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Original Research

Copper transporter Ctr1 contributes to enhancement of the sensitivity of cisplatin in esophageal squamous cell carcinoma

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ABSTRACT

Increasing evidence has demonstrated that Ctr1 plays a crucial role in the regulation of cisplatin uptake in a variety of tumors. The purpose of this study was to investigate its role in mediating cisplatin sensitivity in ESCC cells. Immunohistochemistry (IHC), In situ hybridization (ISH) and semi-quantitative RT-PCR were used to detect Ctr1 expressions in ESCC tissues. qRT-PCR and Western blot was performed to investigate the levels of Ctr1 mRNA and protein in ESCC cells. CCK-8, Flow cytometry and Transwell chamber assay were carried out to examine cell proliferation, apoptosis, migration and invasion abilities in ESCC cells. We found that ESCC tissues and cells had higher Ctr1 level than normal tissues and Het-1A cell. Ctr1 expression was correlated with histological grade, invasion depth, TNM staging and lymph node metastasis in ESCC patients. Ctr1 depletion reduced the suppressive role of proliferation, migration and invasion as well as the inductive role of cell apoptosis and Caspase-3 activity evoked by cisplatin, whereas Ctr1 upregulation combined with cisplatin exerted the synergistic role in regulation of proliferation, apoptosis, Caspase-3 activity, migration and invasion in ESCC. In conclusion, Ctr1 is implicated in ESCC development and progression and its expression may be a novel predictor for assessment of cisplatin sensitivity in ESCC.

Introduction

Esophageal cancer (ESCA) is one of the most common tumor types in the world, and esophageal squamous cell carcinoma (ESCC) as the main histological type of ESCA possesses more than 90% of all ESCA [1]. ESCC patients mainly distribute in some Asian countries, especially in China [2], and the overall survival rate of ESCA patients remains quite poor, with 5-year survival rate of approximate 20%, which may be due to the diagnosis at an advanced stage [3,4]. Currently, standard treatment strategies for ESCA patients contain surgery, chemotherapy and radiotherapy. Cisplatin as a conventional chemotherapeutic drug was firstly approved as antitumor drug by US Food and Drug Administration in 1978 [5], and has been widely applied to treat a number of tumors in clinic, including ESCC patients [6–10], however, its intrinsic and acquired resistance as well as serious nephrotoxicity, ototoxicity and hematological toxicity greatly hindered its therapeutic efficacy and further clinic application [5,11,12]. Therefore, it is very imperative to seek for novel biomarker for auxiliary evaluation of cisplatin efficacy for ESCC patients in clinic.

Copper transport 1 (Ctr1) gene was preliminarily discovered due to its high-affinity for copper in Saccharomyces cerevisae [13], and then human Ctr1 was cloned according to its complementation characteristic [14]. Human Ctr1 consists of four key domains such as an extracellular N-terminus, three transmembrane helices, an intracellular loop of variable length and an intracellular C-terminal tail. Human Ctr1 is ubiquitously expressed in a variety of tissues, including choroid plexus, connective tissues of eye, renal tubules, ovary and testis, etc. [15]. Previous investigation revealed that Ctr1 was responsible for copper

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transport by its extracellular N-terminus domain rich in Met-and His--residues [16,17], but in the last decade, many studies have confirmed that Ctr1 plays an essential role in facilitating cisplatin intake and improving therapeutic efficacy of cisplatin [18]. Most notably, some cisplatin-resistant tumors displayed low Ctr1 level [19], whereas the utilization of copper chelator efficiently enhanced the sensitivity of cisplatin on tumor cells [20]. Given the crucial role of Ctr1 in the intake of cisplatin in tumor cells, targeting Ctr1 may be a novel strategy for improving the antitumor efficacy of cisplatin, which will contribute to the achievement of individualized treatment by detecting Ctr1 expression in different ESCC patients or other tumor patients based on cisplatin to evaluate the sensitivity to cisplatin.

In the present study, we explored the expression pattern of Ctr1 in ESCC tissues and cell lines, and verified the relationship between its expression and clinicopathological features of ESCC patients. Furthermore, the role of Ctr1 in mediating cisplatin sensitivity was expounded by loss-of-function and gain-of-function experiments in ESCC cells. Our current data suggest that Ctr1 may be a novel predictor for cisplatin efficacy in ESCC patients.

Methods

Tissue samples

One hundred and eight cases of ESCC samples and corresponding normal esophageal epithelial tissues were obtained from the First Affiliated Hospital of Zhengzhou University. All ESCC patients without chemotherapy and radiotherapy have signed informed consent form. The experiments were approved by the Research and Ethics Committee of the First Affiliated Hospital of Zhengzhou University.

In situ hybridization (ISH)

The Ctr1 probe sequence (5′-GCAATGTTCTATGAAGGACTCAAGATAGCCCGAG AGAGCCTGCT GCGTAAG-3′) was labeled by Digoxin and was synthesized by Beijing Aoke Biotechnology Co., Ltd. ISH was carried out according to our previous reports [21,22]. The ESCC tissue sections deparaffinized were placed in 10 mM citrate buffer (pH 6.0) for 1 h in a microwave oven at 60 °C, and then 10 μg/ml proteinase K (Boehringer Mannbeim Biochemicals, Indianapolis, IN) was applied to the tissue sections for 30 min at room temperature. Afterwards, Ctr1 probe was applied to tissue sections for 16 h in a moisturized chamber. Finally, NBT/BCIP stock solution was used for the development of staining signals. PBS was used as negative control instead of Ctr1 probe.

Immunohistochemistry (IHC)

IHC was performed according to our previous publication [21]. Briefly, tissue samples fixed by formalin and embedded by paraffins were continuously cut into 4–6 μm. Antigen retrieval was performed by microwave heating in 10 mM citrate buffer (pH 6.0) for 20 min, and then 3% hydrogen peroxide was applied to tissue sections to block the activity of endogenous perxidase for 5 min at 25 °C. Subsequently, primary antibody against Ctr1 (GeneTex company, USA) diluted 1:200 was incubated with tissue slides overnight at 4 °C. After rinsing, the tissue slides were incubated with the corresponding second antibody for 60 min at room temperature. Finally, DAB reagent was used for development of staining signals.

Scores of staining

The staining results of ISH and IHC were independently assessed in a double-blinded manner by two pathologists. The standard of staining score was performed according to our previous publication [22]. Briefly, positive cell numbers were counted in the following: 0 (<5%); 1 (5–25%); 2 (25%–50%); 3 (50%–75%) and 4 (> 75%). Standard of staining intensity was in the following: 0 (no signal); 1 (weak); 2 (moderate); and 3 (intense). The score of positive cell number multiplied by the score of staining intensity" made the final staining score, and the score <1 was considered as negative, and other scores were defined as positive.

Semi-quantitative RT-PCR

Total RNA was isolated from ESCC tissues and paired normal tissues by Trizol reagent (Invitrogen company, USA) according to the manufacturer's instructions, which was subjected to cDNA synthesis (TIANGEN BIOTECH CO., LTD, Beijing, China). The specific primers including Ctr1F: 5′-AAGATAGCCCGAGAGAGCCT-3′, Ctr1R: 5′-TGGATGATGTGCAGCACTGC-3′ (product size: 176 bp), GAPDHF: 5′-CGGAGTCAACGGATTTGGTCGTAT-3′, GAPDHR: 5′-AGCCTTCTCCATGGTG GTGAAGAC-3′ (product size: 307 bp) was used to amplify the specific band. Semi-quantitative assay was performed to detect the band intensity using Gene Tools software (UVP., Lnc., Upland, CA, USA).

Cell lines, cell culture and transfection

ESCC cell lines including Eca109, Kyse70, Kyse450 cells as well as normal esophageal epithelial cell Het-1A were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin (Sigma-Aldrich, USA), and 100 μg/ml streptomycin (Sigma-Aldrich, USA) at 37 °C in an incubator with 5% CO_2. Control siRNA (si-Con) (Santa Cruz company, USA), Ctr1 siRNA (si-Ctr1) (Santa Cruz company, USA), pcDNA3.1 and pcDNA3.1-Ctr1 were transfected to Eca109, Kyse70 and Kyse450 cells by Lipofectamine™ 2000 (Invitrogen Life Technologies, Carslbad, CA, USA) according to manufacturer's instruction.

Real-time quantitative PCR (qRT-PCR)

Total RNA was extracted using Trizol reagent (Invitrogen Life Technologies, Carslbad, CA, USA) according to the manufacturer's protocol. qRT-PCR was performed by Quant one step qRT-PCR Kit (SYBR Green) (TIANGEN BIOTECH CO., LTD, Beijing, China) in an ABI 7500 series PCR machine Applied Biosystems using the following primers: Ctr1F: 5′-AAGATAGCCCGAGAGAGCCT-3′, Ctr1R: 5′-TGGATGATGTG CAGCACTGC-3′ (product size: 176 bp), GAPDHF: 5′-GGGAGCCAAAAGGGT CATCA-3′, GAPDHR: 5′-AGTGATGGCATGGACTGTGG-3′.

Western blot

Total proteins were isolated from Eca109, Kyse70 and Kyse450 cells with various treatments by RIPA and PMSF (Solarbio, Beijing, China). Protein concentrations were measured using Coomassie Brilliant Blue G250 kits (Solarbio, Beijing, China) by Bradford method according to manufacturer's protocol. Whereafter, SDS-PAGE was performed, and the proteins were electro-transferred to PVDF membrane (Millipore Corporation, USA). After blocking with skimmed milk, primary antibody against Ctr1 and β-actin (Abcam, Cambridge, MA, USA) was incubated with PVDF membrane (Roche, Switzerland) overnight at room temperature, followed by the addition of the second antibody (ZSGB-BIO, Guangzhou, China). Finally, signal of protein expression was developed using AzureC300 Darkroom Eliminator (Azure Biosystems, USA).

CCK-8 assay

Transfected ESCC cells were digested using trypsinase and counted. Around 2×10^3 ESCC cells were seeded into 96-well plate for each well in triplicate. Different concentrations of cisplatin (0, 1, 2, 3, 4 and 5 μg/ml) was added to corresponding well. Cell viability was determined at 48 h using CCK-8 kit according to manufacturer's protocol by detecting 450 nm in a microplate reader.

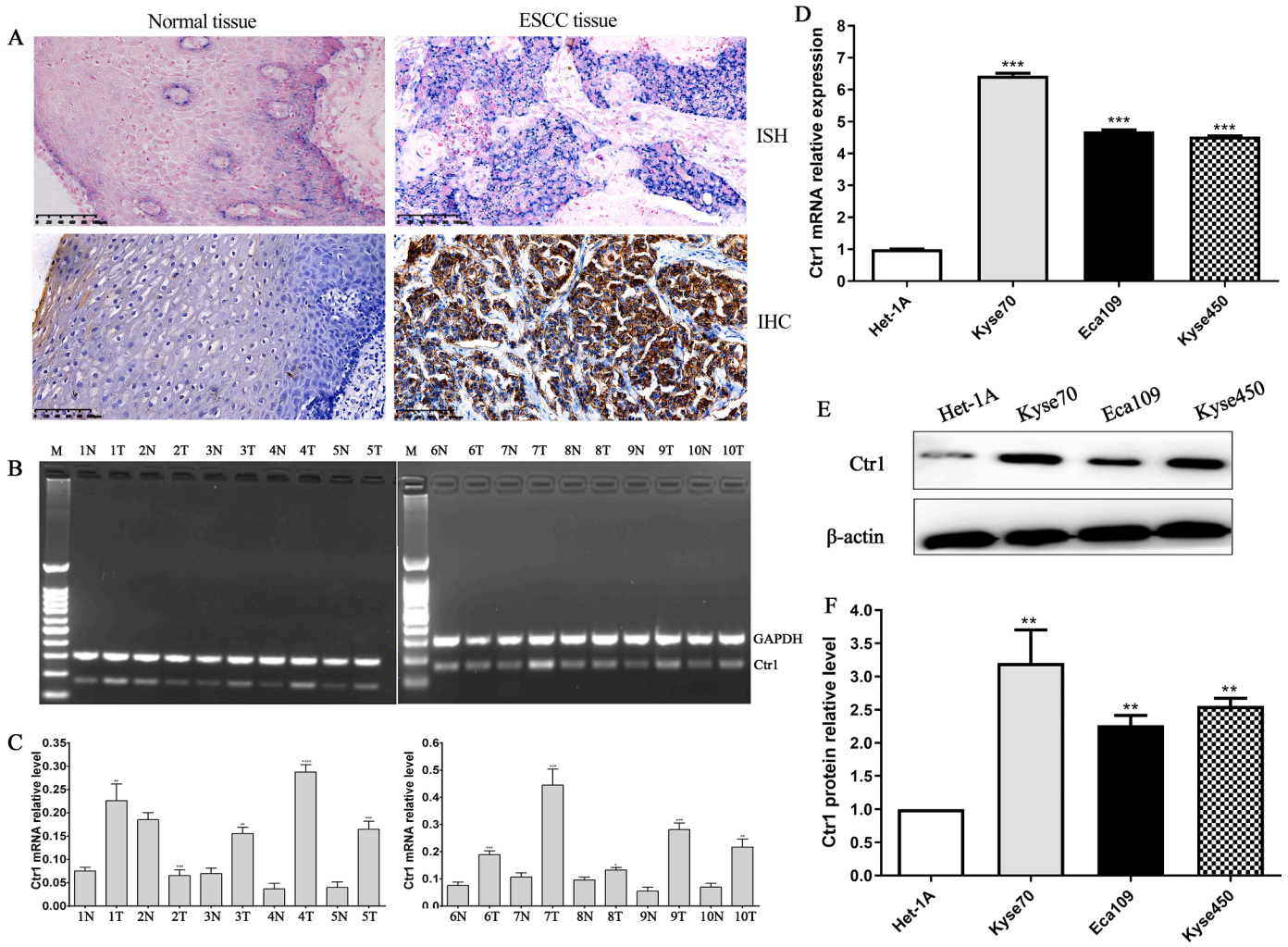


Fig. 1. High expressions of Ctr1 mRNA and protein in ESCC tissues and cells.
A: Representative image of Ctr1 high expression in ESCC tissues by ISH and IHC; ISH and IHC assay for Ctr1 mRNA and protein expressions in 108 cases of ESCC tissues and paired normal esophageal epithelial tissues, Bar=100 μm. B: Semi-quantitative RT-PCR detection for Ctr1 mRNA expression in randomly selected 10 cases of ESCC tissues and paired normal tissues using Ctr1 specific primers. C: Statistical assay for Ctr1 relative level in ESCC tissues and paired normal tissues, *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001, compared para-carcinoma tissues. D: qRT-PCR assay for Ctr1 mRNA expression in ESCC cell lines (Kyse70, Eca109, and Kyse450 cells), **P<0.01 and ***P<0.001, compared with Het-1A cell. E: Western blot analysis for Ctr1 protein expression in ESCC cell lines (Kyse70, Eca109, and Kyse450 cells), and β-actin was used as loading control. F: Relative Ctr1 protein level in various ESCC cell lines (Kyse70, Eca109, and Kyse450 cells), **P<0.01, compared with Het-1A cell.

Cell apoptosis

Cell apoptosis was performed according to the manufacturer's protocol using Annexin V FITC/PI (Beyotime Biotechnology, Shanghai, China). In brief, transfected ESCC cells were collected using trypsinase and seeded into 6-well plate. Subsequently, specific cisplatin concentration for each ESCC cell in triplicate was added to corresponding well. Annexin V/PI reagents were added to Eppendorf tube for 30 min. Finally, Flow cytometry (BD Biosciences, USA) was utilized to determine cell apoptosis.

Detection of Caspase-3 activity

Caspase-3 activity was determined with a commercial kit according to the manufacturer's protocols (APOP-CYTO Caspase-3 Colorimetric Assay Kit; Medical and Biological Laboratories, Japan) as described in a previous study [23].

Cell migration

Cell migration experiment was performed using Transwell chamber without Matrigel. Briefly, transfected ESCC cells combined with specific concentration cisplatin for each ESCC cell (around 1×10^5) were added to upper layer of chamber, and 20% FBS was added to underlayer of chamber. Invasive cells were fixed using methanol and stained with crystal violet at 48 h. Finally, invading cell numbers were counted under the field of 200 × magnification.

Cell invasion

Cell invasion experiment was performed using Transwell chamber with Matrigel (BD Biosciences, USA). Briefly, transfected ESCC cells combined with specific concentration cisplatin for each ESCC cell (around 1×10^5) was added to upper layer of chamber, and 20% FBS was added to underlayer of chamber. Invasive cells were fixed using methanol and stained with crystal violet at 48 h. Finally, invading cell numbers were counted under the field of 200 × magnification.

Table 1
The expressions of Ctr1 mRNA and protein in ESCC tissues and normal tissues.

Histological types	Ctr1 mRNA			χ^2	*P*	Ctr1 protein		χ^2	*P*
	n	+	–			+	–		
ESCC tissues	108	71	37	24.008	0.000	65	43	21.675	0.000
Normal tissues	108	35	73			31	77		

Table 2
The associations of Ctr1 mRNA and protein expressions with clinicopathological features in ESCC.

Features	n	Ctr1 mRNA				Ctr1 protein			
		+	–	χ^2	*P*	+	–	χ^2	*P*
Age (years)									
>60	62	45	17	3.024	0.082	39	23	0.449	0.503
≤60	46	26	20			26	20		
Gender									
Male	67	47	20	1.523	0.217	41	26	0.075	0.784
female	41	24	17			24	17		
Histological grade									
I	32	11	21	20.803	0.000	9	23	21.930	0.000
II	53	40	13			36	17		
III	23	20	3			20	3		
Invasive depth									
Superficial layer	48	25	23	7.155	0.007	20	28	12.365	0.000
Deep layer	60	46	14			45	15		
TNM staging									
I+II	64	31	33	20.883	0.000	30	34	11.614	0.001
III+IV	44	40	4			35	9		
Lymph node metastasis									
No	69	40	29	5.122	0.024	35	34	7.137	0.008
Yes	39	31	8			30	9		

Statistical analysis

All data, expressed as mean±SD, were investigated using Graphpad Prism 8.0 software. The results of IHC and ISH were evaluated using Chi square test, the comparison of two groups was analyzed using t-test, and the comparison of three groups or above was investigated using one-way ANOVA. A *P* value less than 0.05 was regarded as statistical significance.

Results

High expressions of Ctr1 mRNA and protein in ESCC tissues

To understand the role of Ctr1 in ESCC development and progression, we firstly investigated the expressions of Ctr1 mRNA and protein in 108 cases of ESCC tissues and paired normal tissues. We found that 71 cases of ESCC tissues displayed the positive expression of Ctr1 mRNA and 65 cases of ESCC tissues exhibited the positive expression of Ctr1 protein (Fig. 1A, Table 1), which was validated by semi-quantitative RT-PCR in randomly selected 10 cases of ESCC tissues and paired normal tissues, and only 1 cases displayed converse results (Fig. 1B and C). We selected 3 ESCC cell lines and normal esophageal epithelial cell Het-1A to further investigate the Ctr1 expression. qRT-PCR assay demonstrated that Ctr1 expression in 3 ESCC cell lines was significantly higher than that in Het-1A cell (all $P<0.01$) (Fig. 1D), which was verified by Western blot (Fig. 1E and F). These findings suggest that high Ctr1 expression may participate in ESCC development and progression.

The association of Ctr1 expression with clinicopathological features in ESCC

To address the question whether Ctr1 expression was related to the development and progression of ESCC? We investigated the correlations of the expressions of Ctr1 mRNA and protein with clinicopathological features in ESCC. We found that Ctr1 mRNA and protein expressions were both correlated with histological grade, invasion depth, TNM staging and lymph node metastasis in ESCC (all $P<0.05$), but not associated with age and gender of ESCC patients (all $P>0.05$) (Table 2). These data imply that Ctr1 may play an important role in the development and progression of ESCC.

Ctr1 depletion decreases the sensitivity of cisplatin in ESCC cells

It is well documented that Ctr1 plays an essential role in cisplatin mediated cytotoxicity [18,24-26]. Therefore, we further observed the effect of Ctr1 downregulation on cell proliferation in ESCC cells. We found Ctr1 siRNA significantly reduced Ctr1 mRNA level at 24 h, 48 h and 72 h in Eca109, Kyse70 and Kyse450 cells (Fig. 2A). Western blot revealed that Ctr1 siRNA markedly suppressed Ctr1 protein expression at 24 h, 48 h and 72 h in Eca109, Kyse70 and Kyse450 cells (Fig. 2B and C). Based on these results, CCK-8 was used to identify the role of Ctr1 downregulation in mediating the cytotoxicity of cisplatin on ESCC cells. We found that Ctr1 downregulation reduced the sensitivity of ESCC cells on cisplatin with IC50 value for Eca109 (si-Con vs si-Ctr1 1.997 vs 2.241), Kyse70 (si-Con vs si-Ctr1 1.470 vs 2.339) and Kyse450 (si-Con vs si-Ctr1 1.615 vs 2.713) (Fig. 2D). These findings suggest that Ctr1 level may be an important predictive marker for cisplatin efficacy in ESCC cells.

Ctr1 downregulation suppresses cell apoptosis triggered by cisplatin in ESCC cells

To further explore the role of Ctr1 downregulation in the apoptosis mediated by cisplatin in ESCC cells, Flow cytometry was used to determine the apoptotic cell numbers. Here, we found that different doses of cisplatin combined with si-Con markedly induced cell apoptosis in varying ESCC cells, compared with si-Con alone (Fig. 3A and B). However, compared with cisplatin plus si-Con, cisplatin combined with si-Ctr1 extremely reduced the apoptotic cell numbers in different ESCC cells (Fig. 3A and B). Similarily, compared with cisplatin plus si-Con, cisplatin combined with si-Ctr1 extremely reduced the activity of

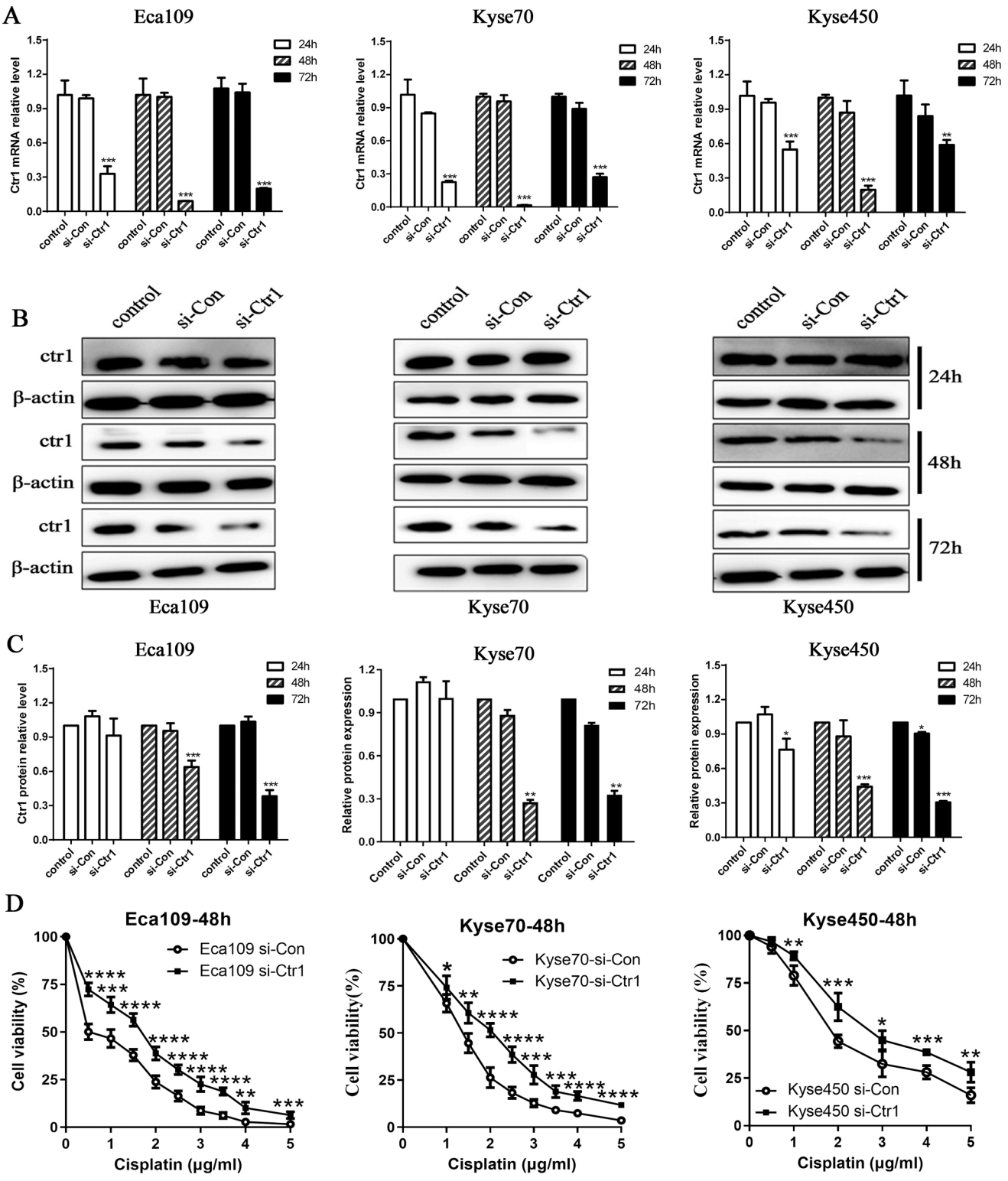


Fig. 2. The Ctr1 downregulation reduces the sensitivity of cisplatin in ESCC cells.
A: Ctr1 siRNA significantly suppressed Ctr1 mRNA expression at 24 h, 48 h and 72 h in various ESCC cells (Eca109, Kyse70 and Kyse450), si-Con and si-Ctr1 were transfected to Eca109, Kyse70 and Kyse450 cells by Lipofectamine™ 2000, and Semi-quantitative RT-PCR was used to determine the relative level of Ctr1 at 24 h, 48 h and 72 h after transfection, $^{**}P<0.01$ and $^{***}P<0.001$, compared with control group and si-Con group. B: Western blot was performed to investigate the Ctr1 protein expression at 24 h, 48 h and 72 h after transfection with si-Ctr1 and si-Con in different ESCC cells (Eca109, Kyse70 and Kyse450), and β-actin was employed as a loading control. C: The relative level of Ctr1 was counted using the rate of Ctr1 protein level to β-actin level in diverse ESCC cells (Eca109, Kyse70 and Kyse450), $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$, compared with control group and si-Con group. D: The Ctr1 downregulation reduced the cytotoxicity of cisplatin in distinct ESCC cells. ESCC cells (Eca109, Kyse70 and Kyse450) were harvested at 48 h after transfection, different doses of cisplatin was applied to ESCC cells above, and CCK-8 was used to determine cell viability, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ and $^{****}P<0.0001$, indicating statistical significance, compared with si-Con.

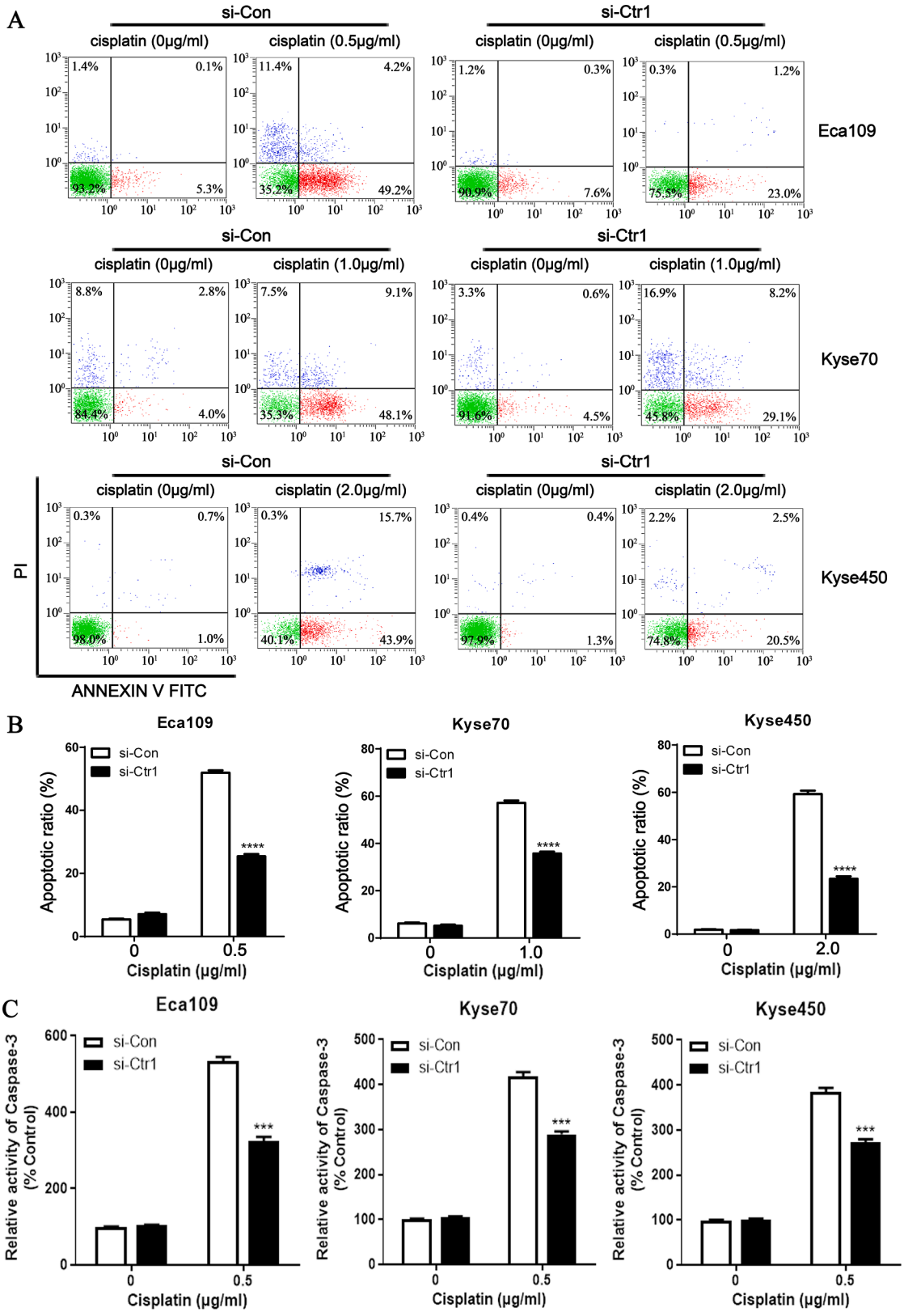


Fig. 3. Ctr1 depletion prominently repressed cell apoptosis evoked by cisplatin in ESCC cells.
A: Flow cytometry assay for cell apoptosis in different ESCC cells treated with si-Con, si-Con plus cisplatin, si-Ctr1 as well as si-Ctr1 plus cisplatin; ESCC cells (Eca109, Kyse70 and Kyse450) were harvested at 48 h after transfection, different doses of cisplatin was applied to ESCC cells above, and Flow cytometry was employed to determine cell apoptosis. B: Statistical assay for apoptotic cell numbers in different ESCC cells treated with si-Con, si-Con plus cisplatin, si-Ctr1 as well as si-Ctr1 plus cisplatin, ***$P<0.001$ and ****$P<0.0001$, compared with si-Con plus cisplatin group. C: Ctr1 depletion combined cisplatin significantly suppressed the activity of Caspase-3 induced by cisplatin plus si-Con; ESCC cells (Eca109, Kyse70 and Kyse450) were harvested at 48 h after transfection, different doses of cisplatin was applied to ESCC cells above, and Caspase-3 activity kit was utilized to determine the activity of Caspase-3, ***$P<0.001$, compared with si-Con plus cisplatin group.

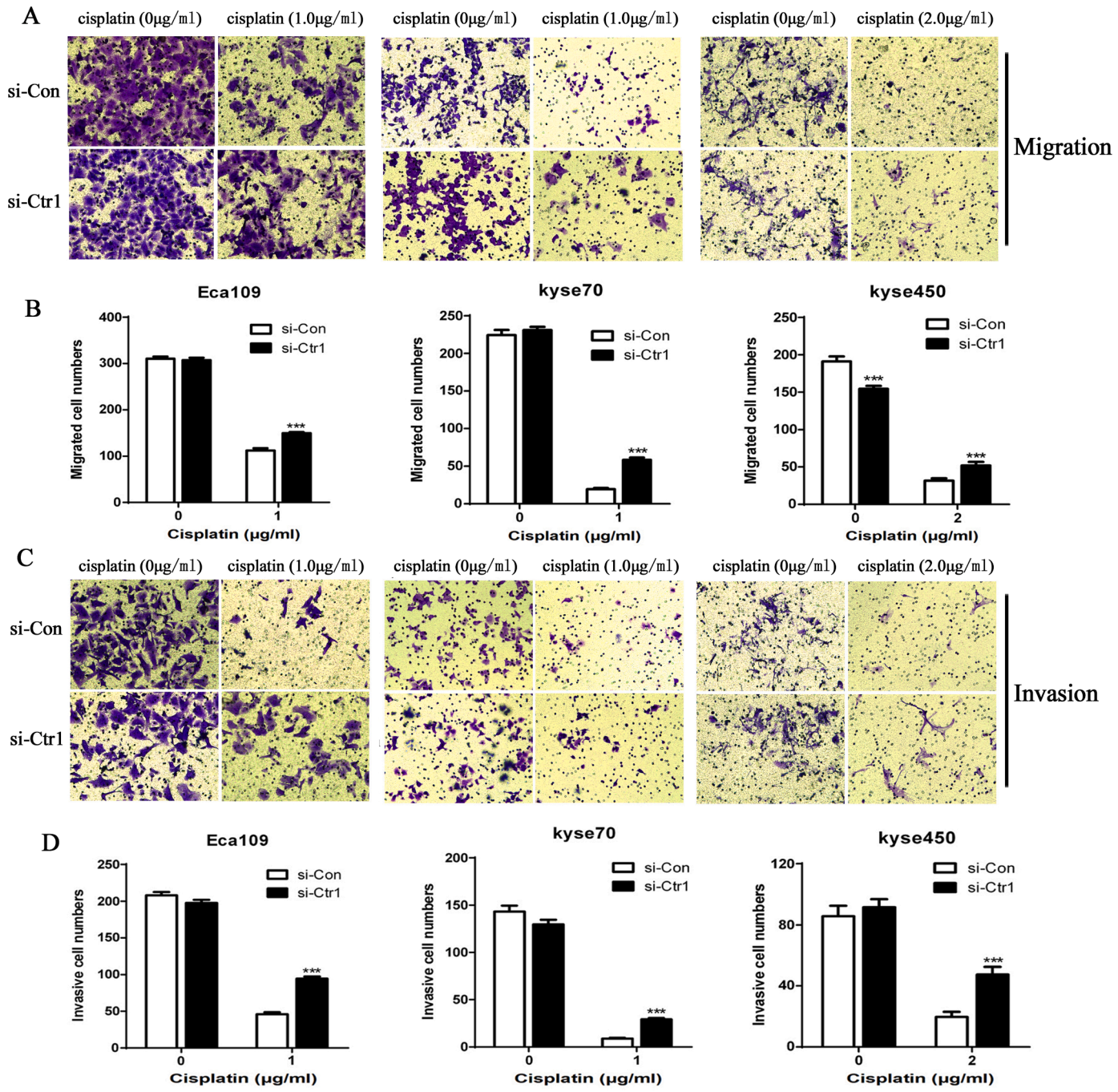


Fig. 4. The Ctr1 downregulation meliorated cell migration and invasion abilities inhibited by cisplatin in number of ESCC cells.
A: Transwell assay for cell migration in various ESCC cells treated with si-Con, si-Con plus cisplatin, si-Ctr1 and si-Ctr1 plus cisplatin; ESCC cells (Eca109, Kyse70 and Kyse450) were harvested at 48 h after transfection, different doses of cisplatin was applied to ESCC cells above, and Transwell chamber without Matrigel was employed to determine cell migration. B: Statistical assay for migratory cell numbers in different ESCC cells treated with si-Con, si-Con plus cisplatin, si-Ctr1 and si-Ctr1 plus cisplatin, ***$P<0.001$, compared with si-Con plus cisplatin group. C: Transwell assay for cell invasion in varied ESCC cells treated with si-Con, si-Con plus cisplatin, si-Ctr1 and si-Ctr1 plus cisplatin; ESCC cells (Eca109, Kyse70 and Kyse450) were harvested at 48 h after transfection, different doses of cisplatin was applied to ESCC cells above, and Transwell chamber with Matrigel was used to determine cell invasion. D: Statistical assay for invasive cell numbers in a variety of ESCC cells treated with si-Con, si-Con plus cisplatin, si-Ctr1 and si-Ctr1 plus cisplatin, ***$P<0.001$, compared with si-Con plus cisplatin group.

Caspase-3 in different ESCC cells (Fig. 3C). These findings imply that Ctr1 downregulation represses cell apoptosis induced by cisplatin in ESCC cells.

Ctr1 downregulation reversed cell migration and invasion abilities suppressed by cisplatin in ESCC cells

To further dissect the role of Ctr1 downregulation in cell migration and invasion mediated by cisplatin in ESCC cells, Transwell chambers without or with matrigel were utilized to indagate the cell migration and invasion abilities in a variety of ESCC cells. Our results revealed that there was no alteration in cell migration and invasion ability between ESCC cells transfected with si-Con and si-Ctr1 (Fig. 4A-D). However, compared with si-Con plus cisplatin, the migratory and invasive cell numbers were strikingly increased in si-Ctr1 plus cisplatin group ($P<0.001$) (Fig. 4A-D). These data suggest that Ctr1 plays a pivotal role

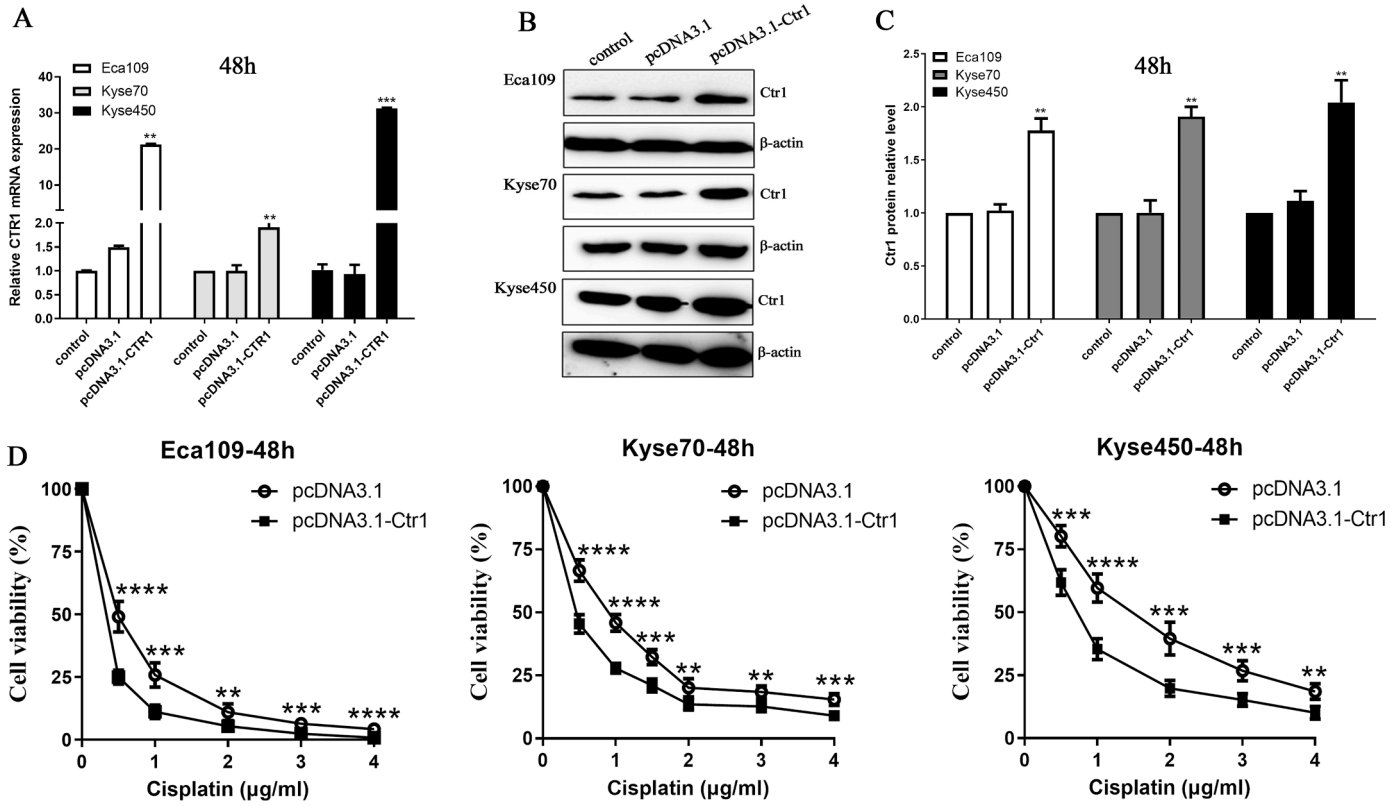


Fig. 5. The Ctr1 upregulation potentiated the killing efficacy of cisplatin in ESCC cells.
A: pcDNA3.1-Ctr1 remarkablely enhanced the Ctr1 mRNA level at 48 h in various ESCC cells (Eca109, Kyse70 and Kyse450), pcDNA3.1 and pcDNA3.1-Ctr1 were transfected to Eca109, Kyse70 and Kyse450 cells by Lipofectamine™ 2000, and Semi-quantitative RT-PCR was used to determine the relative level of Ctr1 at 48 h after transfection,$^{**}P<0.01$ and $^{***}P<0.001$, compared with control group and pcDNA3.1 group. B: Western blot was performed to investigate the Ctr1 protein expression in a number of ESCC cells 48 h after transfection with pcDNA3.1 and pcDNA3.1-Ctr1, and β-actin was used as a loading control. C: The relative level of Ctr1 protein in untreated ESCC cells, ESCC cells transfected with pcDNA3.1 and pcDNA3.1-Ctr1, $^{**}P<0.01$, compared with control group and pcDNA3.1 group. D: CCK-8 was used to examine the cell proliferative ability in different concentration of cisplatin with pcDNA3.1 or pcDNA3.1-Ctr1, ESCC cells (Eca109, Kyse70 and Kyse450) were harvested at 48 h after transfection, different doses of cisplatin was applied to ESCC cells above, and CCK-8 was used to determine cell viability, $^{**}P<0.01$, $^{***}P<0.001$ and $^{****}P<0.0001$, indicating statistical significance, compared with pcDNA3.1.

in regulating cell migration and invasion mediated by cisplatin in ESCC cells.

Ctr1 upregulation reenforces the cytotoxicity of cisplatin in ESCC cells

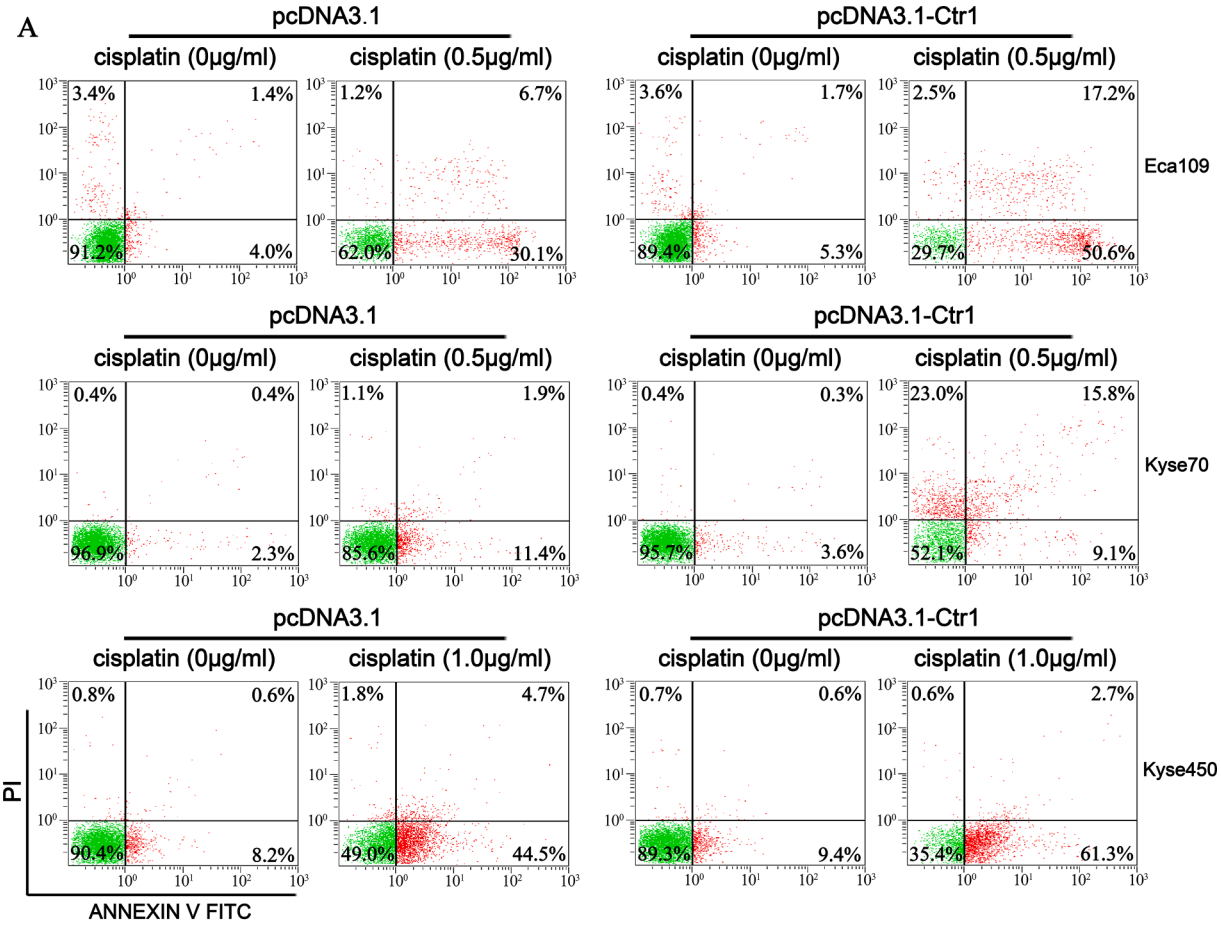
To verify the role of Ctr1 overexpression in cisplatin-mediated cytotoxicity in ESCC cells, we firstly constructed the pcDNA3.1-Ctr1 overexpression vector, which was transfected to ESCC cells. qRT-PCR assay revealed that pcDNA3.1-Ctr1 dramatically upregulated the levels of Ctr1 mRNA and protein in 3 ESCC cells, such as Eca109, Kyse70 and Kyse450, compared with control group and pcDNA3.1 group (Fig. 5A, B and C). Further CCK-8 data revealed that Ctr1 overexpression evidently increased cytotoxicity efficacy of cisplatin in Eca109, Kyse70 and Kyse450 cells with IC50 value for Eca109 (pcDNA3.1 vs pcDNA3.1-Ctr1 0.499 vs 0.001), Kyse70 (pcDNA3.1 vs pcDNA3.1-Ctr1 1.081 vs 0.708) and Kyse450 (pcDNA3.1 vs pcDNA3.1-Ctr1 3.814 vs 0.002) (Fig. 5D). These data indicate that high Ctr1 level contributes to enhance the cytotoxicity efficacy of cisplatin in ESCC cells.

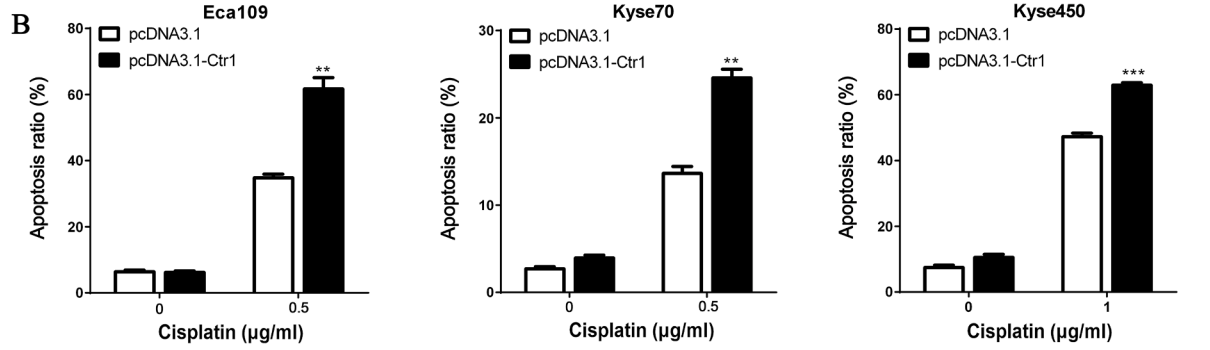
The synergistic role of Ctr1 upregulation combined with cisplatin in the induction of cell apoptosis in ESCC cells

To further make an assay of the role of Ctr1 upregulation plus cisplatin in regulation of cell apoptosis, we performed the Flow cytometry assay for cell apoptosis in ESCC cells. The results indicated that compared with pcDNA3.1 alone, pcDNA3.1-Ctr1 alone didn't induce cell apoptosis in various ESCC cells ($P>0.05$) (Fig. 6A and B). However, the apoptotic cell numbers in pcDNA3.1 plus cisplatin were obviously higher than that in pcDNA3.1 without cisplatin ($P<0.01$), and further investigation demonstrated that pcDNA3.1-Ctr1 combined with cisplatin significantly enhanced apoptotic cell numbers, compared with pcDNA3.1 plus cisplatin ($P<0.01$) (Fig. 6A and B). Besides, pcDNA3.1-Ctr1 combined with cisplatin significantly increased the activity of Caspase-3 in various ESCC cells, compared with pcDNA3.1 plus cisplatin ($P<0.01$) (Fig. 6C). These data state clearly that Ctr1 combined with cisplatin possesses the synergistic role in the induction of cell apoptosis in ESCC cells.

Ctr1 upregulation plus cisplatin remarkably represses migration and invasion abilities in ESCC cells

To further dissect whether Ctr1 upregulation enhances migration and invasion abilities suppressed by cisplatin, Transwell chamber without or with matrigel was used to investigate the cell migration and invasion abilities in ESCC cells, respectively. Our results indicated that there was no difference in cell migration and invasion ability between ESCC cells transfected with pcDNA3.1 and pcDNA3.1-Ctr1 (Fig. 7A-D). However, the migratory and invasive cell numbers in pcDNA3.1 plus cisplatin were obviously lower than that in pcDNA3.1 without cisplatin ($P<0.01$), and stepwise studies demonstrated that pcDNA3.1-Ctr1 combined with cisplatin markedly repressed the migratory and invasive cell numbers, compared with pcDNA3.1 plus cisplatin ($P<0.01$) (Fig. 7A-D). These results suggest that Ctr1 plus cisplatin plays a synergistic role in the suppression of cell migration and invasion in ESCC





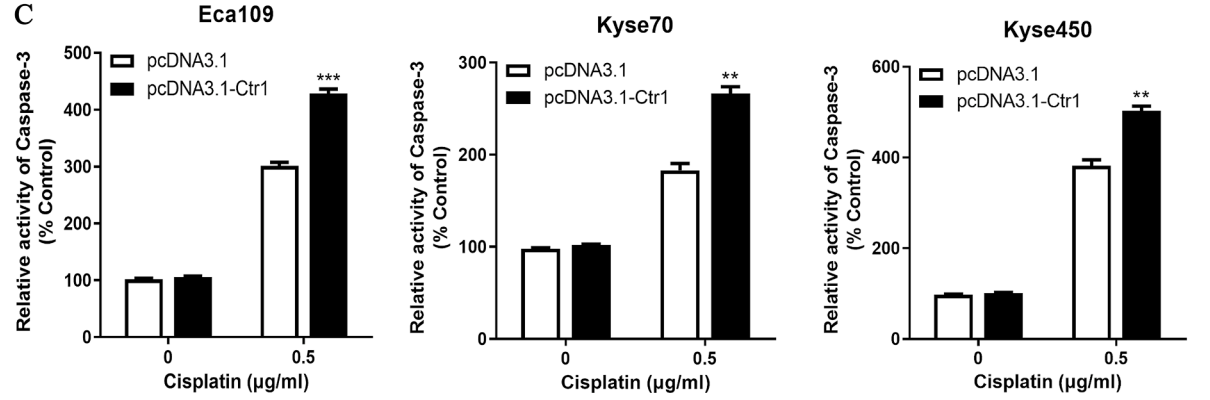


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Fig. 6. Ctr1 upregulation combined with cisplatin displayed the synergistic role in the induction of cell apoptosis in ESCC cells.
A: Flow cytometry assay for cell apoptosis in different ESCC cells treated with pcDNA3.1, pcDNA3.1 plus cisplatin, pcDNA3.1-Ctr1 and pcDNA3.1-Ctr1 plus cisplatin, ESCC cells (Eca109, Kyse70 and Kyse450) were harvested at 48 h after transfection, different doses of cisplatin was applied to ESCC cells above, and Flow cytometry was employed to determine cell apoptosis. B: Statistical assay for apoptotic cell numbers in different ESCC cells treated with pcDNA3.1, pcDNA3.1 plus cisplatin, pcDNA3.1-Ctr1 and pcDNA3.1-Ctr1 plus cisplatin, $^{**}P<0.01$ and $^{***}P<0.001$, compared with pcDNA3.1 plus cisplatin group. C: pcDNA3.1-Ctr1 combined cisplatin significantly promoted the activity of Caspase-3 induced by cisplatin plus pcDNA3.1; ESCC cells (Eca109, Kyse70 and Kyse450) were harvested at 48 h after transfection, different doses of cisplatin was applied to ESCC cells above, and Caspase-3 activity kit was utilized to determine the activity of Caspase-3, $^{**}P<0.01$ and $^{***}P<0.001$, compared with pcDNA3.1 plus cisplatin group.

cells.

Discussion

Evaluation of the efficacy of cisplatin-based chemotherapy in clinic has caused great attention in the world. However, drug-resistance and serious toxicity have greatly blocked the further treatment of tumor patients, including ESCC, further resulting in therapeutic failure [27,28]. In this study, we found that Ctr1 expression in ESCC tissues was significantly higher than that in normal esophageal epithelial tissues, and its expression was tightly associated with histological grade, invasion depth, TNM staging and lymph node metastasis, suggesting Ctr1 may be implicated in ESCC development and progression. Stepwise investigation revealed that Ctr1 depletion reduced the sensitivity of cisplatin on ESCC cells, suppressed cell apoptosis and the activity of Caspase-3 mediated by cisplatin and reversed migration and invasion cell numbers inhibited by cisplatin. Conversely, Ctr1 overexpression combined with cisplatin exhibited the synergistic role in suppressing cell proliferation, migration and invasion as well as inducing cell apoptosis and enhancing the activity of Caspase-3 in ESCC cells. Our data presented herein suggest that Ctr1 is an important mediator in regulating cisplatin entry into ESCC cells, and thus Ctr1 level may be an ancillary predictor for the therapeutic efficacy of cisplatin in ESCC patients.

The convincing evidence is the fact that high copper concentration in serum has linked to tumor incidence, progression and recurrence in a variety of tumors, including ESCC [29]. Porcu C, et al. demonstrated that the addition of copper promoted cell proliferation, migration and invasion ability in hepatocellular carcinoma cells HepaRG and HepG2 [30], which was manipulated by Ctr1 level. Ctr1 as copper transporter is responsible for entry of copper into cells [16], and the connection of copper and Ctr1 level implies that Ctr1 may be associated with tumor development and progression. Recent studies demonstrated that transcription factor MYC regulated Ctr1 transcription by binding to its promoter region, which further promoted Ctr1 upregulation in hepatocellular carcinoma [30]. Landon CD, et al. found that Ctr1 protein expression was dramatically increased in most cisplatin-sensitive bladder cancer cell lines [31]. Increasing data have indicated that the prognosis of patients treated using platinum chemotherapy is positively correlated with Ctr1 expression in non-small cell lung carcinoma, ovarian cancer and cervical cancer [20,32,33]. In addition, Ctr1 expression is also verified to be an underlying independent prognostic factor for the prediction of the recurrence and patients' survival in clear cell renal cell carcinoma [34]. Current reports impel us to assess the Ctr1 expression pattern in ESCC, which will provide novel insight into the clinic value of Ctr1 in therapy of ESCC patients. Our data revealed that Ctr1 expression in ESCC tissues and cells was markedly higher than that in normal tissues and cells, and its expression was closely associated with histological grade, invasion depth, TNM staging and lymph node metastasis, implying its involvement in ESCC development and progression. Although current data present the high Ctr1 expression in ESCC tissues and cells, what evoked this phenomenon remains to be under investigation. Meanwhile, it is regretful that there is currently a lack of the expression patterns of Ctr1 in ESCC patients without or with cisplatin treatment due to none of ESCC patients administrated by chemotherapy, therefore, the role of the Ctr1 expression and chemotherapy based on cisplatin in the prognosis of ESCC patients remains be further explored.

Widespread investigations have demonstrated that Ctr1 overexpression modestly promoted the intake of cisplatin [35], whereas its downregulation blocked the entry of cisplatin into tumor cells. However, several groups reported that there was no association of Ctr1 expression with cisplatin uptake [36,37]. Although a large number of results have confirmed the underlying clinic value of Ctr1 in many different tumors, especially in mediating cisplatin intake [38–40], its role in ESCC remains to be unveiled. Based on these conflicting reports, it was badly need of addressing whether the cytotoxicity of cisplatin was dependent on Ctr1 expression in ESCC cells. To this end, a series of experiments was carried out by loss-of-function and gain-of-function. We found that Ctr1 downregulation reduced the cytotoxicity of cisplatin in a number of ESCC cells. Conversely, Ctr1 overexpression enhanced the sensitivity of cisplatin on ESCC cells. These findings imply that Ctr1 level may harbor the cardinal value to assess cisplatin therapeutic efficacy in ESCC, and may be a novel predictor of the efficacy of chemotherapy based on cisplatin for ESCC patients.

Conclusions

In conclusion, Ctr1 at high level enhances the cisplatin efficacy in ESCC cells, including synergistic regulation in cell proliferation, apoptosis, migration and invasion. Although being lack of the data regarding animal experiment, our preliminary results have proved the cytotoxicity efficacy of cisplatin mediated by Ctr1 depletion and overexpression in ESCC cells. These key phenotypes including cell proliferation, apoptosis, migration and invasion have been evaluated the relationship between cisplatin and Ctr1 expression in ESCC cells. The Ctr1 expression pattern and its regulatory role in cisplatin sensitivity elaborated in this study will drive us further to elucidate Ctr1 function and its possible molecular mechanisms in animal experiment and clinical trials in the future, which will contribute to achieve personalized therapy for ESCC patients during chemotherapy.

Data availability statement

The datasets and material used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Approved by the Medical Ethics Committee of the First Affiliated Hospital of Zhengzhou University.

Consent for publication

Not applicable.

CRediT authorship contribution statement

Xin Wang: Conceptualization, Methodology, Validation, Formal analysis, Writing – original draft. **Qianqian Lou:** Software, Validation, Formal analysis. **Tianli Fan:** Software, Validation, Formal analysis. **Qing Zhang:** Investigation, Resources. **Xiangxiang Yang:** Investigation, Resources. **Hongtao Liu:** Supervision, Conceptualization, Writing – review & editing, Funding acquisition. **Ruitai Fan:** Supervision,

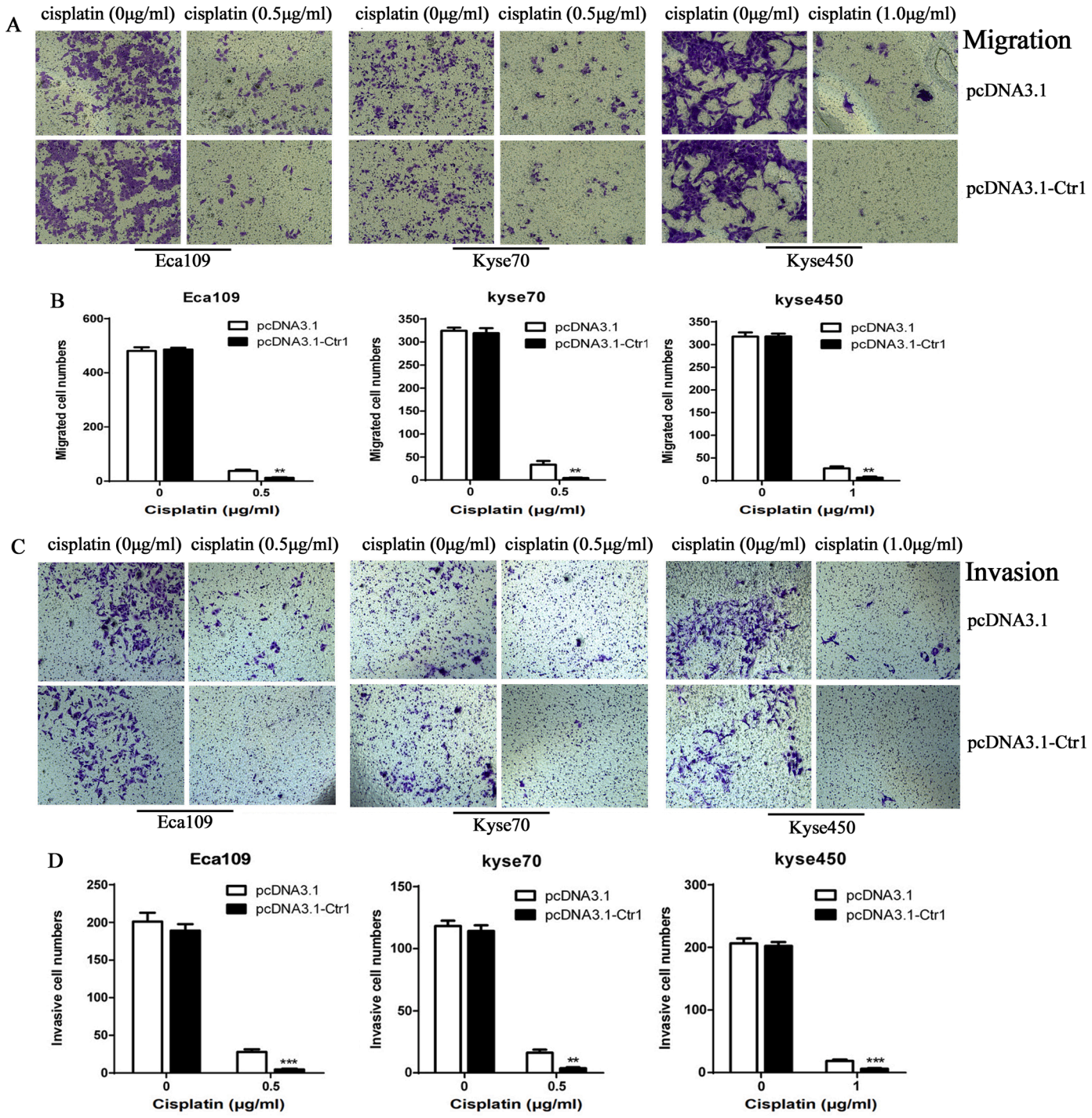

Fig. 7. Ctr1 upregulation exerted the synergistic role in the repression of cell migration and invasion with cisplatin in ESCC cells.
A: Transwell assay for cell migration in various ESCC cells treated with pcDNA3.1, pcDNA3.1 plus cisplatin, pcDNA3.1-Ctr1 and pcDNA3.1-Ctr1 plus cisplatin; ESCC cells (Eca109, Kyse70 and Kyse450) were harvested at 48 h after transfection, different doses of cisplatin was applied to ESCC cells above, and Transwell chamber without Matrigel was employed to determine cell migration. B: Statistical assay for migratory cell numbers in different ESCC cells treated with pcDNA3.1, pcDNA3.1 plus cisplatin, pcDNA3.1-Ctr1 and pcDNA3.1-Ctr1 plus cisplatin, $^{**}P<0.01$ and $^{****}P<0.0001$, compared with pcDNA3.1 plus cisplatin group. C: Transwell assay for cell invasion in varied ESCC cells treated with pcDNA3.1, pcDNA3.1 plus cisplatin, pcDNA3.1-Ctr1 and pcDNA3.1-Ctr1 plus cisplatin; ESCC cells (Eca109, Kyse70 and Kyse450) were harvested at 48 h after transfection, different doses of cisplatin was applied to ESCC cells above, and Transwell chamber with Matrigel was employed to determine cell invasion. D: Statistical assay for invasive cell numbers in a variety of ESCC cells treated with pcDNA3.1, pcDNA3.1 plus cisplatin, pcDNA3.1-Ctr1 and pcDNA3.1-Ctr1 plus cisplatin, $^{**}P<0.01$ and $^{***}P<0.001$, compared with pcDNA3.1 plus cisplatin group.

Conceptualization, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

[1] H. Liang, J.H. Fan, Y.L. Qiao, Epidemiology, etiology, and prevention of esophageal squamous cell carcinoma in China, Cancer Biol. Med. 14 (1) (2017) 33–41.
[2] L.A. Torre, F. Bray, R.L. Siegel, J. Ferlay, J. Lortet-Tieulent, A. Jemal, Global cancer statistics, 2012, CA Cancer J. Clin. 65 (2) (2015) 87–108.
[3] H. Chen, L. Zhou, Y. Yang, L. Yang, L. Chen, Clinical effect of radiotherapy combined with chemotherapy for non-surgical treatment of the esophageal squamous cell carcinoma, Med. Sci. Monit. 24 (2018) 4183–4191.
[4] C. Wang, J. Wang, Z. Chen, Y. Gao, J. He, Immunohistochemical prognostic markers of esophageal squamous cell carcinoma: a systematic review, Chin. J. Cancer 36 (1) (2017) 65.
[5] S. Manohar, N. Leung, Cisplatin nephrotoxicity: a review of the literature, J. Nephrol. 31 (1) (2018) 15–25.
[6] P.J. Loehrer, L.H. Einhorn, Drugs five years later. Cisplatin, Ann. Intern. Med. 100 (5) (1984) 704–713.
[7] M.S. Kies, S.T. Rosen, T.K. Tsang, R. Shetty, P.A. Schneider, C.B. Wallemark, T. W. Shields, Cisplatin and 5-fluorouracil in the primary management of squamous esophageal cancer, Cancer 60 (9) (1987) 2156–2160.
[8] J.A. Ajani, B. Ryan, T.A. Rich, M. McMurtrey, J.A. Roth, L. DeCaro, B. Levin, C. Mountain, Prolonged chemotherapy for localised squamous carcinoma of the oesophagus, Eur. J. Cancer 28A (4–5) (1992) 880–884.
[9] A.D. Hilgenberg, R.W. Carey, E.W. Wilkins Jr., N.C. Choi, D.J. Mathisen, H. C. Grillo, Preoperative chemotherapy, surgical resection, and selective postoperative therapy for squamous cell carcinoma of the esophagus, Ann. Thorac. Surg. 45 (4) (1988) 357–363.
[10] B.D. Minsky, T.F. Pajak, R.J. Ginsberg, T.M. Pisansky, J. Martenson, R. Komaki, G. Okawara, S.A. Rosenthal, D.P. Kelsen, INT 0123 (Radiation Therapy Oncology Group 94-05) phase III trial of combined-modality therapy for esophageal cancer: high-dose versus standard-dose radiation therapy, J. Clin. Oncol. 20 (5) (2002) 1167–1174.
[11] Y.H. Chen, H.I. Lu, C.Y. Chien, C.M. Lo, Y.M. Wang, S.Y. Chou, Y.Y. Su, L.H. Shih, S.H. Li, Treatment outcomes of patients with locally advanced synchronous esophageal and head/neck squamous cell carcinoma receiving curative concurrent chemoradiotherapy, Sci. Rep. 7 (2017) 41785.
[12] R. Katoh, Y. Takebayashi, S. Takenoshita, Expression of copper-transporting P-type adenosine triphosphatase (ATP7B) as a chemoresistance marker in human solid carcinomas, Ann. Thorac. Cardiovasc. Surg. 11 (3) (2005) 143–145.
[13] A. Dancis, D.S. Yuan, D. Haile, C. Askwith, D. Eide, C. Moehle, J. Kaplan, R. D. Klausner, Molecular characterization of a copper transport protein in S. cerevisiae: an unexpected role for copper in iron transport, Cell 76 (2) (1994) 393–402.
[14] B. Zhou, J. Gitschier, hCTR1: a human gene for copper uptake identified by complementation in yeast, Proc. Natl. Acad. Sci. U. S. A. 94 (14) (1997) 7481–7486.
[15] Y.M. Kuo, B. Zhou, D. Cosco, J. Gitschier, The copper transporter CTR1 provides an essential function in mammalian embryonic development, Proc. Natl. Acad. Sci. U. S. A. 98 (12) (2001) 6836–6841.
[16] E.B. Maryon, S.A. Molloy, K. Ivy, H. Yu, J.H. Kaplan, Rate and regulation of copper transport by human copper transporter 1 (hCTR1), J. Biol. Chem. 288 (25) (2013) 18035–18046.
[17] C.J. De Feo, S.G. Aller, V.M. Unger, A structural perspective on copper uptake in eukaryotes, Biometals 20 (3–4) (2007) 705–716.
[18] S. Ishida, J. Lee, D.J. Thiele, I. Herskowitz, Uptake of the anticancer drug cisplatin mediated by the copper transporter Ctr1 in yeast and mammals, Proc. Natl. Acad. Sci. U. S. A. 99 (22) (2002) 14298–14302.
[19] M.D. Hall, M. Okabe, D.W. Shen, X.J. Liang, M.M. Gottesman, The role of cellular accumulation in determining sensitivity to platinum-based chemotherapy, Annu. Rev. Pharmacol. Toxicol. 48 (2008) 495–535.
[20] S. Ishida, F. McCormick, K. Smith-McCune, D. Hanahan, Enhancing tumor-specific uptake of the anticancer drug cisplatin with a copper chelator, Cancer Cell 17 (6) (2010) 574–583.
[21] X. Wang, X. Li, X. Zhang, R. Fan, H. Gu, Y. Shi, H. Liu, Glucose-6-phosphate dehydrogenase expression is correlated with poor clinical prognosis in esophageal squamous cell carcinoma, Eur. J. Surg. Oncol. 41 (10) (2015) 1293–1299.
[22] H.T. Liu, N. Wang, X. Wang, S.L. Li, Overexpression of Pim-1 is associated with poor prognosis in patients with esophageal squamous cell carcinoma, J. Surg. Oncol. 102 (6) (2010) 683–688.
[23] M. Li, H. Li, C. Li, S. Zhou, L. Guo, H. Liu, W. Jiang, X. Liu, P. Li, M.A. McNutt, et al., Alpha fetoprotein is a novel protein-binding partner for caspase-3 and blocks the apoptotic signaling pathway in human hepatoma cells, Int. J. Cancer 124 (12) (2009) 2845–2854.
[24] X. Lin, T. Okuda, A. Holzer, S.B. Howell, The copper transporter CTR1 regulates cisplatin uptake in Saccharomyces cerevisiae, Mol. Pharmacol. 62 (5) (2002) 1154–1159.
[25] K.M. Bompiani, C.Y. Tsai, F.P. Achatz, J.K. Liebig, S.B. Howell, Copper transporters and chaperones CTR1, CTR2, ATOX1, and CCS as determinants of cisplatin sensitivity, Metallomics 8 (9) (2016) 951–962.
[26] C.A. Larson, B.G. Blair, R. Safaei, S.B. Howell, The role of the mammalian copper transporter 1 in the cellular accumulation of platinum-based drugs, Mol. Pharmacol. 75 (2) (2009) 324–330.
[27] P. Phatak, K.A. Byrnes, D. Mansour, L. Liu, S. Cao, R. Li, J.N. Rao, D.J. Turner, J. Y. Wang, J.M. Donahue, Overexpression of miR-214-3p in esophageal squamous cancer cells enhances sensitivity to cisplatin by targeting survivin directly and indirectly through CUG-BP1, Oncogene 35 (16) (2016) 2087–2097.
[28] L. Goense, P.S. van Rossum, D. Kandioler, J.P. Ruurda, K.L. Goh, M.D. Luyer, M. J. Krasna, R. van Hillegersberg, Stage-directed individualized therapy in esophageal cancer, Ann. N Y Acad. Sci. 1381 (1) (2016) 50–65.
[29] N.A. Dar, M.M. Mir, I. Salam, M.A. Malik, G.M. Gulzar, G.N. Yatoo, A. Ahmad, A. Shah, Association between copper excess, zinc deficiency, and TP53 mutations in esophageal squamous cell carcinoma from Kashmir Valley, India–a high risk area, Nutr. Cancer 60 (5) (2008) 585–591.
[30] C. Porcu, L. Antonucci, B. Barbaro, B. Illi, S. Nasi, M. Martini, A. Licata, L. Miele, A. Grieco, C. Balsano, Copper/MYC/CTR1 interplay: a dangerous relationship in hepatocellular carcinoma, OncoTargets Ther. 9 (10) (2018) 9325–9343.
[31] C.D. Landon, S.E. Benjamin, K.A. Ashcraft, M.W. Dewhirst, A role for the copper transporter Ctr1 in the synergistic interaction between hyperthermia and cisplatin treatment, Int. J. Hyperthermia 29 (6) (2013) 528–538.
[32] H.H. Chen, J.J. Yan, W.C. Chen, M.T. Kuo, Y.H. Lai, W.W. Lai, H.S. Liu, W.C. Su, Predictive and prognostic value of human copper transporter 1 (hCtr1) in patients with stage III non-small-cell lung cancer receiving first-line platinum-based doublet chemotherapy, Lung Cancer 75 (2) (2012) 228–234.
[33] Y.Y. Lee, C.H. Choi, I.G. Do, S.Y. Song, W. Lee, H.S. Park, T.J. Song, M.K. Kim, T. J. Kim, J.W. Lee, et al., Prognostic value of the copper transporters, CTR1 and CTR2, in patients with ovarian carcinoma receiving platinum-based chemotherapy, Gynecol. Oncol. 122 (2) (2011) 361–365.
[34] Y. Xia, L. Liu, Q. Bai, Q. Long, J. Wang, W. Xi, J. Xu, J. Guo, Prognostic value of copper transporter 1 expression in patients with clear cell renal cell carcinoma, Oncol. Lett. 14 (5) (2017) 5791–5800.
[35] I.S. Song, N. Savaraj, Z.H. Siddik, P. Liu, Y. Wei, C.J. Wu, M.T. Kuo, Role of human copper transporter Ctr1 in the transport of platinum-based antitumor agents in cisplatin-sensitive and cisplatin-resistant cells, Mol. Cancer Ther. 3 (12) (2004) 1543–1549.
[36] K.D. Ivy, J.H. Kaplan, A re-evaluation of the role of hCTR1, the human high-affinity copper transporter, in platinum-drug entry into human cells, Mol. Pharmacol. 83 (6) (2013) 1237–1246.
[37] G.L. Beretta, L. Gatti, S. Tinelli, E. Corna, D. Colangelo, F. Zunino, P. Perego, Cellular pharmacology of cisplatin in relation to the expression of human copper transporter CTR1 in different pairs of cisplatin-sensitive and -resistant cells, Biochem. Pharmacol. 68 (2) (2004) 283–291.
[38] E.Y. Ilyechova, E. Bonaldi, I.A. Orlov, E.A. Skomorokhova, L.V. Puchkova, M. Broggini, CRISP-R/Cas9 mediated deletion of copper transport genes CTR1 and DMT1 in NSCLC Cell Line H1299. biological and pharmacological consequences, Cells 8 (4) (2019).
[39] I. Buss, A. Hamacher, N. Sarin, M.U. Kassack, G.V. Kalayda, Relevance of copper transporter 1 and organic cation transporters 1-3 for oxaliplatin uptake and drug resistance in colorectal cancer cells, Metallomics 10 (3) (2018) 414–425.
[40] M.C. Akerfeldt, C.M. Tran, C. Shen, T.W. Hambley, New EJ: interactions of cisplatin and the copper transporter CTR1 in human colon cancer cells, J. Biol. Inorg. Chem. 22 (5) (2017) 765–774.