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Original Article

CTEN-induced TGF-β1 expression facilitates EMT and enhances paclitaxel resistance in bladder cancer cells

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Abstract: Objectives: To investigate the role of C-terminal tensin-like (CTEN) in mediating chemotherapy resistance via epithelial-mesenchymal transition (EMT) in bladder cancer (BC) cells, through the regulation of transforming growth factor-β1 (TGF-β1) expression. Methods: Lentiviral vectors were used to create CTEN overexpression and knockdown constructs, which were then introduced into paclitaxel-resistant BC cell lines. The effects of CTEN manipulation on cell proliferation and drug sensitivity was assessed using the CCK-8 assay, and apoptosis was evaluated by flow cytometry. The expression levels of CTEN, TGF-β1, and EMT markers were quantified by RT-qPCR and Western blot analysis. The interaction between CTEN and TGF-β1 and its effect on TGF-β1 methylation were studied using bisulfite sequencing PCR and co-immunoprecipitation. Results: Overexpression of CTEN in BC cells was associated with decreased paclitaxel efficacy, reduced apoptosis, and elevated levels of TGF-β1 and EMT-related proteins. CTEN was found to bind TGF-β1, inhibiting its methylation and thereby promoting TGF-β1 upregulation. This increase in TGF-β1 expression facilitated the EMT process and enhanced drug resistance in BC cells. Conclusions: The induction of TGF-β1 expression by CTEN promotes EMT and increases chemotherapy resistance in BC cells. Targeting CTEN or the EMT pathway could improve chemosensitivity in treatment-resistant BC, suggesting a novel therapeutic strategy to enhance chemotherapy effectiveness.

Keywords: C-terminal tensin-like, epithelial-mesenchymal transition, drug resistance, bladder cancer

Introduction

Bladder cancer (BC) is the most prevalent malignant tumor of the urinary system and holds the highest incidence among urinary tumors in China [1, 2]. Approximately 25% of BC patients initially present with muscle-invasive forms of the disease. While the majority of BC cases are non-muscle-invasive, these patients are at high risk of recurrence or progression to muscularis propria invasion post-treatment [3]. Consequently, early surgical intervention combined with postoperative intravesical chemotherapy is critical for managing BC, effectively reducing recurrence rates and enhancing patient outcomes and survival. Despite these efforts, two-thirds of patients experience recurrence, and 15-20% progress to more advanced disease stages [4]. The predominant causes of mortality in BC are local infiltration and distant metastasis from muscle-invasive forms [5]. The etiology of BC involves multiple factors and mutations, remaining largely unclear. Conventional treatments for BC include surgical resection, radiotherapy, and chemotherapy [6]. Despite chemotherapy's effectiveness, resistance to anticancer agents significantly undermines treatment success, contributing to metastasis and increased mortality [7, 8].

Paclitaxel, a chemotherapy agent derived from Taxus plant bark, acts by promoting microtubule polymerization, inhibiting their depolymerization, and inducing cell death by blocking mitosis [9-11]. It is particularly effective against cells in the G2 and M phases of the cell cycle, thus widely used in BC chemotherapy [12, 13]. However, resistance to paclitaxel in BC treatment reduces its therapeutic effectiveness and

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adversely affects chemotherapy outcomes [14]. The mechanisms underlying this resistance are not well understood, highlighting the need for research into the biological and molecular mechanisms of drug resistance. Investigating how BC cells develop resistance to paclitaxel is essential for devising effective molecular therapies that can complement traditional chemotherapy and reverse resistance. The integration of molecular therapy with standard chemotherapy represents a promising research area in BC treatment.

C-terminal tensin-like (CTEN, also known as TNS4) is a tensin family member protein crucial for cell adhesion, migration, and signal transduction [15, 16]. Recent studies indicate that CTEN is upregulated in various cancers, including colorectal, gastric, and breast cancers, where it correlates with deeper tumor invasion, lymph node metastasis, and poor prognosis [17-19]. In colorectal cancer, reduced CTEN expression diminishes cell migration and invasion, whereas increased CTEN levels downregulate E-cadherin, promoting epithelial-mesenchymal transition (EMT) and consequently enhancing tumor cell migratory and invasive capabilities [20]. However, the role of CTEN in paclitaxel resistance in BC remains unexplored.

Transforming growth factor-β1 (TGF-β1) is a cytokine essential for mammalian development, with roles in cell migration, proliferation, and tissue repair [21]. It has recently become a focus in cancer research due to its ability to induce EMT in various epithelial cells, including mammary epithelial cells, liver cells, and proximal renal tubules [22]. TGF-β1 activation also facilitates EMT progression in T24 cells by upregulating Vimentin mRNA and protein levels, thereby increasing their invasive potential [23]. Yet, the implications of TGF-β1 in BC chemotherapy resistance and its underlying mechanisms have not been documented.

This study investigates the impact of CTEN on EMT-mediated chemotherapy resistance in drug-resistant BC cells by regulating TGF-β1 expression. We aim to clarify CTEN's role and molecular mechanism in BC chemotherapy resistance and identify potential targets for reversing BC chemotherapy resistance.

Materials and methods

Cell culture

The human BC cell line was purchased from Shanghai BlueFBio Biotechnology Development Co., Ltd., China. Cells were maintained in Roswell Park Memorial Institute 1640 medium, supplemented with 10% fetal bovine serum, and incubated at 37°C in a 5% CO_2 atmosphere. Paclitaxel, obtained from Sigma, USA, was diluted to the desired concentration with dimethyl sulfoxide. Based on prior studies, paclitaxel concentrations were set between 0 and 100 nM. For this study, cells were treated with varying concentrations of paclitaxel (0, 0.1, 0.2, 0.4, 0.8, and 1.6 μM) over a 48-hour period [24].

Cell transfection

Transient transfection of plasmids was performed using Lipofectamine 2000 (Invitrogen), following the manufacturer's protocol. The RNA sequences used for CTEN silencing were: sh1-CTEN: 5'-GTCTGATGTCAGCT-ATATGTT-3', sh2-CTEN: 5'-CAGTGTCTGATGTCAGCTATA-3', and sh3-CTEN: 5'-GCCTCAGTTTCCTCAATCATA-3'. Both the CTEN overexpression and interference vectors were developed by HanBio Biotechnology Co., Ltd., China. Lipofectamine 2000 and vector solutions were each diluted in serum-free and antibiotic-containing cell culture mediums, respectively. After a 5-minute room temperature incubation of each solution, they were mixed and further incubated at room temperature for 20 minutes. Paclitaxel-resistant BC cells were seeded in 6-well culture plates and incubated for 24 hours before the transfection mixture was added.

RT-qPCR

Cells were lysed and incubated at room temperature for 5 minutes, followed by RNA extraction via centrifugation. RNA concentration was assessed through gel electrophoresis and the RNA was stored at -80°C. Complementary DNA synthesis was conducted via reverse transcription, followed by PCR amplification using an RT-qPCR kit (Shanghai Enzyme-linked Biotechnology Co., Ltd., China). mRNA expression levels of the target gene were quantified, with GAPDH serving as the internal control. The genetic primer sequences was showed in **Table 1**.

Table 1. Genetic primer sequences

Genes	Sequences (5'-3')
TGF-β1	F: CAGATCCTGTCCAAGCTA
	R: CCTTGGCGTAGTAGTCG
N-cadherin	F: GAGATCCTACTGGACGGTTCG
	R: TCTTGGCGAATGATCTTAGGA
Vimentin	F: GACCTCTACGAGGAGGAGAT
	R: TTGTCAACATCCTGTCTGAA
Fibronectin	F: TGGAACTTCTACCAGTGCGAC
	R: TGTCTTCCCATCATCGTAACAC
E-cadherin	F: GGTGAATTTTTAGTTAATTAGCGGTAC
	R: CATAACTAACCGAAAACGCCG
β-actin	F: TGGCACCCAGCACAATGAG
	R: CTAAGTCATAGTCCGCCTAG

Western blot

Cells were lysed using RIPA buffer (Solarbio Life Science, Beijing, China) and proteins were collected from the supernatant. Protein concentrations were determined by the bicinchoninic acid method. Proteins were mixed with SDS-PAGE loading buffer and heated at 100°C for 5 minutes to ensure complete denaturation. Proteins were then separated by SDS-PAGE and transferred to a PVDF membrane. The membrane was blocked with blocking solution for 1 hour and washed three times with Tris-buffered saline containing 0.1% Tween® 20 (TBST). It was then incubated overnight at 4°C with primary antibodies diluted in TBST. The primary antibodies used included anti-TGF-β1 (1:2000, Cell Signaling Technology, USA), anti-N cadherin (1:3000, Abcam, UK), anti-Vimentin (1:200, Abcam), anti-Fibronectin (1:1000, Abcam), and anti-E-cadherin (1:5000, Abcam). The following day, the membrane was incubated with a secondary antibody (1:1000 dilution, Solarbio) at room temperature for 1 hour. Protein bands were visualized using a chemiluminescent substrate and an imaging system.

Evaluation of drug resistance index by cell counting kit-8 (CCK-8) assay

Cells were seeded in 96-well plates at a density of 5 × 10^5 cells per well and cultured for 24 hours. They were then treated with various drug concentrations for 12 hours. Subsequently, 20 μL of CCK-8 solution (MedChemExpress, China) was added to each well, and absorbance was measured at 450 nm using a microplate reader (DeTie Experimental Equipment Co., Ltd., China) at 0.5, 1, 2, and 4 hours post-treatment. Blank controls were set up by adding an equal volume of cell culture medium and CCK-8 solution to wells without cells. Absorbance values were used to calculate the half-maximal inhibitory concentration (IC50) of the drug.

Annexin-V/PI staining

After 72 hours of treatment with drugs or following transfection, cells were harvested and transferred into flow tubes. The cells were then washed with pre-cooled PBS, and 100 μL of 1 × Binding buffer was added. To this suspension, 5 μL of FITC-conjugated Annexin V (Aibixin Biotechnology Co., Ltd., China) and 5 μL of propidium iodide (PI) were added. The cells were incubated for 15 minutes at room temperature in the dark. An additional 300 μL of 1 × binding buffer was added before analyzing cell apoptosis using flow cytometry (Sartorius Trading Ltd., Shanghai).

Bisulfite sequencing PCR (BSP)

DNA was extracted from the cells and treated with bisulfite using the EpiTect Bisulfite Kit (Qiagen, USA). Primers for the PCR were provided by Invitrogen, Shanghai. A small amount of the bisulfite-treated DNA was used for PCR amplification. The amplified DNA was then subjected to gel electrophoresis, followed by purification and sequencing using an ABI 3730 DNA sequencer (Applied Biosystems). The methylation status of CpG sites within the DNA was determined based on the sequencing results.

CO-immunoprecipitation assay (CO-IP)

The assay was performed according to the protocol of the IP/CO-IP Kit (Nanjing ACE Biotechnology Co., Ltd., China). Proteins were extracted from cells using RIPA lysis buffer containing Tris-HCl, 1% Triton X-100, protease inhibitor, and phosphatase inhibitor. Extracted proteins were divided into two groups: one treated with TGF-β1 antibody (10-20 μg/mL, Cell Signaling Technology) and the other with Rabbit IgG (CST 2729), both incubated overnight at 4°C. Subsequently, 50 μL of Protein G Agarose (Millipore, IP05) was added to each group. Magnetic beads were pre-cooled,

washed twice with PBS, and blocked with 3% BSA for 30 minutes. Antibody-protein complexes were then added to the beads and incubated overnight at 4°C. After washing twice with cold wash buffer, complexes were eluted with 3× SDS-PAGE loading buffer, heated at 95°C for 10 minutes, and then cooled on ice. The supernatant was collected for Western blot analysis after centrifugation at 12,000 rpm at 4°C.

Statistical analysis

Statistical analyses were conducted using SPSS version 22.0. Results were expressed as mean ± standard deviation. The t-test was applied for comparisons between two groups, while one-way ANOVA or repeated measures ANOVA, followed by a Bonferroni correction, was used for analyses involving multiple groups. Given the small sample size and potential deviations from normal distribution, non-parametric tests such as the Mann-Whitney U test or Kruskal-Wallis test were also utilized. A significance level of $P < 0.05$ was adopted to identify statistically significant differences.

Results

Effect of CTEN overexpression on chemotherapy resistance in BC cells

The CTEN overexpression and interference vectors were utilized to infect drug-resistant BC cells. Assessment of CTEN expression revealed upregulation in cells transfected with the CTEN overexpression vector. In contrast, all groups with silenced CTEN expression showed decreased levels, with the most significant reduction observed in the group transfected with the CTEN-sh3 interference vector (**Figure 1A**, **1B**). A stable CTEN silencing vector (CTEN-sh3) was subsequently constructed for further experimentation. After stable transfection, paclitaxel-resistant BC cells were cultured with various paclitaxel concentrations. Notably, the inhibitory effect of paclitaxel was minimal in the CTEN overexpression group and most pronounced in the CTEN silencing group (**Figure 1C**). The apoptosis rate was reduced in BC cells overexpressing CTEN, whereas it was increased in cells where CTEN was silenced (**Figure 1D**). Moreover, elevated CTEN expression upregulated the mRNA and protein levels of TGF-β1, N-cadherin, Vimentin, and Fibronectin, while downregulating E-cadherin. Conversely, suppressing CTEN expression reversed these effects and effectively hindered the EMT process in the CTEN-sh3 group (**Figure 2A**, **2B**).

Molecular mechanism of CTEN regulation of TGF-β1

Exploring the impact of CTEN on TGF-β1 expression revealed that increased CTEN expression was linked to reduced methylation of TGF-β1, H3K4, and H3K9. Inhibition of CTEN expression resulted in increased methylation levels of these markers. Elevated CTEN levels inhibited methylation of TGF-β1 and histones, promoting the transcriptional activity of the TGF-β1 gene and enhancing its expression (**Figure 3A**). Additionally, CO-IP analysis showed enhanced binding affinity between the CTEN protein and TGF-β1 with increased CTEN expression. Reduced CTEN levels led to weaker protein-protein interactions with TGF-β1 (**Figure 3B**). These results suggest that the CTEN protein in paclitaxel-resistant BC cells interacts with TGF-β1 protein, facilitating its downstream signaling cascade.

CTEN regulates chemotherapy resistance in BC cells by regulating TGF-β1 expression

The CTEN silenced expression vector and TGF-β1 overexpression vector were transfected into BC cells. Following this, the stable, drug-resistant cell lines were cultured with an equal concentration of paclitaxel. Analysis showed that silencing CTEN expression enhanced the chemotherapy's inhibitory effect on drug-resistant cells, while increased TGF-β1 expression diminished this effect (**Figure 4A**). Additionally, silencing CTEN improved apoptotic capabilities in these cell lines, whereas upregulating TGF-β1 reduced apoptosis and increased survival (**Figure 4B**). We also assessed the expression of EMT-related proteins; silencing CTEN decreased mRNA and protein levels of TGF-β1, N-cadherin, Vimentin, and Fibronectin, but increased levels of E-cadherin. In contrast, elevated TGF-β1 expression negated the effects of CTEN silencing (**Figure 4C**, **4D**). These findings indicate that CTEN and TGF-β1 collaboratively activate the EMT pathway in BC cells, thereby reducing their sensitivity to chemotherapy.

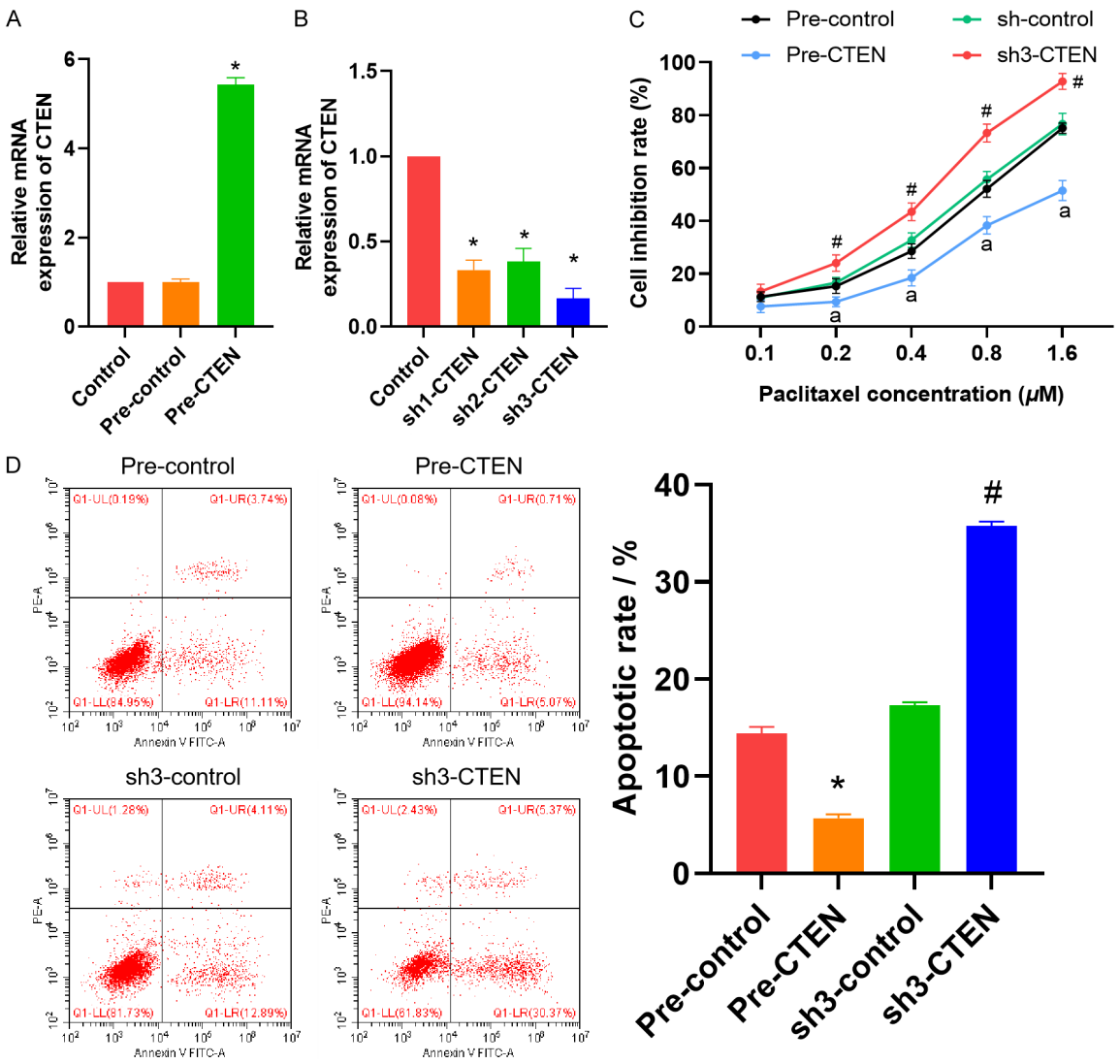

Figure 1. Effect of C-terminal tensin-like (CTEN) expression on bladder cancer (BC) cell line. A. RT-qPCR was used to assess the efficiency of CTEN overexpression. B. The efficacy of the CTEN silenced vector was evaluated using RT-qPCR. C. CCK-8 assay was conducted to determine the drug inhibition rate. D. Annexin-V/PI staining was performed to assess apoptosis. $^{*}P < 0.05$, compared with control group; $^{a}P < 0.05$, compared with pre-control group; $^{\#}P < 0.05$, compared with sh-control group.

Discussion

Tumor recurrence due to drug resistance in BC cells contributes significantly to overall BC recurrence rates. Despite advances in medical treatments and an expanding repertoire of therapeutic strategies for BC, drug resistance and subsequent tumor recurrence continue to pose significant challenges [25]. Paclitaxel, known for its efficacy against various malignancies including breast, prostate, lung, gastric, ovarian, cervical, and head and neck cancers, currently serves as a second-line chemotherapy agent in BC management due to its broad anticancer activity [26, 27]. While the effectiveness of paclitaxel as a first-line chemotherapy agent is approximately 50%, its efficacy drops to 20-30% when used as a second or third-line treatment [28, 29]. Recent trends indicate increasing resistance rates to paclitaxel over time. The incomplete understanding of the mechanisms underlying this resistance underscores the need for further research to elucidate the pathways involved in paclitaxel resistance.

EMT is a critical process in tumor invasion and metastasis, characterized by the transforma-

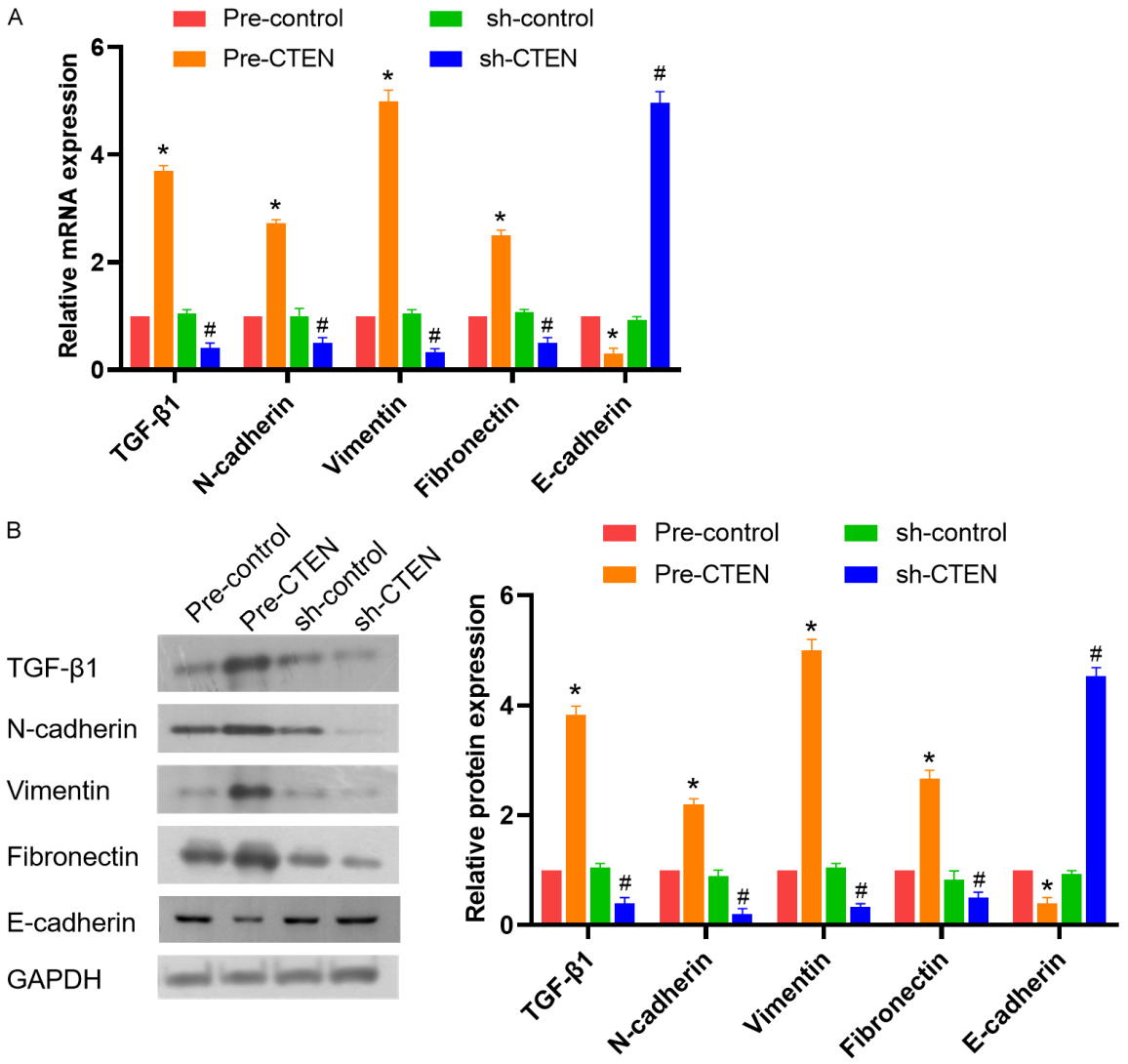

Figure 2. Effect of CTEN expression on epithelial-mesenchymal transition (EMT)-related protein expression. A. mRNA levels of TGF-β1, N-cadherin, Vimentin, Fibronectin, and E-cadherin were quantified using RT-qPCR. B. Protein levels of TGF-β1, N-cadherin, Vimentin, Fibronectin, and E-cadherin were measured using Western blot analysis. $^{*}P < 0.05$, compared with pre-control group; $^{\#}P < 0.05$, compared with the sh-control group.

tion of epithelial cells into mesenchymal cells under specific physiological and pathological conditions [30]. During EMT, mesenchymal cells exhibit increased expression of markers such as N-cadherin, Vimentin, and Fibronectin, while epithelial markers like E-cadherin are downregulated [31, 32]. A strong correlation between EMT, chemotherapy resistance, and distant metastasis in epithelial tumors has been well-documented [33, 34]. Recent studies have linked the occurrence of EMT with resistance to paclitaxel/docetaxel in tumor cells. Furthermore, elevated levels of multidrug resistance associated protein-1 (MRP-1) have been shown to facilitate EMT induction [35, 36]. Transfection of the transglutaminase (TG) gene in tumor cells has led to upregulation of EMT-related genes such as Twist1, ZEB1, and Snail, inducing EMT and reducing chemotherapy sensitivity [37]. Highly invasive cancer cell lines exhibit concurrent changes in EMT status and increased Twist expression, correlating with heightened resistance to paclitaxel [38]. This study proposes a potential link between EMT alterations and paclitaxel resistance in BC cell lines.

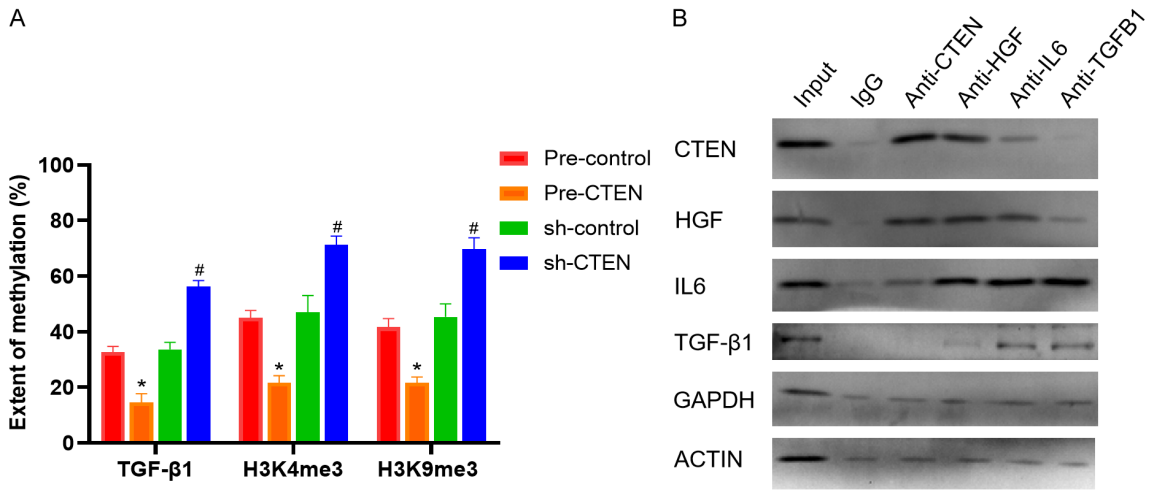

Figure 3. Molecular mechanism of TGF-β1 regulation by CTEN. A. The gene methylation levels were quantified using Bisulfite sequencing PCR (BSP). B. CO-immunoprecipitation (CO-IP) was employed to assess the binding affinity between CTEN and TGF-β1. $^{*}P < 0.05$, compared with pre-control group; $^{\#}P < 0.05$, compared with the sh-control group.

TGF-β1 is a cytokine crucial for the development of mammals, including human embryos. It regulates cell migration and proliferation and promotes tissue repair among other functions [39]. Studies by Matsuda et al. have shown that TGF-β1 contributes to the resistance of hepatocellular carcinoma to sorafenib [40]. Kim et al. reported that TGF-β1 induces EMT in lung cancer cells, enhancing their migration and invasion and resistance to apoptosis [41]. Additionally, the CTEN protein is implicated in various cancer-related genes or signaling pathways, such as epidermal growth factor, mouse sarcoma virus oncogene, and fibroblast growth factor. CTEN plays a significant role in the biological activities of malignant tumors, either as a regulator of gene expression or as a mediator of signal transduction [42, 43].

We successfully constructed expression vectors for CTEN overexpression and silencing, which were transfected into paclitaxel-resistant human BC cell lines. Our findings indicated that CTEN overexpression promoted cell proliferation in these drug-resistant cells, which is consistent with the results by Lu et al. who observed similar effects in non-small cell lung cancer cells [44]. Moreover, CTEN overexpression inhibited TGF-β1 methylation, enhancing TGF-β1 expression and activating the EMT process [45]. These results align with the findings that CTEN synergistically enhances TGF-β1 expression, promoting EMT and increasing chemotherapy resistance in tumor cells [46].

Conversely, silencing CTEN expression suppressed the EMT process and improved the cells' response to chemotherapy. Increasing TGF-β1 expression counteracted these effects, promoting EMT, restoring proliferation, and reducing chemotherapy efficacy. CTEN facilitated TGF-β1 expression by inhibiting its methylation, suggesting a synergistic role with TGF-β1 in promoting EMT, as supported by the results of Asiri et al. [47]. Therefore, elevated TGF-β1 levels could further stimulate EMT, potentially contributing to tumor cell drug resistance.

The role of CTEN as a focal adhesion molecule varies across different cancer tissues, indicating its diverse functional implications. For instance, CTEN expression is inversely related to paclitaxel resistance in prostate cancer, while it correlates with tumor progression in lung and colon cancers [48-50]. These findings suggest that CTEN may have both oncogenic and tumor suppressor functions, depending on the cancer type. Thus, targeting the EMT process may enhance the sensitivity of BC to chemotherapy drugs.

In conclusion, we demonstrate that CTEN and TGF-β1 synergistically promote EMT in BC cells, contributing to drug resistance. Modulating the

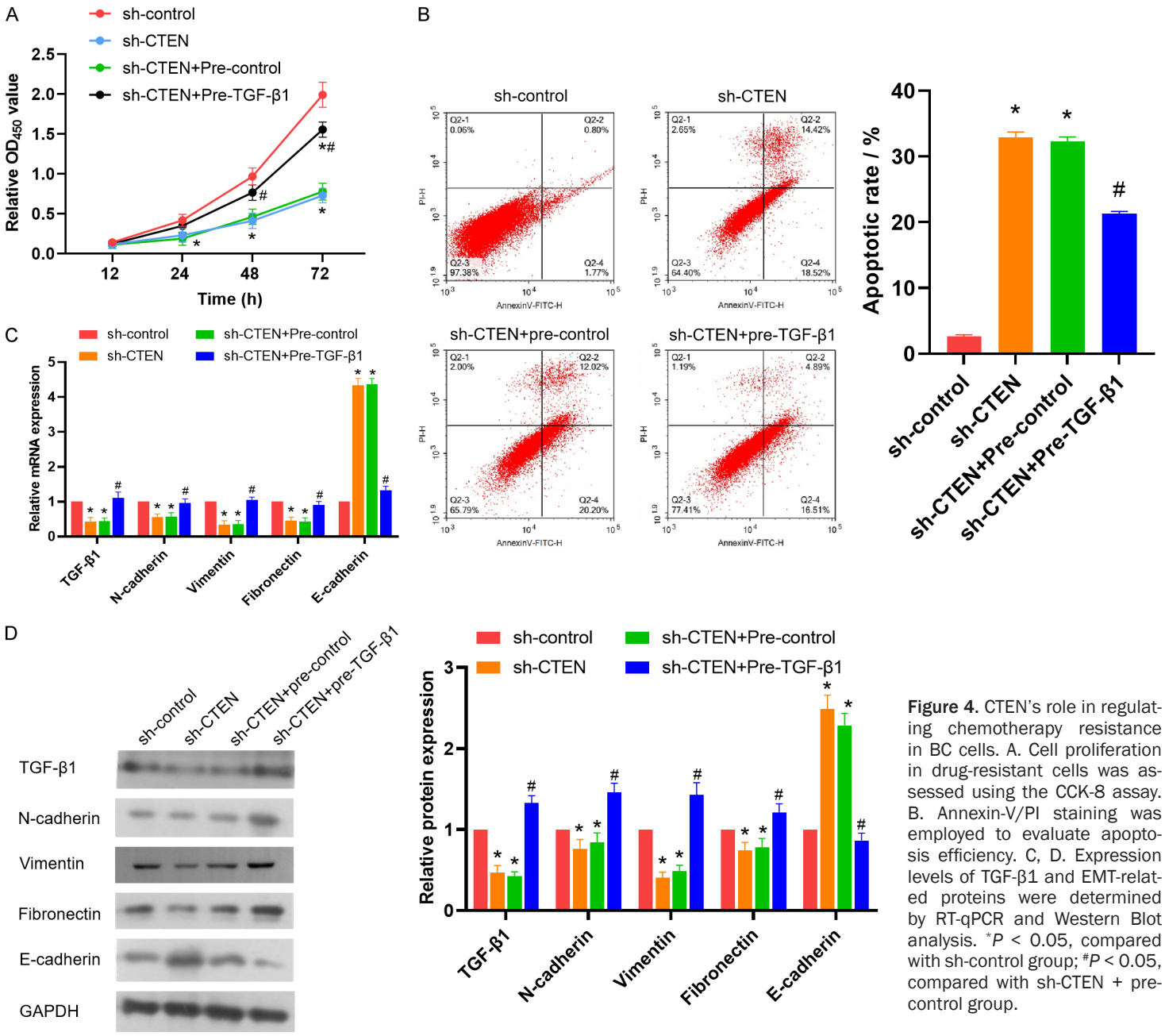

Figure 4. CTEN's role in regulating chemotherapy resistance in BC cells. A. Cell proliferation in drug-resistant cells was assessed using the CCK-8 assay. B. Annexin-V/PI staining was employed to evaluate apoptosis efficiency. C, D. Expression levels of TGF-β1 and EMT-related proteins were determined by RT-qPCR and Western Blot analysis. $^{*}P < 0.05$, compared with sh-control group; $^{\#}P < 0.05$, compared with sh-CTEN + pre-control group.

expression of CTEN and TGF-β1 and inhibiting EMT can potentially enhance the sensitivity of BC cells to chemotherapy, thereby improving treatment outcomes. Additionally, this research elucidates the mechanisms underlying paclitaxel resistance, offering valuable insights for improving BC treatment strategies.

However, this study has limitations, including the sole use of drug-resistant BC cells as experimental models. Future research should include parental BC cells and normal bladder cells to validate and extend these findings. Moreover, expanding this research to include animal models of bladder cancer drug resistance could further clarify the roles of CTEN and TGF-β1 in mediating paclitaxel resistance. Additionally, analyzing peripheral blood samples from patients with drug-resistant BC may help identify CTEN and TGF-β1 as potential biomarkers for paclitaxel resistance in BC.

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Disclosure of conflict of interest

None.

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