Sipi soup inhibits cancer-associated fibroblast activation and the inflammatory process by downregulating long non-coding RNA HIPK1-AS

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Abstract. Sipi soup (SPS), the aqueous extract derived from the root bark of *Sophora japonical L.*, *Salix babylonica L.*, *Morus alba L.*, as well as *Amygdalus davidiana (Carr.) C. de Vos*, is a traditional Chinese medicine frequently used to prevent and treat infection and inflammation. However, the role of SPS in cancer-associated fibroblasts (CAFs) require further investigation. In the present study, the effects of SPS on fibroblast inactivation and the underlying mechanism were investigated. Reverse transcription-quantitative polymerase chain reaction was used to analyze the mRNA expression levels of fibroblast activation protein (FAP), interleukin (IL)-6, α-smooth muscle actin (α-SMA) and programmed cell death 4 (PDCD4). Flow cytometry was used to evaluate cell apoptosis. Immunofluorescence was used to determine the number of activated fibroblasts. The present study reported that SPS treatment did not affect the proliferative apoptotic potential of fibroblasts. Treatment with HeLa cell culture medium (CM) induced a significant increase in the expression levels of FAP, IL-6 and α-SMA, but reduced the expression of PDCD4. Flow cytometry was used to evaluate cell apoptosis. Immunofluorescence was used to determine the number of activated fibroblasts. The present study reported that SPS treatment did not affect the proliferative apoptotic potential of fibroblasts. Treatment with HeLa cell culture medium (CM) induced a significant increase in the expression levels of FAP, IL-6 and α-SMA, but reduced the expression of PDCD4. SPS reversed the effects of HeLa CM on the expression of these genes. Analysis with a long non-coding (lnc)RNA array of numerous differentially expressed lncRNAs revealed that the expression levels of the lncRNA homeodomain-interacting protein kinase 1 antisense RNA (HIPK1-AS) were increased in cervicitis tissues and cervical squamous cell carcinoma tissues compared with in normal cervical tissues. HIPK1-AS expression levels were upregulated in response to HeLa CM, but were decreased under SPS treatment. The down-regulation of HIPK1-AS expression via short hairpin RNA abolished the effects of HeLa CM on the expression of inflammation-associated genes. The findings of the present study suggested that SPS may prevent the progression of cervical cancer by inhibiting the activation of CAF and the inflammatory process by reducing HIPK1-AS expression.

Introduction

Cervical cancer has become the leading cause of cancer-associated mortality in the female population (1); the etiopathogenesis of cervical cancer is complex. Thus, improving understanding of the molecular mechanisms underlying cervical cancer is of primary concern (2). Chronic inflammation has been reported to be a promoting factor in the majority of human malignancies and has been directly associated with various steps involved in tumorigenesis (3). Inflammatory cytokines, interleukins (ILs), interferons, transforming growth factors, chemokines and adhesion molecules from stromal cells, such as fibroblasts, have been associated with chronic inflammation and the promotion of cervical cancer (4).

Cancer-associated fibroblasts (CAFs) were reported to support tumorigenesis by stimulating angiogenesis, cancer cell proliferation and invasion; CAFs may also mediate tumor-promoting inflammation (5). Additionally, normal dermal fibroblasts may be affected by carcinoma cells to induce the expression of proinflammatory genes (5). Thus, inhibiting the activation of CAFs may be a potential strategy for cancer management. Long non-coding RNAs (lncRNAs) have been reported to contribute to the tumor-promoting phenotype of CAFs (6,7). For example, LINC00092 binds a glycolytic enzyme, 6-phophofructo-2-kinase/fructose-2,6-bisphosphate, thereby promoting metastasis by altering glycolysis and sustaining the local supportive function of CAFs (8); however, the role of lncRNA homeodomain-interacting protein kinase 1 antisense RNA (HIPK1-AS) on CAF activation remains unknown.
Sipi soup (SPS), the aqueous extract derived from the root bark of Sophora japonica L., Salix babylonica L., Morus alba L., as well as Amygdalus davidiana (Carr.) C. de Vos, is a traditional Chinese medicine frequently used to prevent and treat inflammation (9-11). The extract from the leaves of M. alba L. have been reported to exhibit a highly inhibitory effect against acute inflammation, which may be associated with the presence of chlorogenic acid and flavonoids (12); however, its role in CAFs remains unknown. In the present study, the effects of SPS on fibroblasts and the potential underlying mechanism of SPS-regulated fibroblast activation were investigated.

Materials and methods

Tissues. Cervical samples (n=184) were collected from women (age, 25-76-years-old) who underwent routine cervical cancer investigation at the Longyan First Hospital from May 2015 to April 2016 (Fujian, China). The subjects with any other types of cancer and systemic inflammatory diseases, including diabetes, sepsis, nephritis, hepatitis and lupus erythematosus were excluded. Written informed consent was obtained from all participants in the present study. Additionally, the present study was approved by the Ethics Committee of the Longyan First Hospital. The specimens were grouped according to the histological diagnosis. Samples analyzed in the present study consisted of normal cervical (n=30), cervicitis (n=40), cervical intraepithelial neoplasia-I (CIN I; n=34), CIN II-III (n=38) and cervical squamous cell carcinoma tissues (SCC; n=42), which were graded using the World Health Organization grading system (13). Classification of tissue samples was conducted in a blind manner by two pathologists. Samples were stored at -80°C until use.

Preparation of SPS. Dried forms of root bark of S. japonical L., S. babylonica L., M. alba L., as well as A. davidiana (Carr.) C. de Vos were purchased from a Traditional Chinese pharmacy (Baicaotang, Putian, China). SPS was prepared by adding 20 g of each dried plant into 100 ml distilled water, which was heated for 20-30 min until it started to boil. After 30 min of boiling, the aqueous extract was filtered with filter screen (10x10-cm mesh) to remove any solid particles. SPS was then cooled down and used for subsequent analysis.

Cell culture and treatment. HeLa cells and human normal cervical fibroblasts were purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). The cell lines were cultured routinely for 48 h prior to passageing in Dulbecco's modified Eagle’s medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) and cultured in a 37°C humidified atmosphere containing 5% CO2.

For activation, the fibroblasts (1x10^5/ml) were treated with the DMEM culture media (CM, 2 ml) from the HeLa cell line for 48 h at 37°C. Knockdown of homeodomain-interacting protein kinase 1 antisense RNA (HIPK1-AS) in fibroblasts was achieved via transfection with lentivirus containing short hairpin RNA (shRNA) against HIPK1-AS: 5'-TGC TGTACAGCGGCGAGTCTGTTCACAGTTTGCCACTG ACTGACGTGGAACACTGCGCTGTA-3' (multiplicity of infection=10) using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). The plasmid construction and lentivirus package were performed by Shanghai Genechem Co. Ltd. (Shanghai, China). The untreated cells were used as control. Cells were plated in 6-well clusters and transfected for 48 h. Transfected cells were used in further assays; qPCR was performed to measure HIPK1-AS expression to determine whether transfection was successful.

SPS treatment. Fibroblasts (1x10^5/ml) were treated with SPS (0.1, 0.5, 1, 2, 3 and 4 mg/ml) for 48 h or 2 mg/ml SPS for 0, 24, 48, 72 and 96 h at 37°C. As the proliferation rate of the control group was similar to that of cells treated with SPS (2 mg/ml), this concentration was selected for further analysis. Cells untreated with SPS served as the control.

Long non-coding (Inc)RNA array. The Arraystar Human LncRNA Microarray v4.0 (Affymetrix; Thermo Fisher Scientific, Inc.) was used for the global scanning of IncRNA expression in total RNA samples, which were extracted with TRIzol® (Thermo Fisher Scientific, Inc.) from untreated fibroblasts (control), HeLa-CM-treated fibroblasts and HeLa-CM together with SPS treated fibroblasts. Total RNA was analyzed by Kangchen BioTech Inc. (Shanghai, China) using Arraystar Human LncRNA Microarray v4.0 (Affymetrix; Thermo Fisher Scientific, Inc.). A total of 600 ng total RNA from each sample was employed; sample labeling (cyanine 3; Quick Amp Labeling kit, cat no. 5190-0442, Agilent Technologies, Santa Clara, CA, USA), microarray hybridization (Agilent Gene Expression Hybridization kit, cat no. 5188-5242, Agilent Technologies) and washing (Gene Expression Wash Buffer 1 and 2, cat no. 5188-5325 and 5188-5326, respectively, Agilent Technologies) were performed based on the manufacturer's standard protocols. The raw data were normalized with the quantile algorithm (Kangchen BioTech). Differentially expressed IncRNAs were then identified by analyzing fold change, as well as the P-value. The threshold set for significantly up- and downregulated genes was a fold change >2.0 and P<0.05.

Reverse transcription-quantitative polymerase chain (RT-qPCR) analysis. Total RNA was extracted from cells, including control (untreated) cells, cells treated with HeLa CM, cells treated with HeLa-CM plus SPS or HIPK1 shRNA, and the samples obtained from patients using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The expression levels of HIPK1-AS, fibroblast activation protein (FAP), IL-6, α-smooth muscle actin (α-SMA), programmed cell death protein 4 (PDCD4) were measured with a OneStep RT-PCR kit (Qiagen, Inc., Valencia, CA, USA) according to the manufacturers’ protocols on CFX96 Touch™ Deep Well Real-Time PCR Detection system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The expression of β-actin served as an endogenous control. The primer sequences were as follows: HIPK1-AS, sense 5'-GCCCTTACAGGAGGAGCT-3', antisense, 5'-CCACACTTGGGATAGTGAAC-3'; FAP, sense 5'-TTGAAATTTGATAGTGAAC-3', antisense, 5'-CCG ATCAATTCGCCGAATTA-3'; IL-6, sense 5'-TCTCAA CCCCATAATATAGGAC-3', antisense, 5'-GATGCC
GTCGAGGATGTACC-3′; α-SMA, sense 5′-TCCGCTTCAATCTCTGGCCGTA-3′, antisense, 5′-CAGGATTCCGTGCTTATTGCC-3′; PDCD4, sense 5′-ACCTGCTAGATCTCTGATAACT-3′, anti-sense, 5′-TTGGAGCTGGTGACAGT-3′; and β-actin, sense 5′-TGTTACAGGAGTTCCCCCTGCG-3′, anti-sense, 5′-ATGCTATCCACTCCCCGTGTG-3′. qPCR was performed as follows: 95°C for 3 min, and 39 cycles of 95°C for 10 sec and 60°C for 30 sec. The experiment was repeated in triplicate. Data were processed using the 2-ΔΔCT method (14).

CCK-8 cell proliferation assay. Cell proliferation rates of fibroblasts were measured using a Cell Counting Kit-8 (CCK-8; Beyotime Institute of Biotechnology, Hangzhou, China). A total of 0.5x10^4 cells treated with SPS (2 mg/ml for 0, 24, 48, 72 and 96 h, or 0.1, 0.5, 1, 2, 3 and 4 mg/ml for 48 h) were seeded in each 96-well plate for 24 h, and further incubated for 24, 48 and 72 and 96 h at 37°C, respectively. CCK-8 reagent (10 µl) was added to each well at 1 h at 37°C prior to the endpoint of each respective incubation. The optical density value in each well was determined at a wavelength of 490 nm using a microplate reader. Proliferation rate was calculated as (optical density value of SPS treatment group/optical density value of control group)×100%.

Annexin V-fluorescein isothiocyanate (FITC) staining and flow cytometry. Staining was performed with Annexin V-FITC kit according to the manufacturer’s protocols (Nanjing KeyGen Biotech, Co., Ltd., Nanjing, China). Briefly, 2x10^5 cells were harvested by centrifugation at 1,000 x g for 5 min at room temperature and resuspended in 100 µl binding buffer (contained in the kit), followed by a 15 min incubation with 5 µl Annexin V-FITC in the dark at 37°C. Subsequently, 10 µl propidium iodide was added with gentle agitation for 10 min in the dark at 37°C. A flow cytometer (BD Biosciences, San Jose, CA, USA) was employed for detecting apoptotic events and FlowJo (version 10.4.2, FlowJo LLC, Ashland, OR, USA) was used to analyze the apoptotic rate.

Immunofluorescence assays. Cells, including control (untreated), cells treated with HeLa CM and cells treated with HeLa-CM plus HIPK1 shRNA, cultured on coverslips (1x10^5/ml) for 48 h at 37°C were fixed with 4% formaldehyde for 30 min at room temperature and then permeabilized with 0.5% Triton-X-100 in PBS for 20 min. Subsequently, cells were blocked with non-fat 5% milk in tris-buffered saline and 0.1% Tween-20 for 60 min at 37°C, incubated with anti-α-SMA antibody (cat. no. ab5694, 1:200, Abcam, Cambridge UK) at 4°C overnight. The cells were incubated with Cy3 Goat Anti-Rabbit IgG (cat. no. C2821, 1:200, Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at 37°C for 1 h. The coverslips were stained with DAPI (1:2,000, SC-3598, Santa Cruz Biotechnology, Inc., Dallas, TX, USA) for 2 min at room temperature and mounted on slides using anti-fade mounting medium (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China). Immunofluorescence images were acquired using a fluorescence microscope (Nikon Eclipse 80i, Nikon Corporation, Tokyo, Japan). An excitation wavelength of 552 nm and x100 magnification were applied for immunofluorescence analysis. ImageJ (version 1.8, National Institutes of Health, Bethesda, MD, USA) was used to analyze fluorescence intensity.

Statistical analysis. In the present study, all experiments were repeated at least three times, and all data were expressed as the mean ± standard error of the mean. SPSS software, version 18.0 (SPSS, Inc., Chicago, IL, USA) was used to perform statistical analysis. Differences between two groups were compared with an independent-samples t-test. Differences among three or more groups were compared with one-way analysis of variance with Tukey’s post-hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of SPS on fibroblast proliferation and apoptosis. To investigate the role of SPS on the proliferation and apoptosis of fibroblasts, cells treated with different concentrations SPS and the effects of various durations of treatment with 2 mg/ml SPS were analyzed. Fibroblasts were treated with SPS at 0.1 to 4 mg/ml for 48 h. The results of the present study revealed that SPS did not induce significant inhibition of fibroblast proliferation; however, a notable reduction with 4 mg/ml SPS was observed (Fig. 1A). Cells were treated with SPS (2 mg/ml) for various durations. The results revealed that there were no significant differences in cell viability between the control and SPS-treated groups (Fig. 1B). In addition, the role of SPS in fibroblast apoptosis was investigated in the present study. The results demonstrated that treatment with serial concentrations of SPS and treatment of varying durations of 2 mg/ml SPS did not significantly induce cell apoptosis (Fig. 1C and D, respectively).

SPS inactivates fibroblasts to reduce the release of proinflammatory factors. As significant effects of SPS on fibroblast proliferation and apoptosis were not observed, the present study investigated whether SPS may regulate the activation of fibroblasts. The fresh CM obtained from 48 h of culture from HeLa cells was used to induce the activation of fibroblasts. The results of the present study revealed that HeLa CM induced a significant increase in the expression levels of FAP, IL-6 and α-SMA (Fig. 2A-C, respectively), but significantly reduced the expression of PDCD4 (Fig. 2D) compared with the control group. Significant inhibition in the expression of FAP, IL-6 and α-SMA expression, and significantly increased the expression of PDCD4 (Fig. 2) compared with the HeLa CM group, suggesting that SPS may reverse HeLa CM-induced activation of fibroblasts.

SPS regulates IncRNA expression in fibroblasts. To investigate the underlying mechanism by which SPS may inactivate and prevent activation of fibroblasts, an IncRNA array was performed to screen genes and IncRNAs that may be associated with the underlying molecular mechanism in cells. Numerous differentially expressed IncRNAs were analyzed in HeLa CM-treated cells and HeLa CM together
with SPS-treated cells compared with the control group. The results of the present study revealed that lncRNA HIPK1-AS expression may be induced by HeLa CM, whereas treatment with SPS was associated with the reduction in HIPK1-AS expression (Fig. 3A). To further investigate this difference, cervical tissue samples were obtained, including cervicitis, CIN I, CIN II-III and cervical squamous cell carcinoma (SCC). HIPK1-AS expression levels were significantly increased in cervicitis tissues compared with in normal cervical tissues; the expression levels of HIPK1-AS were significantly higher in SCC tissues and CIN II-III tissues than in cervicitis tissues (Fig. 3B). In addition, the results of the present study indicated that HIPK1-AS expression levels were significantly increased in fibroblasts in response to HeLa CM compared with in the control; this upregulation was also significantly inhibited by treatment with SPS.
Knockdown of HIPK1-AS by shRNA inactivates fibroblasts and reduces the release of proinflammatory factors. The role of HIPK1-AS in fibroblast activation was investigated in the present study. HIPK1-AS expression was significantly downregulated via lentiviral infection with HIPK1-AS compared with the control (Fig. 4A). The present study reported that the downregulation of HIPK1-AS significantly inhibited HeLa CM-mediated upregulation of FAP, IL-6 and α-SMA expression levels, and the downregulation of PDCD4 (Fig. 4B-E). The immunofluorescence results also indicated that HIPK1-AS knockdown reduced the number of HeLa CM-activated fibroblasts (Fig. 4F). These results indicate that HIPK1-AS knockdown may inhibit HeLa CM-mediated activation of fibroblasts and the release of proinflammatory factors.

Discussion

In the present study, the proliferative and apoptotic abilities of fibroblasts were not markedly altered under treatment with serial concentrations of SPS, or treatment with SPS for various durations; however, SPS may reverse the activation of fibroblasts and release of proinflammatory factors mediated by HeLa CM.

The use of *S. japonica* (Fabaceae), also known as Huai (Chinese), has been recorded in classical medicinal treatises of ancient China, and it is currently recorded in the Chinese Pharmacopoeia and European Pharmacopoeia (10). Numerous flavonoids and isoflavonoids comprise the active constituents of *S. japonica*. These chemical compounds exhibit a wide range of biological activities *in vitro* and *in vivo*, including anti-inflammatory, antioxidant and antitumor properties (15,16). Oxymatrine, a potent monosomic alkaloid extracted from *S. japonica*, may inhibit the production of tumor necrosis factor-α, IL-1β and IL-6, and may also inhibit the extracellular signal-regulated kinase, p38 and c-Jun N-terminal kinase signaling pathways in lipopolysaccharide-stimulated microglial cells (17). In addition, oxymatrine was reported to exhibit anti-inflammatory properties in septic shock-induced myocardial injury via the inhibition of the Janus kinase 2/signal transducer and activator of transcription 3 signaling pathway (18). Sophoricoside was isolated from immature fruits of *S. japonica* and was reported to inhibit the bioactivity of IL-6 (19). UP1306, a proprietary extract of *M. alba*, was demonstrated to reduce bone and cartilage degradation via the reported inhibition of catabolic proinflammatory signaling pathways (20). Morusin, a prenylated flavonoid isolated from the root bark of *M. alba*, may reduce tissue damage in an animal model of 2′4′6-trinitrobenzene-induced colitis (21). In the present study, it was
revealed that SPS may attenuate HeLa CM-mediated fibroblast activation and the release of proinflammatory factors; however, the specific chemical components in SPS that serve a role in fibroblast activation require further validation.
Furthermore, the present study investigated the potential mechanism underlying fibroblast inactivation and prevention of proinflammatory factor release mediated by SPS. By using an lncRNA array, the expression levels of HIPK1-AS were observed to be induced by HeLa CM and inhibited by SPS in fibroblasts. In addition, the findings of the present study demonstrated that HIPK1-AS expression levels were increased in cervicitis, CIN I-III and SCC tissues. Loss of function experiments revealed that knockdown of HIPK1-AS inhibited the activation of fibroblasts and proinflammatory factor release mediated by HeLa CM. HIPK1-AS is a novel lncRNA that has been located on the host gene HIPK1 (chromosome 1: 113,924,001-113,929,258 reverse strand); the transcript contains 5 exons and maps to 276 oligo probes (22,23). HIPKs regulate cell differentiation, proliferation and apoptosis (24). HIPK1 was identified as a oncoprotein in lung adenocarcinoma cells (25). Additionally, HIPK1 mRNA was reported to be a translational target of PDCD4 (26). HIPK1 stimulates the translation of its own mRNA; PDCD4 may suppress the translation of HIPK1 mRNA by interfering with this auto-regulatory feedback mechanism (26). In the present study, it was reported that HIPK1-AS knockdown increased the expression of PDCD4; however, the underlying mechanism requires further investigation. IL-6 has been associated with several stages of tumor development by mediating epithelial-stromal interactions (27). The upregulation of IL-6 has been reported to serve a significant role in the pathogenesis of cervical cancer, and has been frequently detected in the stromal region of cervical cancer tissues (28). Additionally, IL-6 was reported to be co-expressed with α-SMA in fibroblasts (29,30); combined with transforming growth factor-β, α-SMA and IL-6 have been suggested to interact with PDCD4 in the tumor stroma (27).

As a possible limitation of the present study, the dried components of SPS were not ground during the preparation of SPS. Further investigation may be performed in the future to investigate whether more notable effects occur with various methods of SPS preparation; the effects of SPS in vivo require further study.

In conclusion, the present study demonstrated that SPS attenuated HeLa CM-mediated fibroblast activation and the release of proinflammatory factors by inhibiting HIPK1-AS expression. HIPK1-AS may be associated with cervical lesions, as well as cervicitis; the upregulation of HIPK1-AS in the tumor stroma may serve a role in the inflammatory process and the progression of cervical cancer. Therefore, HIPK1-AS and SPS may be considered as a therapeutic target and agent, respectively, in the treatment of cervical cancer; however, further investigation is required.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Author contributions

RQ and LY made substantial contributions to the design of the study. BZ and YY analyzed and interpreted the patient data. BQ and YY performed cell biological experiments. BZ and BQ performed quantitative polymerase chain reaction and immunofluorescence staining. All authors contributed to writing the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of the Longyan First Hospital (Fujian, China). Written informed consent was obtained from all patients.

Consent for publication

Not applicable.

Conflict of interest

The authors declare that they have no competing interests.

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