Activation of the LKB1-SIK1 signaling pathway inhibits the TGF-β-mediated epithelial-mesenchymal transition and apoptosis resistance of ovarian carcinoma cells

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Abstract. Ovarian cancer is the most common and lethal type of gynecological malignancy, due to its invasiveness. The present study aimed to analyze the molecular mechanism underlying chemoresistance in ovarian carcinoma cells, which may lead to local migration toward adjacent tissues and long-distance metastasis to other organs. A total of 12 patients with ovarian fibroma were used to evaluate chemoresistance and chemosensitivity. The sensitivity and resistance of ovarian carcinoma cells was measured using apoptosis analysis, morphological observation, survival rate analysis, immunohistochemistry and immunostaining. The mechanism underlying the interaction between the epithelial-mesenchymal transition (EMT) and liver kinase B1 (LKB1)-salt-inducible kinase 1 (SIK1) signaling pathways was additionally investigated in ovarian carcinoma. The results of the present study demonstrated that ovarian carcinoma cells isolated from patients exhibited apoptosis resistance. Inhibition of TGF-β expression led to an inhibition of growth, migration and invasion, in addition to a promotion of apoptosis, in ovarian carcinoma cells treated with paclitaxel. Studies have indicated that the LKB1-SIK1 signaling pathway may be suppressed in ovarian carcinoma cells compared with normal ovarian cells, leading to activation of the EMT signaling pathway. The results of the present study demonstrated that upregulation of LKB1 promoted SIK1 expression and markedly suppressed the growth and aggressiveness of ovarian cancer cells. Upregulation of LKB1 additionally promoted apoptosis in ovarian carcinoma cells. In addition, the results of the present study demonstrated that the knockdown of LKB1 further promoted the expression of transforming growth factor-β and EMT, which downregulated the chemosensitivity of ovarian carcinoma cells. Additionally, overexpression of LKB1 in ovarian carcinoma cells increased chemosensitivity, resulting in a significant inhibition of migration and invasion. The present findings indicated that the enhancement of LKB1-SIK1 suppressed the growth and aggressiveness of ovarian carcinoma cells isolated from clinical patients, which subsequently contributed to an inhibition of metastatic potential. In conclusion, targeting the LKB1-SIK1 signaling pathway to inhibit EMT may provide potential therapeutic benefits in ovarian carcinoma.

Introduction

Worldwide, ovarian cancer is one of the most common types of human gynecological tumor, and the morbidity and mortality rate is increased compared with other gynecological malignancies (1,2). A number of factors may lead to tumorigenesis in ovarian cancer, demonstrating the complex pathology of the disease (3). A previous clinical study indicated that the incidence of ovarian cancer is increasing worldwide (4). Currently, tumorectomy, radiotherapy and chemotherapy are the primary therapies for patients with ovarian cancer in the clinic. However, ineffective treatments and treatment noncompliance frequently contribute to a worsening of symptoms due to the apoptosis resistance of ovarian malignant cells in patients with cancer (5). Although novel treatments to improve clinical medicine have been investigated, the improvements have had little effect on the survival rate of patients with ovarian cancer (6).

Apoptosis resistance is important for tumor growth and aggressiveness, and is caused by regulatory disorders of the apoptotic signaling pathway (7). Numerous molecules and proteins have been identified to be associated with the biochemical processes underlying apoptosis resistance, and may regulate the apoptotic signaling pathway in various tumor cells (8-10). Cellular studies and clinical data have suggested a direct association between the expression of the anti-apoptotic gene survivin and the apoptotic susceptibility of human ovarian cancer cells (11-13). Resistance to radiotherapy and chemotherapy is the principal challenge for the treatment of recurrent ovarian cancer, and frequently causes varying degrees of immune system damage, treatment failure and tumor metastasis. Therefore, understanding the underlying mechanisms of radioresistance and chemoresistance in ovarian cancer may contribute to the inhibition of apoptosis.
resistance and the benefits of cancer therapy, in addition to the development of innovative systemic therapies.

Apoptosis resistance, particularly increased epithelial-mesenchymal transition (EMT), remains an intractable clinical problem in the treatment of ovarian cancer (14). A previous study revealed that EMT may affect the cell cycle, differentiation, survival and apoptosis, regulated by transforming growth factor (TGF)-β in tumor cells (15). In addition, TGF-β upregulation may suppress mothers against decapentaplegic homolog (Smad) and non-Smad signaling in mammary epithelial cells, leading to EMT and the inhibition of growth arrest and apoptosis (16). Additionally, Chorna et al (17) demonstrated that TGF-β production may act as a natural immunosuppressor, which is regarded as an anti-apoptotic protein for doxorubicin. Research has indicated that the liver kinase B1 (LKB1)-salt-inducible kinase 1 (SIK1) signaling pathway is associated with lung cancer cell growth, and previous results have suggested that attenuating LKB1-SIK1 may promote tumor invasion via upregulation of TGF-β production in non-small cell lung cancer cells (18). However, the signaling pathway and molecular mechanisms of LKB1-SIK1 has not been investigated in ovarian cancer.

In the present study, to determine the role of LKB1-SIK1 in ovarian cancer, the activity and expression levels of LKB1-SIK1 were analyzed in ovarian cancer tissues with normal adjacent tissues as the control. Migratory and invasive capacities were evaluated using Transwell. The association between LKB1-SIK1, EMP, apoptosis resistance and ovarian cancer cell growth was investigated using western blotting, small interfering RNA (siRNA), protein overexpression and immunofluorescence.

Materials and methods

Ethics statement. The present clinical investigation (no. HMCH2010072508) was performed in strict accordance with the recommendations in the Guide for Haidian Maternal and Child Healthcare Center (Beijing, China) between May 2010 and October 2015. A total of 12 female patients with ovarian fibroma were required to review trial protocols and amendments, and to provide written informed consent. The present study was approved by the ethics committee of Haidian Maternal and Child Healthcare Center. The demographic and clinical pathological characteristics of the patients are summarized in Table I.

Cell culture and reagents. Ovarian cancer cells (1x10⁷) were isolated from patients with ovarian cancer using tumor cell separation methods (19). Ovarian cancer tissues and adjacent normal tissues were washed with PBS. Tumor tissue was cut into pieces and separated into individual cells using minimum essential medium (MEM) containing 5% pancreatin and penicillin/streptomycin (2 mM) (both from Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Cells were maintained for 12 h at 37˚C in the presence of 5% CO₂. Subsequently, cells were filtered, collected and identified by microscopic investigation (20). Ovarian cancer cells were cultured in MEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc.).

Table I. Characteristics of patients with ovarian cancer.

| Characteristic | Value |
|---------------|-------|
| Patient no.   | 12    |
| Age range, years | 32.4-53.8 |
| Cancer type   | Ovarian fibroma |
| Tumor stage, n |     |
| I             | 8     |
| II            | 3     |
| III           | 1     |
| IV            | 0     |
| History of cancer | None |
| History of allergy | None |
| Prior treatment | None |

Cells were cultured in a humidified atmosphere containing 5% CO₂ at 37˚C in a cell culture incubator (Gibco; Thermo Fisher Scientific, Inc.).

Western blotting. Ovarian cancer cells transfected with siRNA or the eukaryotic expression vector for LKB1 were homogenized in lysate buffer containing protease-inhibitor (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and were centrifuged at 8,000 x g at 4˚C for 10 min. The supernatant was used for analysis of the total protein using a bicinonicinic protein assay kit (Gibco; Thermo Fisher Scientific, Inc.). Protein samples (20 µg) were separated on 12% sodium dodecyl sulfate polyacrylamide gels and transferred onto polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA) as previously described (21). Protein was blocked with 5% bovine serum albumin reagent (Roche Diagnostics, Basel, Switzerland) for 1 h at 37˚C. For western blotting, primary goat anti-human antibodies against TGF-β (cat. no. ab31013), zinc-finger protein SNAI1 (cat. no. ab53519), Snai2 (cat. no. ab187109), Twist-related protein 1 (cat. no. ab50887), zinc finger E-box-binding homeobox 1 (ZEB1; cat. no. ab71286), E-cadherin (cat. no. ab76319), vimentin (VIM; cat. no. ab137321), VEGF (cat. no. ab27278), angiotensin-1 (Ang-1; cat. no. ab53951) and β-actin (cat. no. ab8226) (all 1:500 dilution; Abcam, Cambridge, UK), were incubated overnight at 4˚C, followed by incubation with horseradish peroxidase-conjugated polyclonal anti-rabbit immunoglobulin G antibody (1:10,000, cat. no. HAF008; R&D Systems, Inc., Minneapolis, MN, USA) for 1 h at room temperature. A Ventana Benchark automated staining system was used for analyzing protein expression (Olympus BX51; Olympus, Tokyo, Japan).

siRNA transfection. Ovarian cancer cells (1x10⁶) were transfected with HiPerFect reagent (Qiagen GmbH, Hilden, Germany), according to the manufacturer's instructions (22). siRNA-TGF-β (100 pmol), siRNA-LKB1 (100 pmol) and siRNA-vector (100 pmol) were transfected into ovarian cancer cells for 24 h at 37˚C. The sequences of siRNA-TGF-β, siRNA-LKB1 and siRNA-vector were designed and are listed in Table II. siRNA oligonucleotide pools containing three

isotopic methylation (24).
sequences targeting TGF-β or LKB1 were purchased from Eurogentec, Ltd. (Liège, Belgium).

**Endogenous expression of LKB1.** In order to establish stable ovarian cancer cells with endogenous LKB1 expression, the eukaryotic expression vector pCMVp-NEO-BAN (Takara Biotechnology Co., Ltd., Dalian, China) was used to construct recombinant plasmids. LKB1 was cloned, sequenced and recombined into pCMVp-NEO-BAN to construct pCMVp-NEO-LKB1 (pLKB1). The recombinant plasmid pCMVp-NEO-LKB1 was subsequently transfected into ovarian cancer cells. pHK1 (1.0 μg) or pvector (1.0 μg) was transfected into cultured ovarian cancer cells (5x10⁶) using Lipofectamine 2000 (Sigma-Aldrich; Merck KGaA), according to manufacturer's instructions. Stable LKB1-overexpressing ovarian cancer cells were selected by G418 screening (23). After 48 h transfection, LKB1-overexpressing ovarian cancer cells were used to subsequent experimentation.

**Apoptosis analysis.** Apoptosis analysis of ovarian tumor cells was performed using flow cytometry. Human ovarian tumor cells (5x10⁶) were cultured in 6-well plates with paclitaxel (2.0 mg/ml; Sigma-Aldrich; Merck KGaA) for 48 h to achieve the maximal apoptosis rate. Ovarian tumor cells were harvested at 48 h post-treatment by trypsinization. Ovarian tumor cells were subsequently washed in cold PBS and adjusted to 1x10⁶ cells/ml with PBS. Following double staining with fluorescein isothiocyanate (FITC)-Annexin V and propidium iodide using the FITC Annexin V Apoptosis Detection kit (Chemicon; EMD Millipore), all sections were observed in Cell Quest software (version 3.3; BD Biosciences, San Jose, CA, USA), according to manufacturer's instructions, to detect apoptosis in ovarian tumor cells. All experiments were performed in triplicate.

**MTT cytotoxicity assays.** Ovarian cancer cells were incubated (1x10⁶) with paclitaxel or PBS in 96-well plates for 48 h in triplicate. Subsequently, 20 μl MTT (5 mg/ml) in PBS solution was added to each well, the plate was further incubated for 4 h. Most of the medium was removed and 100 μl dimethyl sulfoxide was added into the wells to solubilize the crystals. The OD was measured using an ELISA reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at wavelength of 450 nm.

**Histological, immunohistochemical and immunofluorescence staining analyses.** Ovarian tumor tissues were fixed in situ overnight in 10% buffered formalin. The fixed tissues were cut mid-saggital and being embedded in paraffin (4 μm thickness) using standard protocols. Hematoxylin and eosin staining was used to visualize the area of myocardial infarction after treatment with matrine. Immunohistochemical staining was performed using an avidin-biotin-peroxidase technique. Tumor sections (4 μm) were deparaffinized in xylene, dehydrated through graded ethanol and treated with 0.3% hydrogen peroxide in methanol for 30 min at 37°C. Paraffin-embedded ovarian normal tissue and tumor sections were prepared and epitope retrieval was performed at 95°C for 15 min for further analysis. The paraffin sections were treated with hydrogen peroxide (3%) for 10-15 min, which subsequently was blocked with a blocking solution (5% skim milk powder) for 10-15 min at 37°C. Subsequently, the sections were incubated in goat anti-human anti-Snai2 (1:1,000, cat. no. ab187109), Twist (1:1,000, cat. no. ab50887), ZEB1 (1:1,000, cat. no. ab71286), LKB1 (1:1,000, cat. no. ab15095), SIK1 (1:1,000, cat. no. ab64428), phosphorylated (p-)LKB1 (1:1,000, cat. no. ab63473), p-SIK1 (1:1,000, cat. no. ab217809), TGF-β (1:1,000, cat. no. ab31013) and VIM (1:1,000, cat. no. ab137321) at 4°C for 12 h. All sections were washed three times and incubated with secondary rabbit anti-goat antibodies (1:2,000, cat. no. ab150117; Abcam) for 1 h at 37°C. For immunofluorescence, ovarian tumor cells were stained with goat anti-human vascular endothelial growth factor and angiopoietin-1 antibodies. Additionally, a terminal deoxynucleotidyl transferase dUTP nick end labeling assay was performed using a Peroxidase Apoptosis Detection kit (Chemicon; EMD Millipore). All sections were observed in six random fields in the confocal microscope at magnification, x40 (Nikon E400, Nikon Corporation, Tokyo, Japan).

**Analysis of the cell cycle.** To analyze the effects of LKB1 overexpression on the cell cycle stage of ovarian cancer cells, flow cytometry was performed. Exponentially, culturing ovarian cancer cells (1x10⁶) or LKB1 overexpression were cultured for 24 h at 37°C. Cells were washed and trypsinized and rinsed with phosphate-buffered saline (PBS). All cells were fixed in 75% ice-cold ethanol for 5 min and then washed with PBS three times. The fixed cells were washed with RNase A (20 μg/ml, Fermentas; Thermo Fisher Scientific, Inc.) and stained with propidium iodide (20 μg/ml, Sigma-Aldrich; Merck KGaA) for 10 min at 37°C. The percentages of cells in G1 phase were analyzed using BD FACSCalibur (Becton Dickinson; BD Biosciences, San Jose, CA, USA).

| Name | Sense | Antisense |
|------|-------|-----------|
| TGF-β | 5'-GGATACCAACTATGCTTCAGCTCC-3' | 5'-AGGGCTCATAATATAGGGGCAGGGTC-3' |
| LKB1 | 5'-CTAGCTCAGACCCTTAGACGCC | 5'-AAAAAGGCAGGCACCTGAGACGCCGT |
| Vector | 5'-AGAAGGAATCATGGCTCAGC-3' | 5'-CAATAAGTAGCATGACCTGCTCGGT-3' |

siRNA, small interfering RNA; TGF-β, transforming growth factor-β; LKB1, liver kinase B1.
Cell invasion and migration assays. Ovarian tumor cells subjected to different treatments (LKB1 overexpression or TGF-β inhibition) were used to analyze invasion and migration. Migration and invasion assays in ovarian tumor cells were conducted in a 24-well MEM culture plate with chamber inserts (BD Biosciences) with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) for 12 h at 37°C. For migration assays, 1x10^3 cells/well ovarian tumor cells were placed into the upper chamber with a non-coated membrane. For the invasion assays, cells (1x10^3 cells/well) were placed into the upper chamber with a Matrigel-coated membrane. Matrigel were fixed with 4% formaldehyde and stained with 4',6-diamidino-2-phenylindole as well as counted in 6 random fields under a microscope. Invasion and migration were calculated in at least three randomly stained fields under a light microscope (Nikon E400; Nikon Corporation).

Statistical analysis. All data are presented as the mean ± standard error of triplicate samples, and analyses were performed using Prism 6.0 software (GraphPad Software, Inc., La Jolla, CA, USA). Statistical differences between experimental groups were analyzed using Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

Analysis of the expression levels of EMT proteins, LKB1 and SIK1 in ovarian tumor tissues. The expression levels of EMT molecules, LKB1 and SIK1 were measured in ovarian tumor tissues. As presented in Fig. 1A, expression of the EMT pathway components Snai2, Twist and ZEB1 was upregulated in ovarian tumor tissues compared with normal ovarian tissues. The results in Fig. 1B and C demonstrated that the expression and phosphorylation levels of LKB1 and SIK1 were downregulated in ovarian tumor tissues compared with normal ovarian tissues. It was additionally observed that the expression levels of TGF-β and VIM were markedly increased in ovarian tumor tissues compared with normal ovarian tissues (Fig. 1D). The results of the present study demonstrated that the knockdown of LKB1 expression promoted TGF-β and Snai1 expression in ovarian tumor cells (Fig. 1E). Additionally, it was observed that the knockdown of LKB1 promoted the apoptosis resistance of ovarian tumor cells treated with paclitaxel (Fig. 1F). The present results suggested that the expression levels of EMT, LKB1 and SIK1 were increased in ovarian tumor tissues, which may be associated with the aberrant growth and aggressiveness of ovarian tumor cells.

Analysis of growth and apoptotic resistance of ovarian tumor cells following inhibition of TGF-β expression. As presented in Fig. 2A, the growth rate of ovarian tumor cells isolated from clinical patients was increased compared with normal ovarian cells. Proliferation and migration assays demonstrated that growth and aggressiveness was inhibited by the inhibition of TGF-β expression in ovarian tumor cells (Fig. 2B and C). Apoptosis experiments demonstrated that the inhibition TGF-β expression by siRNA promoted apoptotic sensitivity in cells treated with paclitaxel for 48 h (Fig. 2D). It was observed that the inhibition of TGF-β expression by siRNA inhibited the proliferation of ovarian tumor cells compared to si-vector-transfected cells (Fig. 2E). Additionally, it was observed that the inhibition of TGF-β expression suppressed the expression levels of Sna1, Twist and ZEB1, as determined by western blotting (Fig. 2F). The results of the present study
suggested that TGF-β expression may be associated with the growth and apoptotic resistance of ovarian tumor cells.

**LKB1 upregulation stimulates SIK1 expression and inhibits the EMT signaling pathway in ovarian tumor cells.** The present study further analyzed the influences of LKB1 on TGF-β expression and the EMT signaling pathway in ovarian tumor cells. The results in Fig. 3A demonstrated that SIK1 expression was promoted by LKB1 upregulation in ovarian tumor cells, as determined by immunofluorescence analysis.
Western blotting demonstrated that TGF-β expression was decreased by LKB1 (Fig. 3B). The apoptosis assay indicated that the apoptosis sensitivity of ovarian tumor cells was increased following upregulation of LKB1 (Fig. 3C). In addition, the results demonstrated that the expression of important regulatory factors in the EMT pathway, E-cad, VIM, Snail2 and γH2AX, was downregulated by LKB1 upregulation in ovarian tumor cells (Fig. 3D). Growth, migration and invasion assays demonstrated that growth and aggressiveness was inhibited following overexpression of LKB1 expression in ovarian tumor cells (Fig. 3E and F). The present findings suggested that activation of the LKB1 signaling pathway may inhibit the TGF-β-mediated EMT pathway and decrease growth, aggressiveness and apoptosis resistance in ovarian carcinoma cells.

**LKB1 overexpression inhibits cell cycle and apoptosis resistance-associated protein expression in ovarian tumor cells.** The present study investigated the effects of LKB1 on cells survival-and apoptosis resistance-associated protein expression in ovarian tumor cells. The results demonstrated that LKB1 overexpression inhibited the survival and cell cycle progression of tumor cells treated with paclitaxel, compared with control cells (Fig. 4A and B). Western blotting demonstrated that the expression of Bcl-2, Bax and p53 was inhibited by LKB1 overexpression in ovarian tumor cells compared with control cells (Fig. 4C). However, caspase-3, caspase-8 and Apaf-1 expression was increased by LKB1 overexpression in ovarian tumor cells compared with control cells (Fig. 4D). The expression of aggressiveness-associated proteins FN and RNF-8 was decreased by LKB1 overexpression in ovarian tumor cells (Fig. 4E). Additionally, the results demonstrated that the expression levels of vascular endothelial growth factor (VEGF) and Ang-1 were downregulated by LKB1 overexpression, as determined by immunofluorescence analysis (Fig. 4F). The present results suggested that LKB1 overexpression may inhibit aggressiveness-and apoptosis resistance-associated protein expression in ovarian tumor cells.

**Discussion**

Ovarian cancer is associated with poor prevention, intractable malignancy and a poor prognosis (24). Traditional treatments, including radiotherapy, chemotherapy and surgery, are limited to palliative approaches for patients with advanced ovarian cancer (25,26). Although novel therapeutic agents and protocols for patients with ovarian cancer have been proposed in previous reports, the mortality and survival rates remain poor due to an increased rate of recurrence and metastasis following surgical resection (27). It has been reported that intrinsic and acquired resistance to anticancer treatments has been recognized to be a notable impediment to favorable outcomes in the clinic (28,29). The present study investigated the growth and aggressiveness of clinical ovarian cancer cells and analyzed the potential molecular mechanisms of apoptotic resistance in cells treated with a chemotherapeutic drug. Previous studies have suggested that ovarian cells may survive exposure to chemotherapeutic drug treatment and may display cancer stem cell and EMT-positive phenotypes (30,31). Consistent with the results in the published literature, an additional previous
study demonstrated that the apoptotic resistance of ovarian cancer was induced by the EMT phenotype and expression of EMT-associated proteins (32). Notably, the present findings suggested that the inhibition of LKB1-SIK1 may reverse apoptotic resistance in ovarian cancer cells through the TGF-β-mediated EMT signaling pathway.

Previous studies have indicated that the development of anti-angiogenic drugs for the treatment of patients with ovarian cancer is an emerging field of oncology hoping to enter the preclinical stage of clinical trials (33-35). Additionally, many patients with advanced ovarian cancer respond poorly to traditional treatments or experience limited benefit from these treatments (36). The present study demonstrated an association between the EMT and SIK1 signaling pathways in ovarian carcinoma cells. EMT leads to cellular heterogeneity and supports tumor engraftment, which has been associated with underlying tumor heterogeneity and growth, metastasis and progression of ovarian cancer (37). Tang et al (38) demonstrated that inhibiting vasculogenic mimicry formation by reducing EMT may contribute to tumor apoptosis in ovarian cancer. In the present study, the results indicated that key regulatory factors in the EMT signaling pathway were upregulated in clinical ovarian tumor tissues, resulting in rapid growth and aggressiveness of tumor cells. Inhibition of TGF-β expression led to an inhibition of growth and aggressiveness, in addition to a promotion of apoptosis, in ovarian carcinoma cells treated with paclitaxel. The present results demonstrated that VEGF and Ang-1 were downregulated by LKB1 overexpression, suggesting that LKB1 may exert regulatory effects on ovarian cancer cell growth.

A previous study demonstrated that the LKB1-SIK1 signaling pathway was suppressed in ovarian carcinoma cells compared with normal ovarian cells, which led to activation of the EMT signaling pathway (18). In the present study, it was observed that inhibition of TGF-β expression promoted paclitaxel-induced apoptosis and suppressed tumor metastasis-associated protein expression (Snai2, Twist and ZEB1) in ovarian tumor cells. Cha et al (39) suggested that the binding of transcription elongation factor A protein 3 to TGF-β receptor I may induce apoptosis regulated by the Smad- and mitogen-activated protein kinase-dependent pathways in ovarian carcinoma cells. Notably, it was suggested that enhanced LKB1-SIK1 signaling may inhibit the TGF-β-mediated EMT signaling pathway and the chemoresistance of ovarian cancer cells, which may subsequently contribute to limited metastatic potential, suggesting that targeting the LKB1-SIK1-TGF-β-EMT signaling pathways may be a promising therapeutic option for promoting the chemosensitivity and inhibiting the growth and metastasis of ovarian cancer cells.

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