Int J Clin Exp Pathol 2016;9(10):10028-10035
www.ijcep.com /ISSN:1936-2625/IJCEP0026034

Original Article

Silencing of CTHRC1 inhibits proliferation and metastasis of endometriotic stromal cells

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Received February 16, 2016; Accepted May 20, 2016; Epub October 1, 2016; Published October 15, 2016

Abstract: Collagen triple helix repeat containing-1 (CTHRC1) is aberrantly overexpressed in a variety of malignant human cancers. However, effects of CTHRC1 on endometriosis (EM) remain unclear. Herein, we aimed at exploring the effects of CTHRC1 on EM. We found that CTHRC1 protein expression was significantly increased in EM tissues compared with normal control as indicated by immunohistochemistry staining and western blot. Then, human endometriotic stromal cells (ESC) were isolated and transfected with siRNA targeting CTHRC1. Cell viability was identified by CCK8 and the capacity of cell metastasis was determined by Transwell assay. qRT-PCR and western blot assays were used to measure the expression levels of CTHRC1 and several tumor-related genes. Results showed that compared with the mock and negative controls knockdown of CTHRC1 expression significantly inhibited cell proliferation at 24 h, 48 h and 72 h in a time-dependent manner; suppressed cell migration, invasion and adhesion of ESC (n=3, $P<0.01$). Further, siRNA-CTHRC1 down-regulated the expression levels of several tumor cellular processes related proteins, matrix metallopeptidase 9 (MMP9), matrix metallopeptidase 2 (MMP2), CD44, TGF-β1 and ICAM1, while up-regulated E-cadherinexpression. Also, the phosphorylation level of ERK1/2 was inhibited. This study indicated that CTHRC1 may promote the progression of EM.

Keywords: Endometriosis (EM), CTHRC1, proliferation, migration, invasion, adhesion

Introduction

Endometriosis (EM) is a common gynecological disorder which may lead to symptoms such as dysmenorrheal and even infertility. Histologically, EM is defined as stroma outside the uterine cavity and the presence of endometrial glands [1]. It is the most common gynecological malignancy affecting 8-10% of the total women population, mainly postmenopausal women [2]. The main clinical problems of EM contain a variable severity of pain, recurrence after surgical or medical treatment and difficulty in pregnancy [1, 3, 4]. It have been suggested that endometriotic cells are invasive and able to metastasize [5, 6], which is similar to the mechanisms in the metastasis of malignant tumors.

Collagen triple helix repeat containing-1 (CTHRC1) is an overexpressed gene screened from the process of arterial damage [7]. It is reported that CTHRC1 can reduce collagen deposition and promote cell migration [8]. CTHRC1 may involve in the tissues remediation process during vascular remodeling, and increasing research has proved that tissues remediation is closely associated with tumorigenesis [9, 10]. There are abundant reports about the function of CTHRC1 on various kinds of cancers. For instance, overexpression of CTHRC1 in hepatocellular carcinoma increases tumor cell invasion and predicts poor prognosis [11]. In human gastric cancer, CTHRC1 may be associated with metastasis and it is up-regulated by transforming growth factor-β1 or promoter demethylation [12]. CTHRC1 has been reported to affect peritoneal carcinomatosis and serve as a new predictor of prognosis for patients with colorectal cancer [13]. Overexpression of CTHRC1 is associated with tumor aggressiveness and poor prognosis in human non-small cell lung cancer [14]. Although CTHRC1 is involved in various cancers, the relationships between CTHRC1 and EM remain unclear.

In the current study, we found that CTHRC1 protein expression was significantly increased in EM tissues compared with normal control. CTHRC1 protein expression was much higher in ectopic endometrial tissues than in eutopic endometrial tissues. We then isolated endometriotic stromal cells (ESC) and knockdown CTHRC1 expression by small interfering RNA (siRNA) transfection to explore the effects of CTHRC1 on the proliferation and invasion of ESC. Furthermore, the possible mechanism was studied, which may explain the mechanism of CTHRC1 promoting the progression of EM.

Materials and methods

Patients and tissues

Endometrial tissue samples were obtained from 30 patients who underwent surgery between January 2013 and December 2013 for treatment of EM or for other benign gynaecological disorders (controls). The expression levels of CTHRC1 in normal tissues, eutopic and ectopic endometrial tissues were detected by immunohistochemical staining and western blot assays. Informed and written consent was obtained from all patients or their advisers according to the ethics committee guidelines of Women's Hospital (Hangzhou, China).

Immunohistochemistry assay

Immunohistochemistry assay was performed to measure the protein levels of CTHRC1 in EM tissues and adjacent normal tissues. Briefly, the formalin-fixed and paraffin-embedded (FFPE) 5 μm tissue sections were deparaffinized and rehydrated. The rehydrated sections were treated with 0.01 M citrate buffer (pH 6) to unmask antigen. Then sections were washed three times with 0.01 M PBS, incubated with 5% (w/v) bovine serum albumin (BSA) at 37°C for 30 min, and subsequently at 4°C overnight with CTHRC1 primary antibody (Ab85739, Abcam, 1:50). Then the sections were washed three times with PBS and incubated with horseradish peroxidase-conjugated secondary antibody for 1 hat room temperature. The sections were developed with diaminobenzidine tetrahydrochloride and then counterstained with haematoxylin.

Isolation and culture of human endometriotic stromal cells (ESC)

Endometriotic stromal cells (ESC) were isolated under sterile conditions as previously reported [15]. Then the tissues were washed with PBS to remove blood clots mucus, cut into small pieces with the size of less than 1 mm^3, and incubated with 1% collagenase solution (GIBO, USA) at 37°C for 2.5 h. After confirming the glands and stoma were separated under microscopic, tissues were centrifuged at 800 rpm, washed twice with PBS, suspended in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Rockville, MD, USA), and filtered with a 200 mesh stainless steel screen to remove the undigested tissues. Then the obtained cells were cultured in DMEM supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin and 10% fetal bovine serum (FBS, Gibco BRL). And the incubator (Thermo, Fisher Scientific Inc., Waltham, MA, USA) was set to 37°C, 100% humidity and 5% CO_2. The purity of the ESC was assessed by immunocytochemical staining with anti-vimentin (Abcam, ab8978).

Small interfering RNA (siRNA) transfection

Three siRNAs targeting human CTHRC1 mRNA (5'-GCGGAGUGUACAUUUACAA-3'; 5'-GCUCAUCUGUGGGAAGUAU-3'; 5'-CUGGGUUGGUACUUGUUCA-3') and a non-specific scramble siRNA sequence (NC: 5'-UUGUACUACACAAAAGUACUG-3') were synthesized by Genepharma (Shanghai, China). Cells in logarithmic growth phase were collected, digested by trypsin, counted and added to a 6-well culture plate with 1 ml/well containing 5×10^5 cells/mL. And then cells were seeded in antibiotic-free medium the day before transfection. After that, cells were transfected with 400 nmol/L siRNA-CTHRC1 or siRNA (negative control) using lipofectamine™ 2000 (Invitrogen, Shanghai, China) according to the instructions. After 24 h, 48 h, and 72 h, cells were collected respectively, with each of the time regarded as one treated group and 0h as a control for proliferation assay. The transfected cells at 48 h Cells were used for the following cell adhesion assay, transwell assay, qRT-PCR and western blot assay.

Quantitative real-time PCR (qRT-PCR) assay

Quantitative real-time PCR (qRT-PCR) was used to determine the protein levels of CTHRC1 in different groups after transduction. Total RNA samples were isolated using Trizol Reagent (Invitrogen, Japan). cDNA was synthesized from approximately 5 μg of RNA using AMV reverse transcriptase (Fermentas, USA). qRT-PCR reactions in a 25 μL total volume were performed

using SYBR→Green 10 × Supermix (Takara, Japan) on Roche Light Cycler→480II System (Roche Diagnostics Ltd., Switzerland). The Primer Express Software (Applied Biosystems, Shanghai, China) were used to design primer pairsfor human genes (Primer F: 5'-TTGTTCA-GTGGCTCACTTC-3', Primer R: 5'-TCATTTCAGG-GCTTCCTTG-3') with GAPDH as the internal control. The PCR procedure was: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 45 s; one cycle of 95°C for 15 s, 60°C for 1 min; one cycle of 95°C for 15 s, 60°C for 15 s. All PCR reactions were repeated three times and the relative expression levels of different groups were calculated by normalizing to the mRNA expression level of GAPDH using the $2^{-\Delta\Delta CT}$ method [16].

Western blot analysis

The relationship between CTHRC1 and tumor-regulated genes were explored by western blot assays. Transfected cells were harvest, washed twice with PBS, lysed in ice-cold radio in ice-cold radio immunoprecipitation assay buffer (RIPA, Beyotime, Shanghai, China) containing 0.01% protease and phosphatase inhibitor (Sigma, Shanghai, China), and incubated on ice for 30 min. Then cell lysis was obtained, centrifuged 12,000 × g at 4°C for 10 mins. Proteins in supernatant were obtained and quantified by BCA protein quantitation kit (thermo, PICPI23223). About 20-30 μg protein samples in each load were separated by 10% SDS-PAGE gel, and transferred electrophoretically to a polyvinylidene fluoride membrane (Millipore, Shanghai, China). The membrane were blocked with 5% BSA in PBST and incubated with primary antibodies against CTHRC1 (Abcam, Ab-85739, 1:1000 dilution), MMP2 (Abcam, Ab-92536, 1:1000 dilution), MMP9 (Abcam, Ab-119906, 1:1000 dilution), CD44 (CST, #5640, 1:1000 dilution), ICAM1 (Abcam, Ab53013, 1:500 dilution), E-cadherin (CST, #14472, 1:1000 dilution), TGFβ1 (Abcam, Ab92486, 1:500 dilution), ERK1/2 (CST, #4695, 1:500 dilution), p-ERK1/2 (CST, #4376, 1:500 dilution) and GAPDH (CST, #5174, 1:1500 dilution). Blots were incubated for 1 hour at 37°C with goat anti-mouse or anti-rabbit secondary antibody (Beyotime, Shanghai, China) and Intensities were measured using enhanced chemiluminescence (ECL, Thermo Scientific, Shanghai, China).

Cell growth and proliferation assay

Cell Counting Kit (CCK)-8 Kit (Tongren, Shanghai, China) was used to assess the effects of siRNA-CTHRC1 on the viability of endometriotic stromal cells in different group (control, negative control and siRNA-CTHRC1 treatment group). Briefly, CCK-8 reagent was added to each treated group of wells with 1:10 (v/v) per 100 μL medium when cells were transfected for 0 h, 12 h, 48 h and 72 h. After the endpoint of incubation, optical density (OD) at 450 nm was determined for the supernatant of each well by a microplate reader. Experiments were performed at least three times with each time in triplicate.

Transwell migration and invasion assays

Transwell assays were performed to assess the cell ability of migration and invasion after the cells were transfection with siRNA-CTHRC1. For invasion assay, the inserts were pre-coated with matrigel (BD Bioscience) and solidified at 37°C incubator for 30 min. Briefly, cells in different groups were starved with serum-free DMEM medium for 24 h. Then, cells were trypsinized, washed with serum-free medium and resuspended in medium containing 1% FBS. After counting, cells were seeded into Transwell at a density of 5×10^4 per well, and the lower chamber was added with 0.75 mL complete medium containing 10% FBS. After incubation for another 48 h, cells that did not migrate were completely removed with a cotton swab. Migrated cells were fixed for 10 min in 4% paraformaldehyde (JRDUN Biotech, Shanghai, China), washed by 1 × PBS and stained by 1 mL 0.5% crystal violet for 30 min. The numbers of migrated and invaded cells in five randomly selected fields were counted under a microscope (OLYMPUS, Shenzhen, China).

Adhesion assays

To detect the effect of siRNA-CTHRC1 on cell adhesion, 12-well plates was used for determining the number of adhesive cells. Cell suspension (1×10^5 cells/well) was added to the well and incubated for 1 h at 37°C, 5% CO_2. After centrifugation, the supernatant was discarded and cells were washed with PBS twice. Adherent cells were fixed with 4% methanol for 15 min and stained with crystal violet for 30

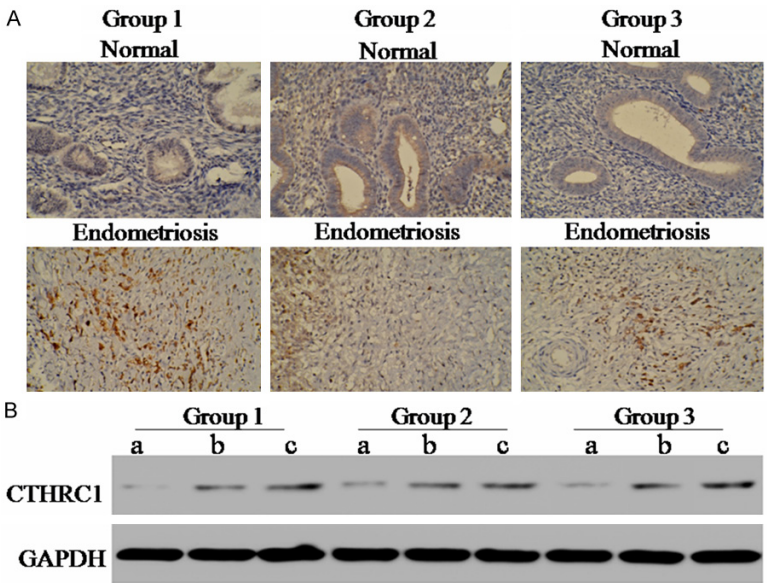

Figure 1. High expression of CTHRC1 in tumor tissues compared with normal tissues. A. The signal distribution of CTHRC1 in EM was much stronger than in normal tissues as assessed by immunohistochemistry staining. B. Protein expression of CTHRC1 was progressively increased with the increasing grade of disease severity. "a" represents normal tissues, "b" represents eutopic endometrial tissues and "c" represents ectopic endometrial tissues.

min. Photographed and counted the number of adherent cells from three random selected 200 × fields of microscope.

Statistical analysis

Experiments were performed with values expressed as mean ± SD. All statistical analyses were conducted using the Graphpad Prism 6.0 software. Data were analyzed by T tests. In all statistical comparisons, a *P* value of less than 0.05 was considered to be statistically significant.

Results

High expression of CTHRC1 in EM tissues compared with normal tissues

The expression levels of the endogenous CTHRC1 in EM tissues and normal tissues were detected by immunohistochemistry staining. Our results showed that the signal of CTHRC1 in EM was much stronger than in normal tissues (**Figure 1A**). Moreover, compared with eutopic endometrial tissues, ectopic endometrial tissues had an obviously higher expression of CTHRC1 (**Figure 1B**). The results suggested that CTHRC1 probably played a role in the development of EM.

Isolation of endometriotic stromal cells (ESC)

In order to explore the possible effects of CTHRC1 on the progression of EM, ESC was isolated from ectopic endometrial tissues. The purity of ESC was determined by immunohistochemistry staining with anti-vimentin (**Figure 2A**). The percentages of vimentin-positive cells were over 90% and the cells can be used in the following assays.

Detection of CTHRC1 levels after treatment with siRNA-CTHRC1

Three siRNAs targeting human CTHRC1 mRNA (siCTHRC1-1, -2 and -3) and a non-specific scramble siRNA sequence (NC) were synthesized and transfected ESC. In order to detect whether the siRNA-CTHRC1 can knockdown CTHRC1 expression, qRT-PCR and western blot assays were performed. As shown in **Figure 2B** and **2C**, relative mRNA and protein expression levels of CTHRC1 in CTHRC1 siRNA-treated group were decreased notably. siCTHRC1-3 had the highest knockdown efficiency and was chosen for the following assays.

siRNA-CTHRC1 inhibited ESC viability

Cell growth (OD/450 nm) and proliferation rates were measured by CCK8. Results showed that after transfection with siRNA for 24 h, 48 h and 72 h, cell viability was weakened markedly in siRNA-CTHRC1-3 group cells in a time-dependent manner (n=3, P<0.001) (**Figure 3**). It suggested that siRNA-CTHRC1 inhibited cell viability.

siRNA-CTHRC1 inhibited cellular adhesion, migration and invasion

Transwell assays were performed to explore the effects of siRNA-CTHRC1-3 on cell migration and invasion. As shown in **Figure 4**, treatment with siRNA-CTHRC1-3 obviously suppressed the adhesion, migration and invasion ability of ESC compared with the NC group and control. The number of cell migration in the control, NC

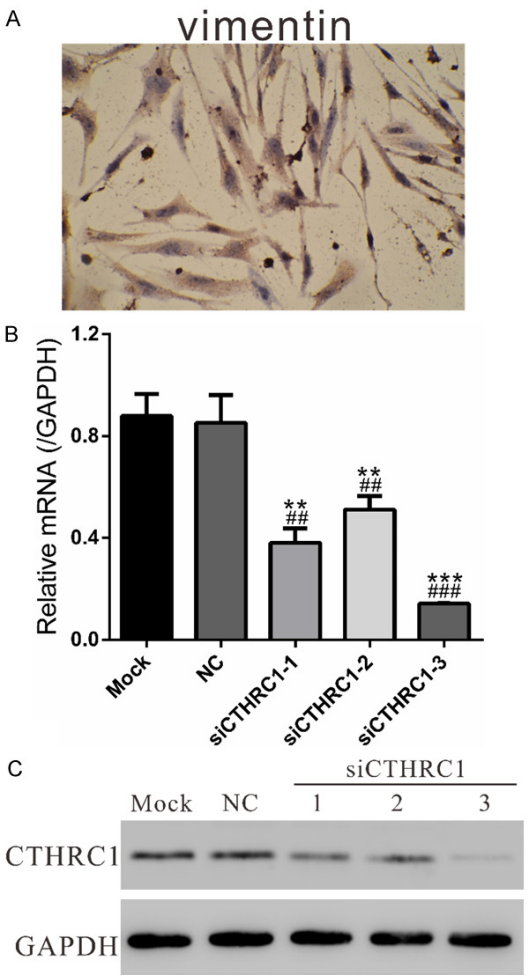

Figure 2. Detection of CTHRC1 expression after treatment with siRNA-CTHRC1. (A) Identification of ESC by immunocytochemical staining with anti-vimentin. Magnification: × 200. (B, C) Three siRNAs targeting human CTHRC1 mRNA (siCTHRC1-1, -2 and -3) and a non-specific scramble siRNA sequence (NC) were synthesized and transfected ESC. Relative mRNA (B) and protein (C) expression levels of CTHRC1 in siRNA-CTHRC-treated group were notably decreased. **P<0.01, ***P<0.001 *vs* Mock, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 *vs* NC.

and siRNA-CTHRC1 group was 108 ± 7, 98 ± 8 and 35 ± 7, respectively. The number of cell invasion in the three groups was 60 ± 6, 58 ± 6 and 17 ± 2, respectively. The number of cell adhesion in the three groups was 31 ± 4, 34 ± 3 and 15 ± 3, respectively. The results indicated that siRNA-CTHRC1-3 inhibited adhesion, migration and invasion of ESC.

siRNA-CTHRC1-1regulated the expression of tumor-related protein

In order to further understand the mechanism through which cells proliferation and metastasis of the endometriotic stromal cells were inhibited by siRNA-CTHRC1, the protein levels of several tumor-related genes were detected western blot. As revealed in **Figure 5A**, the protein levelsof matrix metallopeptidase 9 (MMP9), matrix metallopeptidase 2 (MMP2), CD44, transforming growth factor-β1 (TGF-β1), ICAM1 (intercellular cell adhesion molecule-1) were all down-regulated by siRNA-CTHRC1, while E-cadherin was up-regulated. Moreover, treatment with siRNA-CTHRC1 inhibited the phosphorylation of ERK1/2 (**Figure 5B**).

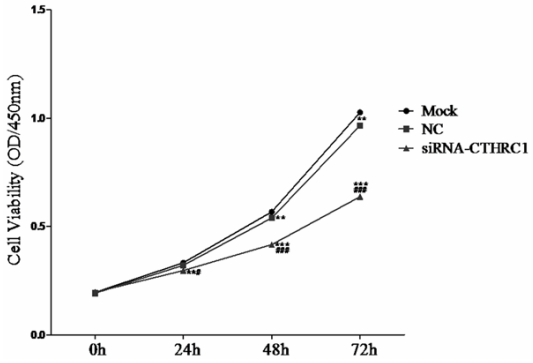

Figure 3. siRNA-CTHRC1-3 inhibited ESC viability. After transfection with siRNA-CTHRC1-3 or negative control (NC) siRNA for 24 h, 48 h and 72 h, cell viability was markedly weakened in a time-dependent manner. n=3, mean ± SD. **P<0.01, ***P<0.001 *vs* Mock, $^{\#}$P<0.05, $^{\#\#\#}$P<0.001 *vs* NC.

Discussion

Recently, CTHRC1 has been found to be aberrantly up-regulated in various human solid tumors and may be closely associated with migration and invasion of tumors [17, 18]. Also it is reported that CTHRC1 could be considered as an effective and novel prognostic marker for the prediction of recurrence or metastasis of colorectal cancer [19]. However, the function and regulation of CTHRC1 in EM and the relationship between EM and CTHRC1 have not been reported. In this current study, we demonstrated for the first time that a higher expression of CTHRC1 occurs in the endometriosis tissues than in normal tissues. Our in vitro experiments demonstrated that CTHRC1 inhibited cell proliferation, migration, invasion and adhesion of ESC. The present study indicated that CTHRC1 may promote the progression of EM.

In exploring the function of CTHRC1 at the molecular level, the expression levels of sever-

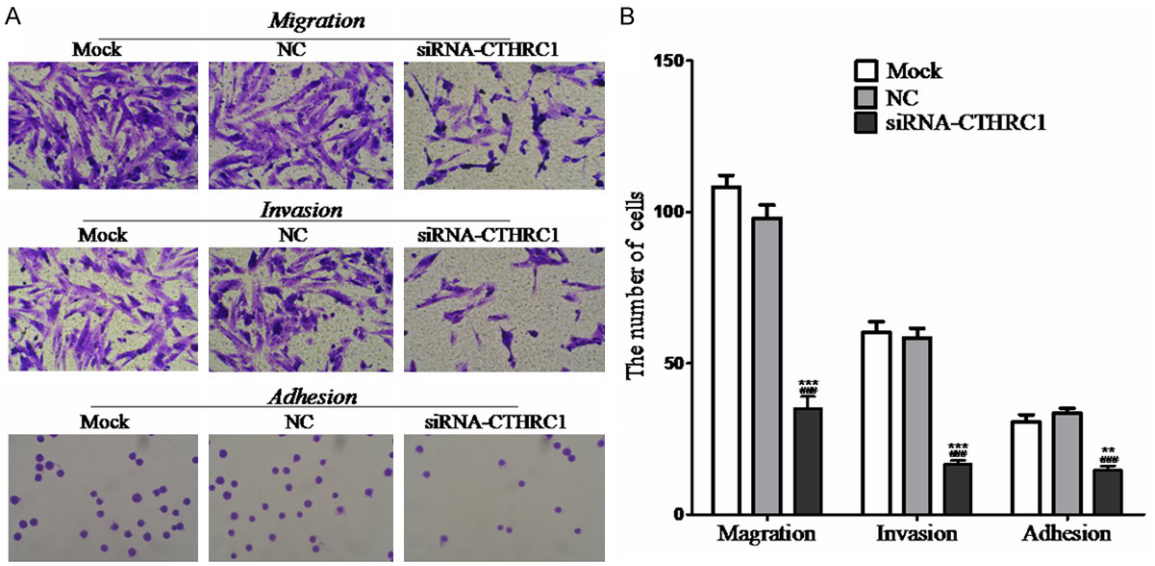

Figure 4. siRNA-CTHRC1-3 inhibited cellular adhesion, migration and Invasion. A. Treatment with siRNA-CTHRC1 obviously suppressed the adhesion, migration and invasion ability of EM cells compared with the NC group and control. B. Data were presented as mean ± SD from three independent experiments. ***P<0.001 *vs* Mock, ###P<0.001 *vs* NC.

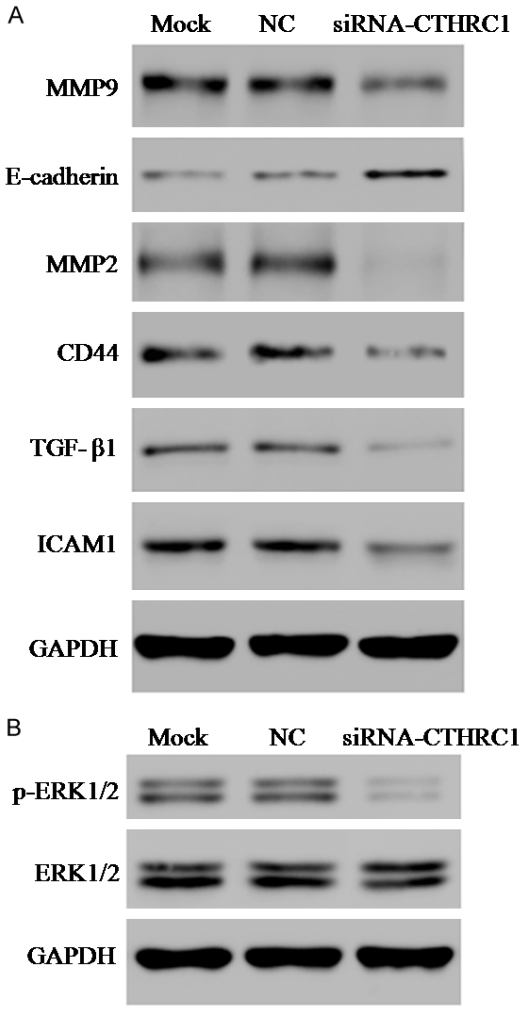

Figure 5. siRNA-CTHRC1 regulated the expression of tumor related genes. A. The protein levelsof MMP9, MMP2, CD44, TGF-β1 and ICAM1 were all down-regulated by siRNA-CTHRC1, while E-cadherin was up-regulated. B. Treatment with siRNA-CTHRC1 inhibited the phosphorylation of ERK1/2.

al tumor-related genes were detected in our study. It is reported MMPs are associated with cancer-cell invasion and metastasis. Activation of MMPs, including MMP2 and MMP9, have been detected in almost all types of human cancer and are closely correlated with advanced tumor stage, increased invasion and metastasis, and shortened survival time [20, 21]. Here, our results showed that the protein levels of MMP2 and MMP9 were inhibited in siRNA-CTHRC1 treated EM cells. Besides, E-cadherin [22] and TGF-β1 signaling [23] play a vital role during the EMT (epithelial-mesenchymal transition) process, which is closely associated with tumor cell metastasis [24, 25]. In this study, siRNA-CTHRC1 treatment resulted in a significant increase in E-cadherin expression and a notable decrease in TGF-β1 expression, which suggested that CTHRC1 may affect EM cell invasion through regulating EMT. CD44 has multiple functions and plays vital roles in enhancing the integrin-mediated tumor cell metastasis [26] and promoting tumor cell invasion by regulating the activity of metalloproteinase [27, 28]. ICAM1 plays an important role in

embryo development, differentiation, normal tissue maintenance, inflammation and immunity response [29]. In this study, both CD44 and ICAM1 were down-regulated in siRNA-CTHRC1 group cells. Our study suggested CTHRC1 may affect cell adhesion through regulating CD44 and ICAM1. Further, we found that the ERK1/2 phosphorylation status was inhibited by siRNA-CTHRC1. ERK1/2 are important mediators of cell proliferation [30]. It has been reported that activation of ERK1/2 was enhanced in various kinds of cancers [31]. Our results showed that p-ERK1/2 was down-regulated in siRNA-CTHRC1 treated EM cells. Considering the above reports, we inferred that inhibition of CTHRC1 resulted in the inhibition of p-ERK1/2, thus repressing cell proliferation.

In conclusion, we first demonstrated the higher expression of CTHRC1 in EM tissues than in normal tissues. Knockdown of CTHRC1 expression significantly inhibited cell proliferation, cell migration, invasion and adhesion of ESC. Further, the expression levels of several tumor cellular processes related proteins and phosphorylation level of ERK1/2 were affected by CTHRC1 knockdown. Our findings have uncovered a novel role of CTHRC1 in regulating the pathological processes of EM and suggested a novel therapeutic target for EM.

Acknowledgements

This study was supported by Zhejiang Province Natural Science Funds Grant (No. Y13H-040003) and National Natural Science Funds of Youth Science Foundation Grant (No. 814-01181).

Disclosure of conflict of interest

None.

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