Knockdown of LKB1 Sensitizes Endometrial Cancer Cells via AMPK Activation

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Abstract
Metformin is an anti-diabetic drug and has anticancer effects on various cancers. Several studies have suggested that metformin reduces cell proliferation and stimulates cell-cycle arrest and apoptosis. However, the definitive molecular mechanism of metformin in the pathophysiological signaling in endometrial tumorigenesis and metastasis is not clearly understood. In this study, we examined the effects of metformin on the cell viability and apoptosis of human cervical HeLa and endometrial HEC-1-A and KLE cancer cells. Metformin suppressed cell growth in a dose-dependent manner and dramatically evoked apoptosis in HeLa cervical cancer cells, while apoptotic cell death and growth inhibition were not observed in endometrial (HEC-1-A, KLE) cell lines. Accordingly, the p27 and p21 promoter activities were enhanced while Bcl-2 and IL-6 activities were significantly reduced by metformin treatment. Metformin diminished the phosphorylation of mTOR, p70S6K and 4E-BP1 by accelerating adenosine monophosphate-activated kinase (AMPK) in HeLa cancer cells, but it did not affect other cell lines. To determine why the anti-proliferative effects are observed only in HeLa cells, we examined the expression level of liver kinase B1 (LKB1) since metformin and LKB1 share the same signalling system, and we found that the LKB1 gene is not expressed only in HeLa cancer cells. Consistently, the over-expression of LKB1 in HeLa cancer cells prevented metformin-triggered apoptosis while LKB1 knockdown significantly increased apoptosis in HEC-1-A and KLE cancer cells. Taken together, these findings indicate an underlying biological/physiological molecular function specifically for metformin-triggered apoptosis dependent on the presence of the LKB1 gene in tumorigenesis.

Key Words: Metformin, LKB1, AMPK, HeLa, Endometrial cancer, Apoptosis

INTRODUCTION
Metformin (1,1-dimethylbiguanide hydrochloride) is generally an inexpensive and well-tolerated member of the biguanide class (Triggle and Ding, 2017). Around the world, it is the most widely prescribed anti-diabetic drug, and it also helps prevent cardiovascular disease and suppress metabolic syndrome (Evans et al., 2005; Ben Sahra et al., 2010). Various preclinical studies have indicated that metformin can suppress cell proliferation, trigger apoptotic cell death, provoke cell cycle arrest in vitro, and diminish the occurrence and growth of tumors in an in vivo orthotopic or xenograft mouse model system (Buzzi et al., 2007; Ben Sahra et al., 2008; Zhao et al., 2015; Zì et al., 2015). Metformin can be used as a sensitizer or can be combined with standard chemo-therapeutic agents and radiotherapy to treat tumors (Fasih et al., 2014; Qu et al., 2014; Zhang et al., 2014; Lengyel et al., 2015; Uehara et al., 2015; Zhu et al., 2021). Furthermore, metformin plays an important role in targeting tumor stem cells and in reverting the epithelial-mesenchymal transition, a key process in tumor metastasis (Barriere et al., 2013; Nangia-Makker et al., 2014; Lei et al., 2017). Thus, metformin has been recognized as a potential anticancer agent. However, there is a need to conduct further studies on why patients show differences in response to metformin and with the anticancer action and mechanism of metformin.

AMPK is an enzyme that plays an important role in cellular energy homeostasis, largely regulating glycolysis and fatty acid oxidation, and it is also associated with cell growth and proliferation in eukaryotes (Steinberg and Kemp, 2009; Hardie, 2011; Yuan et al., 2013; Garcia and Shaw, 2017). Functionally, AMPK is accelerated by the AMP:ATP ratio contributed by the
metabolic conditions, including obstructing ATP production due to deficiency of oxygen or glucose. The acceleration in response to the promotion of AMP involves phosphorylation by an up-stream target kinase, the liver kinase B1 (LKB1) (Towler and Hardie, 2007).

The LKB1 protein is the main up-stream kinase of AMPK, implying that the inhibitory effects of LKB1 on cancer can be controlled by AMPK (Shaw et al., 2004; Li et al., 2017). In cancer, AMPK can be a promising target to treat tumors. Tumor cells have characteristic metabolic transformations from the metabolic state of the healthy original cell, and as a major metabolic modulator, AMPK can regulate metastasis. In addition, AMPK can act to suppress tumorigenesis by controlling the cell proliferation, cellular stress responses, cell growth, autophagy, and cell polarity (Wang and Guan, 2009). Therefore, the possible anti-tumorigenic function of metformin can be regulated by its role in accelerating AMPK, which in turn suppresses the phosphorylation of a mammalian target of rapamycin (mTOR) (Li et al., 2017; Mallik and Chowdhury, 2018). Considering the defined role of AMPK in tumorigenesis, the goal of our research is to elucidate the potential biological effects of metformin on the cervical and endometrial cancers, as well as demonstrate the underlying molecular function.

In this manuscript, we found that metformin showed different inhibitory effects on the growth of HeLa (low LKB1 expression), HEC-1-A and KLE cells (high LKB1 expression). We also found that gene silencing of LKB1 expression recovers the metformin-induced growth inhibition in HEC-1-A and KLE cells.

MATERIALS AND METHODS

Cell lines, reagents, and antibodies
Human cervical carcinoma cell lines HeLa, endometrial cancer cell lines (HEC-1-A and KLE) and human embryonic kidney 293T (HEK293T) cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and were grown in monolayer cultures in accordance with ATCC recommendations. Metformin was purchased from Sigma (St. Louis, MO, USA). The following antibodies used in this study were anti-caspase-3, anti-LKB1, anti-Bcl-2, anti-Bcl-xl, anti-Bax, anti-AMPK, anti-phospho-AMPK, anti-mTOR, anti-phospho-mTOR (Ser2448), anti-p70S6K, anti-phospho-p70S6K (Thr421), anti-4E-BP1, anti-phospho-4E-BP1 (Thr70) (Cell Signaling, Beverly, MA, USA), anti-PARP (BD Biosciences, San Jose, CA, USA), anti-GAPDH, anti-cyclin D1, anti-CDK4, anti-p27, anti-p16, anti-IL-6 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-p21 (Ab-1; Oncogene, Cambridge, MA, USA), and anti-β-actin (Sigma).

Cell proliferation assay and flow cytometric analysis of apoptosis
Cell viability was evaluated using the CellTiter-Glo luminescent assay kit (Promega, Madison, WI, USA), according to the supplier’s instructions. Briefly, cells were grown at a density of 4.6×10^3 per well in 96-well plates. After 24 h, the cells were transfected with various concentrations of metformin. Cell viability was assessed using the CellTiter-Glo reagent (Promega) according to the manufacturer’s instructions.

Apoptosis of HeLa, HEC-1-A, and KLE cells was analyzed utilizing flow cytometry as previously reported (Byun et al., 2012). In brief, 2.8×10^3-3.2×10^5 cells/well were seeded in 60 mm plates. Whole cells were incubated with FITC-labeled Annexin V and propidium iodide (PI) for 15 min in accordance with supplier’s instructions (BD Pharmingen, Mississauga, ON, Canada) and were developed utilizing fluorescence activated cell sorting (FACS) Vantage BD FACSCalibur flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA).

Assay of caspase-3 and PARP activity, construction of small interfering RNA (siRNA) for LKB1 gene
The activity of caspase-3 and PARP in HeLa, HEC-1-A, and KLE cancer cells were developed as previously reported (Zhang and Snyder, 1992; Kang et al., 2012). Various siRNA sequences from the LKB1 RNA sequence were prepared utilizing a siRNA Construction kit (Ambion, Austin, TX, USA). After selection of two clones, the prepared sequences were then transfected with oligofectamine (Thermo Fisher Scientific, Carlsbad, CA, USA) in accordance with supplier’s recommendation. TRIZOL Reagent (Thermo Fisher Scientific) was used to isolate whole RNA and reverse transcription (RT) PCR was then conducted. LKB1 expression was measured via western blot analysis.

Western blot analysis
Western blot analysis was conducted in accordance with a previous report (Kang et al., 2020). Briefly, whole cells were lysed in standard RIPA lysis buffer in ice for 30 min. After centrifugation, the protein concentration was evaluated utilizing the Bradford assay. About 15-20 µg protein extracts were separated by 10-12% SDS-PAGE and were transferred onto a nitrocellulose membrane (BioRad, CA, USA). After blocking, the membranes were probed for caspase-3, PARP, β-actin, p21, p27, p16, cyclin D1, CDK4, IL-6, Bcl-2, Bcl-xl, Bax, AMPK, phospho-AMPK, mTOR, phospho-mTOR, p70S6K, phospho-p70S6K, 4E-BP1, and phospho-4E-BP1, respectively. The protein bands were visualized utilizing an ECL chemiluminescence detection kit (GE Healthcare, Piscataway, NJ, USA).

Luciferase activity assay
In vitro luciferase activity was assessed using a dual luciferase reporter assay system (Promega). Cells were introduced with plasmids containing p27-Luc, p21-Luc, Bcl-2-Luc, and IL-6-Luc, in which the promoter of p27, p21, Bcl-2, and IL-6 control the expression of luciferase, respectively (Kang et al., 2012; Rho et al., 2012). In brief, cells were seeded to 85% confluence and co-transfected with the promoter-luciferase plasmids containing the Renilla luciferase reporter for 24 h. Following lysis with the radio-immunoprecipitation buffer, the lysates were cleared by centrifugation at 12,000 rpm for 20 min, and the cell extracts were incubated with the luciferase substrate reagent for 30 min at room temperature according to the supplier’s instructions. Then, a 5 µL aliquot of each sample was quantified using a MicroLumat Plus LB96V luminometer (Berthold Technologies, Bad Wildbad, Germany). The ratio was normalized for Renilla luciferase activity to correct for variation in transfection efficiency.

Statistical analysis
All the data are expressed as the mean ± SD and were estimated using Student’s t-test and analyses of variance according to the number of groups compared. Significant differences (p<0.05) are depicted with asterisks in each figure. The analy-
Fig. 1. Metformin selectively activates apoptotic cell death in human cervical and endometrial cancer cell lines. (A, B) Cell proliferation assay (A, left panel), FACS analysis (A, right panel), and caspase-3 and PARP assay (B). HeLa, HEC-1-A and KLE cells were treated with 0, 10, 20 and 50 mM metformin, respectively. Relative values of cell proliferation were calculated as described in the supplier’s protocols employing CellTiter-Glo assay system. Results represent the mean ± SD of at least three independent experiments conducted in triplicate. *p<0.05; **p<0.01 compared with the control (0 mM). Subsequently, early- (▲) and late-stage (◼) apoptotic cell death triggers by metformin was performed with fluorescein isothiocyanate (FITC)-labeled Annexin V assay. All experiments were repeated at least three times with similar results. And then, caspase-3 and PARP cleavages induced by metformin treatment. Soluble protein extracts were detected by western blot analysis for cleaved caspase-3 and cleaved PARP. β-actin served as a control. All results seen are representative of three independent experiments.
Metformin regulates the expression level of cell cycle- and apoptosis-associated proteins

The underlying molecular mechanism by which metformin played its role in attenuating HeLa cervical cell proliferation is explored by conducting a Western blot analysis to demonstrate the important signals related to the cell cycle and apoptosis function. As presented in Fig. 2A, treatment with metformin considerably suppressed the expression of cyclin D1 (CDK1), CDK4, and IL-6, but it accelerated the expression of p16 and p21 or p27. Next, we investigated metformin-treated cell growth by observing the expression of apoptosis-associated proteins Bcl-2, Bcl-xL, and Bax. These proteins are well-known essential regulators involved in cell growth and apoptosis. As seen in Fig. 2B, Bcl-2 and Bcl-xL expression were significantly diminished, whereas Bax was considerably accelerated by metformin treatment, compared with the control. Furthermore, the luciferase reporter-gene assay validated that metformin dramatically accelerated the transcription levels of p27 and p21, while Bcl-2 and IL-6 promoter activities were remarkably diminished (Fig. 2C). Collectively, these results possibly indicate that metformin can be used as an anti-tumor drug in cervical cancer therapy.

LKB1 overexpression desensitizes HeLa cells to 20 mM metformin

To investigate why 20 mM metformin specifically inhibits the proliferation of HeLa cells, we examined the differences in expression of LKB1 in HeLa, HEC-1-A, and KLE cells by a reverse transcriptase-polymerase chain reaction (RT-PCR) and western blotting. We found that the LKB1 is not expressed only in HeLa cancer cells but expressed in HEC-1-A and KLE cells (Fig. 3A). LKB1 overexpression was confirmed in HeLa cells using plasmid containing LKB1 (LKB1) (Fig. 3B). Then, the effect of LKB1 overexpression on 20 mM metformin-induced growth inhibition of HeLa cells was examined using flow cytometry. The overexpression of LKB1 in the HeLa cancer cells reduced 20 mM metformin-induced apoptosis (Fig. 3C). After 20 mM metformin treatment, cleaved caspase-3 and PARP levels were significantly higher, but the ectopic expression of LKB1 reduces metformin-induced cleavage of caspase-3 and PARP (Fig. 3D). These results indicate that a loss of LKB1 is essential for 20 mM metformin-induced growth inhibition and apoptosis of HeLa cancer cells.

A loss of LKB1 expression by LKB1 siRNA sensitizes HEC-1-A and KLE cells

Then, we examined the effects of LKB1 siRNA (siLKB1) on HEC-1-A and KLE cells, which expresses LKB1. Reduced expressions of LKB1 by siLKB1 in HEC-1-A, sensitized to 20 mM metformin, leading to the increases in portions of apoptotic cells and the cleavage of caspase-3 and PARP (Fig. 4A, 4B). Same trends were observed in KLE cells (Fig. 4C, 4D). LKB1 inhibition might sensitize nonresponsive HEC-1-A, and KLE cancer cells to cells vulnerable to 20 mM metformin treatment.

Metformin enhances phosphorylation of AMPK in siLKB1-treated KLE cells

We examined whether phosphorylation of AMPK was ob-
**Fig. 3.** Expression of *LKB1* gene in HeLa and endometrial cancer cell lines and effects of metformin on the LKB1-overexpressed HeLa cells. (A) Expression levels of *LKB1* mRNA and protein was analyzed by RT-PCR and western blot analysis. GAPDH and β-actin included as an mRNA or protein loading control, respectively. (B) LKB1 overexpression was confirmed by western blot analysis. (C) After treated/transfected with metformin or metformin plus LKB1, flow cytometric analysis was conducted to calculate the early- (∆) and late- (■) stage apoptotic death cells in HeLa cell lines. (D) Cells were collected and treated with lysis buffer. Total cell lysates were subsequently subjected to western blot analysis. Expression levels of cleaved caspase-3 and cleaved PARP protein were observed using indicated specific antibodies. All data were repeated at least three times with similar results.

**Fig. 4.** siLKB1 sensitizes HEC-1-A and KLE cells. After treated/transfected with metformin or metformin plus siLKB1, flow cytometric analysis was conducted to calculate the early- (∆) and late- (■) stage apoptotic cell death cells in HEC-1-A (A) and KLE cells (C). (B, D) Caspase-3 and PARP assay. HEC-1-A (B) and KLE cells (D) were collected and treated with lysis buffer. Total cell lysates were subsequently subjected to western blot analysis. Expression levels of cleaved caspase-3 and cleaved PARP protein were observed using indicated specific antibodies. All data were repeated at least three times with similar results.
Metformin inhibits the mTOR signaling pathway through AMPK activation in HeLa cells lacking LKB1 expression

To elucidate the effects of metformin on AMPK signaling in low-dose metformin-sensitive HeLa cells and metformin-non-responsive HEC-1-A tumor cells, we examined the phosphorylation of AMPK, mTOR, and mTOR down-stream targets such as p70S6K and 4E-BP1. Total cell lysates from the control HeLa cells and metformin-treated HeLa cells were examined through western blot analysis. As shown in Fig. 6A, metformin remarkably induced the activation of AMPK phosphorylation and reduced mTOR phosphorylation, as well as the phosphorylation of p70S6K and 4E-BP1 in HeLa cells (metformin sensitive). By contrast, no changes in the phosphorylation of AMPK, mTOR, p70S6K, and 4E-BP1 were observed in HEC-1-A endometrial cancer cell (metformin nonresponsive) (Fig. 6B). Therefore, metformin might suppress the proliferation of LKB1-deficient HeLa cells through inhibition of the mTOR signaling pathway by activating AMPK.

DISCUSSION

Metformin is used as an oral anti-diabetic drug, and it is well-known to regulate cell growth and protein synthesis by suppressing the mTOR signaling cascade by accelerating ATM (ataxia telangiectasia mutated), LKB1, and then AMPK (Shaw et al., 2005; Towler and Hardie, 2007; Lee et al., 2019). According to the previously reported, the anti-cancer effects of metformin are generally exhibits on various human cancer types including breast (Zakikhani et al., 2006; Dowling et al., 2007), ovary (Aligire et al., 2008; Gottlieb et al., 2008), lung (Aligire et al., 2008; Brancher et al., 2021), colon (Buzzi et al., 2021) and prostate tumor cell lines (Ben Sahra et al., 2008).

LKB1 is inactivated approximately 30% through a somatic mutation in non-small cell lung cancer (NSCLC) cells and is regarded as tumour suppressor (Liu et al., 2013). However, the biological and molecular mechanism dependent on LKB1 gene that provides the anti-cancer effect of metformin for endometrial cancers remain unclear.

Our data showed a growth inhibition effect of metformin on HeLa cells is consistent with the previous report (Fig. 1) (Xiao et al., 2012). Furthermore, metformin showed growth inhibition in HEC-1-A cells (Zhang et al., 2017) and invasion inhibitory effects in KLE cells (Suh et al., 2020), the growth inhibition effect of metformin in these cell lines was not observed in our data (Fig. 1).

AMPK/mTOR signaling cascade is usually under the control of LKB1. LKB1 is a major up-stream kinase responsible for AMPK phosphorylation. Loss of LKB1 results in loss of AMPK signaling pathway. Therefore, loss of LKB1 prevents the therapeutic effects of metformin, indicating that metformin treatment of mice accelerated AMPK activity in the liver and lowered blood glucose levels in an LKB1-dependent manner (Shaw et al., 2005; Li et al., 2018). However, in this study, we have discovered that LKB1 overexpression can specifically prevent metformin-induced growth inhibition and apoptotic cell death in HeLa cancer cells (Fig. 3C, 3D). Interestingly, siLKB1 can considerably induce apoptotic cell death in metformin-treated HEC-1-A and KLE tumor cells (Fig. 4). Therefore, the results in Fig. 3 and 4 suggests that metformin-induced cell death via LKB1/AMPK axis is not valid in HeLa, HEC-1-A, and KLE tumor cells.

Interestingly, siLKB1 enhanced the phosphorylation of AMPK in metformin-treated KLE cells (Fig. 5B). In line with this observation, metformin enhanced the phosphorylation of AMPK also in HeLa cells deficient for LKB1 (Fig. 6A). These results clearly show that AMPK is activated by metformin even in the deficiency of LKB1. Kinases other than LKB1, namely
CAMKK, ATM, and TAK1 have been reported to be able to activate AMPK (Sun et al., 2007; Herrero-Martin et al., 2009; Sanli et al., 2010; Gotou et al., 2013; Fogarty et al., 2016; Loubiere et al., 2017; Chen et al., 2018).

LKB1 can act as a low energy sensor and acts as a negative regulator of apoptosis in normal cells (Shaw et al., 2004). Therefore, under the basic conditions, LKB1 acts as a sensor of low energy and maintains the ATP consumption process including protein synthesis through check via phosphorylation of TSC2 by AMPK (Shaw et al., 2004). In response to stress such as hypoglycemia, hypoxia, malnutrition, or mitochondrial toxins, LKB1 phosphorylates AMPK, blocking the ATP consumption process to offset the elevated AMP level and upregulating ATP production (Shaw et al., 2004). LKB1-induced activation of AMPK prevents normal cells from entering apoptosis in response to elevated AMP. However, in the case of cancer, metformin treatment suppressed mTOR activation and S6K phosphorylation in LKB1-deficient HeLa cells via TSC activity induced by AMPK phosphorylation (Fig. 6). Metformin induces cell death in HeLa and LKB1-knockdown KLE and HEC-1A cells presumably through this mechanism.

In general, since LKB1 is a tumor suppressor, LKB1 deficiency likely induces an increase in the oncogenic potential or growth of cancer cells (Shaw et al., 2004). Therefore, LKB1 deficient cells show high mitochondrial metabolism, as indicated by increased mitochondrial potential and oxygen consumption rate (Whang et al., 2016). Accordingly, these cells appear to be vulnerable to substances that affect the mitochondrial function. Therefore, metformin treatment induces LKB1-independent activation of AMPK, thereby affecting mitochondria (Vial et al., 2011) and activating TSC through phosphorylation (Green et al., 2011), which seems to inhibit the AMPK pathway.

In all, we propose loss of LKB1 is involved in the anti-proliferative effects of metformin in HeLa cervical cancer cells. si-LKB1 converts metformin non-responsive HEC-1-A and KLE cells to responsive to metformin. These results suggested that expression level of LKB1 might be a marker for responsiveness to metformin.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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