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Tanshinone IIA inhibits cervix carcinoma stem cells migration and invasion via inhibiting YAP transcriptional activity

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ABSTRACT

This study aims to explore the effects and related mechanisms of Tanshinone IIA in cervix carcinoma (CC) stemness-like cells migration, invasion, stemness and chemotherapeutical sensitivity. Here, we found that Tanshinone IIA suppressed CC stemness-like cells migration and invasion in a concentration- and time-dependent manner. And consistent results were obtained in CC cells stemness characterized as the decrease of CC stemness markers expression and cells spheroid formation ability. Mechanistically, we found that Tanshinone IIA suppressed RNA binding protein HuR translocation from nuclear to cytoplasm, and thus reduced YAP mRNAs stability and transcriptional activity. Importantly, overexpression YAP-5SA rescued the inhibition of Tanshinone IIA on CC cells stemness. Furthermore, Tanshinone IIA enhanced adriamycin sensitivity in CC stemness-like cells, this effect was attenuated by YAP-5SA overexpression too. Therefore, Tanshinone IIA could suppress CC stemness-like cells migration and invasion by inhibiting YAP transcriptional activity.

1. Introduction

Cervical cancer (CC) is one of the common gynecological malignant tumors that seriously threaten women health, and is closely related with the human papillomavirus (HPV) infection and the HPV vaccine has achieved remarkable results in the prevention of cervical cancer [1]. Although surgery, radiotherapy and chemotherapy have exerted better effects on early CC treatment, the efficacy on local stage and metastatic CC is limited and the survival rate of recurrent CC is lower [2]. Cancer stem cells (CSCs) have been confirmed to be involved in tumor initiation, metastasis and recurrence [3], thus, finding novel drugs that could eliminate CC CSCs or suppress CC cells stemness may provide potential methods or new clues for treating CC patients.

Danshen (*Salvia miltiorrhiza Bunge*) has been used extensively and historically in China to treat various diseases, including cardiovascular diseases, cerebrovascular diseases and cancer [4]. TanshinoneIIA is one of the major monomer extracted from root of *Salvia miltiorrhiza* (RSM), which holds various activities, especially in tumors progression [5] and inflammation [6]. Previous studies have shown that TanshinoneIIA could inhibit the growth of glioma and breast cancer stem cells [7,8]. However, the roles and related mechanisms of Tanshinone IIA in CC stemness have never been reported.

Hippo pathway is a conservative signaling in mammals, and consists of MST1/2 (mammalian Sterile 20-like kinase 1/2), LATS1/2 (large tumor suppressor 1/2) which could phosphorylate and inactivate the downstream transcriptional effectors YAP/TAZ [9]. YAP/TAZ has been shown to contribute to tumor cells stemness and inhibition of YAP/TAZ transcriptional activity could attenuate CSCs progression [10,11]. Notably, LATS1/2 could suppress breast cancer EMT and metastasis via inactivating YAP/TAZ activity [12]. However, effectors in facilitating the development of YAP/TAZ are frustrated. Therefore, it is an urgent need to find novel YAP/TAZ inhibitors.

RNA binding proteins are a kind of proteins that could bind to and enhance mRNAs stability [13]. HuR, as an RNA binding protein, has been shown to facilitate CSCs progression, such as HuR facilitates cancer stemness of lung cancer cells via regulating miR-873/CDK3 and miR-125a-3p/CDK3 axis [14], and miR-146b-5p overexpression attenuates stemness and radioresistance of glioma stem cells by targeting HuR/lincRNA-p21/beta-catenin pathway [15]. Here, we found that Tanshinone IIA could inhibit HuR translocation from nuclear to cytoplasm. Furthermore, we indicated that HuR directly bound to YAP and enhanced YAP transcriptional activity. Importantly, we showed that Tanshinone IIA attenuated CC cells stemness, CC stem cells migration and invasion in a concentration- and time- dependent manner, these effects were attenuated by YAP-5SA overexpression which could not be phosphorylated by LATS1/2. Finally, we found that Tanshinone IIA

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enhanced adriamycin sensitivity in CC cells. Therefore, our results suggest that Tanshinone IIA could suppress CC stem cells formation, migration and invasion by activating Hippo pathway.

2. Material and methods

2.1. Cells culture and reagents

CC cells Hela, and C33 A, and healthy primary normal cervical epithelial cells HcerEpic were purchased from the Chinese Academy of Sciences Cell Bank. All of the cell lines were cultured in Dulbecco's Minimum Essential Medium (DMEM) medium (Gibco,USA) with 10% FBS (fetal bovine serum, Gibco) plus 80 U/ml penicillin and 0.08 mg/ml streptomycin at 37 °C under humidified atmosphere with 5% CO_2. Tanshinone IIA (Cat # S2365) and adriamycin (S1208) were purchased from Selleck.cn. pQCXIH-Myc-YAP-5SA (YAP-5SA) plasmid (Plasmid # 33093), 8xGTIIC-luciferase plasmid (Plasmid # 34615), a YAP-responsive synthetic promoter driving luciferase expression plasmid and pFRT_TO_eGFP_ELAVL1 (Plasmid #106105), a plasmid inducing HuR expression, were purchased from Addgene.

2.2. Transfection

Cells were seeded at the density of 5×10^5 cells/well in a 6-well plate. After 24 h, 2.5 μg of YAP-5SA plasmid was transfected into cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) following the manufacturer's instructions before Tanshinone IIA treatment.

2.3. Cell spheroid formation assay

CC cells with YAP-5SA transfection or not were cultured in ultra-low attachment 24-well plates (Corning, Union City, CA) at 500 cells/well with MammoCult™ Human Medium Kit (Cat#05620, Stemcell Technologies, Vancouver, BC, Canada), followed by Tanshinone IIA treatment. After 10 days culture, mammospheres number and size were evaluated using a microscope fitted with a ruler. Random six areas were chosen for quantifying spheres number.

2.4. Cell migration and invasion assay

Cell spheres formed by CC cells were digested and re-suspended, and 8×10^4 cells were added to the upper chamber of 24-well MILLIcell Hanging Cell Culture inserts 8 mm PET (MILLIPORE) pre-coated with BD BioCoat Matrigel followed by Tanshinone IIA treatment or not, 800 μl medium containing 20% FBS was used as a chemo-attractant in the bottom chamber. After 24 h for migration and 36 h for invasion, cells migrating and invading into the underside were fixed in methanol for 15 min, and stained with 0.1% viola crystalline solution for 15 min. Five random fields from each of the triplicate were counted by using phase contrast microscopy. Quantification was carried out by measuring with Microplate Reader (OD 570 nm) after being destained with 30% glacial acetic acid.

2.5. Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted using TRNzol (Cat # DP405-02, TIANGEN, Beijing, China) according to the manufacturer's protocols. Then total RNA was reverse transcribed into cDNA using TIANScript RT Kit (Cat # KR104-01, TIANGEN) following the standard protocols. Afterwards, mRNA expression was examined using Quant qRT-PCR kit(SYBR Green) (Cat # FP302-01, TIANGEN) and carried out on an ABI Prism 7500 Detection System (Applied Biosystems, Inc., USA). GAPDH was used as an internal reference. mRNA expression was measured using $2^{-\triangle\triangle ct}$ method.

2.6. Western blot

The detailed procedure was referred to the previous study [16]. The primary antibodies against HuR (ab200342), YAP (ab52771), ALDH1 (ab9883), Cleaved caspase 3 (ab2302), Cleaved PARP1 (ab32064), Nanog (ab80892) and CTGF (ab6992) were purchased from Abcam. The primary antibodies against Caspase 3 (Cat # 19677-1-AP), PARP1 (Cat # 13371-AP), E-cadherin (Cat 20874-1-AP) and Vimentin (Cat # 10366-1-AP) were purchased from proteintech, GAPDH primary antibody (Cat # AF1186) was purchased from Beyotime (Beijing, China). Secondary peroxidase-conjugated goat anti-rabbit (Cat # A0208) and Peroxidase-conjugated goat anti-mouse (A0216) were purchased from Beyotime. Immunoblots were exposed using the ECL Plus (Solarbio Life Sciences, Beijing, China), following manufacturer's instructions using IVIS-Lumina imaging system (Caliper Life Sciences, MA, USA).

2.7. RNA binding protein immunoprecipitation (RIP) assay

The detailed procedure was mentioned before [17]. Briefly, CC cells were lysed with 25 mM Tris−HCl buffer (pH 7.5) and 100 U/ml RNase inhibitor (Sigma), and then incubated with protein-A Sepharose beads pre-coated with 3 μg anti-HuR (ab200342) antibody or control rabbit IgG for 1.5 h at 4 °C. The RNA-protein complexes were pulled-down by protein A/G agarose beads and RNA was extracted with TRNzol, then YAP mRNA level was detected via qRT-PCR assay.

2.8. Immunofluorescent assay

Cells were cultured on glass bottom plates and treated with Tanshinone IIA. After 24 h, cells were washed with PBS, and then fixed with 4% paraformaldehyde for 15 min, followed by the permeabilization with 0.1% Triton X-100 for 10 min and blocked with 5% BSA in PBS for 1 h at room temperature. Then cells were incubated with antibody against HuR (ab200342) overnight at 4 °C, and washed with PBS for 5 min three times and incubated with FITC-conjugated secondary antibody (Cat # A0562, Beyotime) for 1 h at room temperature, and washed with PBS for three times again and observed under the confocal microscopy.

2.9. Luciferase reporter assay

YAP transcriptional activity was assayed via luciferase reporter assay in cells with different treatment. Briefly, 8xGTIIC-luciferase plasmid was co-transfected into CC cells with β-gal (Ambion, USA) plasmid using Lipofectamine^{TM2000} followed by Tanshinone IIA treatment for 72 h. After that, the luciferase activity of 8xGTIIC-luciferase plasmid was measured using a Luciferase Reporter Assay Kit (cat. no. K801-200, BioVision, Inc., Milpitas, CA, USA). β-gal activity was determined using a β-Galactosidase Enzyme Assay System with Reporter Lysis Buffer (cat. no. E2000, Promega Corporation) following the manufacturer's protocols, and was used as a normalization control for luciferase activity.

2.10. Cell viability assay

Cell spheres were digested and 3×10^3 cells were seeded into 96-well plates, followed by the treatment of adriamycin plus Tanshinone IIA or not, after 24 h, 48 h and 72 h, cell viability was examined by CCK8 assay kit (Cat # HY-K0301, MedChemExpress, USA) according to the manufacturer's recommendation.

2.11. Statistical analysis

All data were expressed as mean ± SD. Means were compared using Student's unpaired test. $P < 0.05$ was considered statistically significant. All experiments were repeated at least three times.

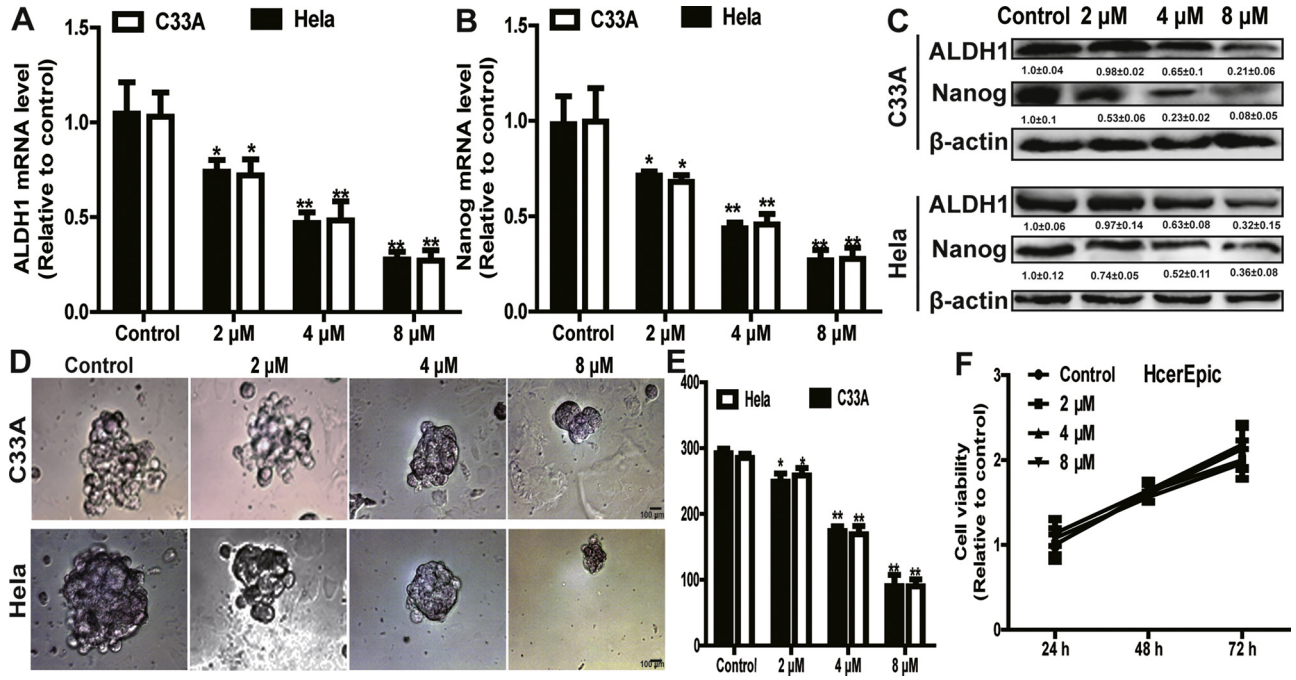

Fig. 1. Tanshinone IIA inhibits CC cells stemness in a concentration dependent manner. (**A** and **B**) mRNA level of ALDH1 (**A**) and Nanog (**B**) was detected in C33 A and Hela cells with the treatment of different concentration of Tanshinone IIA as indicated for 48 h. (**C**) Protein level of ALDH1 and Nanog was examined in cells depicted in (**A**). (**D** and **E**) The potential of CC cells spheroid formation was evaluated with the treatment of different concentration of Tanshinone IIA as indicated for 10 days by measuring the spheroid size (**D**) and numbers (**E**). Data were presented as mean ± s.d; **P < 0.01 vs. control.

3. Results

3.1. Tanshinone IIA inhibits CC cells stemness in a concentration dependent manner

Firstly, we investigated whether Tanshinone IIA could attenuate CC cells stemness. As expected, Tanshinone IIA decreased the expression of CSCs-related makers (ALDH1 and Nanog) in a concentration dependent manner (Fig. 1**A**–**C**). Furthermore, the ability of cell spheroid formation was attenuated by Tanshinone IIA in a concentration-dependent manner, characterized as the decrease of the spheres number and size (Fig. 1D and E). Notably, we detected whether Tanshinone IIA held toxicity on healthy primary normal cervical epithelial cells (HcerEpic), as shown in Fig. 1F, Tanshinone IIA had no toxicity on HcerEpic cells. Thus, these results indicate that Tanshinone IIA could inhibit CC cells stemness.

3.2. Tanshinone IIA inhibits CC stem cells migration and invasion in a concentration-dependent manner

Since CSCs have been shown to contribute to tumors progression and cell spheres were enriched for CSCs [18], we tried to detected whether Tanshinone IIA could directly suppress CC cells spheres viability, migration and invasion. CC cells spheres were digested, re-suspended and seeded into 96-well plates, after cells were attached, Tanshinone IIA was added into wells. After 24 h, 48 h and 72 h, cells viability was examined and showed that Tanshinone IIA significantly decreased cells viability (Fig. 2**A** and **B**). Additionally, after CC cells spheres were digested and re-suspended, cells were added into the upper chamber of transwell culture inserts pre-coated with matrigel or not. 24 h later, cells migration and invasion were evaluated and we found that Tanshinone IIA remarkably decreased cells migration and invasion ability (Fig. 2**C** and **D**). Moreover, the effects of Tanshinone IIA on EMT (Epithelial-mesenchymal Transition) process of CC stem cells were explored. As shown in Fig. 2E–G, indeed CC spheres held higher expression of mesenchymal marker (Vimentin) and lower expression of epithelial marker (E-cadherin), which was rescued by Tanshinone IIA treatment. Therefore, these results suggest that Tanshinone IIA inhibits CC stem cells progression.

3.3. Tanshinone IIA suppresses RNA binding protein HuR translocation from nuclear to cytoplasm and thus attenuates YAP transcriptional activity

Since another component of Danshen, cryptotanshinone could inhibit the nuclear-cytoplasmic translocation of RNA binding protein HuR [19], we assumed that Tanshinone IIA held the same effects. As shown in Fig. 3**A** and **B**, the nuclear-cytoplasm translocation of HuR was suppressed in CC cells with Tanshinone IIA treatment. Then we tried to explore the targets of HuR in this process, and informatics methods (http://starbase.sysu.edu.cn/index.php) predicted that YAP was a potential target of HuR. Firstly, qRT-PCR and western blot analysis indicated that YAP expression was increased in CC cells with HuR overexpression (Fig. 3**C** and **D**). Additionally, luciferase reporter assay showed that HuR overexpression increased YAP transcriptional activity characterized as the increase of 8xGTIIC-luciferase activity, a YAP-responsive synthetic promoter driving luciferase expression plasmid and the expression of CTGF, a target of YAP. (Fig. 3**E** and **F**). Importantly, RIP assay showed that YAP mRNA abundance was significantly increased in RNA immunoprecipitated with HuR antibody (Fig. 3G), indicating that HuR could directly bind to YAP mRNA and thus enhanced YAP transcriptional activity. We further investigated whether Tanshinone IIA could inhibit YAP transcriptional activity too, and consistent results were obtained characterized as the decrease of 8xGTIIC-luciferase activity, YAP expression and the expression of CTGF (Fig. 3**H**–J). And Tanshinone IIA attenuated the binding of HuR to YAP mRNA (Fig. 3K).

3.4. Overexpression of YAP-5SA rescues the inhibition of tanshinone IIA on CC cells stemness

Then we continue investigating whether resuming YAP transcriptional activity could attenuate the inhibition of Tanshinone IIA on CC

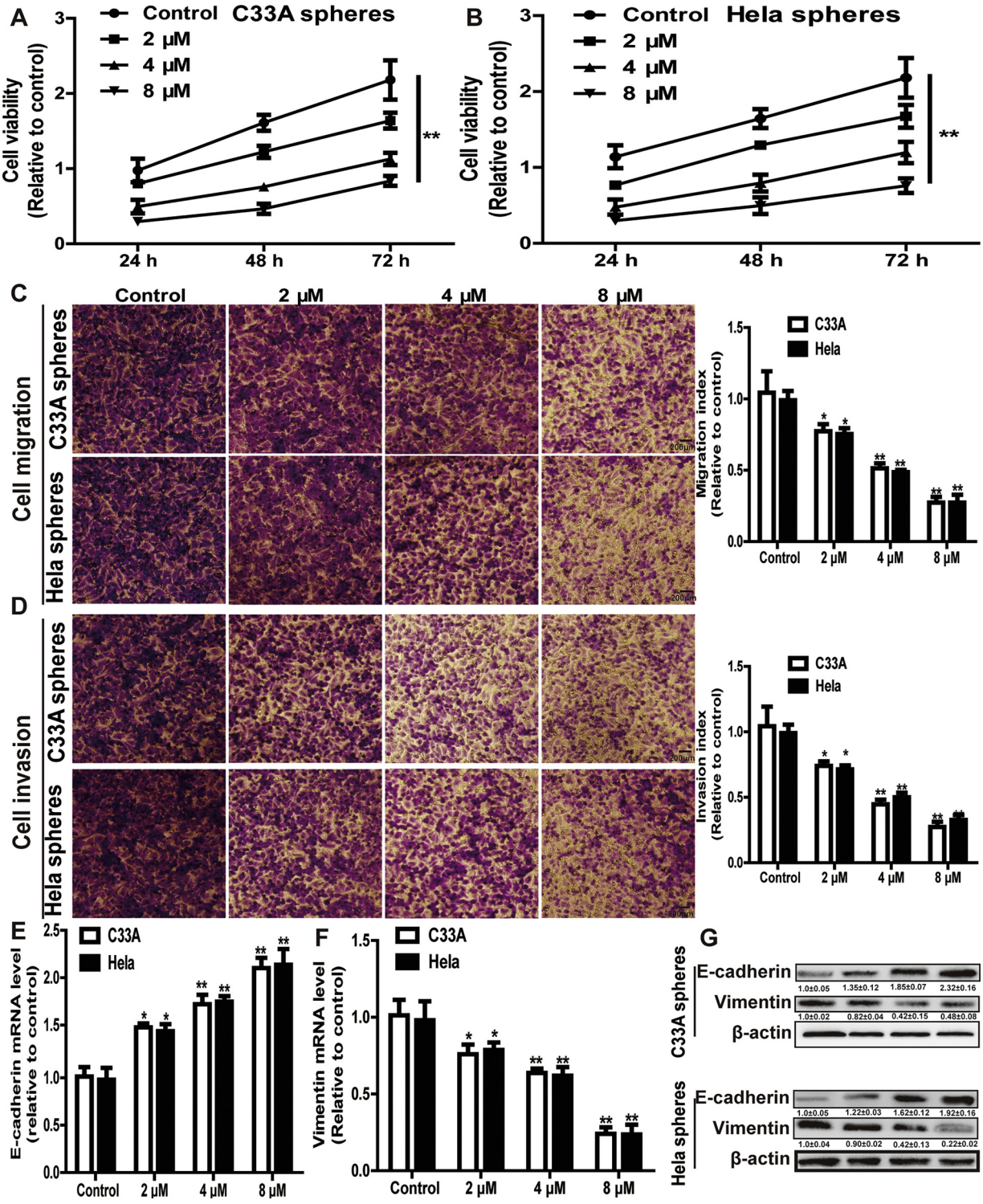

Fig. 2. Tanshinone IIA inhibits CC stem cells migration and invasion in a concentration-dependent manner. (**A** and **B**) Cell viability of cells derived from CC spheres was measured with the treatment of different concentration of Tanshinone IIA as indicated. (**C** and **D**) Cell migration (**C**) and invasion (**D**) ability of cells derived from CC spheres was determined within the treatment of different concentration of Tanshinone IIA as indicated. (**E** - **G**) The expression of E-cadherin and Vimentin was detected in cells depicted in (**A**). Data were presented as mean ± s.d; **P < 0.01 vs. control.

cells stemness. YAP-5SA was overexpressed in CC cells followed by Tanshinone IIA treatment, which could avoid the phosphorylation of LATS1/2 [20]. qRT-PCR and western blot results showed that overexpression of YAP-5SA rescued the expression of stemness markers (ALDH1 and Nanog) expression (Fig. 4**A** and **B**). Furthermore, the decrease of the ability of cell spheroid formation induced by Tanshinone IIA was partly resumed by YAP-5SA overexpression in CC cells (Fig. 4**C** and **D**). Additionally, YAP-5SA overexpression attenuated the migration and invasion of CC spheres suppressed by Tanshinone IIA (Fig. 4**E** and **F**), and hence of the EMT process in CC spheres (Fig. 4G). Therefore, our results indicate that Tanshinone IIA inhibits cervix carcinoma stem cells migration and invasion via inhibiting YAP transcriptional activity.

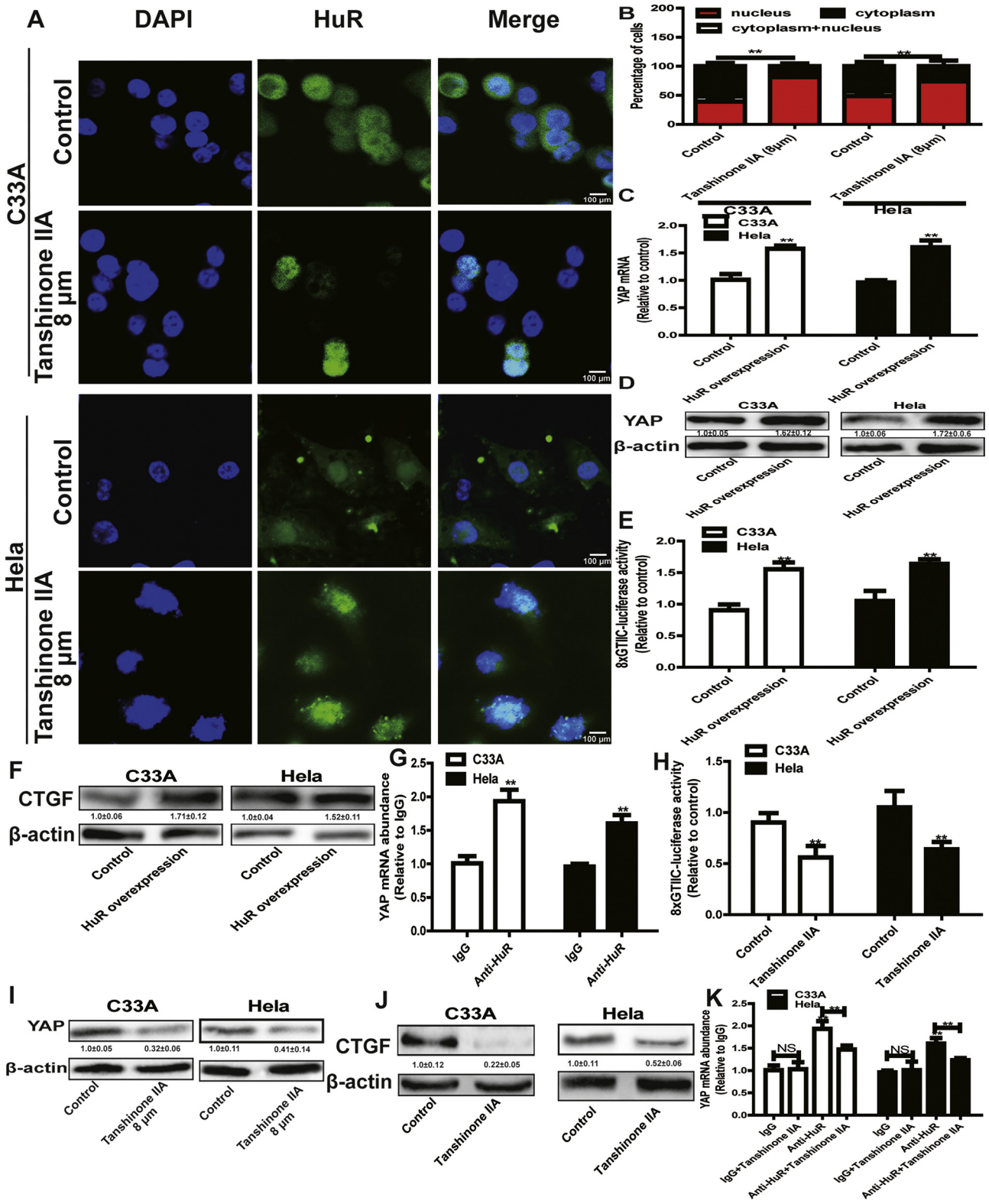

Fig. 3. Tanshinone IIA suppresses RNA binding protein HuR translocation from nuclear to cytoplasm and thus attenuates YAP transcriptional activity. (**A** and **B**) The nuclear-cytoplasmic translocation of HuR was examined in CC cells with Tanshinone IIA treatment by Immunofluorescent assay, and quantified (**B**). (**C** and **D**) YAP mRNA (**C**) and protein (**D**) level was measured in CC cells with HuR overexpression or not. (**E**) The luciferase activity of 8xGTIIC-luciferase activity, a YAP-responsive synthetic promoter driving luciferase expression plasmid, was evaluated in cells depicted in (**C**). (**F**) CTGF protein level was determined in cells depicted in (**C**). (**G**) YAP mRNA level was detected in RNA after being immuoprecipitated by anti-HuR via qRT-PCR analysis. (**H**) The luciferase activity of 8xGTIIC-luciferase activity was examined in CC cells with Tanshinone IIA (8 μM) treatment. (**I**) CTGF protein level was evaluated in CC cells with Tanshinone IIA (8 μM) treatment. (J) YAP mRNA level was detected in RNA after being immuoprecipitated by anti-HuR after different treatment as indicated via qRT-PCR analysis. Data were presented as mean ± s.d; **P < 0.01 vs. control.

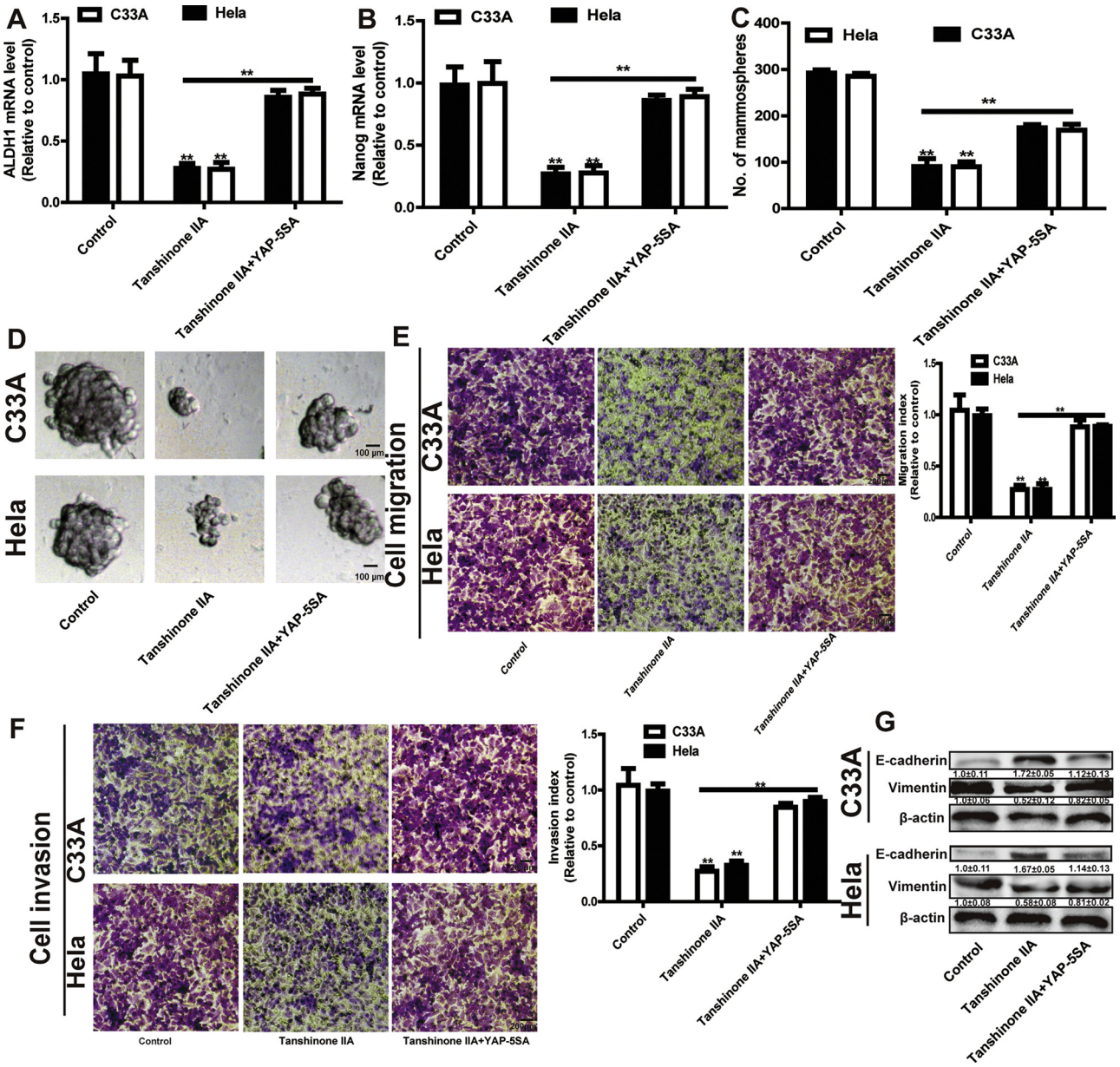

Fig. 4. Overexpression of YAP-5SA rescues the inhibitory effects of Tanshinone IIA on CC cells stemness. (**A** and **B**) ALDH1 (**A**) and Nanog (**B**) mRNA level was measured in CC cells with YAP-5SA overexpression before Tanshinone IIA treatment or not. (**C** and **D**) CC cells sphere size (**D**) and numbers (**C**) were detected in cells depicted in (**A**). (**E** and **F**) The ability of cell migration and invasion cells derived from CC spheres with YAP-5SA overexpression before Tanshinone IIA treatment or not was examined. (**G**) E-cadherin and Vimentin protein expression was evaluated in cells depicted in (**E**). The concentration of Tanshinone IIA used was 8 μM. Data were presented as mean ± s.d; **P < 0.01 vs. control.

3.5. Tanshinone IIA enhances adriamycin sensitivity in CC cells

As CSCs confers to chemoresistance [21], we finally detected whether Tanshinone IIA could enhance adriamycin sensitivity in CC spheres. Cell spheres were digested and seeded into 96-well plates followed by adriamycin treatment plus Tanshinone IIA or not. Indeed, CC cells were sensitive to adriamycin or Tanshinone IIA treatment, but CC cells digested from cells spheres were resistant to adriamycin treatment, and the resistance of adriamycin was attenuated by Tanshinone IIA treatment (Fig. 5**A** and **B**). Meanwhile, the expression of apoptotic executors (Cleaved caspase3 and Cleaved PARP) was increased compared with cells treated with adriamycin or Tanshinone IIA alone (Fig. 5**C** and **D**). Thus, we conclude that Tanshinone IIA could enhance adriamycin sensitivity in CC cells.

4. Discussion

CSCs have been proved to be involved in tumor initiation, metastasis, recurrence and chemoresistance [22]. Previous studies have shown that targeting flavin-containing enzymes could eliminate CSCs by inhibiting mitochondrial respiration [23], and CDK4/6 inhibitor could target CSCs in breast cancer [24]. However, there is no drugs reported to target CSCs in CC cells. To the best of our knowledge, this is the first study revealing that Tanshinone IIA could eliminate CC CSCs and enhance adriamycin sensitivity in CC cells.

As previous studies have shown that cells spheres were enriched CSCs [25], cells spheres were collected and chosen for investigation in this work. Here, we found that Tanshinone IIA could not only attenuate the ability of CC cells spheroid formation, but also directly decrease the viability of cells digested from spheres formed by CC cells. Furthermore,

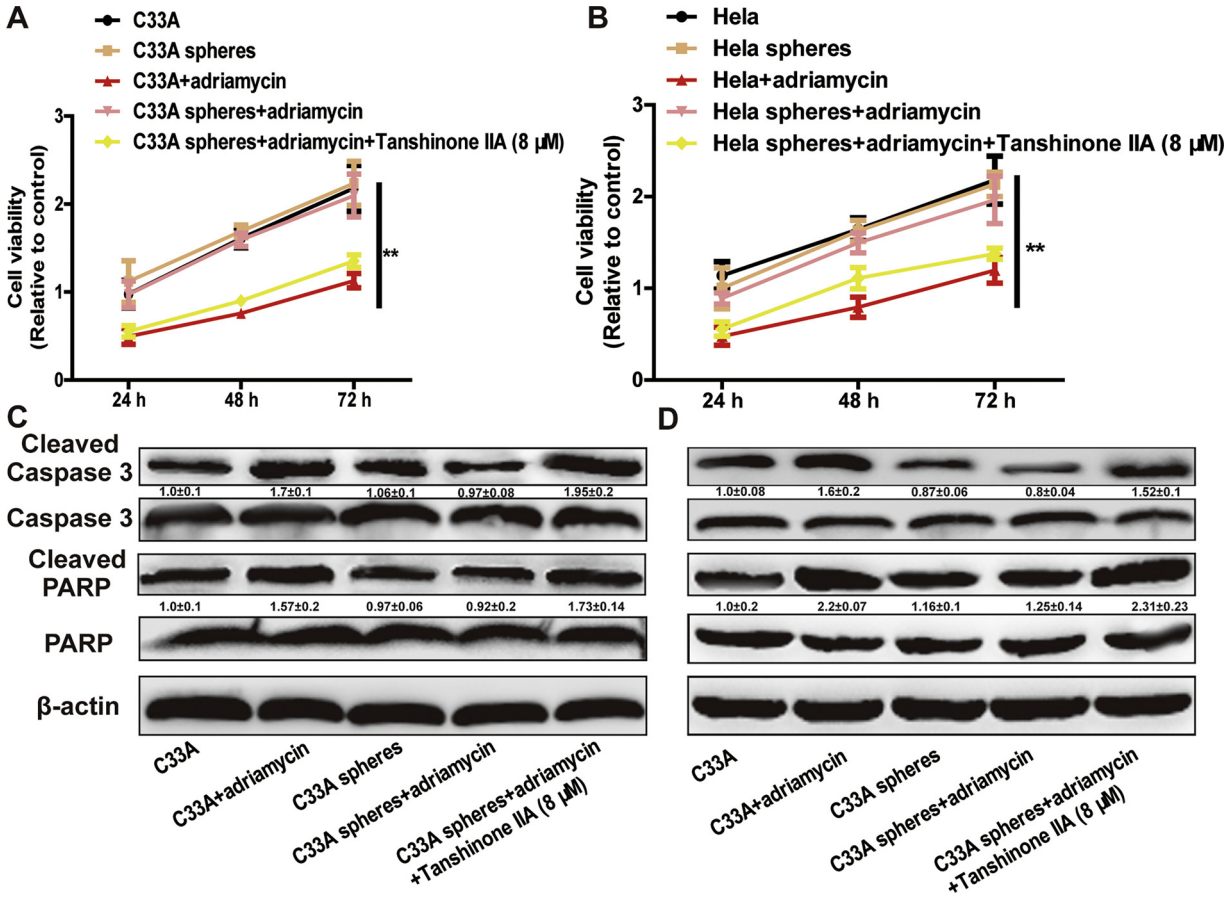

Fig. 5. Tanshinone IIA enhances adriamycin sensitivity in CC cells. (**A** and **B**) Cell viability of CC cells with adriamycin treatment, and cells derived CC spheres with adriamycin treatment as well as Tanshinone IIA or not was determined by CCk8 assay. (**C** and **D**) The expression of apoptosis executors (Cleaved caspase 3 and cleaved PARP) was detected in cells depicted in (**A**). Data were presented as mean ± s.d; **P < 0.01 vs. control.

we found that cells digested from spheres formed by CC cells were indeed resistant to adriamycin treatment, this effect was rescued by Tanshinone IIA treatment. Thus, we may conclude that Tanshinone IIA could directly kill CC CSCs.

RNA binding protein HuR plays promotive roles in tumor progression, including tumor cells stemness [14]. And another component of Danshen, cryptotanshinone has been shown to inhibit the nuclear-cytoplasmic translocation of HuR [19]. Since Tanshinone IIA is another main component of Danshen and our results showed that Tanshinone IIA could also exert the similar effects on HuR translocation from nuclear-cytoplasmic with cryptotanshinone, future studies could be performed to explore whether cryptotanshinone or even Danshen has the same effects with Tanshinone IIA. Notably, since YAP could be phosphorylated by then endogenous LATS1/2, YAP-5SA which could avoid the phosphorylation by LATS1/2, YAP-5SA was used to rescue YAP expression to elucidate the inhibition of Tanshinone IIA on CC cells stemness is partly dependent on YAP transcriptional activity. And we must admit that this work was just carried out *in vitro*, further in vivo experiments should be performed to confirm this effect. And it is still unclear whether Tanshinone IIA could affect normal stem cells function, this issue needs to be further explored.

In conclusion, as Tanshinone IIA could kill CC CSCs viability and resume the sensitivity of adriamycin in CC CSCs, Tanshinone IIA might be used to treat CC chemoresistant patients combined with chemotherapy.

Conflicts of interest

The authors declare no conflict of interest.

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