Abstract. Musk ketone exerts antiproliferative effects on several types of cancer, such as lung and breast cancer. However, the effects and underlying mechanisms of action of musk ketone in gastric cancer (GC) are poorly understood. The present study aimed to investigate the effects of musk ketone in GC cells. The present study indicated that musk ketone exerted significant anticancer effects on GC cells. The IC_{50} values of musk ketone were 4.2 and 10.06 µM in AGS and HGC-27 cells, respectively. Low dosage of musk ketone significantly suppressed the proliferation and colony formation of AGS and HGC-27 cells. Cell cycle arrest and apoptosis were induced by musk ketone. Furthermore, microarray data indicated that musk ketone treatment led to downregulation of various genes, including sorbin and SH3 domain containing 2 (SORBS2). Reverse transcription-quantitative PCR and immunoblotting results indicated that musk ketone repressed mRNA and protein expression levels of SORBS2. It was also shown that knockdown of SORBS2 inhibited the proliferation and colony formation of HGC-27 cells. The antiproliferative effects of musk ketone were decreased in HGC-27 cells with SORBS2 silencing. In summary, the present study indicated that musk ketone suppressed the proliferation and growth of GC partly by downregulating SORBS2 expression.

Introduction

Gastric cancer (GC) is the fourth most frequent malignant tumor and the second leading cause of cancer-related mortality globally (1). Over 950,000 patients are diagnosed with GC annually and ~750,000 cases succumb to this malignancy (2). Helicobacter pylori (H. pylori) infection is a major risk factor for GC (3). Following H. pylori infection, chronic inflammation is induced in the stomach, which is accompanied by abnormal cell proliferation, apoptosis and certain genetic or epigenetic changes, eventually leading to carcinogenesis (4). Following diagnosis, the preferred treatment for patients with GC is surgery. However, the majority of these patients relapse, and other treatment strategies, including endoscopic therapy, radiotherapy and systemic therapy, may be beneficial for certain patients (5). Unfortunately, the 5-year overall survival of GC is poor and the mortality rate is very high (1). The development of novel drugs may improve the efficacy of therapeutic options for patients with GC.

Traditional Chinese medicine (TCM) is widely used in China for the treatment of various illnesses, such as cancer and depression (6-8). Musk is an essential material used in the perfume industry (9). Musk ketones are the major components of musk and are included in the TCM concoctions. Recently, musk ketone has attracted the attention of researchers from different scientific fields (10,11). Increasing evidence has demonstrated that musk ketone may be helpful for the prevention of certain diseases. For example, musk ketone was found to significantly repress the growth and induce the apoptosis of lung cancer cells, whereas the expression levels of IL-24 and DNA damage-inducible transcript 3 protein were upregulated following musk ketone treatment (11). In addition, musk ketone was shown to induce the growth and differentiation of neural stem cells in cerebral ischemia by activating the PI3K/AKT signaling pathway (10). However, the effects and underlying mechanisms of musk ketone in GC remain unclear.

The present study aimed to explore the antitumor effects of musk ketone in GC. The IC_{50} value of musk ketones was assessed in AGS and HGC-27 cells. Based on the results, a specific concentration was used for GC cell treatment. Cell proliferation and colony growth were examined following musk ketone treatment, as were cell cycle progression and apoptosis. It was also investigated whether this compound
induced dysregulation of numerous genes at the molecular level, including sorbin and SH3 domain containing 2 (SORBS2). The present study sought to determine whether musk ketone can suppress GC cell growth by regulating SORBS2 expression.

Materials and methods

Cell lines and cell culture. The human GC cells AGS and HGC-27 were purchased from the American Type Culture Collection. AGS and HGC-27 cells were cultured in Dulbecco’s modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.), which was supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin and streptomycin solution (Gibco; Thermo Fisher Scientific, Inc.). All the cells were maintained at 37°C with 5% CO₂.

Measurement of the IC₅₀ of musk ketone in AGS and HGC-27 cells. Musk ketone (purity ≥98%) was purchased from Sigma-Aldrich (Merck KGaA). The IC₅₀ of musk ketone was detected in AGS and HGC-27 cells. Briefly, a total of 2,000 AGS and HGC-27 cells were seeded in triplicate in 96-well plates. Different dosages (0, 0.0031, 0.031, 0.31, 3.1, 31 µM) of musk ketone were added into each well. After 48 h, the culture medium was removed and each well was added with 100 µl culture medium and 10 µl Cell Counting Kit-8 (CCK-8; Beyotime Institute of Biotechnology). After incubation for 3 h, cell viability was analyzed by measuring the OD450.

SORBS2 interference. Small interfering (si)RNAs against siCtrl (5'-UAUUCUCCGAUCGUACGUU-3'), siSORBS2-1 (5'-AUACCCCAUCGUUACUAGU-3') and siSORBS2-2 (5'-GGGCAUCUUCGCAUCUAC-3') were synthesized from Huzhou Hippo Biotechnology Co., Ltd. A total of 3x10⁵ cells were seeded in 6-well plates and transfected with siRNAs (50 nM/well) using RNAiMAX (4 µl/well; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocols. After 48 h, knockdown efficacy was determined and the cells were subjected to cell function experiments.

SORBS2 overexpression. pCDNA3.1 plasmids (Tianyi Huiyuan Biotech Co., Ltd.) were used to overexpress SORBS2 in GC cells. AGS and HGC27 cells were seeded in 6-well plates at a density of 5x10⁵ cells/well. Cells were transfected with pCDNA3.1-empty and pCDNA3.1-SORBS2 plasmids (5 µg/well) using VigoFect for 6 h at room temperature (2 µg/well; Vigorous Biotechnology Beijing Co., Ltd.). After 48 h, cells were subjected to immunoblotting and apoptosis analysis.

Cell viability analysis. To determine the anticancer effects of musk ketone in GC cells, equal numbers of AGS and HGC-27 cells were seeded in 96-well plates, which contained 100 µl DMEM. Musk ketone was added and the cells were maintained for 1-3 and 4 days. Finally, 10 µl CCK-8 solution was added to each well and the wells were incubated at 37°C for 3 h. Cell viability was analyzed by measuring the OD450.

Colony formation assay. A total of 1,000 AGS and HGC-27 cells were seeded in triplicate in 6-well plates to observe colony formation. The cells were incubated with vehicle or musk ketone. Following 7 days of culture, the colonies were washed with PBS three times. Subsequently, the colonies were fixed with 100% methanol at room temperature for 15 min and stained with 0.2% crystal violet solution at room temperature for 30 min. Images were captured using a camera (Nikon Corporation). Cell colonies (>50 cells) were counted manually.

Reverse transcription-quantitative PCR (RT-qPCR). RNA extracted from indicated cells were subjected to reverse transcription and cDNA quantification, according to the methods as described previously (12). qPCR was performed using the SYBR Premix Ex Taq™ II kit (Takara Biotechnology Co., Ltd.). The following thermocycling conditions were used for qPCR: 94°C for 5 min, 40 cycles of 94°C for 5 sec and 60°C for 1 min. Relative expression levels were calculated using the 2⁻ΔΔCq method (13). The primer sequences were as follows: SORBS2 forward, 5'-AAAGACCCATGAGTT CTGCAAG-3' and reverse, 5'-GCTCGCACCTTTGATCTCC CA-3; β-actin forward, 5'-GAGCTGCGTGGTGGTCC-3' and reverse, 5'-CCAGAGCCGTACAGGAGATGCA-3'; and GAPDH forward, 5'-GACTCATGACCACTCATTGC-3' and reverse, 5'-AGAGGCCAGGATGATTTCTG-3'.

Western blotting. Total proteins were extracted from GC cells treated with vehicle or musk ketone using lysis buffer (Beyotime Institute of Biotechnology). The concentration of total proteins was detected by the BCA Protein Assay Kit. A total of 30 µg protein per lane were separated on 12% SDS-PAGE, and were subsequently transferred onto PVDF membranes. Following blocking with 5% skimmed milk at room temperature for 2 h, the membranes were incubated with the indicated primary antibodies at 4°C overnight, followed by incubation with HRP-conjugated secondary antibodies at room temperature for 2 h. Protein expression was detected by SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific, Inc.) on a Fusion system. Image-Pro Plus software v6.0 (Media Cybernetics, Inc.) was used to analyze the protein signals. The antibody against SORBS2 (1:1,000; cat. no. 24643-1-AP) was obtained from ProteinTech Group, Inc. Antibodies against cleaved (cle)-caspase 3 (1:500; cat. no. ab32042) and caspase 3 (1:500; cat. no. ab184787) were from Abcam. The GAPDH primary antibody (1:4,000; cat. no. sc-47724) and horseradish peroxidase-conjugated secondary antibodies (both 1:10,000; normal mouse IgG, cat. no. sc-2748; normal rat IgG, cat. no. sc-2750) were purchased from Santa Cruz Biotechnology, Inc.

Cell cycle analysis. The cell cycle was examined using propidium iodide (PI) staining (Cell Cycle and Apoptosis Analysis kit; Shanghai Yeasen Biotech Co., Ltd.). Briefly, a total of 2x10⁶ GC cells were seeded in 6-well plates and treated with vehicle or musk ketone. Following 48 h of incubation, the cells were fixed with 70% alcohol overnight on ice. Subsequently, the cells were stained with staining buffer at 37°C for 30 min and the cell cycle was analyzed by flow cytometry (cytoFlex, Beckman Coulter, Inc.). The data were analyzed by CytExpert (version 2.4.0.28; Beckman Coulter, Inc.).

Apoptosis assay. The induction of apoptosis was detected using PI/Annexin V staining (Annexin V-FITC/PI Apoptosis Detection kit, Shanghai Yeasen Biotech Co., Ltd.), according
to the manufacturer's protocols. The cells were washed with PBS and stained with PI and Annexin V in room temperature for 15 min in the dark. The level of apoptosis was analyzed by flow cytometry (cytoFlex, Beckman Coulter, Inc.). The data were analyzed by cytexpert (Version 2.4.0.28; Beckman Coulter, Inc.). The FITC+/PI− cells represent early apoptosis, while FITC+/PI+ cells represent late apoptosis.

**Transwell assay.** A total of 8x10⁴ HGC27 cells in 200 µl FBS-free DMEM were seeded in upper surface of 8.0-µm filter migration chambers (Corning, Inc.). A total of 500 µl complete DMEM with 10% FBS (both Gibco; Thermo Fisher Scientific, Inc.) was added in lower compartment of 24-well plates. The plates were maintained in the cell incubator. After 24 h, the cells attached on the upper surface were removed and the cells attached on the lower surface were fixed with 100% methanol and were stained with 0.2% crystal violet solution at room temperature for 30 min.

**Microarray analysis.** Total RNA was extracted from AGS cells treated with vehicle or musk ketone using TRIzol® reagent (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocols. Microarray analysis was performed by Shanghai OE Biotech Co., Ltd. The dysregulated genes were identified based on statistical significance at P<0.05 and fold-change >1.5.

**Statistical analysis.** All the quantification results and statistical significance were analyzed using GraphPad Prism software 6.0 (GraphPad Software, Inc.). The enrichment analysis of signaling pathways were assessed by Gene Set Enrichment Analysis (GSEA; version 4.0.3) (14). The results are shown as the mean ± standard error of the mean for three independent experiments. Unpaired Student's t-test was applied to analyze the differences between the two groups. One-way ANOVA followed by a Tukey's post hoc test was applied to analyze the differences among groups. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**Musk ketone significantly represses the proliferation of GC cells.** To explore the inhibitory effects of musk ketone on GC cells, the IC₅₀ of this compound was evaluated in AGS and HGC27 cells. These cell lines were seeded in 96-well plates and were treated with different concentrations of musk ketone. Cell viability was detected by the CCK-8 assay. The IC₅₀ values of musk ketone in AGS and HGC27 cells were 4.2 and 10.06 µM, respectively. Subsequently, the time-dependent inhibitory effects of musk ketone on GC cells were examined. Briefly, equal numbers of AGS and HGC27 cells were seeded in 96-well plates and the cells were incubated with vehicle or musk ketone. Cell proliferation was assessed by CCK-8 on days 1-3 and 4. The data indicated that musk ketone inhibited the proliferation of AGS and HGC27 cells on day 1 following treatment. This inhibitory effect was more prominent when the cells were treated for longer time periods (Fig. 1C and D).
Figure 2. Musk ketone induces cell cycle arrest and apoptosis of AGS and HGC-27 cells. (A and B) AGS cells were incubated with vehicle or 4.2 µM musk ketone for 48 h. The induction of apoptosis was detected by PI/Annexin V staining; (A) representative images and (B) quantification of apoptosis. (C and D) HGC-27 cells were incubated with vehicle or 10.06 µM musk ketone for 48 h. The induction of apoptosis was detected using PI/annexin V staining; (C) representative images and (D)  quantification of apoptosis. (E) Immunoblotting analysis of Cle-caspase 3 and caspase 3 in cells described in (A) and (C). (F and G) The cells described in (A) were analyzed using cell cycle analysis by PI staining; (F) representative images and (G) quantification of the cell cycle. (H and I) The cells described in (C) were analyzed by PI staining; (H) representative images and (I) quantification of the cell cycle. **P<0.01, ***P<0.001 vs. vehicle group. PI, propidium iodide; Cle-, cleaved.
To confirm this hypothesis, the colony formation assay was performed in AGS and HGC-27 cells treated with or without musk ketone. The data indicated that AGS and HGC-27 cells incubated with vehicle formed a significantly higher number of colonies compared with those incubated with musk ketone (Fig. 1E-H). These results suggested that musk ketone exerted a suppressive effect on GC cells.

**Musk ketone promotes cell cycle arrest and apoptosis.** Cell cycle and apoptosis deregulation are hallmarks of cancer. It was herein examined whether musk ketone regulated cell cycle arrest and apoptosis. AGS and HGC-27 cells were treated with vehicle and musk ketone for 48 h. The cells were harvested for cell cycle and apoptosis analyses. It was demonstrated that musk ketone treatment enhanced apoptosis of AGS and HGC-27 cells (Fig. 2A-D). Moreover, musk ketone treatment resulted in increased ratio of Cle-caspase 3 to caspase 3 in both cells (Fig. 2E). Furthermore, musk ketone treatment increased the percentage of cells at the G0/G1 phase and decreased the percentage of cells at the S phase in both cell lines (Fig. 2F-I). These results indicated that musk ketone treatment led to cell cycle arrest and increased apoptosis of GC cells.

**Gene expression profiling following musk ketone treatment.** To profile the downstream effectors of musk ketone, AGS cells were treated with vehicle or musk ketone and subjected to microarray analysis. Thousands of genes were regulated by musk ketone, including 2,657 upregulated and 1,728 downregulated genes (Fig. 3A and Table S1). GSEA indicated that ‘Pathways In Cancer’ and ‘Cell Cycle’ were negatively regulated by musk ketone (Fig. 3B and C). In addition, microarray analysis indicated downregulation of SORBS2 (Fig. 3A). Furthermore, western blotting and RT-qPCR analyses confirmed that musk ketone repressed the expression of SORBS2 (Fig. 3D and E).

**Knockdown of SORBS2 suppresses the proliferation and growth of GC cells.** To investigate the role of SORBS2 downregulation in GC, the SORBS2 gene was knocked down in HGC-27 cells. Western blotting and RT-qPCR analyses indicated that SORBS2 was efficiently silenced by siRNA treatment (Fig. 4A and B). The cells were subsequently analyzed by CCK-8 and colony formation assays. The data indicated that SORBS2 knockdown significantly suppressed the proliferation and colony formation of HGC-27 cells (Fig. 4C-E). Furthermore, apoptosis was induced by SORBS2 knockdown (Fig. 4F and G). By contrast, SORBS2 overexpression, which was verified via western blotting (Fig. S1A), suppressed the apoptosis of GC cells (Fig. S1B and C). Transwell results indicated that SORBS2 knockdown suppressed the migration of HGC-27 cells (Fig. 4H and I).
Figure 4. Knockdown of SORBS2 inhibits the proliferation of gastric cancer cells. (A) Reverse transcription-quantitative PCR analysis of SORBS2 in siCtrl-, siSORBS2-1- and siSORBS2-2-transfected HGC-27 cells. **P<0.01 vs. siNC group. (B) Immunoblotting analysis of SORBS2 in siCtrl-, siSORBS2-1- and siSORBS2-2-transfected HGC-27 cells. (C) Cell proliferation was detected by the Cell Counting Kit-8 assay in siCtrl-, siSORBS2-1- and siSORBS2-2-transfected HGC-27 cells. **P<0.01, ***P<0.001 siSORBS2-1 vs. siCtrl; ##P<0.01, ###P<0.001 siSORBS2-2 vs. siCtrl. (D and E) Colony formation was analyzed in siCtrl-, siSORBS2-1- and siSORBS2-2-transfected HGC-27 cells. Left, images of colonies. Right, quantification results. (F and G) Apoptosis was detected in siCtrl-, siSORBS2-1- and siSORBS2-2-transfected HGC-27 cells. Left, images of apoptosis. Right, quantification results. (H and I) The migratory activity of siCtrl-, siSORBS2-1- and siSORBS2-2-transfected HGC-27 cells was assessed by Transwell assay. Left, images of migration. Right, quantification results. **P<0.01, ***P<0.001 vs. siNC group. SORBS2, sorbin and SH3 domain containing 2; si, small interfering RNA; NC, negative control; Ctrl, control.
These results suggested that SORBS2 functioned as a potential oncogene in GC.

**Knockdown of SORBS2 reduces the anticancer effects of musk ketone.** To validate whether the suppression of GC cell proliferation was dependent on the expression of SORBS2, siNC and siSORBS2-transfected HGC-27 cells were treated with vehicle or musk ketone. It was observed that musk ketone significantly suppressed the proliferation and colony formation of siNC HGC-27 cells, whereas it exerted no obvious effects on siSORBS2 HGC-27 cells (Fig. 5A-C). These results suggested that the anticancer effects of musk ketone may be mediated via regulating SORBS2 expression.

**Discussion**

TCM has long been used in China to treat several diseases, including depression, gastric precancerous lesions and postoperative abdominal adhesions (7,15,16). The most well-known TCM drug is artemisinin (qinghaosu), which has potent therapeutic effects against malaria (17-19). Recently, increasing evidence has demonstrated that TCM is a promising approach in the treatment of malignancies. In the present study, musk ketone, which is a TCM compound, markedly suppressed the proliferation of GC cells. Musk ketone treatment resulted in cell cycle arrest and apoptosis in AGS and HCG-27 cells. Transcript analysis of musk ketone-treated GC cells indicated...
that numerous genes were dysregulated following musk ketone treatment.

The initial evidence indicating that musk ketone may be used for cancer treatment dates back to the 1990s (20). Musk is the major ingredient of this compound. Two years later, Zheng et al (20) demonstrated that the musk residue, which contained musk ketone, could be used as a chemopreventive agent. A toxicity study based on an in vivo mouse lymphoma model and an in vitro unscheduled DNA synthesis and cytogenetics assays revealed that musk ketone did not possess genotoxic potential (21). Recently, Xu and Cao (11) demonstrated that musk and musk ketone exerted suppressive effects on the proliferation and growth of lung cancer cells. However, the role of musk ketone in GC remains poorly understood. Therefore, the present study attempted to determine the IC50 of musk ketone in GC cells. The IC50 values were estimated to be 4.2 and 10.06 µM in AGS and HGC-27 cells, respectively. One dose of musk ketone could significantly repress the proliferation and colony formation of both cell types. Furthermore, musk ketone treatment resulted in cell cycle arrest and enhanced apoptosis in AGS and HGC-27 cells. These results suggested that musk ketone exerted potent anticancer effects on GC.

SORBS2, also referred to as ArgBP2, is located on chromosome 4. Physiologically, SORBS2 regulates actin dynamics, cytoskeleton establishment and signal transduction (22,23). Dysregulation of SORBS2 participates in cancer development. For example, the RNA-binding protein SORBS2 functions as a tumor suppressor in hepatocellular carcinoma (HCC) by regulating nuclear receptor ROR-a mRNA transcription (24). Furthermore, SORBS2 suppresses the metastasis of HCC by inhibiting the ERK signaling pathway (25). In addition, SORBS2 suppresses ovarian cancer metastasis by modulating tumor-suppressive immunomodulatory transcripts (26). However, SORBS2 can promote cell growth and inhibit cell apoptosis in human renal glomerular endothelial cells and human glomerular mesangial cells (27). These studies suggest that SORBS2 may play a distinct role on cell proliferation and apoptosis in a context-dependent manner. In GC, SORBS2 is downregulated by heat shock factor protein 1, which promotes the proliferation and invasion of GC cells (28). However, the association between musk ketone and SORBS2, as well as the precise function of SORBS2 in GC, are largely unknown. Based on the microarray data, the present study indicated that musk ketone significantly reduced the expression levels of SORBS2 in GC cells. Since SORBS2 is important for actin dynamics, maintaining the cell cytoskeleton and signal transduction (22,23), it was predicted that musk ketone suppresses the growth of GC cell at least partly through regulating the expression of SORBS2. Based on loss-of-function and gain-of-function experiments, the present study demonstrated that SORBS2 expression was essential to maintain the proliferation of GC cells. Of note, SORBS2 silencing decreased the sensitivity of GC cells to musk ketone treatment, indicating that SORBS2 may act as an oncogene in GC, and that the expression levels of SORBS2 in GC cells may have a role in the efficacy of musk ketone treatment.

There were certain limitations in the present study. The effect of musk ketone on the tumor growth of GC cells in nude mice was not investigated and the molecular mechanisms by which musk ketone regulates SORBS2 are still unknown. Further studies are required to address these points.

In summary, the present study provided initial evidence that musk ketone is a promising TCM compound for GC treatment. The IC50 of musk ketone was determined in different GC cells. Musk ketone significantly suppressed the proliferation, colony formation and cell cycle progression of GC cells and enhanced apoptosis. At the molecular level, musk ketone downregulated SORBS2 expression in GC cells. Finally, silencing of SORBS2 reduced GC cell proliferation and the sensitivity of GC cells to musk ketone treatment.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Authors' contributions
JA and HYW designed the study. JA, HYW, XMM, BWH, YFY, YPY and ZHS performed the experiments and analyzed the data. JA and HYW wrote the manuscript draft. ZHS revised the manuscript. All authors have read and approved the final version of the manuscript. JA and HYW confirmed the authenticity of all the raw data.

Ethics approval and consent to participate
Not applicable.

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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