

Original Article

COUP-TFII suppresses colorectal carcinoma resistance to doxorubicin involving inhibition of epithelial-mesenchymal transition

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Abstract: Chicken ovalbumin upstream promoter transcription factor II (COUP-TFII) promotes progression of a variety of tumors. The study was designed to explore the role of COUP-TFII in colorectal carcinoma (CRC) resistance to doxorubicin. The sensitivity of CRC cell lines to doxorubicin was calculated by different proliferation rate measured with cell count kit-8 and EdU (5-Ethynyl-2'-deoxyuridine) assay. The expression of COUP-TFII, Vimentin and E-cadherin were verified using western blot. After doxorubicin administration, CRC cell lines presented apparently down-regulated COUP-TFII, E-cadherin expression and increased Vimentin expression. Besides, COUP-TFII knock-down resulted in significantly increased sensitivity to doxorubicin in all of CRC cell lines, but Twist knock-down presented totally reversed results. Furthermore, COUP-TFII knock-down promoted epithelial-mesenchymal transition in (EMT) CRC cell lines. After doxorubicin treatment, immediately decreased COUP-TFII expression significantly promotes CRC cells survival outcomes by suppressing EMT.

Keywords: COUP-TFII, CRC, epithelial-mesenchymal, doxorubicin, resistance

Introduction

Colorectal cancer (CRC) is one of the most common malignancies and the third most frequent cause of cancer deaths in the developed country [1]. Resection is still the most effective therapy for CRC, and post-operation adjuvant chemotherapy significantly reduces the risk of CRC recurrence [2]. The inevitably development of resistance to chemotherapeutic drugs establish a complex hurdle in CRC treatment. The underlying mechanism attribute to the protection of neoplastic cells from harmful molecule commonly caused by the induced overexpression of specific transporters [3]. Furthermore, increasing evidences support the other potential mechanisms participating into the acquisition of anti-cancer drug resistance. Among others, epithelial-mesenchymal transition (EMT) endows tumor cells decreased proliferation, apoptotic tolerance and overexpression of chemoresistance-related genes, these characteristics promote tumor cells survival in chemo-

therapy [4, 5]. Moreover, tumor cells could acquire stemness and secondary tumor initiating property through EMT process [6, 7]. The EMT induces epithelial cells depolarization, cell-cell disconnection and transiting to an elongated, fibroblast-like morphology, previously is considered as a potential mechanism of tumor cells gaining metastatic features. An inability to trace transient and reversible EMT process in vivo result in difficulty to prove the role of EMT tumor cells in distant metastasis. Dingcheng Gao et al created an EMT lineage trace system to monitor EMT or mesenchymal to epithelial transition (MET) in mice and revealed that EMT is not indispensable for the lung metastasis of breast cancer but contributes to chemotherapy resistance/[8]. Raghu Kalluri et al established a mouse pancreatic ductal adenocarcinoma (PDAC) model with deletion of Snail or Twist, two indispensable transcription factors inducing EMT, illustrating that EMT suppression in the primary PDAC did not decreased distant dissemination or metastasis but highlighting that

EMT inhibition improved the chemotherapeutic effect of pancreatic cancer [9]. In field of CRC, there are not published literatures for confirming the null effect of EMT on CRC dissemination likes PDAC and breast cancers, currently researches presented a tendency of EMT promoting CRC metastasis. Undoubtedly, EMT promotes CRC resistance to anti-cancer drug including ingenol 3-angelate, 5-fluorouracil and doxorubicin [10, 11].

Doxorubicin is an anthracycline-based antibiotic and widely used for treating patients with various solid tumors including CRC [12]. The major mechanism of doxorubicin inhibiting tumors is intercalation into the double-stranded DNA resulting in inhibition of DNA combining RNA polymerases and then blocking protein synthesis [12]. Furthermore, doxorubicin promotes free radical formation leading to cancer cell death, and the up-regulation of carbonyl Reductase 1, a member of the short-chain dehydrogenase/reductase superfamily regulating radical production renders development of CRC resistance to doxorubicin [13]. Overcoming doxorubicin resistance is a major challenge in treatment of CRC, and more endeavors have been made in exploring the doxorubicin resistance related genes. In the study, we found that three CRC cell lines administrating doxorubicin presented decreased E-cadherin expression and increased Vimentin expression and down-regulated Chicken ovalbumin upstream promoter transcription factor II (COUP-TFII). The correlation of doxorubicin resistance, EMT and COUP-TFII remains unexplained.

COUP-TFII is a transcription with two highly conserved motifs (a DNA-binding domain and a putative ligand-binding domain) belonging to the steroid/thyroid hormone receptor superfamily and regulating ovalbumin gene expression in chicken oviducts [14]. Recently, increasing evidence indicated that COUP-TFII significantly participated into regulating angiogenesis, metabolism and cell-fate specification attributing to tumorigenesis, tumor progression and metastasis [15]. In addition, COUP-TFII negatively regulates the cadherin-11 to cadherin-6 switch leading to inhibition of EMT and impaired development of kidney during embryonic period [16].

In the study, we used CRC cell lines to investigate the mechanism underlying resistance to

doxorubicin exposure with a focus on the EMT and COUP-TFII expression. Our results suggest that continuous exposure to doxorubicin promoted CRC cell lines EMT through down regulated COUP-TFII expression.

Material and methods

Cell lines and chemical, antibody

Three human CRC cell lines (LOVO, HCT116 and HT29) were purchased from Cell Bank of Type Culture Collection of Chinese Academy of Sciences, Shanghai Institute of Cell Biology, Chinese Academy of Sciences and were grown in 1640 complete medium supplemented with 10% fetal bovine serum (FBS) and 100 U/ml penicillin and streptomycin at 37 °C in a humidified incubator with 5% CO₂. Doxorubicin (Sigma-Aldrich, St. Louis, MO), DAPI (Sigma-Aldrich, St. Louis, MO), COUP-TFII antibody (Abcam, Cambridge, MA, USA), β -actin (Cell Signaling Technology, Beverly, MA, USA), Twist antibody (Cell Signaling Technology, Beverly, MA, USA), E-cadherin antibody (Cell Signaling Technology, Beverly, MA, USA), Vimentin antibody (Cell Signaling Technology, Beverly, MA, USA), Goat anti-Rabbit HRP (Cell Signaling Technology, Beverly, MA, USA), Goat anti-Mouse HRP (Cell Signaling Technology, Beverly, MA, USA).

Small interfering RNA transfection

Untreated LOVO, HCT116 and HT29 cells respectively were plated at 1×10^5 /well in 2 mL of 1640 complete medium for 24 h before transfection. 20-30% confluency of monolayer cells per well were transfected with 50 nM siRNAs targeting genes or a scramble control (GenePharma, Shanghai, China) using Lipofectamine2000 transfection reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions, cells was harvest for western blot and confocal immunofluorescence microscopy after siRNA transfection for 48-72 h. The siRNA sequences targeting Twist, COUP-TFII were listed as follows:

Twist1-homo-1575: 5'GGUGUCUAAAUGCAUUCAUUTT3', 5'AUGAAUGCAUUUAGACACCTT3'; Twist1-homo-810: 5'GGUACAUCGACUCCUCUAT-3', 5'UAGAGGAAGUCGAUGUACCTT3'; Twist1-homo-780: 5'GCAAGAUUCAGACCCUCAATT3', 5'-UUGAGGGUCUGAAUCUUGCTT3'; COUP-TFII-homo-2445: 5'GGCCGUUAUUGGCAAUUCATT3', 5'-

UGAAUUGCCAUUAACGGCCTT3'; COUP-TFII-homo-1971: 5'GCGAGCUGUUUGUGUUGAATT3', 5'-UUCAACACAAACAGCUCGCTT3'; COUP-TFII-homo-2100: 5'GGAUCUCCAAGAGCAAGUTT3', 5'-ACUUGCUCUUGGAAGAUCCTT3'.

Cell proliferation assay

Untreated or siRNA transfection LOVO, HCT116 and HT29 cells respectively were plated at 3×10^3 /well in 100 μ l of 1640 complete medium for 24 h. After doxorubicin or phosphate buffer saline administration, cell proliferation was detected using a cell counting kit-8 (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions after 24, 48 and 72 h incubation, the proliferative ability of above viable cells was identified by the absorbance for optical density at 450 nm through a microplate reader (EI $\times$ 800; BioTek, Winooski, VT, USA). In addition, cell proliferation was further identified by EdU assay (Click-iT EdU Imaging Kit, Invitrogen; Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, DNA was traced with 100 μ l/well mixture of EdU and 1640 medium (50 μ m, 1000:1), and cell nucleus was marked by Hoechst 33344. After incubation, the proliferative ability of cells was observed by a confocal microscopy.

Protein extraction and western blotting analysis

Cells were harvested and lysed in lysis buffer (Cell Signaling Technology, Beverly, MA, USA) at 4°C according to the manufacturer's instructions. Western blotting analysis was performed with the standard protocol. Briefly, protein concentrations were measured using a BCA Protein Assay Kit (Pierce, Rockford, IL, USA) by Bradford assay (Bio-Rad, Hercules, CA, USA). Equal amounts of protein were separated using SDS-PAGE and then transferred electrophoretically to 0.45 μ m polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA). The membrane was blocked with 5% non-fat milk in Tris-buffered saline containing 0.05% Tween-20 (TBST) at room temperature. Membrane was incubated with the primary antibodies overnight at 4°C after washing three times with TBST, and then incubated with the second antibodies at room temperature for 1 hour. The membrane was visualized by Super Signal West

Pico Chemiluminescent Substrate (Pierce, Billerica, MA, USA).

Statistical analysis

Experimental data were expressed as means with standard deviations and the Student's t-test Comparisons were performed for comparison of two groups. All data analysis was conducted with the SPSS software (version 19.0). A value of $P < 0.05$ was set to be statistically significant.

Results

The sensitivity of CRC cell lines to doxorubicin

The sensitivity of three CRC cell lines (LOVO, HCT116 and HT29) to doxorubicin calculated from the change in the cell viability following 24 h drug administration, we found that HT29 presented the most fierce sensitivity to doxorubicin and LOVO had the strongest resistance, and the IC_{50} of LOVO, HCT116 and HT29 cells respectively were 0.8931 0.4546 0.1761 μ g/ml (**Figure 1A**). Exposing above cell lines to doxorubicin of respectively corresponding IC_{50} , the proliferation suppression effect of doxorubicin was further verified by EdU assay (**Figure 1C, 1E**).

COUP-TFII knockdown promoting doxorubicin resistance

Western blot analysis of LOVO, HCT116 and HT29 cells revealed that basal level of COUP-TFII expression is high in LOVO, moderate in HCT116 and low in HT29 (**Figure 1B**). After 24 h doxorubicin administration, CRC cell lines COUP-TFII expression were apparently down-regulated (**Figure 3A**), which suggested that COUP-TFII possibly involves doxorubicin resistance. To further explore the relationship of COUP-TFII and CRC resistance to doxorubicin, a siRNA targeting COUP-TFII and a scramble control were transfected into LOVO, HCT116 and HT29, and the knockdown efficacy was demonstrated by western blotting (**Figure 2D**). Expectedly, COUP-TFII knockdown resulted in significantly increased sensitivity to doxorubicin in all of CRC cell lines (**Figure 2A-C**). Moreover, the doxorubicin IC_{50} of si-LOVO, si-HCT116 and si-HT29 were respectively 0.4430 0.2707 0.1968 μ g/ml, which apparently was inferior to cells transfected control siRNAs.

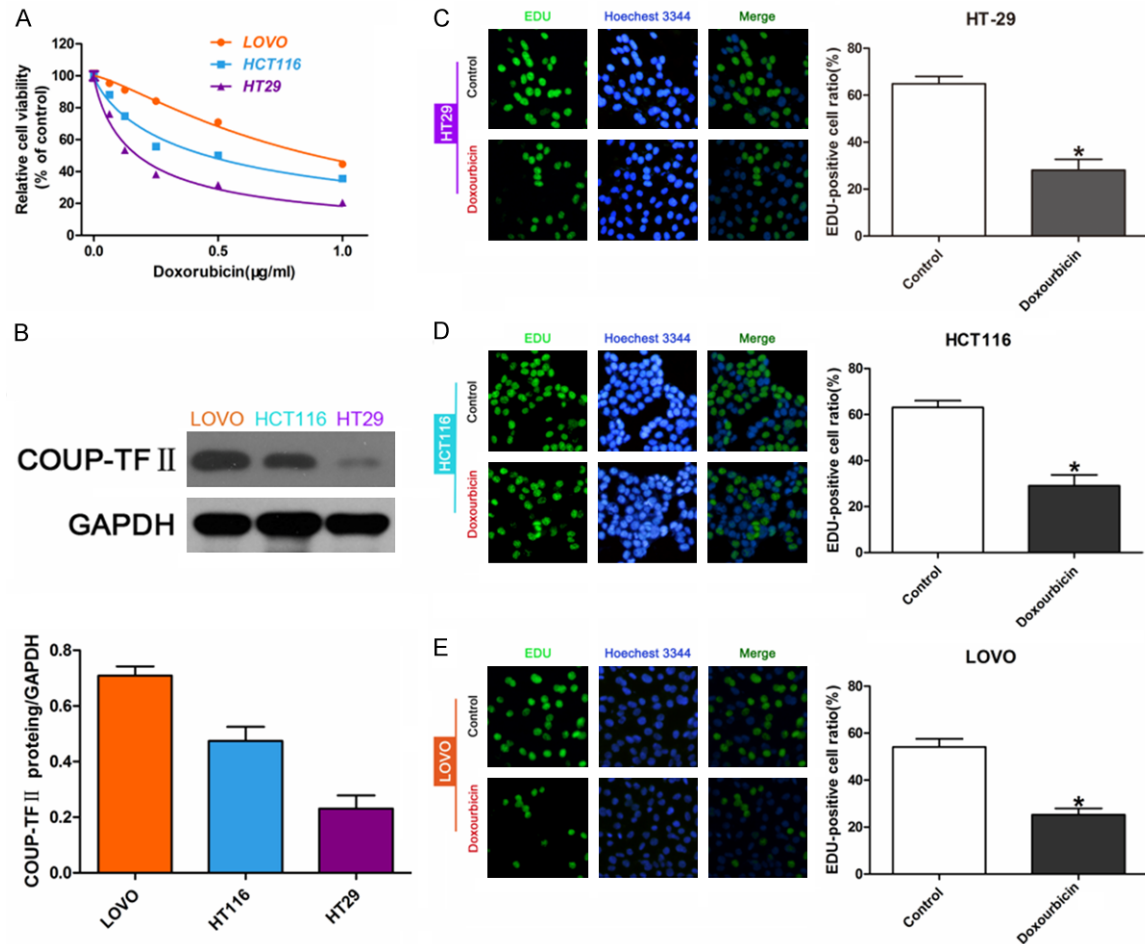


Figure 1. (A) The sensitivity of three CRC cell lines (LOVO, HCT116 and HT29) to doxorubicin was firstly calculated by CCK-8 proliferation assay, and the most fierce sensitivity in HT29, moderate in HCT116 and low in LOVO, and the LD50 of LOVO, HCT116 and HT29 cells respectively were 10, 10, 10 μg/ml. (B) The COUP-TFII expression is high in HT29, moderate in HCT116 and low in LOVO. The proliferation suppression effect of doxorubicin was further verified by EdU assay and relative EdU-positive ratio, respectively, of HT-29 (C), HCT116 (D), and LOVO (E) cells after treatment with doxorubicin or without doxorubicin treatment. *P < 0.05 vs. Control.

EMT resulting in CRC resistance to doxorubicin

Considering the close relationship between EMT and anti-tumor drugs resistance supported by increasing evidence, we tested the EMT in CRC cell lines after treatment with doxorubicin and revealed that doxorubicin significantly decreased the E-cadherin expression and up-regulated Vimentin expression (Figure 3A). Accordingly, the phenomenon of EMT in CRC cell lines following doxorubicin treatment was observable too by confocal microscopy (Figure 3B). Moreover, we established Twist knock-down CRC cell lines. The Twist, Vimentin and E-cadherin expression in protein level were tested by western blot and confocal microscopy to confirm the reduction in EMT of Twist-siRNA

transfection CRC cells (Figure 4D-E). Intriguingly, EMT suppression significantly increased the cytotoxic effect of doxorubicin on CRC cell lines (Figure 4A-C). These data demonstrated that EMT is obviously one of mechanism responsible to the CRC cells survival in doxorubicin treatment.

COUP-TFII promotes EMT leading to CRC resistance to doxorubicin

Given that previously published literatures hinting that COUP-TFII inhibits MET leading to impaired development of embryonic kidney [16]. We measured the markers expression of EMT in siRNA-COUP-TFII transfection CRC cell lines and revealed that COUP-TFII knockdown

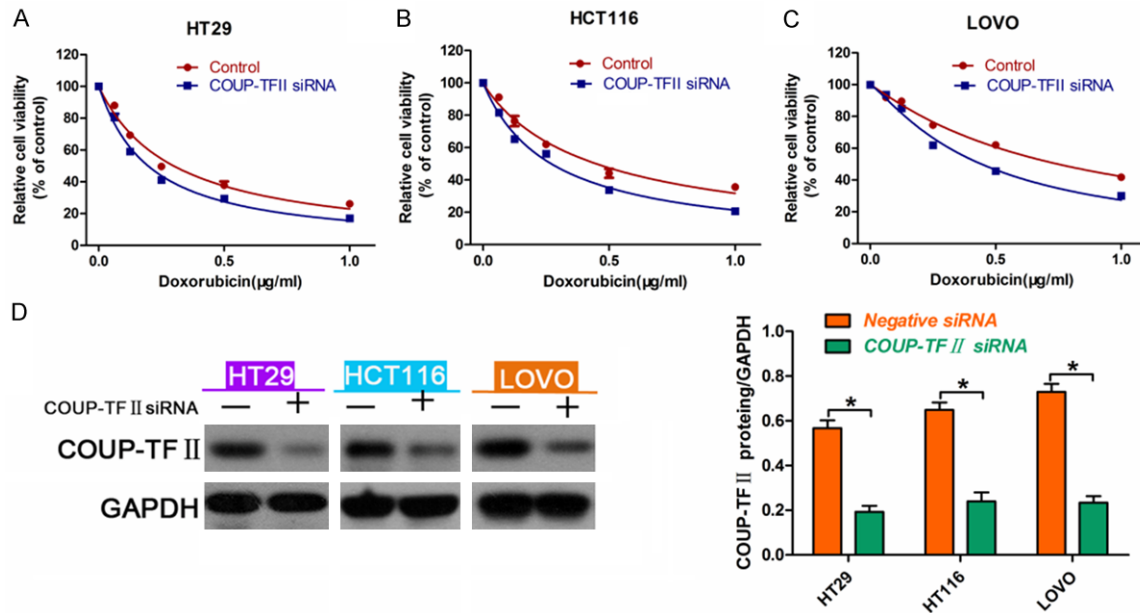


Figure 2. COUP-TFII knockdown resulted in significantly increased sensitivity to doxorubicin in all of CRC cell lines. Negative siRNA transfected (Control) or COUP-TFII siRNA transfected in CRC cells including HT29 (A) HCT116 (B) LOVO (C) were treated with doxorubicin and cell viability was determined using the CCK-8 assays. * $P < 0.05$. (D) The COUP-TFII knockdown efficacy was demonstrated by western blotting. GAPDH served as a loading control, * $P < 0.05$.

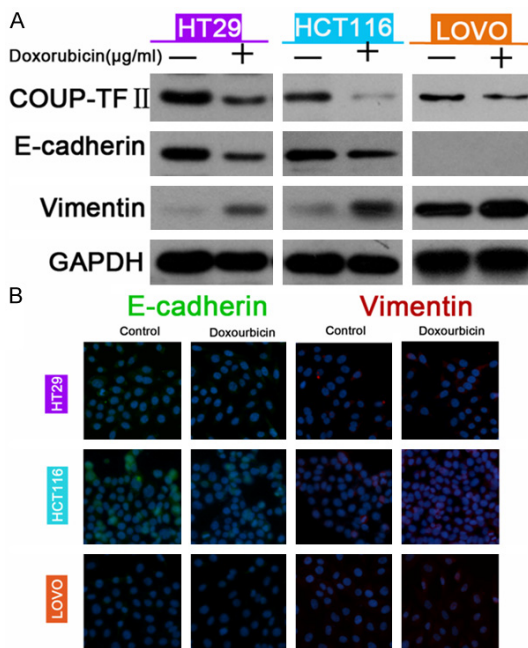


Figure 3. A. Western Blotting was used to detect the expression of COUP-TFII, E-cadherin and Vimentin in CRC cell lines. After doxorubicin administration, CRC cell lines apparently presented down-regulated COUP-TFII, E-cadherin expression and increased Vimentin expression. B. Immunofluorescence staining of CRC cell lines for E-cadherin and Vimentin expression treated with doxorubicin alone or control. The decreased E-cadherin expression and increased Vimentin expression were also observed by a confocal microscopy following doxorubicin treatment.

suppressed Vimentin expression and up-regulated E-cadherin expression (Figure 5E). Furthermore, comparing the cell viability between COUP-TFII knockdown and Twist knockdown CRC cell lines, the results shown no significantly difference between two groups (Figure 5A-C), which hinted that COUP-TFII suppressed CRC resistance to doxorubicin involving inhibition of EMT.

Discussion

In the past few decades, a number of centers demonstrated that EMT could endows primary tumor cells several characteristics such as impaired cell-cell contacts, motile and secondary tumor initiation property, and solidly considered EMT as an initiator of cancer lapse [17]. The metastatic lesions from epithelial tumors commonly exhibited epithelial phenotypes supposedly owing to MET, but deficient method for tracing switch of EMT and MET make the importance of EMT in vivo remain fiercely contested. Recently, the appearance of two models (an EMT lineage tracing model, tri-PyMT/Vim mice, MMTV-PyMT/Rosa26-RFP-GFP/Vimentin-creER, and mouse models of PDAC with deletion of Snail or Twist) for tracing EMT cells in vivo revealed that EMT neither alter the emergence of lung metastasis from breast cancer nor inva-

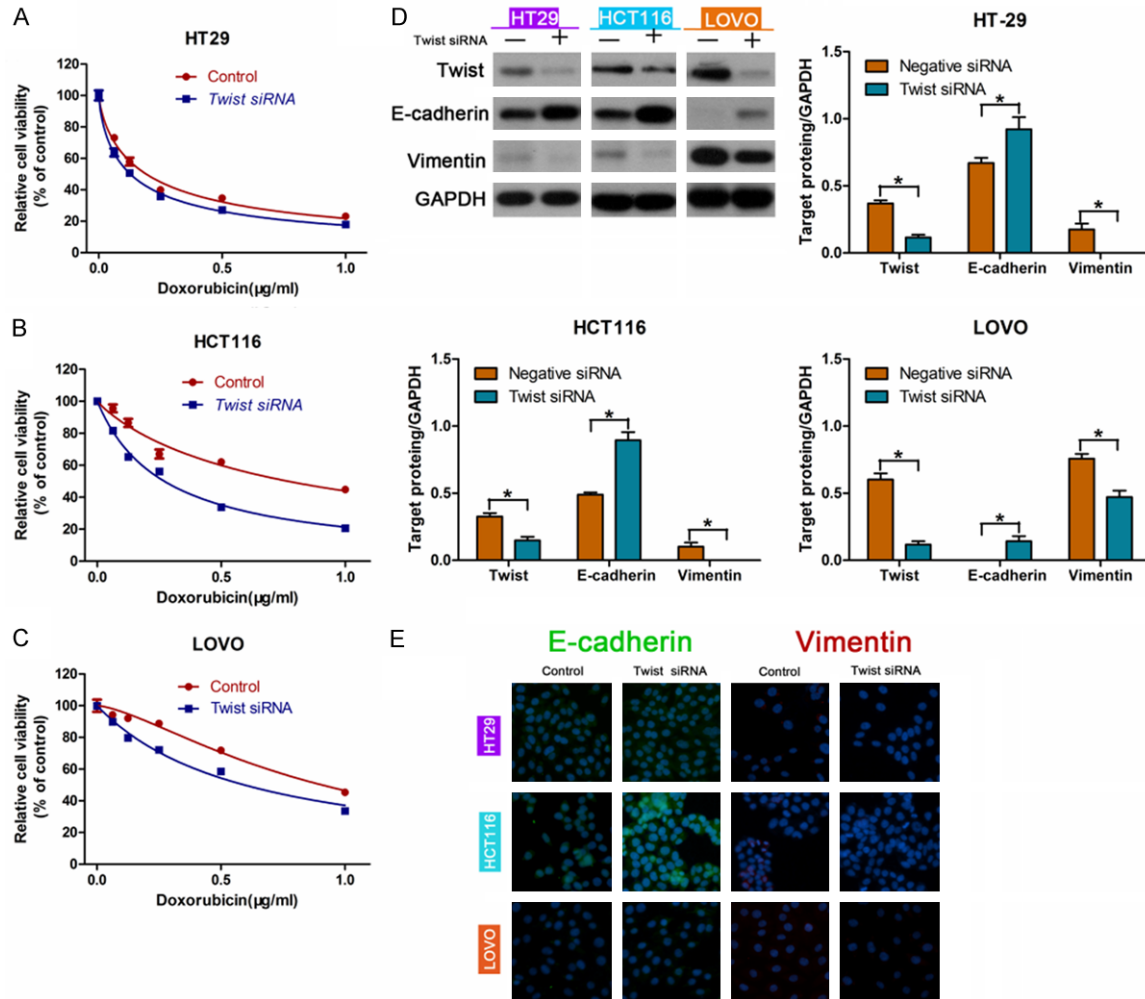


Figure 4. CCK-8 assay was used to analysis the cell viability. After Twist-siRNA transfection, EMT suppression significantly increased the cytotoxic effect of doxorubicin on HT29 (A), HCT116 (B) and LOVO (C). The efficiency of Twist-siRNA was verified by western blot and a confocal microscopy, Twist-siRNA transfection HT29, HCT116 and LOVO significantly decreased Twist expression. (D) In Twist-siRNA transfection cells, decreased Vimentin expression and increased E-cadherin expression were tested by western blotting. Relative protein expression in CRC cells was quantified by band density with GAPDH served as control. * $P < 0.05$. (E) Immunofluorescence was performed to determine the expression of E-cadherin and Vimentin in CRC cell lines.

sive PDAC [18, 19]. The observational time were not as long as several years after operation in clinical practice but the EMT lever undoubtedly was a risk factor for post-operative poor prognosis no matter in breast cancer or PDAC [18, 19], these data potentially suggested that increased capacities likes chemotherapy resistance, immune escape from EMT rather than impaired cell-cell contacts, motile or secondary tumor initiation property responsible for EMT promoting cancer lapse.

Intriguingly, the reports from above two mouse model for tracing EMT in vivo consistently confirmed that EMT significantly contributed to

tumor cells survival during chemotherapy. Similarly, EMT was a fatal barrier to successful treatment of various anti-tumor drugs. Kim AY et al declared that EMT closely associated with 5-Fluorouracil resistance acquisition of HT-29 cell [20]. Moreover, inducing a MET in colon cancer cells given its ability to induced cell cycle arrest, inhibited proliferation and reversed gefitinib, a EGFR inhibitor resistance [21]. Besides, EMT also was responsible for CRC resistance to the protein kinase C β inhibitor enzastaurin [22]. However, rare attention is payed to the association between EMT and doxorubicin resistance in CRC.

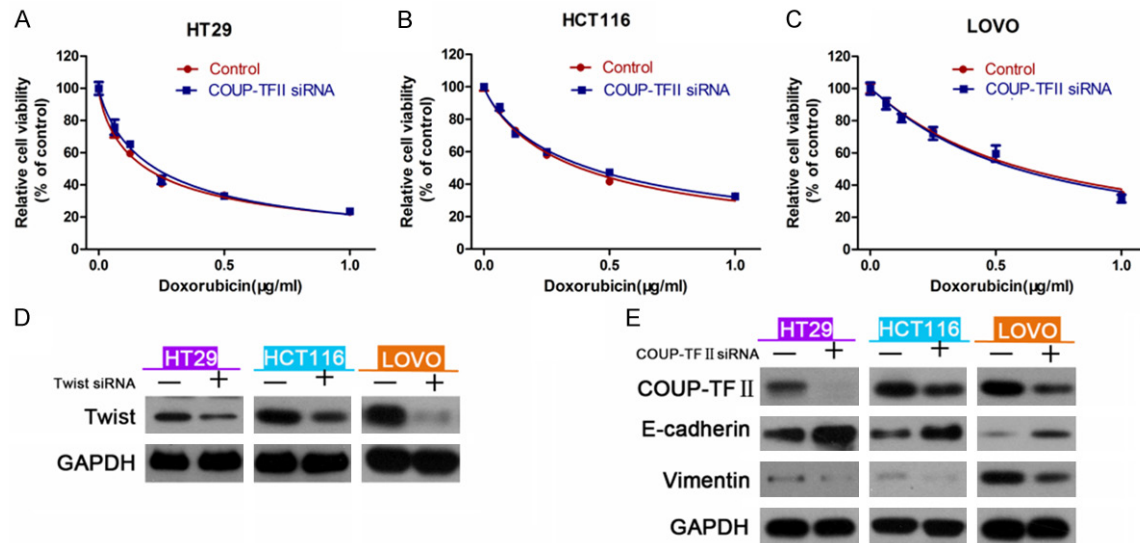


Figure 5. CCK-8 assay was used to determine the cell viability. The cell viability of HT29 (A), HCT116 (B) and LOVO (C) cells under COUP-TFII siRNA, control was under the normal condition. There were no significant difference on cell proliferation between COUP-TFII knockdown and Twist knockdown CRC cells (HT29, HCT116 and LOVO, A-C). Western Blotting showing Twist protein expression in CRC cell lines transfected Twist siRNA or a negative control siRNA (D), detecting the expression of COUP-TFII, Ecadherin and Vimentin under COUP-TFII siRNA or a negative control siRNA (E). COUP-TFII knockdown significantly promoted EMT of HT29, HCT116 and LOVO cells.

Classically, EMT process that presents a morphological change from epithelial to mesenchymal phenotype is marked by the loss or appearance of a group of proteins such as snail, Vimentin, CDH1 and CDH2. Most of these proteins are elaborately regulated by transcription factors and microRNAs at the transcriptional and translational level. MicroRNA200 family is a classical microRNA cluster of inhibiting EMT via a double negative feedback loop regulating mechanism with ZAB1-SIP1, an EMT related transcription factor [23]. SIP1 could downregulate E-cadherin in multiple tumors by binding to conserved E2 boxes from E-cadherin [24]. Besides transcription factors and microRNAs, CD44, a marker of cancer stem cells, is associated with activation of multiple signal pathways and closely linked to the EMT [25]. As a matter of fact, we observed that CRC cell lines (LOVO, HCT116 and HT29) presented increased EMT following treatment with doxorubicin, and Twist knock-down significantly reduced CRC cells survival rate in doxorubicin therapy. These data suggested that EMT was contributed to doxorubicin resistance.

Furthermore, after doxorubicin administration in three CRC cell lines, we found that COUP-TFII expression was immediately down-regulated in survived CRC cells, which hinted that COUP-TFII

was involved in doxorubicin resistance. The speculation was verified by COUP-TFII knockdown cells treated with doxorubicin. COUP-TFII play an important role in the development of embryonic solid organs likes atria and kidney [16, 26]. In addition, COUP-TFII that was unveiled as a pro-oncogenic transcription factor strongly increased the cell growth, angiogenesis and invasiveness, and high COUP-TFII expression significantly correlated with metastasis and poorer prognosis [27]. Deng A et al revealed that elevated expression of COUP-TFII in CRC correlated with metastasis and poorer survival outcomes by promoting expression of Snail1, a well-known protein advancing EMT process [28]. Confusedly, Shou C et al disclosed that high COUP-TFII transcript level improved patients with breast malignancy long-term survival via inhibiting chemo-resistance involving inhibition of TGF- β -dependent EMT [29]. In the study, suppressed Vimentin expression and up-regulated E-cadherin expression were presented in the COUP-TFII knockdown CRC cell lines, which hinted that COUP-TFII was a suppressor of EMT.

In summary, immediately decreased COUP-TFII expression significantly promotes CRC cells survival outcomes following doxorubicin treatment. Moreover, the mechanism underlying

COUP-TFII regulating CRC resistance to doxorubicin is suppressing EMT.

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Disclosure of conflict of interest

None.

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