Casticin inhibits the epithelial-mesenchymal transition in ovarian carcinoma via the hedgehog signaling pathway

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Abstract. Casticin inhibits migration, invasion and induced apoptosis in numerous cancer cells; however, the Hedgehog (Hh) signaling pathway is a key factor in the epithelial-mesenchymal transition (EMT). The present study aimed to assess whether casticin affects the expression of members of the Hh signaling pathway and EMT effectors in ovarian carcinoma. The ovarian cancer SKOV3 cell line was incubated in the presence of various concentrations of casticin or cyclopamine. Next, the expression levels of the main Hh signaling effector glioma-associated oncogene-1 (Gli-1) and EMT-associated factors [Twist-related protein 1 (Twist1), E-cadherin and N-cadherin] were determined by western blotting and reverse transcription-quantitative polymerase chain reaction. Cell proliferation and growth were assessed using MTT and soft agar assays; cell migration and invasion was evaluated using an in vitro migration assay and a transwell invasion assay, respectively. Compared with control group values, Gli-1, Twist1 and N-cadherin expression levels were reduced, whereas E-cadherin levels were increased in the casticin- and cyclopamine-treated groups. Incubation with casticin or cyclopamine resulted in markedly reduced SKOV3 cell viability, migration and invasion, in a dose-dependent manner. To the best of our knowledge, the findings of the present study indicated for first time that casticin may inhibit EMT via Hh signaling in vitro, reducing the migratory ability of ovarian cancer cells.

Introduction

Ovarian cancer, the second-leading cause of gynecological malignancy, is a major cause of cancer-associated mortality among women (1). Although patients usually exhibit an acceptable initial response to first-line therapy (cytoreductive surgery and combined platinum-paclitaxel chemotherapy), the majority of cancer cases recur, are invasive and result in patient mortality (2,3). The underlying mechanism responsible for invasion and metastasis in epithelial ovarian cancer remains unclear (4). Novel therapeutic agents are therefore required to improve patient survival.

The epithelial-mesenchymal transition (EMT), which causes epithelial cells to become similar mesenchymal cells with increased motility, serves a notable role in embryonic development (5). A notable hallmark of EMT is the reduced or lack of expression of the epithelial marker E-cadherin and induction of expression of the mesenchymal marker N-cadherin (6), which promotes metastatic disease (7). EMT is involved in cancer progression, and represents the main event that promotes tumor invasion and metastasis (8,9). Therefore, targeting EMT may aid the development of potentially effective therapies.

Hedgehog (Hh) signaling serves a critical role in embryonic development and its abnormal induction is involved in tumorigenesis. Aberrant Hh activation has been demonstrated in breast, colon, and prostate cancer (10-12), and is closely associated with self-renewal, invasion and migration of cancer stem cells, promoting cancer progression (13,14). It was reported that Hh signaling might induce EMT in pancreatic cancer (9,15). In addition, Yue et al (16) reported that Hh also promoted EMT progression in lung squamous cell carcinoma. Notably, glioma-associated oncogene-1 (Gli-1), a major Hh signaling effector, decreases levels of E-cadherin and increases those of N-cadherin (17,18), indicating that Hh signaling is important for EMT occurrence. Cyclopamine, a smoothened (Smo) Hh signaling pathway inhibitor, is considered to be an anticancer molecule; however, its clinical application is markedly hampered by elevated costs and its instability in aqueous or polar solvents (19). Therefore, developing additional anticancer drugs remains critical.

Fructus viticis, the fruit of Vitex trifolia L. (Verbenaceae), has been used in traditional Chinese medicine for its anti-inflammatory effects for over a thousand years (20,21). Casticin, a biologically active component of Fructus viticis (20), possesses numerous biological and pharmacological effects, including...
immunomodulatory, anti-hyperprolactinemia, neuroprotective and anti-inflammatory properties (22-25), and alters tumorigenesis and tumor progression (26,27). Additionally, it has been demonstrated that casticin suppresses EMT in hepatocellular carcinoma (28). However, studies assessing whether casticin can suppress the EMT via Hh signaling in ovarian carcinoma are scarce. Western blot analysis and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) were used to evaluate the effect of casticin on Hh signaling, which modulates EMT and cell migration in ovarian carcinoma.

**Materials and methods**

**Cell culture and reagents.** Human ovarian cancer SKOV3 cells were obtained from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China), and cultured in Dulbecco’s modified Eagle’s medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin, and 100 U/ml streptomycin (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA), in a humidified atmosphere containing 5% CO₂ at 37°C. Casticin (purity ≥98%) was manufactured by Chengdu Biopurify Phytochemicals Ltd. (Chengdu, China) as yellow crystals (molecular weight, 374.3 Da). Cyclopamine was manufactured by Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Rabbit anti-Gli-1 antibody (1:1,000; cat. no. 2534) and Twist-related protein 1 (Twist1) antibodies (1:1,000; cat. no. 46702) (both from Cell Signaling Technology, Inc., Danvers, MA, USA), and rat anti-E-cadherin (1:1,000; cat. no. SAB4503751) and mouse monoclonal anti-N-cadherin (1:1,000; cat. no. C2542) (both from Sigma-Aldrich; Merck KGaA) antibodies were used in the present study. Goat anti-rabbit IgG, horseradish peroxidase (HRP)-linked antibody (cat. no. 7074; 1:1,000 dilution) and goat anti-mouse IgG, HRP-linked antibody (cat. no. 7076; 1:1,000 dilution) were both purchased from Cell Signaling Technology, Inc.

**Determination of cell proliferation and growth.** Cell proliferation and growth were assessed by MTT and Soft agar assays. For MTT assay, SKOV3 cells (10,000/well) were seeded in 96-well plates and incubated for 24 h prior to treatment with increasing casticin (0.0, 1.0, 2.0, 4.0 and 8.0 µM) or cyclopamine amounts (0.0, 1.25, 25.0, 50.0 and 100.0 µM) for 48 h. Subsequently, 20 µl MTT (5 mg/ml) was added for further 4 h. Dimethyl sulfoxide was used to dissolve the resulting formazan crystals; absorbance was read at 490 nm on Bio-Rad 550 (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The half-maximal inhibitory concentration (IC₅₀) causing 50% cell growth inhibition (IC₅₀) values were determined. Cell growth inhibition rate was determined by the 2ΔΔCq method (30) with GAPDH as an internal reference. The following primers were used: Gli-1 forward, 5'-TTCTTACCCAGGTCCGAGTT-3' and reverse, 5'-CCCTATGTGAACCCATATT-3'; Twist1 forward, 5'-CGAAGCTGAGAAGATGTT-3' and reverse, 5'-CCTTCTGTGGAAACATGAC-3'; N-cadherin forward, 5'-CTGGTGAAGTTTGCAGTGTG-3' and reverse, 5'-GGGATCTGCTTCTATCCTGGCCT-3'; E-cadherin forward, 5'-CTCCAGTTGATCAACATCCAG-3' and reverse, 5'-CGCCCTCTTCTCTACGATAG-3'; GAPDH forward, 5'-GGTGTTGATCCTCTGTTCA-3' and reverse, 5'-CTCAACAGACCTATGTTGT-3'.

**Migration assay.** When SKOV3 cells reached an optimal confluence (80-90%) in a 6-well culture plate, the monolayer was gently and slowly scratched with a clean 100 µl pipette tip across the center of the plate. Following two washes with PBS, casticin (2.0 µM) or cyclopamine (25.0 µM) were added at IC₅₀ values. The artificial wounds were imaged at 0 and 24 h, respectively, under an inverted light microscope at a x10 magnification (CKX41; Olympus Corporation, Tokyo, Japan).

**Transwell invasion assay.** For the invasion assay, 1x10⁶ SKOV3 cells treated with casticin or cyclopamine were seeded in the top chamber onto the Matrigel-coated membrane (24-well insert; pore size, 8 µm; Corning Life Sciences, Corning, NY, USA). Cells were placed in DMEM medium without serum or growth factors in the top chamber, and DMEM medium supplemented with 10% FBS was used in the lower chamber. The cells were incubated for 48 h and then cells that invaded via the pores were removed with a cotton swab. Cells on the lower surface of the membrane were fixed with 4% paraformaldehyde for 10 min at 25°C and stained with 10% Giemsa for 30 min at room temperature. The number of cells invading via the membrane was counted under a light microscope (magnification, x20; three random fields per well).

**RT-qPCR.** SKOV3 Cells were treated with casticin (2.0 µM) or cyclopamine (25.0 µM) for 0, 3, 6, 12 and 24 h, then RNA was extracted and cDNA prepared as previously reported (29). RT-qPCR was conducted with 1 µl cDNA on MyiQ real-time PCR Instrument (Bio-Rad Laboratories, Inc.) using iQ SYBR Green PCR Supermix (Bio-Rad Laboratories, Inc.), following the manufacturer’s instructions. The thermocycling conditions were as follows: 95°C for 3 min, 95°C for 15 sec, and 60°C for 20 sec, for 40 cycles. Relative gene expression levels were determined by the 2^ΔΔCq method (30) with GAPDH as an internal reference. The following primers were used: Gli-1 forward, 5'-TTCTTACCCAGGTCCGAGTT-3' and reverse, 5'-CCCTATGTGAACCCATATT-3'; Twist1 forward, 5'-CGAAGCTGAGAAGATGTT-3' and reverse, 5'-GGGATCTGCTTCTATCCTGGCCT-3'; E-cadherin forward, 5'-CTCCAGTTGATCAACATCCAG-3' and reverse, 5'-CGCCCTCTTCTCTACGATAG-3'; GAPDH forward, 5'-GGTGTTGATCCTCTGTTCA-3' and reverse, 5'-CTCAACAGACCTATGTTGT-3'.

**Western blotting.** SKOV3 cells (1x10⁶) treated with casticin or cyclopamine (at IC₅₀) for 48 h were lysed and analyzed by western blot, as previously reported (31), using the aforementioned antibodies; β-actin was employed as an internal control. Protein bands were visualized by enhanced chemiluminescence kit (GE Healthcare, Chicago, IL, USA), and visualized using the aforementioned instrument (Tanon Inc., Shanghai, China). Data were quantified by ImageJ 1.49v software (National Institutes of Health, Bethesda, MD, USA).

**Statistical analyses.** Experiments were repeated ≥3 times, with data expressed as the mean ± standard deviation. All
One-way analysis of variance was used to perform group comparisons, with a least significant difference test for post hoc multiple comparisons. P<0.05 was considered to indicate a statistically significant difference.

Results

Casticin and cyclopamine inhibit SKOV3 cell proliferation and colony formation. The effects of casticin and cyclopamine on SKOV3 cell viability were determined by the MTT and soft agar assay. The MTT assay revealed that casticin (Fig. 1A) or cyclopamine (Fig. 1B) inhibited SKOV3 cell growth in a dose-dependent manner. IC_{50} values of casticin and cyclopamine were 2.18±0.37 and 27.61±2.16 µM, respectively. Thus, 2.0 µM casticin and 25 µM cyclopamine were used to perform the soft agar assay. As presented in Fig. 1C, the SKOV3 cells treated with casticin or cyclopamine formed ~100% fewer colonies than the control.

Casticin and cyclopamine inhibit SKOV3 cell migration and invasion. Wound-healing assays were performed to assess the effect of casticin and cyclopamine on the migration of SKOV3 cells. As presented in Fig. 2, treatment with casticin (2.0 µM) or cyclopamine (25.0 µM) resulted in wider wounds compared with the control group after 24 h of incubation. Subsequently, quantification was conducted, which revealed that the migration rate of the casticin (72%±1.73) and cyclopamine groups (53.7%±4.37) were significantly lower compared with the control (100%; P<0.05). To analyze whether casticin (2.0 µM) or cyclopamine (25.0 µM) affected cell invasion further, a transwell invasion assay was performed. The results demonstrated that there was a marked difference in the invasion rate of cells that underwent treatment with casticin (2.0 µM) or cyclopamine (25.0 µM; 28.8±7.1 or 35.8±4.4, respectively) compared with the control group (100%; data not shown). These findings indicated that casticin and cyclopamine inhibited the migration and invasion of the human ovarian cancer SKOV3 cell line.

Casticin and cyclopamine alter Gli-1, Twist1, E-cadherin and N-cadherin gene expression levels in SKOV3 cells. To assess whether casticin and cyclopamine affect the mRNA expression of effectors of Hh signaling and EMT markers, SKOV3...
cells were treated for 24 h with complete medium containing 2.0 µM casticin and 25.0 µM cyclopamine, respectively, and were analyzed by RT-qPCR. Compared with the control group, the mRNA expression levels of Twist1 and N-cadherin were significantly reduced.
were markedly decreased (~32 and ~17%, respectively) in the casticin (2.0 µM) groups, and decreased by ~30 and ~38% in the cyclopamine (25.0 µM) groups (Fig. 3A-C). However, the mRNA expression levels of E-cadherin were increased ~4-fold for casticin-treated cells and ~2-fold for cyclopamine-treated cells (Fig. 3D). In addition, the mRNA expression levels of the Hh signaling effector, Gli-1, were also decreased by 35 and 26% in the casticin (2.0 µM) and cyclopamine (25.0 µM) groups, respectively. These results indicated the inhibitory effects of casticin and cyclopamine on Hh signaling and the EMT process.

Effects of casticin and cyclopamine on protein expression levels of Hh signaling effectors and EMT markers in SKOV3 cells. Whether casticin and cyclopamine affect Hh signaling pathway effectors and EMT markers was investigated at the protein level. After 24 h of treatment, E-cadherin protein expression levels were markedly increased in the casticin (2 µM) and cyclopamine (25.0 µM) groups compared with in the control; however, the protein expression levels of Gli-1, Twist1 and N-cadherin were markedly reduced by casticin (2.0 µM) and cyclopamine (25.0 µM) treatments, respectively, in comparison with the control group (Fig. 4A). These findings indicated that casticin and cyclopamine suppressed Hh signaling and the EMT process.

Time-dependent effect of casticin on protein expression levels of EMT markers and Hh signaling effectors. To evaluate the time course of casticin action on protein expression, Gli-1, Twist1, E-cadherin and N-cadherin protein expression levels were determined. The results of the present study were consistent with those of the RT-qPCR analysis: Time-dependent increases in E-cadherin protein levels, concomitant with reduced Gli-1, Twist1 and N-cadherin levels were observed (Fig. 6). Notably, the alterations were most pronounced at 24 h, indicating that casticin may inhibit EMT and Hh signaling to a greater degree over time.

Discussion
Casticin, an anti-inflammatory molecule (32), inhibits numerous cancer cells (27,28,33). Previous studies demonstrated that casticin inhibited EMT in liver cancer stem cells (28) and induced apoptosis in ovarian cancer cells (29). These findings demonstrated that: i) SKOV3 cell viability and migration were inhibited by casticin; ii) casticin suppressed Gli-1 expression; and iii) Twist and N-cadherin protein and mRNA expression were reduced by casticin; and iv) casticin treatment resulted in enhanced expression of E-cadherin.
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Figure 5. Time-dependent effect of casticine treatment on Gli-1, Twist1, N-cadherin and E-cadherin mRNA expression levels. SKOV3 cells were cultured with casticine (2.0 µM) for 0, 3, 6, 12 and 24 h, and analyzed by reverse transcription-quantitative polymerase chain reaction for relative mRNA expression levels of (A) Gli-1, (B) Twist1, (C) N-cadherin and (D) E-cadherin. GAPDH was used for normalization. *P<0.01 vs. Gli-1, Twist1, N-cadherin and E-cadherin expression levels at 0 h; #P<0.01 vs. Gli-1, Twist1, N-cadherin and E-cadherin at 6 h. Gli-1, glioma-associated oncogene-1; Twist1, Twist related protein 1.

Figure 6. Time-dependent effect of casticine on Gli-1, Twist1, N-cadherin and E-cadherin protein expression levels. SKOV3 cells were cultured with DMEM medium containing casticine (2.0 µM) for 0, 3, 6, 12 and 24 h. (A) Western blotting was used to analyze the protein expression levels of Gli-1 Twist1, N-cadherin and E-cadherin, with β-actin used as the internal control. (B-E) Densitometry analysis of Gli-1, Twist1, N-cadherin and E-cadherin protein quantitation, respectively. *P<0.01 vs. levels at 0 h; #P<0.01 vs. levels at 6 h. DMSO, dimethyl sulfoxide; Gli-1, glioma-associated oncogene-1; Twist1, Twist related protein 1.
nRNA and protein. It should be noted that casticin also inhibits cell proliferation and EMT in other types of cancer cell, including hepatocellular carcinoma (28), indicating its therapeutic potential.

The Hh family includes sonic Hh, desert Hh and Indian Hh (34). The Hh gene directly affects embryonic development (35). Gli-1, a potent inducer of downstream target genes, is itself a transcriptional target of Hh signaling (36); however, Gli-1 induction prevents E-cadherin expression in kidney epithelium (37). Additionally, downregulation of Gli-1 induces the invasion and migration of ovarian cancer cells (38), and Gli-1 expression in advanced ovarian cancer may be associated with poor survival (39). It is reported that tumor progression requires a variety of phenotypical alterations that decrease intercellular adhesion, with EMT serving a key role (40). During EMT, epithelial cell markers are lost, whereas mesenchymal markers, including N-cadherin, are expressed. The Hh signaling pathway is closely associated with EMT. It was previously demonstrated that casticin could inhibit the migration and invasion of hepatocellular carcinoma cells by suppressing EMT (28). However, reports that demonstrate these effects in ovarian cancer cells via the Hh signaling pathway are scarce.

In the present study, cyclopamine, an inhibitor of the Hh signaling pathway that directly binds to Smo, was used as positive control. Cyclopamine has been demonstrated to exert effects on numerous types of tumor, including lung, ovarian, and breast cancer (41-43). However, poor oral solubility hinders its clinical development. As aforementioned, casticin may inhibit EMT in SKOV3 cells via the suppression of Hh pathway activity. Casticin exhibited a lower IC50 value than cyclopamine.

The underlying molecular mechanism of casticin in the Hh signaling pathway requires further investigation. The findings of the present study revealed that the metastatic potential of SKOV3 cells incubated with casticin was decreased compared with those incubated with the control. In addition, Gli-1, Twist1 and N-cadherin expression levels were also decreased, whereas those of E-cadherin were increased. This may be due to Gli-1-mediated activation of Twist1, an EMT-inducing transcription factor (44); thus, levels of EMT markers may be affected when Gli-1 expression is reduced. Hence, further investigation is required to understand the clinical efficacy of casticin.

The results of the present study revealed that casticin inhibited ovarian cancer cell migration in vitro by repressing Hh signaling and EMT. Further in vivo studies are necessary to improve the understanding of the relevance of Hh signaling and EMT in ovarian cancer stem-like cells.

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