLA-NHS, 50 µl 100 µM Oligo-NH2, and 100 µl 200mM HEPES ph 8.5 and incubate at 30 °C for 60 minutes. Next, mix 80 µl 3M sodium acetate, 1 µl 20 µg glycogen, and 1 vol 100% Isopropanol to BG coupling reaction. Vortex the tube and store overnight. Centrifuge the Eppendorf tube and wash under 100% ethanol at -20°C, then 80% ethanol, and repeat the spin process. Dissolve the pellet in 200 µl RNase-free sterile water. Transform E. coli with pNS20-SpCas9-SNAP plasmid and incubate on an agar plate at 37 °C. Add 100 µl 50mg/ml kanamycin solution and 33 µl 50mg.ml chloramphenicol solution to sterile LB medium. Pick a colony from the Agar plate and incubate at 37°C overnight. Harvest the cells and save some to store at -80°C for SDS_PAGE analysis. Purify SpCas9-SNAP protein using lyse and centrifuge. Use guild RNAs; purify sgRNAs and run RNA electrophoresis. Culture the cell at 37°C in humidified 5% CO2 environment. Then, thaw the SpCas9-SNAP protein and mix 2.2pmols BG-coupled oligos with 2.2pmols SpCas9-SNAP protein in 1.5 ml microcentrifuge tubes and incubate for 60 at 30°C, then store in ice again. Seed the cells and prepared for transfection reactions. Prepare 2 1.5ml Eppendorf Tube and follow the transfection format to modulate the mixture. Add the mixture to desired wells, and incubate at 37°C with 5% CO2 environment for 24 h. transfer the cells for further investigation [7,10].

2.7 Western Blot Analysis

Cells are being lysed in RIPA (150 mM NaCl, 0.1% SDS, 1% NP-40, or Triton X-100, 1mM ethylene diamine tetra acetic acid (EDTA), 50 mM Tris-HCl, 1% sodium deoxycholate, pH 7.4 −7.8). Extracts will be centrifuged for 10 minutes. Protein concentrations will be determined by SDS_PAGE and transferred to the nitrocellulose membrane. Membranes were blocked in 5% nonfat dry milk for incubation with antibodies. It will be incubated with anti-EGFR monoclonal primary antibodies at 4°C overnight. Lumino-based detection or ECL detection will then be performed [1,11].

2.8 Statistical Analysis

The statistical data collected from western blot, cell viability assay, and xenograft metastasis will be analyzed using the student's T-Test.

3. Results

3.1 Possible Results on BT-549 Cell Lines BAG3 Knockout and Wildtype with or without U0126 Treatment

The following possible results will analyze EGFR levels in BAG3 knockout cells with U0126 treatment(each with a control group). This possible result table is aimed at ensuring that blocking ERK signaling pathway is the major factor that affects the level of EGFR.

BT-549 cell line will be split into four groups, each compared to the control group and therefore generating 16 possible results. There will be only one possible result that fully supports the hypothesis, some partial support and some contradict (or show that my hypothesis is invalid). These possible results are used to show the plausible relationship between BAG3, ERK signaling pathway, and level of EGFR. For simplicity, some results that have similar logic of invalidation will not be discussed wholly.

The following result assumes that under ideal conditions, which means that there are no systematical errors during the experiment, the negative control (wildtype cancer cell) will automatically have a high expression of EGFR. The positive control (docetaxel) will automatically show a low expression of EGFR. Only the result that fully supports my hypothesis will be able to lead on to the next experiment because they together prove my hypothesis.

Table 1 – Group 1 (EGFR Western Blot Analysis) Possible Results on Inhibition of ERK signaling pathway's Effect on EGFR

Treatments	Result 1	Result 2	Result 3	Result 4	Result 5	Result 6	Result 7	Result 8
BAG3 KO (U0126 treatment)	+	+	+	+	+	+	+	+
WT (U0126 treatment)	+	+	+	-	+	-	-	-
BAG3 KO (no U0126 treatment)	+	-	-	+	+	+	-	-
WT (no U0126 treatment)	-	+	-	-	+	+	+	-
Supporting hypothesis	Fully support	Partially support	Partially support	Partially support	Not support	Not support	Not support	Not support
Treatments	Result 9	Result 10	Result 11	Result 12	Result 13	Result 14	Result 15	Result 16
BAG3 KO (U0126 treatment)	-	-	-	-	-	-	-	-
WT (U0126 treatment)	+	+	+	+	-	-	-	-
BAG3 KO (no U0126 treatment)	+	+	-	-	+	+	-	-
WT (no U0126 treatment)	+	-	+	-	+	-	+	-
Supporting hypothesis	Not support	Not support	Not support	Not support	Not support	Not support	Not support	Not support

Possible Result 1

In vitro, using BT-549 cell line: BAG3 knockout cells with U0126 treatment show a decrease in EGFR, wildtype cells with U0126 treatment show a decrease in EGFR, BAG3 knockout cells without U0126 treatment show a decrease in EGFR, and wildtype cells without U0126 treatment show no decrease in EGFR. All of this above supports my hypothesis.

Possible Results 2 and 3

In vitro, using BT-549 cell line: BAG3 knockout cells with U0126 treatment show a decrease in EGFR and wildtype cells with U0126 treatment show a decrease in EGFR. BAG3 knockout cells without U0126 treatment show a decrease in EGFR. wildtype cells without U0126 treatment show no decrease or decrease in EGFR. All of this partially supports my hypothesis.

Possible Result 4

In vitro, using BT-549 cell line: BAG3 knockout cells with U0126 treatment show a decrease in EGFR, wildtype cells with U0126 treatment show no decrease in EGFR, BAG3 knockout cells without U0126 treatment show a decrease in EGFR, and wildtype cells without U0126 treatment show no decrease in EGFR. All of this above partially supports my hypothesis.

Possible Result 5

In vitro, using BT-549 cell line: regardless of the treatment used, all test groups show a decrease in EGFR level. This does not support my hypothesis.

Possible Results 6, 7

In vitro, using BT-549 cell line: BAG3 knockout cells with U0126 treatment show a decrease in EGFR, and wildtype cells without U0126 treatment show a decrease

in EGFR. Wildtype cells without U0126 treatment and BAG3 knockout cells with U0126 treatment either show a decrease or no decrease in EGFR. This does not support my hypothesis.

Possible Result 8

In vitro, using BT-549 cell line: BAG3 knockout cells with U0126 treatment show a decrease in EGFR, wildtype cells with U0126 treatment show no decrease in EGFR, BAG3 knockout cells without U0126 treatment show no decrease in EGFR, and wildtype cells without U0126 treatment show no decrease in EGFR. This does not support my hypothesis.

Possible Result 9, 10, 11, 12, 13, 14, 15, 16:

In vitro, using BT-549 cell line: BAG3 knockout cells with U0126 treatment show no decrease in EGFR. The rest of the test groups either show positive or negative results.

3.2 Possible Results on FAK, AKT, and EGFR Phosphorylation Level in BAG3 Knockout BT-549 Cell Line

The following possible results will analyze the ratio of phosphorylated FAK, AKT, and EGFR proteins compared to non-phosphorylated forms in BAG3 knockout BT-549 cell line, compared to the control group(ratio of above mentioned phosphorylated proteins to their non-phosphorylated forms in a wildtype BT-549 cell). This possible result table tests whether silencing BAG3 will trigger less signaling cascade in ERK pathway, by examining the level of activated signaling proteins that function in the middle—and their final destination, EGFR. Three proteins will each be conducted with Western Blot Analysis and compared to the control group, therefore generating 8 possible results. There will be only one possible result that fully supports the hypothesis, 3 that partially support it, and four that completely contradict it. These possible results show scrutiny of BAG3's effect on the signaling proteins.

The following result assumes that under ideal conditions, which means that there are no systematical errors during the experiment, there will be a higher expression of BAG3 which means more activation of signaling proteins in wildtype cancer cells (negative control), according to the current studies [1]. The positive control with Docetaxel will adversely have lower activation of those proteins.

Table 2. Possible Results on Protein Levels after BAG3 KO

Proteins	Result 1	Result 2	Result 3	Result 4	Result 5	Result 6	Result 7	Result 8
ratio of phosphorylated EGFR compared to normal EGFR	+	+	+	+	-	-	-	-
ratio of phosphorylated FAK compared to normal FAK	+	+	-	-	+	+	-	-
ratio of phosphorylated AKT compared to normal AKT	+	-	+	-	+	-	+	-
Supporting Hypothesis	fully support	partially support	partially support	partially support	not support	not support	not support	not support

Note. "+" represents a significant decrease compared to the wildtype control. "-" represent no decrease or increase compared to the wildtype control.

Possible Result 1

In vitro, using BAG3 KO BT-549 cell line: ratio of phosphorylated EGFR compared to normal EGFR decreases, ratio of phosphorylated FAK compared to normal FAK decreases, and ratio of phosphorylated AKT compared to normal AKT decreases. All of this above fully supports my hypothesis.

Possible Results 2 and 3

In vitro, using BAG3 KO BT-549 cell line: ratio of phosphorylated EGFR compared to normal EGFR decreases, ratio of phosphorylated FAK compared to normal FAK or ratio of phosphorylated AKT compared to normal AKT decreases. All of this above partially supports my hypothesis.

Possible Result 4

In vitro, using BAG3 KO BT-549 cell line: ratio of phosphorylated EGFR compared to normal EGFR decreases, ratio of phosphorylated FAK compared to normal FAK and the ratio of phosphorylated AKT compared to normal AKT shows no decline. All of this above partially supports my hypothesis.

Possible Result 5

In vitro, using BAG3 KO BT-549 cell line: ratio of phosphorylated EGFR compared to normal EGFR shows no decline, ratio of phosphorylated FAK compared to normal FAK and the ratio of phosphorylated AKT

compared to normal AKT decreased. All of the above do not support my hypothesis.
Possible Results 6, 7, 8
In vitro, using BAG3 KO BT-549 cell line: ratio of phosphorylated EGFR compared to normal EGFR shows no decline, ratio of phosphorylated FAK compared to normal FAK and ratio of phosphorylated AKT compared to normal AKT either shows a decrease in only one of the test group or show completely no decrease.

3.3 Possible Results on Cancer Cell Proliferation and Migration after BAG3 KO Treatment

The following possible results table will analyze cancer cell using MTT assay and xenograft metastasis. This experiment is used to show the effect of BAG3 Knockout on cancer cell proliferation and migration on mice.

Table 3. Possible Results on Cancer Cell Growth and Migration after BAG3 KO Treatment

Assays	Result 1	Result 2	Result 3
MTT assay	-	+	+
xenograft metastasis	-	-	+
Supporting Hypothesis	fully support	partially support	not support

Note. "+" represents no change or increase compared to the wildtype control. "-" represent significant decrease compared to the wildtype control.

Possible Result 1
In vivo, using BT-549 cell line: The tumor cells show less proliferation and migration due to BAG3 knockout. This fully supports my hypothesis.
Possible Result 2
In vivo, using BT-549 cell line: The tumor cells show less proliferation, but indicate unchanged migration or increased migration due to BAG3 knockout. This only partially supports my hypothesis.
Possible Result 3
In vivo, using BT-549 cell line: The tumor cells show unchanged or increased proliferation and migration. This negates my hypothesis.

4. Discussion

Possible result 1 fully supports my hypothesis. Under U0126 treatment, ERK signaling pathway is inhibited, therefore, less EGFR is shown in the test sample. BAG3 demonstrates a similar effect with U0126 treatment as it has a similar function of weakening the ERK signaling pathway.
Possible results 2 and 3 partially support my hypothesis. It shows that the inhibition of ERK signaling pathway using U0126 treatment is proved meaningful in reducing the level of EGFR; however, BAG3 does not show significance in reducing the level of EGFR.
Possible result 4 partially supports my hypothesis. In this group, cells treated with BAG3 Knockout all show a reduced level of EGFR regardless of U0126 treatment. This could possibly mean that BAG3 is also functional in other signaling pathways that increase the cancer cell proliferation, therefore, inhibition of ERK pathway is not enough to reduce the EGFR level, while inhibition of BAG3 could.
Possible result 5 does not support my hypothesis. In this group, every group shows a decrease in EGFR level, which means that this result is not plausible and may refezr to systematic error.
Possible results 6 and 7 do not support my hypothesis. Although BAG3 knockout and U0126 treatment do decrease the level of EGFR, however, their function shows no consistency, which means there is no direct correlation between inhibition of ERK signaling pathway and EGFR levels.
Possible result 8 does not support my hypothesis. Only in the group that both BAG3 knockout and U0126 treatment has been used were EGFR levels decreased. This could possibly mean that BAG3 function in other signaling pathways that activates EGFR as well. Therefore, only when we inhibit both ERK signaling pathway and BAG3, a decrease of EGFR will be shown.
Possible results do not support my hypothesis. provides evidence that either shows my hypothesis is the reverse of what is predicted or that BAG3 or ERK signaling pathway is not related to EGFR level.
Possible result 1 fully supports my hypothesis. It shows that inhibition of BAG3 successfully cuts down the signaling pathway and therefore, two of the signaling proteins located at downstream of the pathway also show less activation. As a result, EGFR activation level

is decreased. the more complex relationship could be discussed, however.

Possible result 2 and 3 partially supports my hypothesis. EGFR level is decreased as a result of BAG3 inhibition, however, not all of the signaling proteins indicate a decrease of activation level. This is possibly caused by mutation down the signaling pathway, or that these proteins are activated by other pathways, but still cause a net decrease in EGFR activation level.

Possible result 4 partially supports my hypothesis. In this result group, EGFR activation level is decreased as a result of inhibition of BAG3. However, Unchanged level of FAK and AKT level suggests that BAG3 has no ability to inhibit ERK pathway, or that mutation at other location continued ERK signaling pathway regardless of lacking BAG3. In this case, BAG3 possibly interacts with other ligand that promotes the activation of EGFR. It is still concluded, though not knowing the exact molecular pathway, that BAG3 is able to decrease the EGFR activation level.

Possible result 5 does not support my hypothesis because although FAK and AKT activation level is indeed decreased as a result of inhibition of BAG3, EGFR level does not show any decline. We could conclude that there may exist other signaling pathways that activate EGFR or some signaling proteins could still function even when BAG3, AKT, and FAK are not activated.

Possible results 6, 7, and 8 all shows that either inhibition of BAG3 could not silence ERK signaling pathway or that it is unable to reduce EGFR activation level. Under this condition, it is reasonable to conclude that inhibition of BAG3 is not meaningful in reducing the level of EGFR, which contradicts my hypothesis.

Possible result 1 fully supports my hypothesis. If 3.1.1, 3.2.1, and 3.1.1 all achieve desired results, they together fulfill the hypothesis of this paper. It shows that inhibition of BAG3 is able to reduce the cancer cell proliferation and migration by silencing ERK signaling pathway and therefore activate less EGFR.

Possible results 2 and 3 in animal experiment shows inconsistency. They only partially support my hypothesis, because it is shown that inhibition of BAG3 could not prevent cell migration and possible does not affect proliferation. These results contradict the current research and, therefore, only partially supports, or negate my hypothesis.

5. Conclusion

In general, this study investigates the effect of silencing ERK signaling pathway by inhibition of BAG3. In vitro, examining the U0126 treatment cell EGFR level by the effect of ERK pathway's effect on EGFR is ensured. By examining the signaling proteins activation level the function of the molecule is identified. At last, in vivo test shows the actual effect of the inhibition of BAG3. CRISPR is used to modify the cell gene, western blot is used to monitor the protein level, and MTT assay and xenograft metastasis is used for finding cancer cell proliferation and migration. The result of this study will be used to show whether BAG3 is a good spot for target therapy and other possible clinical value. Future studies could focus on the effect when both BAG3 is inhibited and docetaxel is given. Moreover, the side effect of BAG3 should also be considered because it is a functional protein that also plays an important role at other functions. Other signaling proteins in ERK pathway could as well be used as a targeting point for reducing the level of EGFR.

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