Silibinin attenuates TGF-β1-induced migration and invasion via EMT suppression and is associated with COX-2 downregulation in bladder transitional cell carcinoma

FENG LI1,2*, YI SUN3*, JING JIA1, CHAO YANG1, XIAOSHUANG TANG1, BEN JIN1, KE WANG1, PENG GUO1, ZHENKUN MA1, YULE CHEN1, XINYANG WANG1, LUKE CHANG1, DALIN HE1 and JIN ZENG1

1Department of Urology, The First Affiliated Hospital of Xi’an Jiaotong University, Xi’an, Shaanxi 710061; 2Department of Urology, The Second Affiliated Hospital of Xi’an Jiaotong University, Xi’an, Shaanxi 710004; 3Department of Urology, Shaanxi Provincial People’s Hospital, Xi’an, Shaanxi 710068, P.R. China

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Abstract. Transforming growth factor (TGF)-β1 is highly expressed in bladder transitional cell carcinoma (TCC) and is positively associated with tumor grade. TGF-β1 signaling promotes cell metastasis by inducing epithelial-mesenchymal transition (EMT), however, the underlying mechanisms are not fully understood. Our previous study demonstrated the anti-metastatic effects of silibinin, a natural flavonoid derived from milk thistle, against TCC. The present study investigated the effects of silibinin on TGF-β1-induced EMT in TCC, focusing on the role of prostaglandin-endoperoxide synthase 2 (COX-2). Cell migration was determined by a wound healing assay and Transwell migration assay, and cell invasion was investigated using a Transwell invasion assay. Cell morphology was observed using an inverted microscope. Cell viability was evaluated by an MTT and cell counting assays. EMT markers were detected by reverse transcription-quantitative polymerase chain reaction and western blotting. Specific small interfering RNA was used to knockdown COX-2 gene expression. TGF-β1 promoted cell migration and invasion, induced EMT and upregulated the expression of COX-2. COX-2 knockdown attenuated TGF-β1-induced EMT and invasion by inhibiting EMT, and was associated with COX-2 downregulation. TGF-β1-induced COX-2 upregulation, which was inhibited by silibinin. In addition, TGF-β1-induced EMT was further inhibited when silibinin treatment was combined with COX-2-knockdown. The results suggested that silibinin may be a potential future treatment for metastatic TCC.

Introduction

Non-muscle-invasive bladder cancer (NMIBC) and muscle-invasive bladder cancer (MIBC) are two subtypes of bladder transitional cell carcinoma (TCC), which account for ~95% neoplasms derived from the bladder. The majority of patients with NMIBC receive transurethral resection, whereas early radical cystectomy with pelvic node dissection is recommended for patients with MIBC. Approximately 20% NMIBC cases progress to MIBC and nearly 50% of patients with MIBC die within 5 years due to distant metastasis (1,2). There is an urgent need to understand the molecular mechanisms that lead to TCC metastasis and to identify potential therapeutic drugs to prolong patient survival.

Epithelial-mesenchymal transition (EMT) is among the most well-established theories regarding the mechanism of metastasis, and targeting EMT has achieved notable breakthroughs in basic research and clinical trials (3-7). EMT can be triggered by a number of growth factors and inflammatory mediators. Transforming growth factor (TGF)-β1 is the most studied regulator of EMT (5,8). TGF-β1-induced EMT has been extensively studied in bladder cancer due to high TGF-β1 levels in the blood, urine and tumor tissues of patients with TCC (9). Following TGF-β1-targeted treatment, epithelial cells lose apical polarity and obtain spindle-like mesenchymal morphology with enhanced expression of N-cadherin and decreased expression of E-cadherin (10). However, the underlying mechanism in TGF-β1-induced EMT is not fully understood.

Accumulating evidence has suggested that silibinin, a natural extract from milk thistle, has anti-tumor effects against various cancer types, including breast, renal, prostate...
and bladder cancer (11-17). Silibinin is a potential chemotherapeutic for bladder cancer due to its outstanding anti-neoplasms capacities (18). Beyond its anti-proliferation effect, a study conducted by our group indicated that silibinin exerts significant anti-metastatic effects on TCC through dual-blocking of EMT and stemness (19). However, whether TGF-β1-induced EMT is inhibited by silibinin, and the potential mechanisms involved, are still unclear.

In the present study, the effects of silibinin on TGF-β1-induced metastasis and EMT in TCC were investigated in vitro, focusing on the regulation of prostaglandin-endoperoxide synthase 2 (COX-2).

Materials and methods

Reagents and antibodies. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin and streptomycin cocktail were purchased from HyClone; GE Healthcare Life Sciences (Logan, UT, USA). Primary antibodies anti-E-cadherin (cat. no. 3195), anti-N-cadherin (cat. no. 14215), anti-Vimentin (cat. no. 5741), anti-β-catenin (cat. no. 8480), anti-zinc finger E-box binding homeobox (ZEB)1 (cat. no. 3396), anti-prostaglandin-endoperoxide synthase 2 (COX-2) (cat. no. sc-29279) was from Santa Cruz Biotechnology, MA, USA). Dilutions for primary and secondary antibodies were 1:1,000 and 1:3,000 respectively. COX-2 siRNA (cat. no. sc-15145) and control small interfering (si)RNA (cat. no. 6568) were purchased from Dharmacon (Lafayette, CO, USA). Transwell mini-cells were from EMD Millipore (Billerica, MA, USA). Transwell migration and invasion assay. Cells (3,000/well) were seeded into a 96-well plate and incubated in the cell culture incubator overnight. The medium was replaced with fresh complete medium with/without silibinin (50 µM) and/or TGF-β1 (5 ng/ml). After 48 h, MTT was added to each well (final concentration 0.5 mg/ml) 2-4 h prior to harvesting. The medium was removed and 150 µl dimethyl sulfoxide was added to each well. Samples were vortexed gently to dissolve the precipitates. The optical density value was read using a BioTek plate reader at a 490 nm wavelength (BioTek Instruments, Inc., Winooski, VT, USA).

Cell counting assay. Cells (100,000/well) were seeded into the wells of a 6-well plate and incubated overnight. The medium was replaced with fresh complete medium with/without silibinin (50 µM) and/or TGF-β1 (5 ng/ml) and after 48 h, cells were scraped and resuspended in 5 ml PBS. Cells were then counted using the Beckman Coulter Z2 cell and particle counter (Beckman Coulter, Inc., Brea, CA, USA).

mRNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Protocols used for mRNA isolation, cDNA reverse transcription and RT-qPCR were as previously described (13). Briefly, mRNA was isolated by RNA Fast 200 isolation kit (Fastagen Biotech, Shanghai, China) and then reverse transcribed to cDNA using Takara PrimeScript™ RT Master Mix (Perfect Real-Time) (cat. no. RR036A; Takara Bio, Inc., Otsu, Japan), the thermocycling conditions were 37°C for 15 min (reverse-transcription), 85°C for 5 sec (for heat inactivation of reverse transcriptase) and 4°C (end of reverse transcriptase). A reaction solution which consisted of primers, cDNA and SYBR advantage qPCR premix (cat. no. 639676; Takara Bio, Inc.) was made and loaded to Bio-Rad CFX96 real-time PCR machine (Bio-Rad Laboratories, Inc.). The protocol utilized was initial denaturation (95°C for 30 sec, 1 cycle), PCR (95°C for 5 sec, 55°C for 30 sec and 72°C for 30 sec, 40 cycles). Experiments were conducted in triplicate. Primer sequences are listed in Table I. The 2-ΔΔCq method was used to analyze relative gene expression (21).
Table I. Primer sequences used for reverse transcription-quantitative polymerase chain reaction.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequences (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>F: CGACCACCTTTGTCAAGCTCA</td>
</tr>
<tr>
<td></td>
<td>R: AGGGGAGATTCCAGTGTTGGT</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>F: CGAGAGCTACACGTTCACGG</td>
</tr>
<tr>
<td></td>
<td>R: GGGTGTCAGGGGAAAATAGG</td>
</tr>
<tr>
<td>N-cadherin</td>
<td>F: ACAGTGGGCCACCTAACAAGG</td>
</tr>
<tr>
<td></td>
<td>R: CCGAGATGGGGTGTATAATG</td>
</tr>
<tr>
<td>β-catenin</td>
<td>F: ATGGCTACTCAAGCTGAC</td>
</tr>
<tr>
<td></td>
<td>R: CAGCATTTCAGCAGCTGC</td>
</tr>
<tr>
<td>ZEB1</td>
<td>F: GCACCTGAAGAGACCAGAG</td>
</tr>
<tr>
<td></td>
<td>R: TGCATCTGGTGTTCCATTTT</td>
</tr>
<tr>
<td>Vimentin</td>
<td>F: GAGAACTTTGCCGTTGAAGC</td>
</tr>
<tr>
<td></td>
<td>R: GCTTCCTGTAGGTGGCAATC</td>
</tr>
<tr>
<td>COX-2</td>
<td>F: ATCACAGGCTTCCATGACC</td>
</tr>
<tr>
<td></td>
<td>R: CAGGATAGGCTCCACAGCA</td>
</tr>
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F, forward; R, reverse; COX-2, prostaglandin-endoperoxide synthase 2; ZEB1, zinc finger E-box binding homeobox 1.

Western-blotting. Cells were harvested using 1X radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Jiangsu, China) containing proteinase and phosphatase inhibitor cocktail and boiled with SDS loading buffer. Protein concentration was quantified using a BCA quantification kit (Pierce; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Total protein from each sample (30 µg) was separated by 10% SDS-PAGE. Proteins were then transferred to a PVDF membrane. After blocking for 1 h in 5% skim milk at room temperature, membranes were incubated with primary antibodies for 2 h at room temperature. Membranes were washed 3 times with Tris-buffered saline Tween-20 (TBST) and then incubated with secondary antibodies for 1 h at room temperature. Following 3 washes in TBST, membranes were immersed in ECL mix for 5 min. Protein bands were detected and quantified using a Bio-Rad ChemiDoc system, version 4.0 (Bio-Rad Laboratories, Inc.).

siRNA transfection. siRNAs were transfected into cells using DharmaFECT 1 transfection reagent according to the manufacturer's protocol. Briefly, culture medium in the 6-well plate was replaced with 2 ml serum-free medium 1 h before transfection. A total of 10 µl siRNAs and 4 µl transfection reagents were diluted in 200 µl separate serum-free medium and then mixed together and incubated at room temperature for 10 min. The transfection complex mixture was then added to the culture medium and mixed well by shaking several times to make the final concentration of 50 nM. The medium was replaced by complete medium after 24 h of incubation. mRNA was isolated 48 h after transfection, while protein was harvested at 72 h after transfection.

Statistical analysis. All experiments were performed at least 3 times. SPSS software, version 19.0 (IBM SPSS, Armonk, NY, USA) was used to perform the statistical analysis. Analysis of variance followed by post-hoc Student-Newman-Keuls test was utilized for statistical analysis between or among groups. When the comparison involved only 2 groups, a Student's t-test was used. P<0.05 was considered to indicate a statistically significant difference.

Results

Silibinin attenuates TGF-β1-induced migration and invasion in TCC. TGF-β1 has been reported to promote metastasis in various cancer models in vitro and in vivo, including bladder cancer. Additionally, silibinin has been demonstrated to be a potent metastasis inhibitor. Using TCC cell lines T24 and 253J, with high metastatic and invasive potential, as the model system in vitro, the present study aimed to explore the potential effects of silibinin on TGF-β1-induced migration and invasion in TCC. As determined by the results of a wound healing assay, presented in Fig. 1A and B, 5 ng/ml TGF-β1 promoted cell migration, while 50 µM silibinin inhibited migration, which was consistent with the literature (22). This effect was further confirmed by Transwell migration and invasion assays (Fig. 1C and D). Additionally, TGF-β1 at 5 ng/ml and/or silibinin at 50 µM treatment for 48 h had no impact on cell growth (Fig. 1E and F). These data suggested that TGF-β1 induced migration and invasion, and that these were attenuated by silibinin.

Silibinin attenuates TGF-β1-induced migration and invasion via EMT inhibition. EMT induction is one of the main mechanisms that contributes to increased metastatic potential promoted by TGF-β1. As silibinin attenuated TGF-β1-induced migration and invasion, the present study subsequently investigated the potential mechanisms involved, focusing on EMT signaling. As presented in Fig. 2A, following TGF-β1 treatment, cells exhibited a spindle-like shape with mesenchymal morphology and were more isolated from each other. Interestingly, this change in phenotype was reversed by co-treatment with silibinin and TGF-β1. Silibinin treatment alone did not markedly affect cell morphology. To further confirm the change in phenotype, levels of EMT markers were determined using RT-qPCR. TGF-β1 increased the expression of the mesenchymal markers, N-cadherin, Vimentin, β-catenin and ZEB1, and decreased the expression of the epithelial marker, E-cadherin. When silibinin treatment was combined with TGF-β1, the changes in EMT markers induced by TGF-β1 were attenuated (Fig. 2B). The changes in expression of these EMT-associated genes were further confirmed at the protein level by western blotting analysis, and similar effects were observed (Fig. 2C and D). Notably, E-cadherin was undetectable in the T24 cell line at the mRNA and
protein level, which is in accordance with the literature (23). Collectively, these results indicated that EMT inhibition may be the underlying mechanism involved in the inhibitory effects of silibinin on migration and invasion induced by TGF-β1.

**COX-2 upregulation is essential for TGF-β1-induced EMT.** COX-2 has been reported to be a mediator of TGF-β1-induced EMT (24). However, whether it plays a key role in EMT induced by TGF-β1 in TCC has not been clarified. As presented in Fig. 3A and B, TGF-β1 increased COX-2 expression, upregulated the expression of N-cadherin, Vimentin, β-catenin and ZEB1 and downregulated expression of E-cadherin. To further explore the role of COX-2 in TGF-β1-induced EMT, COX-2 expression was knocked down using specific siRNA. As presented in Fig. 3C, when COX-2 expression was silenced, N-cadherin, Vimentin, β-catenin and ZEB1 expression was decreased, with E-cadherin expression increased. In addition, the mesenchymal morphology induced by TGF-β1 was reversed by COX-2 knockdown (Fig. 3D). These results indicated that COX-2 expression is essential for TGF-β1-induced EMT.

**Silibinin inhibition of TGF-β1-induced EMT is associated with COX-2 downregulation.** As COX-2 was shown to play a pivotal role in TGF-β1-induced EMT, the present study next investigated the potential effects of silibinin on COX-2. As presented in Fig. 4A, in the presence of TGF-β1, expression of COX-2, N-Cadherin, Vimentin, β-catenin and ZEB1 was downregulated by silibinin, and E-Cadherin expression was upregulated. To further confirm the role of COX-2, cells were treated with silibinin with/without COX-2 knockdown in the presence of TGF-β1. As presented in Fig. 4B, silibinin
Figure 2. Silibinin attenuates TGF-β1-induced migration and invasion via EMT inhibition. (A) Representative images of cell morphology (magnification, x200) after 48 h treatment in the presence/absence of TGF-β1 (5 ng/ml) or silibinin (50 µM). Expression of EMT markers E-cadherin, N-cadherin, Vimentin, β-catenin and ZEB1 were determined at (B) the mRNA level using reverse transcription-quantitative polymerase chain reaction after 24 h treatment and (C and D) at the protein level by western blotting analysis after 48 h treatment. GAPDH was used as the internal control in RT-qPCR and western blotting. *P<0.05 vs. TGF-β1 treatment, &P<0.05 vs. control. TGF-β1, transforming growth factor-β1; EMT, epithelial-mesenchymal transition; ZEB1, zinc finger E-box binding homeobox 1.

Figure 3. COX-2 is essential for TGF-β1-induced EMT. (A) Cells were treated with TGF-β1 at 5 ng/ml or 10 ng/ml for 48 h, and the expression of E-cadherin, N-cadherin, Vimentin, β-catenin, ZEB1 and COX-2 were determined using western blotting and (B) statistically analyzed using densitometry. *P<0.05 vs. control. (C) Cells were transfected with control siRNA (siControl) or siCOX-2 for 24 h and then treated with 5 ng/ml TGF-β1 for additional 48 h, and the levels of E-cadherin, N-cadherin, Vimentin, β-catenin, ZEB1 and COX-2 were determined using western blotting and statistically analyzed using densitometry. GAPDH was used as internal control. *P<0.05 vs. siControl. (D) Cells were transfected with siControl or siCOX-2 for 24 h and then treated with/without 5 ng/ml TGF-β1 for another 48 h, representative images of cell morphology (magnification, x200) were captured using a microscope. COX-2, prostaglandin-endoperoxide synthase 2; TGF-β1, transforming growth factor-β1; EMT, epithelial-mesenchymal transition; ZEB1, zinc finger E-box binding homeobox 1; si, small interfering RNA.
Silibinin attenuates TGF-β1-induced metastasis via EMT suppression in TCC.

**Discussion**

TGF-β1-induced cell migration and invasion via EMT induction has been reported in various cancer models, including bladder cancer; however, whether this promotion of metastasis and EMT induction can be modulated by silibinin is unclear. The present study confirmed that TGF-β1 promoted cell migration and invasion via inducing EMT in TCC cells. Additionally, silibinin treatment attenuated the migration and invasion induced by TGF-β1 via EMT suppression.

EMT is a dynamic process and is an initial step in metastasis whereby localized cancer cells become aggressive and migrate to a distant site. During this process, cells lose cuboidal-like epithelial morphology and lose expression of epithelial proteins, such as E-cadherin. Additionally, cells gain an elongated spindle-like mesenchymal morphology, with increased expression of mesenchymal proteins, including N-cadherin (25). Several signaling pathways have been demonstrated to induce EMT, including TGF-β, fibroblast growth factor, epidermal growth factor, hepatocyte growth factor, Wnt/β-catenin and Notch signaling (26). Other crucial regulators, including hedgehog, nuclear factor-κB and activating transcription factor 2 have also been implicated in EMT. However, the signaling pathways or regulators that are involved in EMT induction may vary, as the process is tumor tissue- and cell type-dependent (27-29). Notably, TGF-β1 regulates the function of transcriptional regulators, including Snail1, Slug and Twist, by modulating their expression or altering their binding patterns via canonical or non-canonical signaling pathways, resulting in decreased E-cadherin expression and increased expression of N-cadherin, Vimentin and metalloproteinases in epithelial cells (30-32).

Crosstalk between inflammation and EMT signaling is a current topic of research interest. EMT induced by inflammatory factors, such as interleukin-6 and tumor necrosis factor-α in the tumor microenvironment have been studied in various tumor models (6,33-36). Previously, it was reported that COX-2, a crucial inflammation mediator, is a link between inflammation signaling and EMT in prostate cancer, and that its inactivation represses the expression of genes involved in EMT (37). COX-2 has also been reported to be highly...
expressed in bladder TCC and positively associated with tumor grade (38). However, whether COX-2 plays a role in TGF-β1-induced EMT is unclear. The present study, to the best of the author's knowledge, was the first to demonstrate that COX-2 is a key mediator of EMT induced by TGF-β1 in TCC. COX-2-knockdown inhibited TGF-β1-induced expression of EMT-associated genes and cell morphology transformation. However, how COX-2 mediates TGF-β1-induced EMT in TCC is unclear, and further experiments are required to unmask the underlying mechanisms.

Silibinin has been demonstrated to be a potent EMT suppressor in numerous studies in vitro and in vivo (39), but whether TGF-β1-induced EMT can be inhibited by silibinin is unknown. The present study demonstrated that TGF-β1-induced EMT was suppressed by silibinin, and that COX-2 downregulation was involved in the underlying mechanism. Inhibition of COX-2 by silibinin has also been reported in a previous study that investigated the anti-inflammatory potential of the compound (7). The present study focused on the role of COX-2 in TGF-β1-induced EMT. Silencing of COX-2 in TCC cells together with silibinin treatment resulted in further decrease of COX-2 expression, and increased the inhibition of EMT. This confirmed the vital role of COX-2 in regulating silibinin-induced anti-metastatic effects. However, how COX-2 downregulation by silibinin contributes to TGF-β1-induced EMT suppression is unclear and further investigation is required.

The present study demonstrated that silibinin at 50 µM treatment for 48 h had no impact on cell growth. However, a previous study reported that silibinin (10 µM) significantly suppresses the proliferation of bladder cancer T24 cells (40). Previous studies, in addition to the results of the present study, have revealed that a 50 µM dose of silibinin did not significantly affect cell proliferation after 48 h treatment, including in bladder cancer, prostate cancer, renal cancer and breast cancer (13,16,17,41-44). Specifically, in the study published in Carcinogenesis in 2004, silibinin (50 µM) treatment showed no effect on T24 cell proliferation as determined by cell growth assay and flow cytometry analysis, which was consistent with the results of the present study (43). One possibility was that the batches of silibinin from the supplier used in the present study were different from those used in the aforementioned study.

In conclusion, the findings of the present study demonstrated that TGF-β1 promoted TCC migration and invasion via induction of EMT and upregulation of COX-2. Silibinin inhibited TGF-β1-induced metastasis via inhibition of EMT, which was associated with COX-2 downregulation. The study broadens understanding of TGF-β1-induced EMT and the anti-metastasis capacity of silibinin, and may indicate future treatment strategies for metastatic TCC.

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Availability of data and materials

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

Authors' contributions

FL, YS, PG, LC, DH and JZ conceived and designed the experiments; FL, YS, JJ, CY, XT, BJ, KW, ZM, YC and XW performed the experiments; FL, YS, DH and JZ analyzed the data; FL, YS, DH and JZ wrote the manuscript. All authors read and approved the final manuscript.

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.

References


