The Effect of Metformin on SIK1 and SIK2 in MCF-7 Cell as an Anticancer Agent

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ABSTRACT

Recent studies have shown that the use of metformin prevents the development and spread of cancer. Metformin may show this effect by increasing SIK1 and SIK2 gene expression. For this purpose, MCF-7 cells cultured in appropriate media were divided into 8 groups (1) control, (2) 10 ng/mL TGF-β1, (3) 1.25 mM Metformin, (4) 2.5mM Metformin, (5) 20mM Metformin, (6) 1.25 mM Metformin+10 ng/ml TGF-β1, (7) 2.5mM Metformin+10 ng/ml TGF-β1 and (8) 20mM Metformin+10 ng/ml TGF-β1 doses were administered, respectively. PCR was performed for SIK1 and SIK2 genes, with GAPDH being the reference gene. Application of 10 ng/ml TGF-β1 to MCF-7 cell significantly increased expression level of SIK1 mRNA by 1.6 fold. In non-invasive (TGF-β1 not administered) MCF-7 cell, 2.5 mM and 20 mM metformin increased expression levels of SIK1 mRNA by 1.8, 3.4 fold and SIK2 mRNA by 1.6 and 3.3 fold respectively. In invasive (TGF-β1 administered) MCF-7 cell, 1.25, 2.5 and 20 mM metformin increased expression levels of SIK1 mRNA by 3.5, 3.7, 4 fold; and SIK2 mRNA by 1.9, 2.4, 3.5 fold, respectively. Metformin increased SIK1 and SIK2 gene expression dose-dependently in non-invasive and invasive MCF-7 cells, more significantly in invasive ones. The increase in the SIK1 gene was greater than in SIK2. In the light of these results, investigating the effects of metformin on SIK1 and SIK2 genes in different TGF-β1 sensitive cancer types may open new doors for cancer treatment.

Keywords: MCF-7, metformin, SIK1, SIK2, TGF-β1

INTRODUCTION

In women, breast cancer remains the most common and deadly types of cancer after lung cancer.[1] To curb this disease, prevention of metastasis and invasion as causative agents of mortality and morbidity is important in the treatment of this disease.[2] The studies on this subject have focused primarily on the Transforming Growth Factor Beta 1 (TGF-β1) signaling pathway involved in embryonic development, regulation of cell growth, differentiation and apoptosis. Changes in TGF-β1 signals have been associated with many diseases (fibrosis, cancers and etc.) and have been shown to have an accelerating effect on growth, especially in the advanced stages of the tumor.[3] For transporting the signals from TGF-β1 receptors to the nucleus, Smads are used as mediators[4] and one of the target genes of the TGF-β1 / Smad signal is the Salt Inducible Kinase (SIK) gene.[5]

SIK is a serine / threonine protein kinase that belongs to the 5' AMP-activated protein kinase (AMPK) family isolated from the adrenal glands.[6] It must be phosphorylated for kinase activity by Serine / Threonine Kinase 11 (LKB1) which is a tumor suppressor gene.[7] SIK1 is involved in many different processes such as cell cycle regulation, regulation of gluconeogenesis and lipogenesis, growth and differentiation of muscle, and tumor suppression.

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Its tumor suppression property is related to down-regulating Transforming Growth Factor Beta Receptor 1 (TGFBR1)[5], phosphorylating CREB Regulated Transcription Coactivator 1 (CRTC1/ TORC1) and CREB Regulated Transcription Co-activator 2 (CRTC2 / TORC2)[8] and Tumor Protein P53 (p53 / TP53)-dependent anoikis pathways.[9] On the other hand, SIK2 Inhibits CREB activity which is associated with chemotherapy resistance, tumor progression, and decreased survival.[10] This inhibition occurs via phosphorylation of Serine 794 of insulin receptor substrate 1 (IRS1) and phosphorylation of cAMP Responsive Element Binding Protein (CREB)-specific coactivator CRTC1.[11] In breast cancer patients the increase in SIK2 appears to be significantly associated with better survival[12], while depletion in SIK1 is releated with poor prognosis.[9] Then, it is clear that SIK1 and SIK2 may act synergistically as potential tumors suppressor factors in breast cancer biology.

Metformin (1,1-dimethylbiguanide) is an antidiabetic agent widely used in the treatment of type II diabetes[13] and recent clinical studies show that the risk of cancer is low in type II diabetes patients using metformin.[14] In vivo and in vitro molecular studies have pinpointed the importance of metformin in the treatment and pathophysiology of breast cancer.[15] Moreover, scientists have reported that metformin exerts its anti-carcinogenic effects by affecting cell proliferation and apoptosis[16] via increasing AMPK activation[17] and this later increase is due to the phosphorylation of AMPK via LKB1.[18] Albeit many reports about metformin signaling through AMPK, the signaling which is due to biological association of SIK1 and SIK2 in breast cancer pathophysiology, still it remains to clarify the spectrum of biological effects of metformin on SIK1 and SIK2 .

In this regard, we made the assumption that metformin will act on LKB1 through SIK1 and SIK2, together in coordination with AMPK signaling mechanism.

MATERIALS AND METHODS

Cell Culture Design

MCF-7 cells (established from human breast carcinoma) were used in the this experiment (T.R. Ministry of Agriculture and Rural Affairs, Directorate of Foot and Mouth Disease). They were cultured in the RPMI 1640 medium (Biological Industries) [fetal bovine serum (10%, Sigma-Aldrich), penicillin/ streptomycin (100 U/ 0.1 mg, Sigma-Aldrich), Na-pyruvate (1%, Biological Industries)] in 5 % CO2 in the 25 cm^2 flasks at 37^0. Metformin (D15,095-9) was obtained from Sigma company. TGF-β1 (ab: 50036, lot: GR131362-1) was obtained from Abcam company.

The (1) control, (2) 10 ng/mL TGF-β1, (3) 1.25 mM metformin, (4) 2.5 mM metformin, (5) 20 mM metformin, (6) 1.25 mM metformin and 10 ng/ ml TGF-β1, (7) 2.5 mM metformin and 10 ng/ml TGF-β1 and (8) 20 mM metformin and 10 ng/ml TGF-β1 groups were created. In our study, TGF-β1 is used to make invasive MCF-7 cells. TGF-β1 and metformin concentrations were determined according to the our previous study.[16] Drug doses were performed in all groups in the 25 cm^2 flasks at 70-80% confluence. They were cultured in RPMI 1640 medium in 5% CO$_2$ and at 37^{0}C. Only complete medium was added for control group.

Quantitative RT-PCR

After the 24th hour of drug administration, the medium was discharged from the flasks. Trypsin EDTA was used for detaching of the cells. Using High Pure PCR RNA Isolation Kit (Cat. No: 11828665001, Roche) RNA isolation was performed. Obtained RNAs' quality and quantity were evaluated by Nanodrop spectrophotometer. After this, using Transcriptor First Strand cDNA Synthesis Kit (Cat. No. 04379012001; Roche) mR-NAs were converted to cDNA. Primers and probes were designed for each gene using Universal Probe

Table 1. Sequences of polymerase chain reaction primers

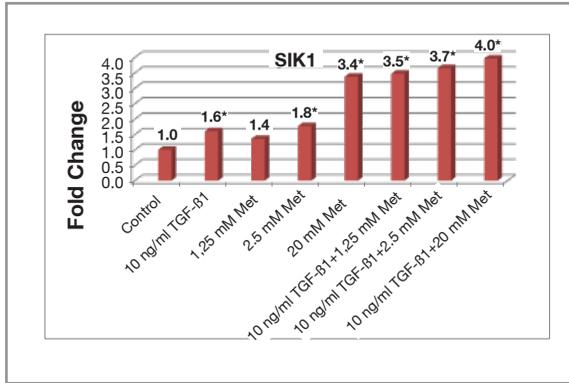| Genes	Forward primer	Reverse primer	Probes
GAPDH	AGCCACATCGCTCAGACAC	GCCCAATACGACCAAATCC	(probe 60, Roche)
SIK1	CATCCCCTTCTTCATGTCTCA	GATCTGGGCGATGGTGAT	(probe 77, Roche)
SIK2	CGTGACCTCAAAGCTGAAAA	TTTCCAAAACCGAAATCTGC	(probe 18, Roche)

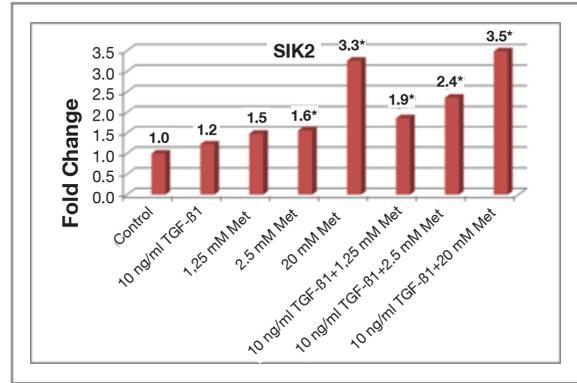**Figure 1.** Gene expression changes of SIK1 in MCF-7 cells.
* P< 0.05

Figure 2. Gene expression changes of SIK2 in MCF-7 cells.
* P< 0.05

Library (UPL Roche) software and confirmed by BLASTn analysis (Table 1). The SIK1 and SIK2 genes expression were measured via Quantitative RT-PCR. GAPDH was accepted as reference gene and control groups were accepted as calibrator. Using QIAGEN 2009 relative expression software (REST) analysis of relative gene expression for the study groups was performed. P< 0.05 was accepted as significant.

RESULTS

SIK1

In the MCF-7 cells, the application of an amount of 1.25 mM metformin does not cause a significant change in the expression level of SIK1 mRNA whereas 2.5 mM and 20 mM metformin increased expression levels of SIK1 mRNA by 1.8 fold (P(H1)= 0.011) and 3.4 fold (P(H1)= 0.001), respectively. Expression level of SIK1 mRNA increased significantly (P(H1)= 0.037) by 1.6 fold at second group. The combination of 10 ng/mL TGF-β1 and 1.25, 2.5 and 20 mM metformin increased expression levels of SIK1 mRNA by 3.5 fold (P(H1)= 0.004), 3.7 fold (P(H1)= 0.001) and 4 fold (P(H1)= 0.001) respectively (Figure 1).

SIK2

In the MCF-7 cells, the sole, application of 1.25 mM metformin caused no significant change in expression level of SIK2 mRNA whereas 2.5 mM and 20 mM metformin increased expression levels of SIK2 mRNA by 1.6 fold (P(H1)= 0.027) and

3.3 fold (P(H1)= 0.001), respectively. Application of 10 ng/mL TGF-β1 caused no significant change in expression level of SIK2 mRNA whereas combinations with 1.25, 2.5 and 20 mM metformin increased expression levels of SIK2 by 1.9 fold (P(H1)= 0.006), 2.4 fold (P(H1)= 0.006) and 3.5 fold (P(H1)= 0.007), respectively (Figure 2).

DISCUSSION

In this study, we found out that metformin dose-dependently upregulated SIK1 and SIK2 in MCF-7 cells. The increase of SIK1 gene expression was more than SIK2 by metformin in both non-invasive and invasive types. Interesingly, metformin induction was much more effective in upregulating of SIK1 and SIK2 genes in invasive MCF-7 cells than non invasive. However we have highlight that the increase in SIK1 gene expression was observed in cells treated with only TGF-β1. This increase may be due to counter-regulatory mechanisms that occur in response to TGF-β1-induced migration.

The association of metformin use with low cancer risk in patients with type 2 diabetes has been demonstrated by epidemiological studies.[14] It is thought that metformin exerts its anticancer effect by affecting cell proliferation and apoptosis.[19] AMPK activation and mechanistic target of rapamycin (mTOR) inhibition are held responsible for its antiproliferative effect.[20] AMPK activation requires LKB1 which is a serine threonine protein kinase.[21] In a study with MCF-7 cells, metformin was shown to increase AMPK phosphorylation via LKB1 in a dose-dependent manner.[18] Increased AMPK causes inhibition of mTOR by phospho-
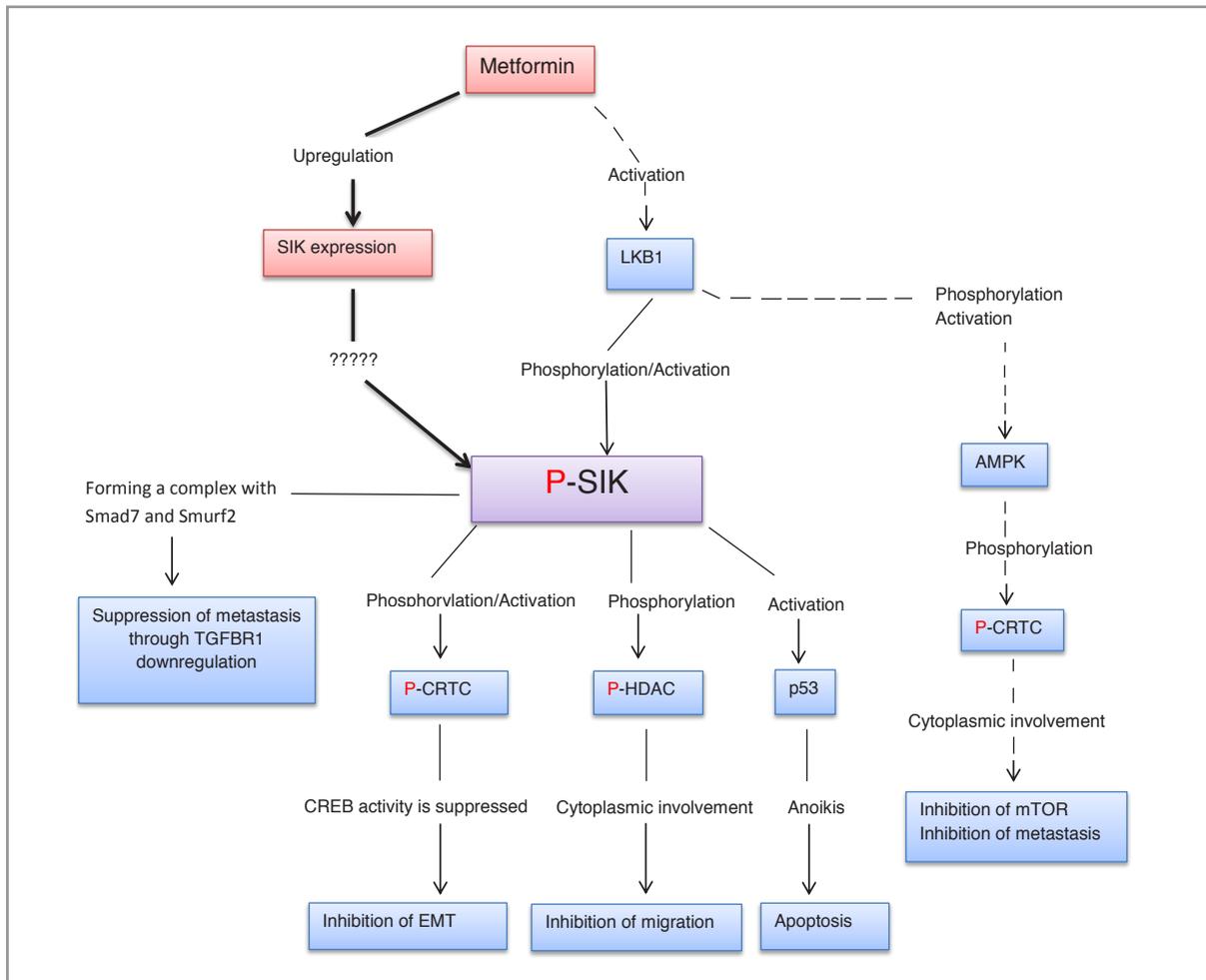
Figure 3. Possible mechanism of metformin on LKB1-SIK signaling pathway ; Arrows indicated by dashed lines show that metformin increases AMPK phosphorylation via LKB1, and AMPK causes inhibition of mTOR by phosphorylating CRTC2. The pathway shown with thick lines shows the findings of our study. Metformin dose-dependently increases SIK1 and SIK2 gene expression. LKB1/STK11: Serine / Threonine Kinase 11; SIK: Salt Inducible Kinase; P-SIK: phosphorylated Salt Inducible Kinase; p53: Tumor protein 53; P-HDAC: Histone Deacetylase; P-CRTC: phosphorylated CREB Regulated Transcription Coactivator; TGFBR1: Transforming growth factor, beta receptor I; AMPK: 5' AMP-activated protein kinase.

rylating CRTC2, which has a critical role in tumor suppression.[22] Inhibition of mTOR may be one of the effective mechanisms to prevent migration and invasion.[23] In addition, the absence of CRTC2 increases angiogenesis, which plays a critical role in tumor progression.[24] Another study has shown that SIK1 suppresses CREB activity, a transcription factor, by phosphorylating CRTC.[25] Similarly, SIK2 also suppresses cAMP-response element-dependent transcription; however, it is thought that the suppression is weaker than SIK1.[6]

Various studies have been conducted on the SIK1 and SIK2 genes in different types of cancer. Studies reported that SIK1 expression level is significant-ly lower in primary breast tumors than in normal breast tissue.[26] These results were later confirmed by other team demonstrating a correlation of low SIK1 expression in human breast and distal metastasis.[9] In agreement with this finding, silencing of the SIK1 gene has been shown to induce metastasis in mouse lungs[11] and overexpression of SIK1 reduced tumor stem cells formation and proliferation in ovarian cancer.[27] A recent study showed that SIK1 is expressed in lower level in hepatocellular carcinoma and increased in SIK1 expression delays hepatocellular carcinoma cell proliferation and epithelial mesenchymal transition (EMT)[28] demonstrating SIK1 key role in p53-dependent anoikis molecular pathways and thus has an important role

in suppressing metastasis.[11] Similarly, SIK2 inhibits melanogenesis by suppressing CRTC1.[29]

SIK1 may exert its tumor suppressive effect by phosphorylating Histone Deacetylase (HDAC) and limiting migration.[30] In addition, SIK1 seems to be effective on EMT.[31] It has been shown that Snail Family Zinc Finger 2 (Snail2), Twist Family BHLH Transcription Factor (Twist), Zinc Finger E-Box Binding Homeobox 1 (Zeb1) and Zinc Finger E-Box Binding Homeobox 2 (Zeb2), genes which are inducable by EMT significantly increased as a result of silencing the SIK1 genes.[32] SIK may also exert its tumor-suppressing effect by blocking TGF-β1 / Smad signaling, which is an effective pathway in carcinogenesis. It is thought that SIK achieves this effect by participating in protein complexes which contain SMAD Specific E3 Ubiquitin Protein Ligase 2 (Smurf2) and SMAD Family Member 7 (Smad7) that down-regulate TGFBR1.[5]

Contrary to the tumor suppressor activities of SIK1 and SIK2, studies have also suggested that both SIK1 and SIK2 would act like oncogenes. In contrast to the suppressive effect of high expression levels of SIK1 in lung and breast tumors, there are also studies suggesting the existence of positive correlation between the SIK1 expression level and other tumors. Indeed, high expression level of SIK1 has been shown to be associated with adrenocortical tumor cells stimulated by adrenocorticotropic hormone in mice.[33] In addition, SIK2 is one of the oncogenes that provides an advantage for the development of cancer cells and correlates with the pathogenesis of ovarian cancer patients.[34] In another study, the absence of SIK2 supported the sensitivity of paclitaxel in ovarian cancers, while the presence of SIK2 correlated with the poor prognosis of patients with advanced ovarian cancer.[35] All these bifunctional effects of SIK1 and SIK2 suggest the existence of tissue factors that will co-modulate the expression of SIK1 and SIK2 are different type of cancer.

In our our study, we reported that metformin boost SIK1 and SIK2 gene expression in non-invasive and invasive MCF-7 cells. These effects can be due in part by its inhibitory effect metastasis activity inhibited by CREB via the LKB1–SIK–CRTC signaling pathway. On the other hand, the upregulation of SIK1 and SIK2 may inhibit metastasis by causing downregulation of TGFBR1. Inhibition in cells migration through HDACs and lastly, metformin may exert its migration inhibitory effect through this pathway, as LKB1-SIK1 signaling is known to inhibit EMT by suppressing Zeb1. Several studies remain to be carry out to address the plausibility of these hypotheses and relate their biological functions and their tumorigenocity (Figure 3).

In conclusion, metformin may exert its anticancer effect by increasing the expression of SIK1 and SIK2 genes in non-invasive and invasive MCF-7 cells. In this context, investigating the effects of metformin on SIK1 and SIK2 genes in different TGF-β1 sensitive cancer types may open new doors for cancer treatment.

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