Article

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EIF5A2 Promotes Doxorubicin Resistance in Bladder Cancer Cells through the TGF-β Signaling Pathway

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Background: Doxorubicin (DOX) is a commonly used chemotherapeutic agent, but bladder cancer (BC) patients often develop resistance that limits therapeutic efficacy. Recent research has demonstrated a link between medication resistance and the expression of eukaryotic translation initiation factor 5A2 (*EIF5A2*) in tumors. This study aimed to investigate whether *EIF5A2* affects the resistance of BC cells to doxorubicin through the transforming growth factor (TGF)-β signaling pathway.
Methods: Doxorubicin-resistant cells in BC (T24/DOX and 5637/DOX) were constructed, then cell viability was detected by cell counting kit-8 (CCK-8); *EIF5A2* mRNA expression was detected using quantitative real-time PCR (qRT-PCR); cell proliferation was detected using clone formation; apoptosis was detected by flow cytometry; and finally, proteins related to the TGF-β signaling pathway (*EIF5A2*, TGF-β1, p-small mothers against decapentaplegic 2 (Smad2)/Smad2, p-Smad3/Smad3) were detected using western blot.
Results: *EIF5A2* was up-regulated in DOX-resistant BC cells, and DOX intervention promoted proliferation and inhibited apoptosis in DOX-resistant BC cells. si-*EIF5A2* reversed the above effects. *EIF5A2* resulted in DOX resistance by activating the TGF-β pathway, and the TGF-β activator SRI-011381 reversed the inhibitory effect of si-*EIF5A2* on DOX resistance.
Conclusions: *EIF5A2* promotes DOX resistance in BC cells through the TGF-β signaling pathway, and *EIF5A2* may be a potential counter-resistance therapeutic strategy in BC chemotherapy.

Keywords: doxorubicin; *EIF5A2*; TGF-β; bladder cancer

Introduction

Bladder cancer (BC) is one of the most common cancer in men [1]. The incidence of BC in China ranks eighth among male malignant tumors and is the highest diagnosed of malignant tumors in the genitourinary system [2,3]. Non-muscle-invasive BC (NMIBC) and muscle-invasive BC (MIBC) are two types of BC that differ in the extent of tumor penetration into bladder tissue [4]. Several treatments, such as chemotherapy, are available for BC. However, the median lifespan of BC patients remains 12 to 15 months as issues related to chemo-resistance can develop, leading to the reduced therapeutic effectiveness of initial chemotherapy treatments during BC recurrence and increased mortality [5]. A barrier that results in treatment failure is resistance to doxorubicin (DOX), a frequently used frontline drug in intravenous and systemic chemotherapy for BC. It is essential to understand the underlying molecular causes of BC's DOX resistance and to find a potent treatment target that can make BC more susceptible to DOX.

Eukaryotic translation initiation factor 5A2 (*EIF5A2*), a member of the Eukaryotic translation initiation factor 5A (EIF5A) family, is an important protein regulating normal function in the nucleus and cytoplasm of cells [6]. *EIF5A2* plays a critical role in the cellular translation process, including the activation and modification of the transfer RNA (tRNA) required to encode proteins and regulates the rate and accuracy of mRNA translation [7,8]. Abnormal expression of *EIF5A2*, such as in colorectal cancer (CRC), is associated with tumorigenesis and progression, resulting in tumor proliferation, invasion, and metastasis [9]. The *EIF5A2*/transforming growth factor (TGF)-β/small mothers against decapentaplegic 2 (Smad2)/3 signaling pathway has also been implicated in the epithelial-mesenchymal transition (EMT) with HERC3, influencing migration, invasion, and metastasis [9]. The overexpression of *EIF5A2* is positively regulated by the E2 promoter binding factor 1 (E2F1)/kruppel-like factor 4 (KLF4) pathway in ovarian cancer cells, making it a potential therapeutic target in the treatment of cancer stem cells (CSCs) [10]. A combination of targeting *EIF5A2* with cisplatin chemotherapy has the potential to overcome hypoxia-induced cisplatin resistance, hindering the development of non-small-cell lung cancer (NSCLC) [11]. The androgen receptor (AR) plays a role in the positive regulation of *EIF5A2* expression in androgen-dependent cells, leading to an increase in prostate cancer (PCa) metastasis through the induction of EMT and

upregulation of *EIF5A2* [12]. Therefore, *EIF5A2* is considered a potential tumor marker and therapeutic target, our previous studies have identified that N1-guanyl-1,7-diaminoheptane (GC7) can enhance chemosensitivity in BC by regulating *EIF5A2* [13]. However, the mechanism of action of *EIF5A2* in BC is unclear.

The TGF-β signaling pathway is a vital signaling cascade [14]. The pathway is expressed ubiquitously in all organisms and regulates various cellular and molecular processes in development and disease [15]. TGF signaling has been well-established as a crucial factor in tumorigenesis. Notably, TGF-β and beta-catenin-bone morphogenetic proteins 2 (BMP2) significantly promote breast cancer (BrC) metastasis by inducing the expression of bone metastasis genes like interleukin-11 (IL11) and connective tissue growth factor (CTGF) *in vivo* [16]. One such miRNA, miR-27a, has been shown to suppress cervical adenocarcinoma progression by targeting the TGF-βRI signaling pathway [17]. Additionally, miR-187 has been shown to regulate cisplatin resistance in gastric cancer cells by regulating the TGF-β/Smad signaling pathway [18]. Lastly, the nucleolar and spindle-associated protein 1 (NUSAP1) has been shown to promote BC progression by modulating the TGF-β signaling pathway [19]. More importantly, *EIF5A2* positively regulated the TGF-β signaling pathway, and TGF-β treatment offset the promoting effects of *EIF5A2* silencing on apoptosis of cisplatin (DDP)-resistant nasopharyngeal carcinoma cells. However, it is unclear whether the expression of *EIF5A2* affects doxorubicin resistance in BC cells through the TGF-β signaling pathway.

In this study, we explored the mechanism of action of *EIF5A2* in chemotherapy resistance of BC by constructing doxorubicin-resistant BC cells, which provides a new target and mechanism of action for the treatment of BC.

Material and Methods

Cell Culture

The T24 and 5637 human BC cell lines were bought from the American Type Culture Collection (ATCC, Manassas, VA, USA). Based on ongoing exposure of the parental cell lines T24 and 5637 to the culture medium containing DOX, respectively, DOX-resistant BC cell lines T24/DOX and 5637/DOX were cultured in our lab. T24, 5637, T24/DOX, and 5637/DOX cells were cultured in DMEM media from Invitrogen (2764637, Thermo Fisher Scientific, Grand Island, New York, USA) that included 10% FBS and 1% penicillin-streptomycin. Prior to the experiment, T24/DOX and 5637/DOX cells were grown with 1 µM DOX to retain the drug-resistant features. All cell lines used in this study are STR authenticated and regularly tested for the absence of mycoplasma infections (Mykoalert kit, LT07, Lonza, Basel, Switzerland).

Cell Viability Assay

T24, T24/DOX, 5637, and 5637/DOX (2×10^3/well) in 96-well plates were allowed to attach overnight. T24, T24/DOX, 5637, and 5637/DOX (2×10^3/well) cells were exposed to DOX (0, 0.1, 1, 10, 20, and 100 µM) for 24 hours to conduct a drug sensitivity investigation. 10 µL Cell Counting Kit-8 (CCK-8) solution (C0038, Invitrogen, Shanghai, China) was added to each cell suspension and incubated in the incubator for 2 hours. An enzyme-labeled instrument measured the absorbance of each cell at 450 nm, and the growth curves of cells in each group were drawn, and the differences between the groups were compared.

Small Interfering RNA (siRNA) Transfection

According to the manufacturer's instructions, siLentFect Lipid Reagent (1703360, BioRad, Hercules, CA, USA) was used to transfect siRNA specific for *EIF5A2* (*EIF5A2*#1, *EIF5A2*#2) and non-specific control (siNC) (A01001, GenePharma, Shanghai, China) when cells reached 30%–50% confluency. The transfection took place for six hours, and quantitative real-time PCR (qRT-PCR) confirmed the transfection effect. The treated cells were employed for several cell tests 48 hours later. The siRNA sequences were as follows: For *EIF5A2*, *EIF5A2#1*: 5′-CCAGCAACAUACCACACAATT-3′; *EIF5A2#2*: 5′-CCAACACCUACUACAUAAUTT-3′. For si-NC: 5′-UUCUCCGAACGUGUCACGUTT-3′.

Quantitative Real-Time PCR

Trizol reagent (15596018, Thermofisher Scientific, Carslbad, CA, USA) was used to extract total RNA from the cultivated cells per the manufacturer's instructions. After being reverse transcribed into cDNA, qRT-PCR was carried out using a 7500 PCR System from Thermo Fisher Scientific and a SYBR Green Master Mix Kit (337619, QIAGEN, Hilden, Germany). Three copies of each response were performed. After normalization with reference controls, the $2^{-\Delta\Delta CT}$ technique was used to determine the gene expression levels. The 20 µL amplification reaction volume contained 0.8 µL amplification primers, 7.2 µL H_2O, 10 µL SYBR Green PCR Master Mix, and 2 µL cDNA. The following primers were used in this study: *EIF5A2* Forward: 5′-GGCTTCCAGCACTTACCCTA-3′ and Reverse: 5′-ATGGTCGTCCTTTGAGCACC-3′; *GAPDH* Forward: 5′-TATGATGATATCAAGAGGGTAGT-3′ and Reverse: 5′-TGTATCCAAACTCATTGTCATAC-3′.

Clonal Formation Experiment

The growth phase of logarithmic cells was subjected to trypsin digestion, and the cells were then inoculated onto 6-well plates (5×10^5 cells/well, in triplicate) and cultured for two weeks in a temperature of 37 °C with 5% CO_2. The process of rinsing the cells with phosphate-buffered saline (PBS) was repeated three times, air-dried, followed by fix-

ation with paraformaldehyde for 30 minutes, and then subjected to three rounds of PBS washes before being stained with crystal violet for 10 minutes. At a stage when the clones could be observed visually, the culture was stopped. After drying, the staining solution was gently removed with PBS, and the number of clones was counted by sight (approximately 50 cells considered one clone).

Flow Cytometry

Cells were washed twice with PBS. Cells were homogeneously mixed with 500 µL of pre-cooled 1× binding buffer, 5 µL of AnnexinV-FITC (Yeasen Biotech Co., Ltd., Shanghai, China), and incubated for 15 minutes at room temperature, then 2.5 µL of PI (Yeasen Biotechnology Co., Ltd., Shanghai, China) for staining. The fluorescence was detected using a Flow cytometry system (FACSCalibur; BD Biosciences, San Diego, CA, USA) with an excitation wavelength of 488 nm and emission wavelength of 525 nm.

Western Blot

Radio immuno precipitation assay (RIPA) lysis buffer (Pierce, Rockford, IL, USA) was used to lyse the cells, and protease inhibitors were added. The supernatant was then collected using high-speed centrifugation. After protein extraction, protein concentrations were measured according to the instructions of the BCA Protein Assay Kit (23225, Thermo Scientific, Rockford, IL, USA). Then, loading buffer was added to the extracted proteins and heated for 10 minutes at 95 °C. 30 µg of protein per well was added to a 10% polyacrylamide gel to separate the proteins. Gel electrophoresis was regulated at a voltage of 80–120 V, and wet transfer and membrane transfer were controlled at a pressure of 100 mV for 45–70 minutes. After the membrane transfer in polyvinylidene difluoride, the proteins were sealed in 5% bovine serum protein and maintained for 1 hour, to which primary antibodies Anti-*EIF5A2* (ab126733, 1:1000, Abcam, Cambridge, UK), Anti-TGF-β1 (ab142139, 1:1000, Abcam, Cambridge, UK), Anti-p-Smad2 (ab280888, 1:1000, Abcam, Cambridge, UK), and Smad2 (ab40855, 1:1000, Abcam, Cambridge, UK), p-Smad3 (ab52903, 1:1000, Abcam, Cambridge, UK), Smad3 (ab40854, 1:1000, Abcam, Cambridge, UK) were incubated overnight at 4 °C. Membrane washing buffer was used to rinse the proteins three times for 5 minutes each, followed by the addition of secondary antibody Goat Anti-Rabbit IgG H&L (ab172730, 1:2000, Abcam Cambridge, MA, USA) and incubation in the greenhouse for 1 hour. After washing the membrane three times, the chemiluminescent reagent enhanced chemiluminescent (ECL) was added to develop the proteins. β-actin was used as an internal reference, and Bio-Rad Gel Dol EZ imager (GEL DOC EZ IMAGER, Bio-Rad, CA, USA) was used to image the proteins. The grey levels of the target bands were analyzed using ImageJ (version 1.46r, National Institutes of Health, Bethesda, MD, USA) software.

Statistical Processing

Data analysis was conducted using SPSS v20.0 (IBM, Armonk, NY, USA) software, while GraphPad Prism Version 9 (GraphPad Software, San Diego, CA, USA) was utilized to generate visual representations of the experimental data. The statistical significance of comparisons between two groups was assessed using a *t*-test. One-way analysis of variance (ANOVA) was employed to compare differences between multiple groups, with p values less than 0.05 denoting a statistically significant difference.

Results

EIF5A2 is Upregulated in DOX-Resistant BC Cells

We treated T24 and T24/DOX cells and 5637 and 5637/DOX cells with different concentrations of DOX for 48 hours. After that, the CCK-8 reagent was used to detect the survival rate of cells. The results showed that DOX had a weak inhibitory effect on T24/DOX and 5637/DOX cells at different concentrations, and the survival rate was high ($p < 0.05$), which proved that drug-resistant cell lines were successfully constructed (Fig. 1A). The expression level of *EIF5A2* mRNA in different cell lines was detected by PCR. The results showed that the expression level of *EIF5A2* mRNA in T24/DOX and 5637/DOX cells was significantly higher than in sensitive cells T24 and 5637 ($p < 0.001$). This indicates that the increased expression of *EIF5A2* in drug-resistant cell lines is related to its drug resistance (Fig. 1B). To evaluate the inhibitory effect of *EIF5A2* siRNA on its gene expression, we used two different siRNA targeting *EIF5A2* (si-*EIF5A2*#1 and si-*EIF5A2*#2) and a negative control (si-NC) to transfect T24/DOX and 5637/DOX cells. The experimental results showed that in the si-*EIF5A2*#1 group, the mRNA level of *EIF5A2* was significantly lower than that in the si-NC group ($p < 0.001$), and the level in the si-*EIF5A2* group was also reduced, but to a slighter extent ($p < 0.01$). Therefore, we chose the more effective si-*EIF5A2*#1 for the follow-up experiments (Fig. 1C).

si-EIF5A2 Inhibits DOX Resistance in DOX-Resistant BC Cells

To clarify the critical role of *EIF5A2* on DOX resistance. We used CCK-8, clone formation and attrition cytometry to validate this. The results showed that DOX intervention promoted proliferation ($p < 0.05$, $p < 0.001$) and inhibited apoptosis ($p < 0.01$) in T24/DOX and 5637/DOX compared to T24+si-NC. si-*EIF5A2* reversed these effects, inhibiting cell proliferation ($p < 0.05$, $p < 0.01$) and promoting cell apoptosis ($p < 0.01$) (Fig. 2A–C). In conclusion, si-*EIF5A2* inhibits DOX resistance in DOX-resistant BC cells.

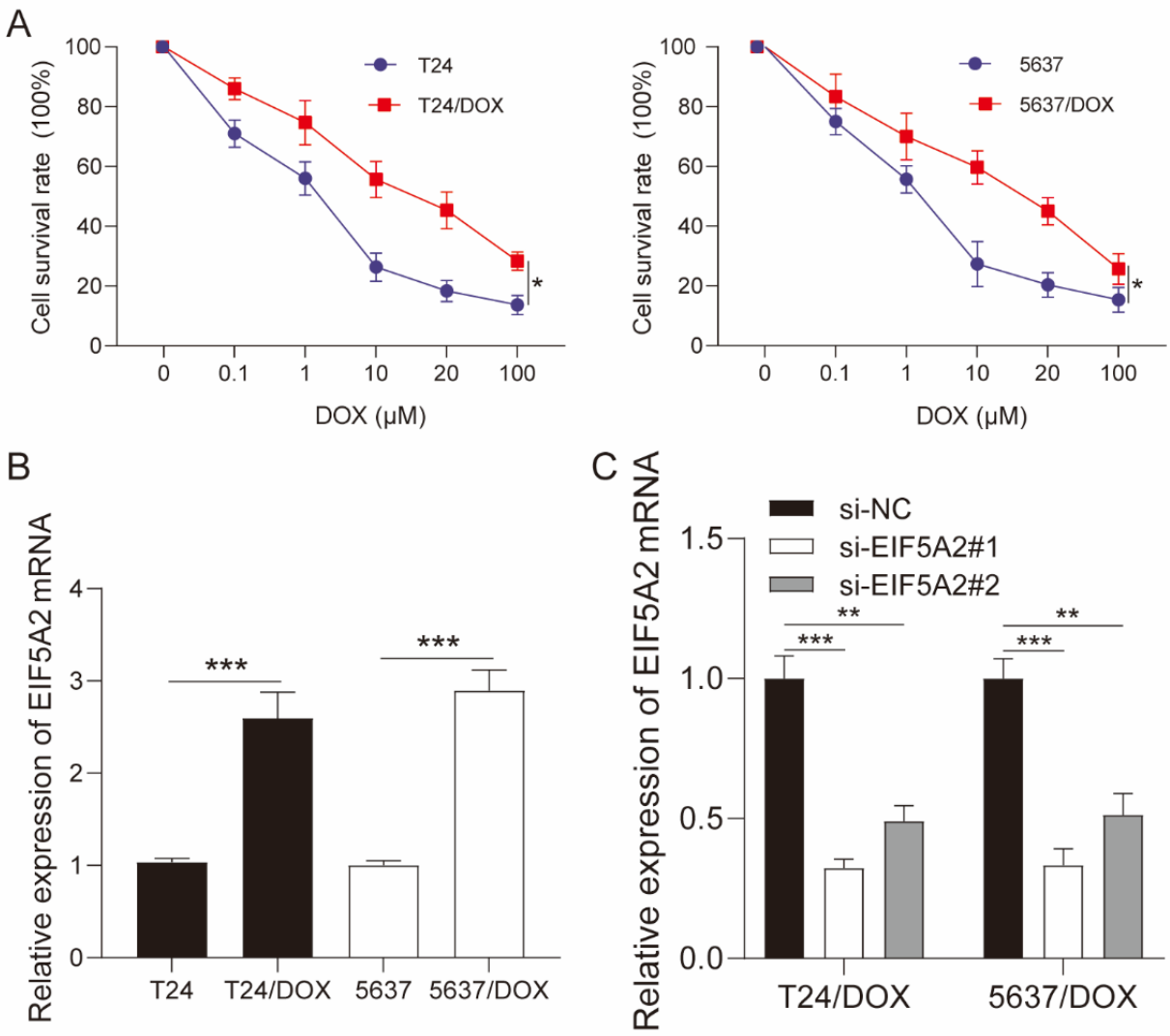

Fig. 1. Eukaryotic translation initiation factor 5A2 (*EIF5A2*) is up-regulated in doxorubicin (DOX)-resistant bladder cancer (BC) cells. (A) The survival rates of T24 and T24/DOX cells and 5637 and 5637/DOX cells treated with different concentrations of DOX for 48 hours were detected by Cell Counting Kit-8 (CCK-8) reagent. (B) The expression level of *EIF5A2* mRNA in different cell lines was detected by PCR. (C) Two different small interfering RNA (siRNA) targeting *EIF5A2* (si-*EIF5A2*#1 and si-*EIF5A2*#2) were used to transfect T24/DOX and 5637/DOX cells to evaluate the inhibitory effect of *EIF5A2* siRNA on their gene expression. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. Data are expressed as Mean ± SD. All samples were done in triplicate and all experiments were performed in triplicate.

EIF5A2 Causes DOX Resistance through Activation of the TGF-β Pathway

To investigate whether *EIF5A2* caused DOX resistance through the TGF-β pathway, we examined it using a western blot (WB) assay. We found that the protein expression levels of *EIF5A2*, TGFβ-1, p-Smad2/Smad2, and p-Smad3/Smad3 were significantly increased after DOX intervention compared with the control group ($p < 0.05$, $p < 0.01$, $p < 0.001$). In contrast, si-*EIF5A2* inhibited *EIF5A2*, TGFβ-1, p-Smad2/Smad2, and p-Smad3/Smad3 expression ($p < 0.05, p < 0.01, p < 0.001$) (Fig. 3). In conclusion, the TGF-β pathway was activated in T24/DOX, and 5637/DOX cells and the TGF-β pathway was inhibited after the knockdown of *EIF5A2*, so we speculated that *EIF5A2* caused DOX resistance by activating the TGF-β pathway.

TGF-β Activator SRI-011381 Reverses the Effect of si-EIF5A2 on DOX Resistance

To further validate the effect of the *EIF5A2*/TGF-β pathway on the mechanism of DOX resistance, we added the TGF-β activator SRI-011381 to verify its effect. CCK-8 ($p < 0.05$) and clone formation ($p < 0.01$, $p < 0.001$) assays demonstrated that the proliferation of T24/DOX and

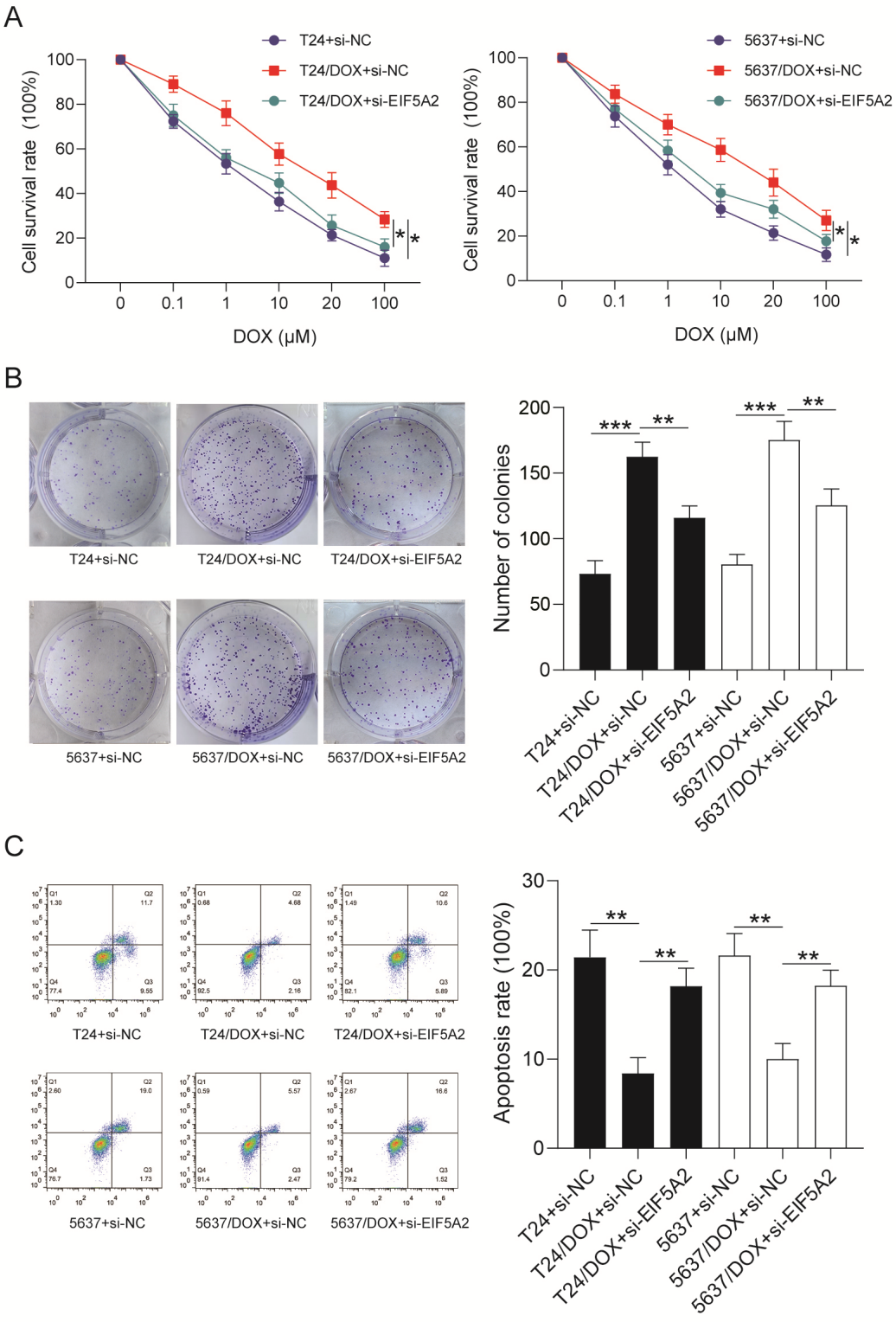

Fig. 2. si-*EIF5A2* inhibits DOX resistance in DOX-resistant BC cells. (A) CCK-8 method was used to evaluate the effects of DOX on the proliferation of T24/DOX, T24+si-NC, T24/DOX+si-*EIF5A2*, 5637/DOX, 5637+si-NC and 5637/DOX+si-*EIF5A2* cells. (B) Clonal formation experiments were used to observe the growth of T24/DOX, T24+si-NC, T24/DOX+si-*EIF5A2*, 5637/DOX, 5637+si-NC and 5637/DOX+si-*EIF5A2* cell lines under the intervention of DOX. (C) The apoptosis levels of T24/DOX, T24+si-NC, T24/DOX+si-*EIF5A2*, 5637/DOX, 5637+si-NC, and 5637/DOX+si-*EIF5A2* cell lines were detected by flow cytometry. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. Data are expressed as Mean ± SD. All samples were done in triplicate and all experiments were performed in triplicate.

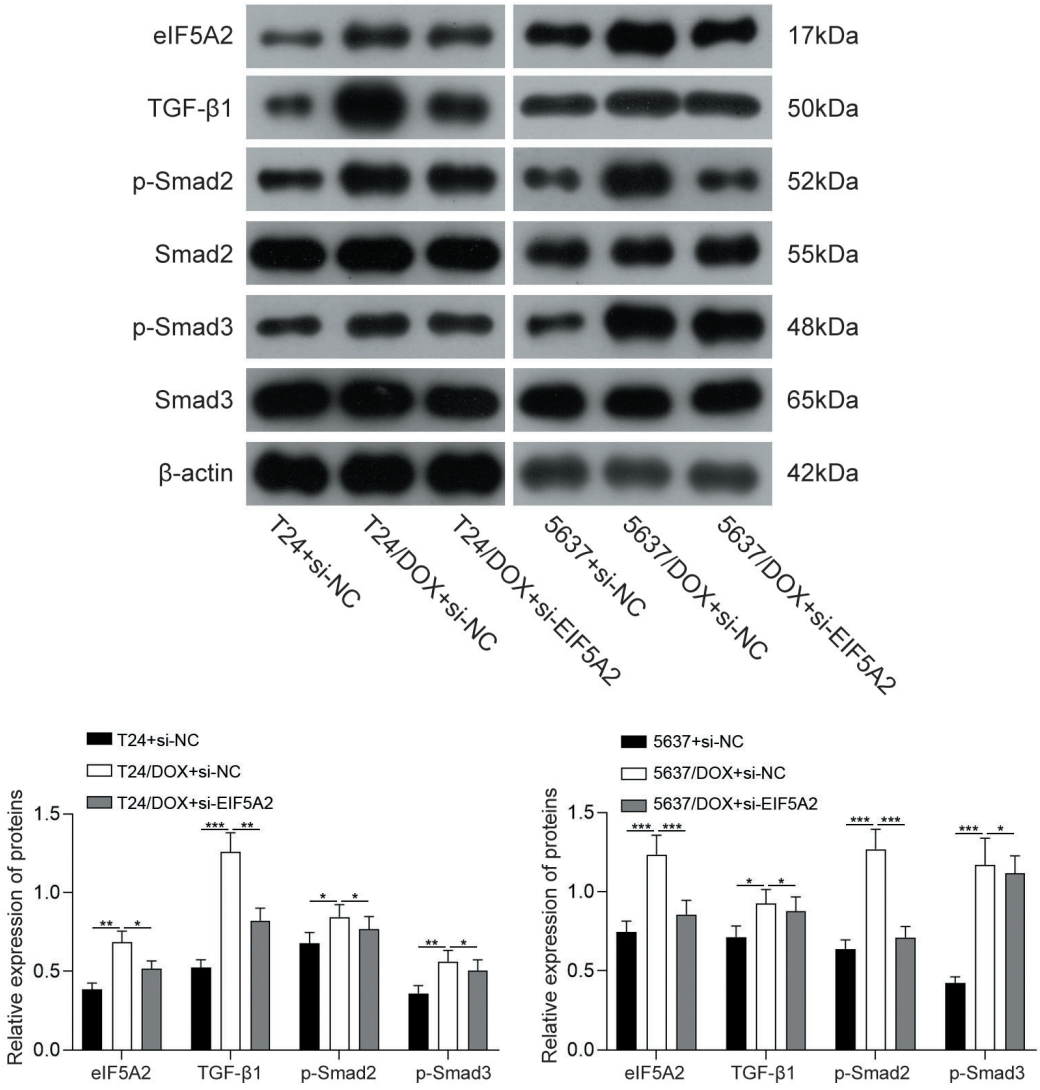

Fig. 3. *EIF5A2* causes DOX resistance through activation of the transforming growth factor (TGF)-β pathway. Western blot was used to detect the changes of *EIF5A2*, TGF-β1, p-small mothers against decapentaplegic 2 (Smad2), Smad2, p-Smad3 and Smad3 protein expression levels in T24/DOX, T24+si-NC, T24/DOX+si-*EIF5A2*, 5637/DOX, 5637+si-NC and 5637/DOX+si-*EIF5A2* cell lines after DOX treatment. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. Data are expressed as Mean $\pm$ SD. All samples were done in triplicate and all experiments were performed in triplicate.

5637/DOX cells was promoted after SRI intervention compared with the control group (Fig. 4A,B). Flow cytometry results showed that T24/DOX and 5637/DOX cell apoptosis was significantly inhibited after SRI intervention ($p < 0.01$, Fig. 4C). Overall, a reversal of the effect of si-*EIF5A2* on DOX resistance upon the addition of the TGF-β activator SRI-011381 was observed.

Discussion

Chemotherapy is an effective method of preventing metastasis and recurrence of BC [20]. DOX is one of the most common and effective drugs widely used in intravesical and systemic chemotherapy for BC [21,22]. DOX belongs to the anthracycline class of antibiotics and integrates with the DNA of the cell nucleus, disrupting the DNA structure and causing apoptosis and cell growth arrest. However, DOX resistance is also a barrier to failed therapy [23].

Overexpression of *EIF5A2*, a gene encoding a protein that plays an important role in cellular translation, has been associated with features such as the malignancy of BC and increased invasive as well as metastatic capacity of the tumor [24,25]. What's more, *EIF5A2* is also associated with chemotherapeutic drug resistance, affecting the sensitivity of tumor cells to treatment through multiple mechanisms [26]. For example, the expression of *EIF5A2* has been shown to promote chemoresistance in colorectal cancer cells via EMT [27]. Further, it has been demonstrated

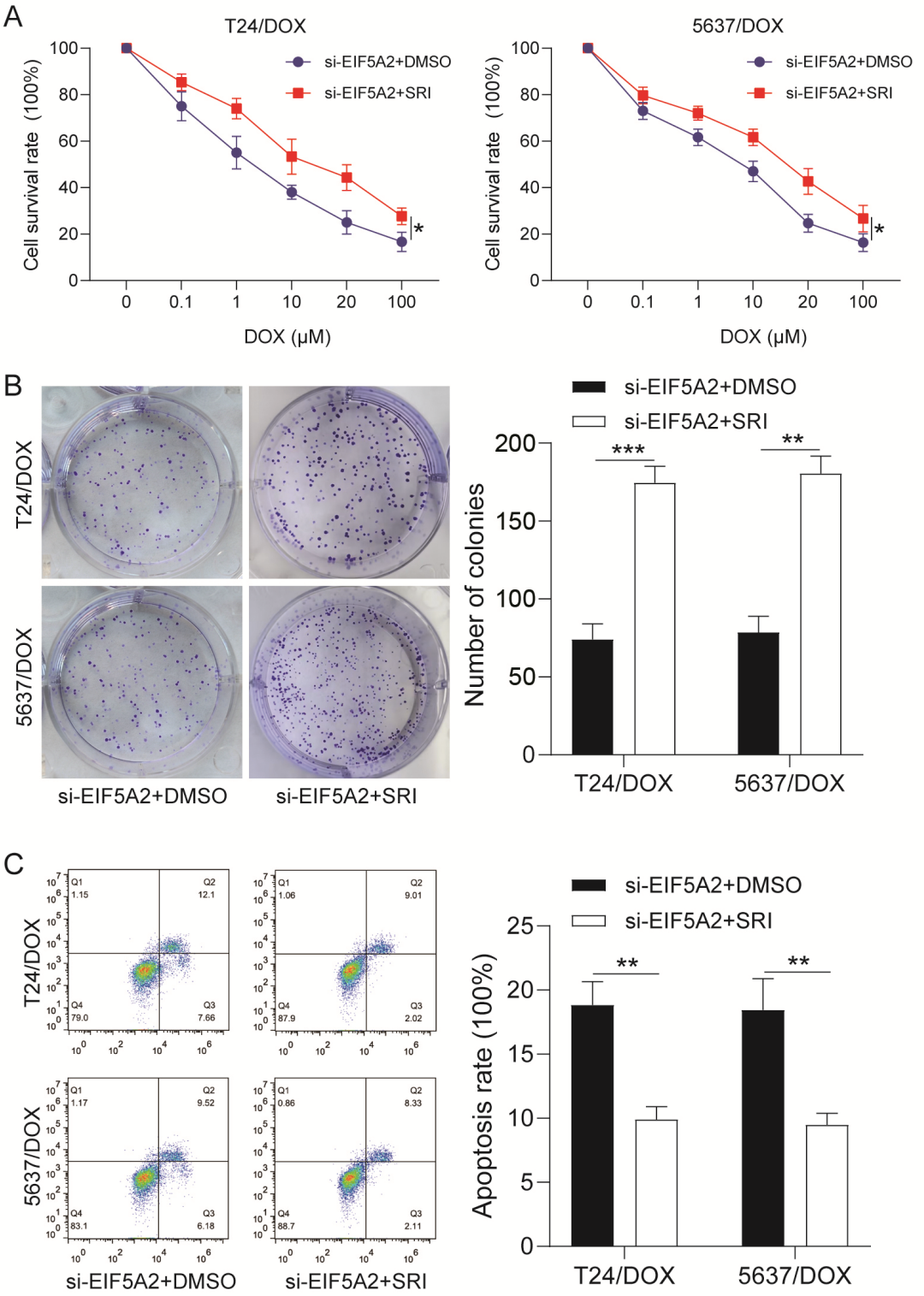

Fig. 4. TGF-β activator SRI-011381 reverses the effect of si-*EIF5A2* on DOX resistance. (A,B) The effects of adding TGF-β activator SRI-011381 on the proliferation of T24/DOX and 5637/DOX cells were demonstrated by CCK-8 and clone formation experiments. (C) The effects of TGF-β activator SRI-011381 on apoptosis of T24/DOX and 5637/DOX cells were detected by flow cytometry. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. Data are expressed as Mean $\pm$ SD. All samples were done in triplicate and all experiments were performed in triplicate.

that *EIF5A2* mediates the resistance of gastric cancer cells to cisplatin by promoting the induction of EMT [28]. Additionally, in hepatocellular carcinoma cells, the knockdown of Circ_0003998 sensitizes cells to doxorubicin by regulating the miR-218-5p/*EIF5A2* axis [29]. Finally, in the case of gastric cancer, miR-9 has been shown to improve the anti-tumor effects of Daunorubicin by inhibiting myeloid cell leukemia-1 (MCL-1) expression and thereby decreasing *EIF5A2* expression [30]. *EIF5A2* has also been studied in BC; for example, GC7 inhibits EMT through inhibition of *EIF5A2*, which sensitizes BC cells to adriamycin [13]. Our findings were similar to them. In this study, we found that *EIF5A2* was upregulated in DOX-resistant BC cells and that si-*EIF5A2* inhibited DOX resistance in DOX-resistant BC cells. However, whether overexpression of *EIF5A2* in normal BC cells induces resistance to DOX is unclear and will be further explored in future studies.

Growth factors of the TGF-β family are at the core of the TGF-β signaling pathway, including TGF-β1, TGF-β2, and TGF-β3 [31]. These growth factors activate TGF-β receptor I and receptor II kinase activity by binding to receptor complexes on the cell membrane and triggering a cascade of signaling responses [32]. The TGF-β signaling pathway plays an important role in regulating several physiological processes in the cell, such as embryonic development, osteoblast differentiation, and fibroblast proliferation [33]. It is also involved in regulating immune cell activation and immune response, as well as the regulation of inflammatory responses [34]. However, abnormal TGF-β signaling pathway activity is closely associated with tumorigenesis, metastasis, and drug resistance [35]. For example, TGF-β promotes heterogeneity and drug resistance in squamous cell carcinoma [36]. TGF-β plays a crucial role in regulating stemness, EMT and apoptosis, thus contributing to the development of drug resistance in triple-negative BrC [37]. Elevated levels of LncRNA UCA1 expression are induced by TGF-β, which ultimately contributes to the development of doxorubicin resistance in BrC cells [38]. Mesenchymal stromal cells have been found to promote the drug resistance of gastrointestinal stromal tumors by activating the phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) pathway through TGF-β2 [39]. The EMT pathway is mediated by ADNP, which prompts the development of cisplatin resistance in BC cells [40]. *EIF5A2* is highly expressed in anaplastic thyroid carcinoma and associated with tumor growth by modulating TGF-signals [41]. *EIF5A2* elevated TGF-β1 expression through STAT3 to induce EMT and promote aggressiveness in BC [42]. Our findings were similar to them, and in this study, we found that *EIF5A2* resulted in DOX resistance through activation of the TGF-β pathway, whereas SRI-011381 intervention reversed the effect of si-*EIF5A2* on DOX resistance.

Conclusions

In summary, *EIF5A2* promotes doxorubicin resistance in BC cells through activation of the TGF-β signaling pathway, suggesting that *EIF5A2* may be a potential sensitizer in BC chemotherapy, which needs to be further determined by a large number of clinical studies to determine the clinical significance of *EIF5A2* and BC. In addition, *EIF5A2* and TGF-β signaling pathways may be important factors affecting doxorubicin resistance in BC cells, and therapeutic strategies targeting these molecular pathways can be designed in the future to improve the therapeutic efficacy of BC. In conclusion, this study provides new evidence for the mechanism of chemoresistance in BC cells and may identify a potential target to increase the sensitivity of BC chemotherapy.

Availability of Data and Materials

The data used to support the findings of this study are available from the corresponding author upon request.

Author Contributions

JSY and LST contributed to the concept. JSY performed the study and wrote the manuscript. XJ acquired and analyzed the data. YC performed help and acquired the data. All authors revised the manuscript. All authors have reviewed and approved this version. All authors are responsible for all aspects of the article.

Ethics Approval and Consent to Participate

Not applicable.

Acknowledgment

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

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