Inhibition of Serum and Glucocorticoid Inducible Kinase 1 (SGK1) in Triple Negative Breast Cancer

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ABSTRACT

Glucocorticoid receptor overexpression leads to poor prognosis in breast cancer, particularly in triple-negative phenotype. This poor prognosis has been shown to be due to the activation of SGK1 (serum and glucocorticoid inducible kinase 1). The aim of this study is to assess SGK1 levels and sensitivity of a panel of TNBC (triple-negative breast cancer) cell lines towards SGK1 inhibitor GSK650394 and to assess the effects of inhibition of SGK1 in TNBC cell lines. Among these cell lines, MDA-MB-436 cells, displaying markedly elevated SGK1 and showing high phosphorylation of the SGK1 substrate NDRG1 (N-Myc downstream regulated gene 1), was unresponsive to the SGK1 inhibitor. The other cell lines with varying SGK1 levels (MDA-MB-231, HCC1937 and BT549) showed marked decrease of NDGR1 phosphorylation due to kinase activity inhibition ($n= 3$, $p< 0.05$). Intriguingly, despite GSK650394 sensitivity in these cells, pharmacological SGK1 inhibition did not decrease GSK3β phosphorylation, exhibiting no effect on GSK3β reactivation ($n= 3$, $p> 0.05$). In addition, SGK1 inhibition did not change E-cadherin and vimentin expression showing that epithelial-mesenchymal transition (EMT) phenotype was not suppressed ($n= 3$, $p> 0.05$). Accordingly, Slug, Snail and Twist mRNA levels were not affected from SGK1 inhibition ($n= 3$, $p> 0.05$). GSK650394 treatment suppressed proliferation in MDA-MB-231 cells and led to a slight decrease in S-phase. The results of this present study supported the hypothesis that SGK1 inhibition strategies could have therapeutic impact in the management of the triple-negative breast cancer.

Keywords: Triple negative breast neoplasms, Epithelial-mesenchymal transition

INTRODUCTION

Breast cancer is the most commonly diagnosed cancer among women, and the second most common in cancer-associated death.[1] Treatment includes surgery, radiation therapy, chemotherapy, hormonal treatment and/or targeted therapies when available.[1]

Triple-negative breast cancer (TNBC) is important among the breast cancer subtypes because it lacks a targeted therapy in contrast to Luminal types (which can benefit from hormonal therapy) and HER2 (human epidermal growth factor receptor 2) positive type (which can benefit from HER2 targeted therapies like trastuzumab). Because triple-negative breast cancers lack targeted therapy, they have a worse prognosis. The search for a targetable molecule for triple-negative breast cancer is an ongoing important topic for researchers.[2]

Glucocorticoid receptor is shown to be overexpressed in triple-negative breast cancer and it correlates with worse prognosis.[3] It has been shown that glucocorticoid receptor overexpression leads to drug resistance and increased proliferation. This effect was proven to be due to SGK1 (Serum and glucocorticoid-regulated kinase 1) activation.[3]

SGK1 is a serin-threonine kinase which is a member of the AGC kinase family, and is closely related to PI3K/Akt/mTOR pathway.[4] SGK1 is activated by phosphorylation by mTORC2 (mammalian/mechanistic target of rapamycin complex 2) and PDK1 (Phosphoinositide-dependent kinase 1), and it protects the cell from apoptosis.[5] SGK1 has been shown to be essential for the proliferation of triple-negative breast cancer cells.[6]

SGK1 upstream molecules PDK1 activation and Glucocorticoid receptor overexpression have been previously shown to be linked with Epithelial-Mesenchymal Transition, which is an important step for metastasis.[7,8] SGK1 was also proven to have a role in the phosphorylation and inhibition of GSK3 beta (Glycogen synthase kinase-3 beta), a molecule which avoids the cell from going through Epithelial-Mesenchymal Transition.[9]

In this study, we aimed to understand the effect of inhibition of SGK1 via GSK650394 on Epithelial-Mesenchymal Transition and thus possibly metastasis in triple-negative breast cancer cells. We also aimed to examine the effect of inhibition of SGK1 on proliferation and cell cycle phase distributions, thus showing the significance of SGK1 for triple-negative breast cancer cells.

MATERIALS AND METHODS

Cell Culture

MDA-MB-231, MDA-MB-468, BT549, MDA-MB-436 and HCC1937 cell lines were chosen with the consideration of their SGK1 levels and PTEN (Phosphatase and TENsin homolog deleted) statuses. These cell lines were obtained from ATCC (American Type Culture Collection) and were cultured regard to the protocols ATCC stated. According to previous literature, MDA-MB-468 had a low SGK1 mRNA and protein expression according to the literature and had a mutation in PTEN, we used this cell line as a negative control.[6] MDA-MB-231 had a high SGK1 protein and mRNA expression according to literature and was PTEN wild-type.[6] BT549, MDA-MB-436 and HCC1937 were high in SGK1 mRNA and protein expression according to the literature and all of them had mutations in PTEN.[6]

SGK1 Pharmacologic Inhibition

SGK1 inhibitor GSK650394 was obtained from Adooq Bioscience. After the trypsinization procedure, cell suspensions were prepared in order to include $1x10^6$ or $2x10^6$ cells. Cells were incubated in the 37^0C %5 CO_2 incubator for 24 hours in order to thoroughly adhere to the surface. SGK1 inhibitor GSK650394 was prepared under the vertical flow hood in sterile conditions. The agent, which was in a crystallized form, was mixed and vortexed in DMSO so that the final concentration was 32 mM. Stock solutions were preserved at -20^0C in volumes of 500 μl. Before removing the medium from the wells, aliquots of the stock solution were serially diluted. 24 hours later the medium was removed from the wells and GSK650394 was added in different doses (which are indicated in the Results section), and incubated for different time periods (which are indicated in the Results section). The wells that weren't incubated with GSK650394 were used as control and 2 ml medium was added to them.

MTT Assay

After cells were removed from the flask and counted, 5000 cells per well were added with 50 μl medium to a 96 well plate. We waited 24 hours for the cells to adhere to the plate surface. We did not add any drug to control wells, and we added 50 μl of twice the molarity of the necessary drug doses (1 μM, 2 μM, 4 μM and 8 μM for 0.5 μM, 1 μM, 2 μM and 4 μM) since there was already 50 μl of media in the wells, which helped us reach the preferred doses. After 48 and 72 hours of drug incubation, 25 μl of MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) was added to wells. 4 hours later, DMF (dimethylformamide) was added, which solubilized tetrazolium crystals made by MTT in viable cells. 24 hours after DMF, plates were analyzed spectrophotometrically.

One-step Real-Time PCR (Polymerase Chain Reaction)

For the mRNA expression analyses, QuantiTect SYBR Green One-Step RT-PCR kit was used according to the protocol (Qiagen). Primers for Snail

were 5'-CGAGTGGTTCTTCTGCGCTA-3' (Forward), 5'-CTGCTGGAAGGTAAACTCTGGA-3' (Reverse), primers for Slug were 5'-CACTGCGATGCCCAGTCTA-3' (Forward), 5'-TTCTCCCCCGTGTGAGTTCTAA-3 (Reverse), primers for Twist were 5'-GCCGGAGACCTAGATGTCATT-3'(Forward), 5'-CCCACGCCCTGTTTCTTTGA-3' (Reverse), primers for β-actin were 5'-GCACTCTTCCAGCCTTCCTT-3'(Forward), 5'-AATGCCAGGGTACATGGTGG-3'(Reverse).

Western Blotting and Protein Analysis

Antibodies used for this study were β-actin Rabbit Anti-human primary antibody (Cell Signaling Technologies-USA), GAPDH Rabbit Anti-human primary antibody (Cell Signaling Technologies-USA), SGK1 Rabbit Anti-human primary antibody (Cell Signaling Technologies-USA), pNDRG1 Rabbit Anti-human primary antibody (Cell Signaling Technologies-USA), total NDRG1 Rabbit Anti-human primary antibody (Cell Signaling Technologies-USA), pGSK3 beta Rabbit Anti-human primary antibody (Cell Signaling Technologies-USA), Vimentin Rabbit Anti-human primary antibody (Cell Signaling Technologies-USA), e-Cadherin Rabbit Anti-human primary antibody (Cell Signaling Technologies-USA), Polyclonal Goat Anti-rabbit immunoglobulins/HRP (Biolegend-USA), Anti-rabbit IgG HRP linked antibody (Cell Signaling Technologies-USA). Total cell lysates were acquired from cells with lysis buffer. For the Polyacrylamide Gel Electrophoresis (PAGE) experiments, Mini-PROTEAN TetraCell System (Bio-Rad) was used. Semi-dry transfer and blotting were completed using Trans-Blot Turbo System according to protocol (Bio-Rad). After the transfer procedure, the PVDF membrane was put in a container comprising 5% nonfat milk solution, after blockage with the milk solution, the membrane was incubated with the primary antibody solution overnight in the cold room on a 100 rpm shaker. The next day, membrane was washed and then incubated with HRP (Horseradish Peroxidase) secondary antibody for an hour. After incubation, membrane was washed again, and lastly for the imaging procedure, a Super Signal West-femto Maximum Sensitivity Substrate ECL kit (Thermo-Fisher) was used and bands were imaged with Kodak Gel Logic 1500 imaging System.

Crystal Violet Assay

After MDA-MB-231 cells were added to a 12-well plate, 40.000 cells per well, drug wells were incubated with GSK650394 in indicated doses, and the medium was added to control wells instead of GSK650394. The drug and medium were changed every other day. 5 days after the initial drug incubation, the drug and medium were removed from the wells. 400 μl crystal violet mixture (0.5% crystal violet dye, 20% methanol) was added to wells and wells were incubated with this dye for 10 minutes. After this procedure, wells were washed with PBS, and macroscopically and microscopically photographed.

Cell Cycle Analysis

MDA-MB-231 cells were plated to a 6-well plate (1 million cells per well). 24 hours after incubation in the incubator, 1 μM GSK650394 was added to drug wells and the medium was added to control wells. 24 hours later, the drug and medium were removed from the wells. Cells were washed with PBS, trypsinized and transferred to a 15 ml centrifuge tube. Ethanol was added to the tube and thoroughly mixed. Tubes were incubated at +4^{0}C, following incubation 2 ml PBS was added and the tubes were centrifuged at 1800 rpm for 5 minutes. 1 ml PBS was added to the resulting pellet. 100 μl RNAse was added to the mixture and vortexed. PI was added and vortexed. Tubes were incubated at 37^{0}C for an hour and analyzed with flow cytometry.

This study was approved by the Non-interventional Clinical Researches Ethics Board of Hacettepe University (date: 25/10/2016, approval number: GO 16/665).

Statistical Analyses

Statistical analyses were conducted with the IBM® SPSS® Statistics Version 23 program. Data were analyzed with independent groups of Student t-test. Acquired values that were $p< 0.05$ were considered statistically significant.

RESULTS

SGK1 Protein and mRNA Expressions in Triple-Negative Breast Cancer Cells

SGK1 protein expressions were shown with the Western blot method. As expected, MDA-MB-468 had a low SGK1 protein expression, and MDA-MB-436 had the highest protein expression. SGK1 mRNA expressions were shown with Real-Time PCR method. As expected, MDA-MB-468 had the lowest mRNA expression among the cell lines, and MDA-MB-436 had the highest mRNA expression.

Determination of Sub-toxic Doses of GSK650394 with MTT Assay

By analyzing the literature and previous articles about the use of GSK650394 on breast cancer cell lines and other cells, we determined that the appropriate dose for our cell lines would be 1 micromolar. Considering this entity, we chose to use 0.5 micromolar, 1 micromolar, 2 micromolar and 4 micromolar doses of GSK650394 for 48 and 72 hours. We did not observe any significant cytotoxicity in MDA-MB-231, MDA-MB-436, HCC1937, BT549, and MDA-MB-468 cell lines with all doses tested for 48 and 72 hours.

Determination of SGK1 Inhibition Through Phospho-NDRG1 Protein Expression Levels in Triple-Negative Breast Cancer Cell Lines

We showed the decrease of phospho-NDRG1 protein with the Western Blot method in the MDA-MB-231 cell line with 1 micromolar dose ($n= 3$, $p< 0.05$). We did not observe any decrease in the BT549 cell line with 1 micromolar dose for 24 hours ($n= 3$, $p> 0.05$). Subsequently, we used 2 micromolar and 4 micromolar doses. We showed a decrease in phospho-NDRG1 expression when we used 2 micromolar GSK650394 ($n= 3$, $p< 0.05$). We did not observe any decrease in the HCC1937 cell line with 1 micromolar dose for 24 hours ($n= 3$, $p> 0.05$). Subsequently, we used 2 micromolar and 4 micromolar doses. We showed a decrease in phospho-NDRG1 expression when we used 4 micromolar GSK650394 ($n= 3$, $p< 0.05$). We did not observe any decrease in phospho-NDRG1 expression when we used 1 micromolar of GSK650394 for 24 hours in the MDA-MB-436 cell line. ($n= 3$, $p> 0.05$) Subsequently, we used 2 micromolar and 4 micromolar doses, we did not observe inhibition with these doses either ($n= 3$, $p> 0.05$). MDA-MB-468 was proven to have low SGK1 expression, and it was planned to be used as a negative control in our experiments. We did not observe any decrease in phospho-NDRG1 expression with 1 micromolar dose for 24 hours ($n= 3$, $p> 0.05$).

Effect of SGK1 Inhibition via GSK650394 on the Proliferation Of MDA-MB-231 Cells

MDA-MB-231 was chosen for the crystal violet (proliferation) experiments, because it was the most sensitive cell line among the chosen cell lines. The effect of GSK650394 was examined macroscopically and microscopically with 1 micromolar and 2 micromolar GSK650394 for 5 days. It was shown that the proliferation of cells was dramatically decreased with the effect of GSK650394 (Figure 1).

Effect of SGK1 Inhibition Via GSK650394 on the Cell Cycle Phases Distribution in MDA-MB-231 Cells

The flow cytometry method was used to show the effect of SGK1 inhibition on the cell cycle. It was shown that with the effect of SGK1 inhibition, the cells that were going through the S phase were decreased ($n= 3$, $p< 0.05$).

Effect of SGK1 Inhibition on Phospho-GSK3 Beta Expression in Triple-Negative Breast Cancer Cell Lines

Effect of SGK1 inhibition on phosphorylated GSK3 beta protein expression was shown with Western Blot method, we used the GSK650394 doses that we found to be inhibitory in previous experiments. We did not see any change in the phosphorylated GSK3 beta expression in MDA-MB-231, HCC1937, BT549, MDA-MB-436 cells before and after treatment with GSK650394 ($n= 3$, $p> 0.05$).

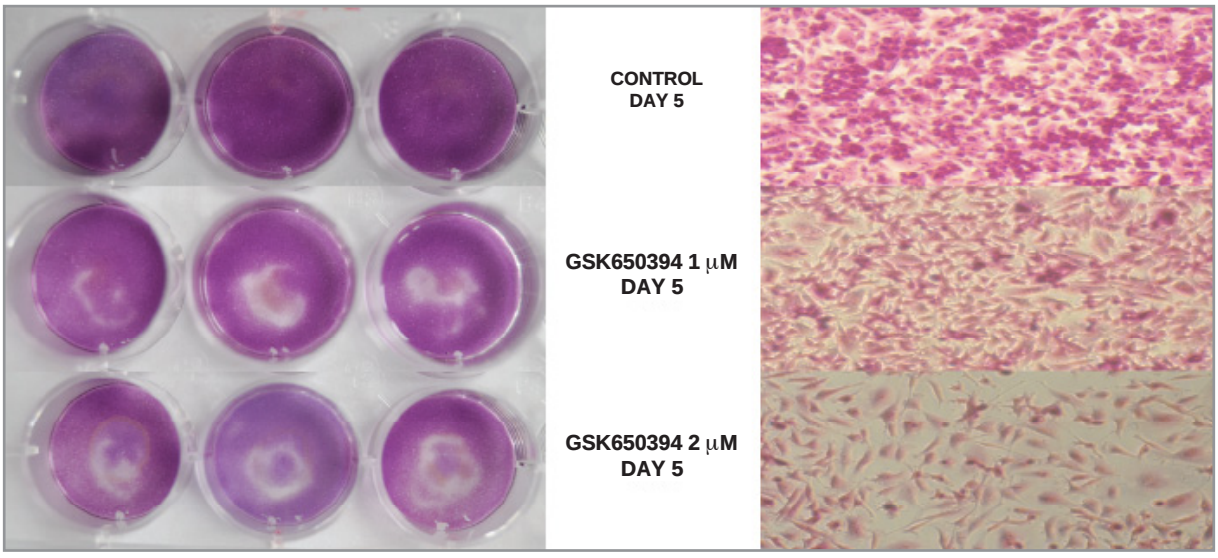

Figure 1. Inhibition of proliferation of MDA-MB-231 cells after treatment with GSK650394 for indicated time and doses, macroscopically and microscopically (10x) imaged.

Effect of SGK1 Inhibition Via GSK650394 on EMT-Related Proteins E-Cadherin and Vimentin Protein Expressions in Triple-Negative Breast Cancer Cell Lines

E-cadherin protein expression was nearly undetectable in MDA-MB-231, BT549 and MDA-MB-436 cell lines, and we did not see any change in the protein expression of E-cadherin after GSK650394 treatment. We did not see any change in the protein expression of E-cadherin in HCC1937 cells after treatment with GSK650394. HCC1937 had a nearly undetectable vimentin expression, in contrast to the other triple-negative breast cancer cell lines. We did not see any change in the protein expression of Vimentin in MDA-MB-231, BT549, MDA-MB-436, HCC1937 cells after treatment with GSK650394 (Figure 2).

Effect of SGK1 Inhibition Via GSK650394 on EMT Related Transcription Factors in MDA-MB-231 and BT549 Cell Lines

The effect of SGK1 inhibition on epithelial-mesenchymal transition was then further examined with Real-Time PCR, showing the changes in mRNA expressions of Snail, Slug and Twist, which are EMT-related transcription factors, with SGK1 inhibition. For the Real-Time PCR experiments, we used the BT549 cell line, which had the most prominent expression of vimentin, thus the cell line with the most mesenchymal characteristics, and the MDA-MB-231 cell line, which was the most sensitive to GSK650394. We did not observe any effect on Snail, Slug, Twist mRNA expression after 6 and 24 hours of 1 micromolar GSK650394 incubations in MDA-MB-231 cells ($n= 3$, $p> 0.05$). We did not observe any effect on Snail, Slug, Twist mRNA expression after 6 and 24 hours of 1 micromolar GSK650394 incubations in BT549 cells ($n= 3$, $p> 0.05$).

DISCUSSION

The aim of this study is to determine the significance of the inhibition of SGK1 molecule in triple-negative breast cancer. Five triple-negative breast cancer cell lines that differ in SGK1 expression and PTEN statuses were grown in cell culture conditions for this aim. In our experiments, we noticed that our cell lines also differed by their epithelial/mesenchymal phenotypes. MDA-MB-231, BT549 and MDA-MB-436 were phenotypically similar with mesenchymal cells, microscopically. However, HCC1937 was similar with epithelial cells, E-cadherin and vimentin protein levels of the cell lines were also consistent with this phenomenon.

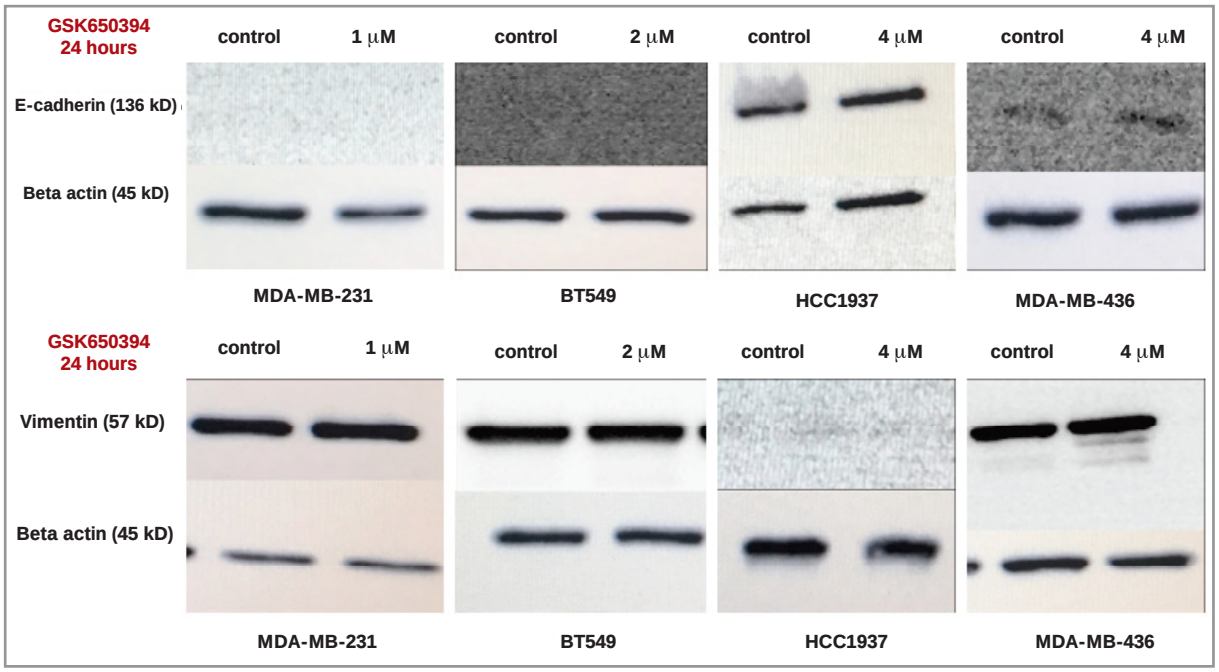

Figure 2. Changes in the protein levels of E-cadherin and Vimentin levels after treatment with GSK650394 in triple-negative breast cancer cell lines (n= 3, p> 0.05).

SGK1 protein and mRNA expressions were consistent with the literature, MDA-MB-468 had the lowest expression of SGK1 among the cell lines.[6] The other cell lines had similar SGK1 mRNA and protein expressions.[6]

To show the effect of GSK650394 on breast cancer cell proliferation, we used the crystal violet assay. For this experiment, we used the MDA-MB-231 cell line, which was the most susceptible cell line among the others. We showed a dramatic decrease in proliferation when we inhibited SGK1 pharmacologically by using 1 micromolar and 2 micromolar of GSK650394 for 5 days. Dependency of the proliferation of triple-negative breast cancer cells to SGK1 is consistent with previous literature[6] and shows that SGK1 is an important target for inhibition in triple-negative breast cancer.

To show the effect of GSK650394 on the cell cycle, we used the flow cytometry-based cell cycle assay. For this experiment, we used the MDA-MB-231 cell line, which was the most susceptible cell line among the others. Consistent with the literature, we showed that there was a decrease in the cells that were in the S phase, which showed that SGK1 had an effect on the cell cycle continuation, and prevented the triple-negative breast cancer cells from going through with the cell cycle, and proliferating.[10] Effects of SGK1 on the cell cycle are important for triple-negative breast cancer, since it has been shown that triple-negative breast cancer cells rely on the function of the transcriptional regulation of the cell cycle.[11]

It was a surprising result that GSK3 beta phosphorylation wasn't affected by inhibition of SGK1, since previous literature showed that SGK1 phosphorylated and inhibited GSK3 beta protein, we expected SGK1 inhibition would lead to the downregulation of phospho-GSK3 beta, and thus increase in its activation.[12] We can explain this effect with the significance of Wnt-beta catenin pathway in triple-negative breast cancer cells. Wnt-beta catenin pathway has been shown to regulate GSK3 beta by phosphorylation and inhibition, and it is a very potent pathway for triple-negative breast cancer.[13] PI3K/Akt pathway is also responsible for phosphorylating and inhibiting GSK3 beta, and it is also a prominent pathway in triple-negative breast cancer.[14] Another explanation for the lack of effect can be the used low dose of GSK650394. But these doses were critical for us, considering the probable

nonspecific kinase inhibitions when we increased the dose.[15] When we specifically inhibited SGK1 with the doses that we used, we did not see any effect on phospho-GSK3.

We showed that inhibition of SGK1 in the chosen triple-negative breast cancer cell lines, did not affect epithelial-mesenchymal transition markers. This could be due to the potent mesenchymal phenotype of the triple-negative breast cancer cells. The cell lines that we chose (except HCC1937) had a very potent vimentin expression, and almost a nonexistent E-cadherin expression. This mesenchymal phenotype can probably be difficult to inhibit with the inhibition of one protein expression. Also, epithelial-mesenchymal transition in triple-negative breast cancer cells has been proven to be affected by many different signaling pathways, among them are PI3K/Akt Pathway and Wnt-beta catenin pathway, which are important signaling pathways in triple-negative breast cancer.[16] Lack of inhibition on Snail, Slug and Twist transcription factors was not a surprising result, considering we did not see any effect on epithelial-mesenchymal transition proteins.

In a study conducted by Liu et al, inhibition of SGK1 was shown to reverse epithelial-mesenchymal transition and impair metastasis in prostate cancer cells in vitro and in vivo.[17] In this study, GSK650394 was used with a 40 micromolar dose for 24 and 48 hours. This study shows that the use of GSK650394 with a higher dose can affect the phenotype differently. Although, it is important not to forget that once we increase the dose, there is a risk of inhibiting different signaling pathways, most importantly the Akt pathway. It has already been shown that Akt is an important regulator of epithelial-mesenchymal transition[14], which means that with non-specific inhibition of Akt, it is probable that epithelial-mesenchymal transition would be affected with higher doses of GSK650394. Also, in this study researchers failed to show the downregulation of vimentin and upregulation of E-cadherin with only pharmacological inhibition, but it was shown via the knockdown of SGK1 with shRNA. Taking this study into focus, we can presume that knockdown of SGK1 can also be effective in regulating epithelial-mesenchymal transition in triple-negative breast cancer cells. This may be an important topic for future research, along with the use of high dose GSK650394.

In a recent study, SGK1 was found to be an important regulator for osteoclastogenesis and bone metastasis, this study used an intracardiac inoculation model on nude mice, and pharmacological inhibition of SGK1.[18] These results also show the importance of SGK1 for metastasis, and animal studies rather than in vitro studies may be beneficial to truly understand the mechanisms behind metastasis. It has also been found that dual inhibition of PDGFR and SGK1 exhibited antitumor activities, especially on metastasis-initiating cells[19] and pharmacologic SGK1 inhibition increased sensitivity for radiotherapy in locally advanced rectal cancer.[20] SGK1 recently emerged as a biomarker and drug target in many human cancers.[21,22]

In conclusion, our present study supported that SGK1 inhibition strategies could have a therapeutic impact in the management of triple-negative breast cancer. Further clinical investigations are needed to determine the exact pathobiological basis of SGK1 during the complicated course of carcinogenesis.

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