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From Cell Biology to Tissue Engineering

Elevated cathepsin K potentiates metastasis of epithelial ovarian cancer

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Summary. Cathepsin K, or CTSK, has been found to be involved in the peritoneal metastasis of ovarian carcinoma. However, the expression and clinicopathological significance of CTSK remains unknown in epithelial ovarian cancer (EOC). The aim of the present study was to investigate the expression of CTSK and its clinicopathological significance in EOC.

TSK expression was evaluated using immunohistochemistry in EOC tissue microarray. The expression of CTSK in EOC was displayed to be markedly higher than that of adjacent normal control. In addition, CSTK expression was shown to be remarkably associated with metastases and inferior overall prognosis of EOC. *In vitro*, Knock-down of CTSD was exhibited to be able to suppress migration and invasion in EOC cell lines OV-2008 but not proliferation in OV-2008. Together, our data showed that elevated CTSD in EOC can potentiate the metastasis of EOC cells, suggesting that targeting CTSD might be used as a novel therapeutic target for EOC.

Key words: Cathepsin K, Epithelial ovarian cancer, Migration, Invasion, Prognosis

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Introduction

Epithelial ovarian cancer (EOC) is the fifth most lethal cancer affecting women, accounting for the majority of mortalities from gynecological cancer (Pendlebury et al., 2017). The high mortality rate of EOC is mainly due to the complications of metastasis. Once the epithelial cells covering the ovaries undergo neoplastic transformation, they exfoliate from the primary tumor and disseminate to the peritoneal cavity through implantation pattern (Yu et al., 2016). Nonetheless, the underlying mechanism by which EOC cells metastasize remains unknown.

Cathepsins are highly expressed in various human cancers, associated with tumor metastasis. It is superfamily, consisting of A, B, C, D, E, F, G, H, L, K, O, S, V, and W family members (Tan et al., 2013). Each member has a different function, playing different roles in distinct tumorigenic processes such as proliferation, angiogenesis, metastasis, and invasion (Nomura and Katunuma, 2005). Among the members belonging to Cathepsins found to be associated with metastasis in various different types of cancer (Nomura and Katunuma, 2005; Gocheva and Joyce, 2007; Husmann et al., 2008; Duong et al., 2014), only Cathepsin K, which is hereafter referred to as CTSK, has been identified to be implicated in the peritoneal metastasis of ovarian cancer (Xu et al., 2016) till now. Despite the finding, the expression and clinicopathological significance of CTSK expression remains to be studied.

In the present study, to understand the expression of CTSK and the clinicopathological significance of CTSK in EOC, EOC tissue microarray was used to detect the status of CTSK with immunohistochemical means,

followed by analysis of association between expression and clinicopathological variables, including overall prognosis. To verify the phenotype we observed on clinical tissue sample, EOC cell lines OV-2008 and ES-2 were used to evaluate the variation of migratory and invasive abilities after knock-down of CTSK. It was shown that CTSK was remarkably up-regulated in EOC relative to paired normal control. Up-regulation of CTSK was significantly associated with metastases and poor overall prognosis. *In vitro*, knock-down of CTSK was presented to be capable of markedly impairing the invasion and migration of EOC cells. Together, our results support that CTSK potentiates the migration and invasion of EOC cells, indicating that targeting against CTSK could be used as underlying therapeutic strategy of EOC.

Materials and methods

Clinical samples

The present study was approved by the Medical Ethics Committee of the Jinan Central Hospital Affiliated to Shandong University. Tissue microarray used for immunostaining analysis of CTSD was commercially purchased from Shanghai Outdo Biotech. Co. Ltd (Catalogue number: HOva-Can90PT-01; Shanghai, China). The tissue microarray consisted of 90 cases of EOC. Of the 90 cases, 41 cases were serous subtype, 9 cases were mucinous subtype, 6 cases were endometrioid, 6 cases clear cell carcinomas, 6 cases ovarian germ cell carcinomas, 3 cases ovarian stromal carcinomas, 15 ovarian serous adenocarcinoma, the remainder mixed type accounting for 4 cases. Staging and grading was assessed according to the WHO classification and grading system 2015 version. None of these patients whose samples were derived received any chemoradiotherapy before ovarectomy, and Informed consent was obtained for each participant, as declared by the Shanghai Outdo Biotech company. Parenthetically, the paired normal control tissue was defined as the healthy tissue that was far away from tumor lesion at least more than 5 centimeters. By this standard, all the paired normal controls were therefore obtained through sampling the ovarian cancer tissues at the same time for medical research after written informed consent was obtained from each participant involved, as declared by the Shanghai Outdo Biotech company.

EOC cell culture and transfection

The human EOC cell lines OV2008 (human, ovary, carcinoma, morphology: Epithelial); JHOS-3(human, Japanese, ovary, serous adenocarcinoma, morphology: Epithelial); JHOS-2(human, Japanese, ovary, serous adenocarcinoma, morphology: Epithelial); OVCAR-3 (human, Caucasian, ovary, mucinous adenocarcinoma, morphology: Epithelial) and SKOV-3 (human, Caucasian, ovary, adenocarcinoma, morphology: Epithelial) were all purchased from Riken Cell Bank (Tsukuba, Japan). ES-2 (human, ovary, Clear cell carcinoma, morphology: Epithelial) was from ATCC organization (ATCC, USA). Cells were cultured in DMEM supplemented with 10% fetal bovine serum and penicillin/streptomycin in a 5% CO_2 humidified incubator at 37°C, unless otherwise stated. For knockdown of CTSK, small interference RNA (siRNA) sequences against homo CTSK (NM_000396.3) were designed and synthesized by Genepharm Company (Genepharm, Shanghai). The sequences were siRNA-CTSK-1: 5'- GGAAGAGAGTTGTATGTACAAC CCAAC-3'; siRNA- CTSK-2:5'-GCCCTGAAGAG GGCAGTGGCCCGAGTG-3';siRNA-scramble:5'-CGTATATGTACTGCGCGTGGAGA-3'. As for the lentiviral-based short hairpin RNA (shRNA) vectors, it were outsourced by GeneChem Company (Genechem, Shanghai). All the EOC cells transfected with lentiviral-based shRNA vectors were subjected to fluorescence-activated cell sorting.

Immunohistochemistry (IHC)

Immunohistochemical stains were performed using heat-induced epitope retrieval, an avidin-biotin complex method. The rabbit polyclonal antibody to homo CTSK (Catalogue number: ab19027; Abcam, Cambridge, USA) was diluted 1:100. The sections were assessed by two pathologists under light microscopic. The staining patterns were scored as follows: negative, weak (less than 30% of cells with positive staining), moderate (less than 60% but more than 30% of cells with positive staining) and strong positive (more than 60% of cells with positive staining) according to the immunostaining intensity. Both moderate and strong positive expression were categorized into high expression, whereas negative and weak staining were classified into low expression.

Western blotting

Seventy-two hours after transfection, OV2008 and ES2 cells were harvested in RIPA lysis buffer (Bioteke, Beijing, China) and 50 μg of total cellular protein were subjected to 12% SDS-PAGE separation. Proteins were transferred to PVDF membrane (Millipore, Boston, MA, USA) and blots were probed with rabbit polyclonal antibodies to CTSK Catalogue number: ab19027; Abcam, Cambridge, USA) and GAPDH (sc-25778, Santa Cruz Biotechnology, CA, USA). GAPDH was used as internal loading control and the blots were visualized with chemiluminescent substrate (Thermo Scientific, USA), and images were captured with a Bio-Rad camera system (Bio-Rad, USA).

MTT assay

Methylthiazolyl blue tetrazolium (MTT; Sigma-Aldrich, St Louis, MO) dye assay was used to evaluate the variation of cell proliferation. OV2008 and ES2 cells

were plated in 96-well plates at a density of 4×10^3 cells per well. After transfection, proliferation was assessed. Cells were incubated with 20 μL MTT at 37°C for 4 hours. The color was developed by incubating the cells in 150 μL dimethyl sulfoxide (DMSO), the absorbance was monitored at 490nm wave length. The data were obtained from three independent experiments.

Wound healing assay

Wound healing assay was used to assay the variation of migration. OV2008 and ES2 cells were plated in 6-well plates at a concentration of 4×10^5 cells/well and allowed to form a confluent monolayer for 24 h. After transfection, the monolayer was scratched with a sterile pipette tip (10 μL), followed by washing with serum free medium to remove floated and detached cells and photographed (time 0 h and 48h) by fluorescent inversion fluorescence microscope (Olympus, Japan).

Transwell assay

The invasion assay was performed using Transwell 24-well dishes with a pore size of 8 μm (Costar, NY, and USA). Approximately 1×10^4 EOC cells in 200 μL of DMEM serum-free medium were placed in the upper chamber, and 300 μL of medium containing 50% serum-free DMEM was placed in the lower chamber. The cells were incubated for 24 h at 37°C in 5% CO_2, then fixed in methanol for 15 min and stained with 0.1% crystal violet in PBS for 15 min. Cells on the upper side of the filters were removed with cotton-tipped swabs and the filters were washed with PBS. Cells on the underside of the filters were examined and counted under a microscope. The assay was repeated at least three times.

Statistical analysis

Data was expressed as mean ± standard deviation (SD), unless otherwise indicated. Data were statistically analyzed using GraphPad Prism 5.0 version (GraphPad Software, Inc., La Jolla, CA, USA). Differences in MTT, Wound-healing and Transwell assays were assessed using the Student t-test or one-way ANOVA test where the data were considered to be normally distributed after Kolmogorov-Smirnov test; otherwise the Mann-Whitney U or Kruskal-Wallis test was utilized if the data was taken to be abnormally distributed after Kolmogorov-Smirnov test. Correlation between CTSK expression and clinicopathological variables was compared using chi-square or Fisher's exact test (expected numbers were less than 5) as appropriate. Univariate survival analyses were plotted using the Kaplan–Meier survival curve and analyzed with the Log-Rank test. Statistical significance was defined as *P<0.05, **P<0.01 or ***P<0.001.

Results

CTSK was remarkably up-regulated in EOC tissues relative to control

In consideration that the correctness and specificity

Table 1. The clinicopathological significance of CTSK expression in EOC.

Parameters		Cases (n=90)	CTSK		χ^2(or F)	P value
			low (-, +)	high (++, +++)		
EOC		90	38	52	14.100	0.000
Normal control		90	63	27		
Age	<50	42	14	28	2.551	0.136
	≥50	48	24	24		
T classification	T_{1+2}	34	15	19	0.080	0.828
	T_{3+4}	56	23	33		
N classification	N0	18	12	6	5.087	0.033
	N1-3	72	26	46		
Clinical stage	I+II	43	21	22	1.477	0.286
	III+IV	47	17	30		
Peritoneal metastasis	Yes	60	19	41	8.221	0.006
	No	30	19	11		
Tumor volume (cm^3)	<4	24	12	12	5.494	0.071
	4-8	40	20	20		
	>8	26	6	20		
Gross classification	Mucinous	6	2	4	5.187	0.149
	Serous	41	11	30		
	Endometrioid	6	1	5		
	Mixed subtypes	27	14	13		

Note: 27 cases of mixed subtypes, here includes 6 cases clear cell carcinomas, 6 cases ovarian germ cell carcinomas, 3 cases ovarian stromal carcinomas, 15 ovarian serous adenocarcinoma. Owing to the relatively limited cases, here we used the term mixed subtype to represent these specific subtypes.

of primary antibody could bias or even mislead the final conclusion (Baker, 2015); we've determined to pre-test the correctness and specificity of the primary antibody we were going to use using antigen pre-adsorption test approach as recommended (Holmseth et al., 2012). Pre-evaluation of the primary antibody to human CTSK commercially from Abcam exhibited that the specificity was adequate to be used in the following analysis (Fig. 1A). Based on which, to investigate the state of CTSK in EOC, we carried out IHC with EOC tissue microarray consisting of 90 paired cases of EOC and its normal control tissues. IHC results revealed that sublocalization of CTSK was mainly membranous and cytoplasmic and that expression of CTSK varied largely among the EOC cases, with CTSK being from weak to mild to strong positive staining. Taken as whole, CTSK was predominantly expressed in EOC compared with paired normal control where CTSK expression was hardly detectable (Fig. 1B). In addition, the expression level of CTSK tends to increase with the increasing of metastasis (Fig. 1C). Clinicopathologically, CTSK expression was found to be significantly associated with metastasis and lymph node metastases (Table 1). To analyze the relationship between CTSK expression and overall prognosis, Kaplan-Meier survival analysis was performed. Kaplan-Meier survival analysis showed that there was significant association between CTSK and inferior overall prognosis (Fig. 1D). These results obtained from clinical tissue suggest that CTSK was a metastasis-associated gene in EOC.

Knock-down of CTSK prevents migration and invasion of EOC cells in vitro

Having understood the clinical phenotype of CTSK expression, subsequently, we extended the analysis of phenotype of CTSK to EOC cell lines *in vitro*. First of all, a panel of human EOC cell lines, including OV2008, JHOS-3, JHOS-2, ES-2, OVCAR-3 and SKOV-3 were enrolled, whose endogenous level of

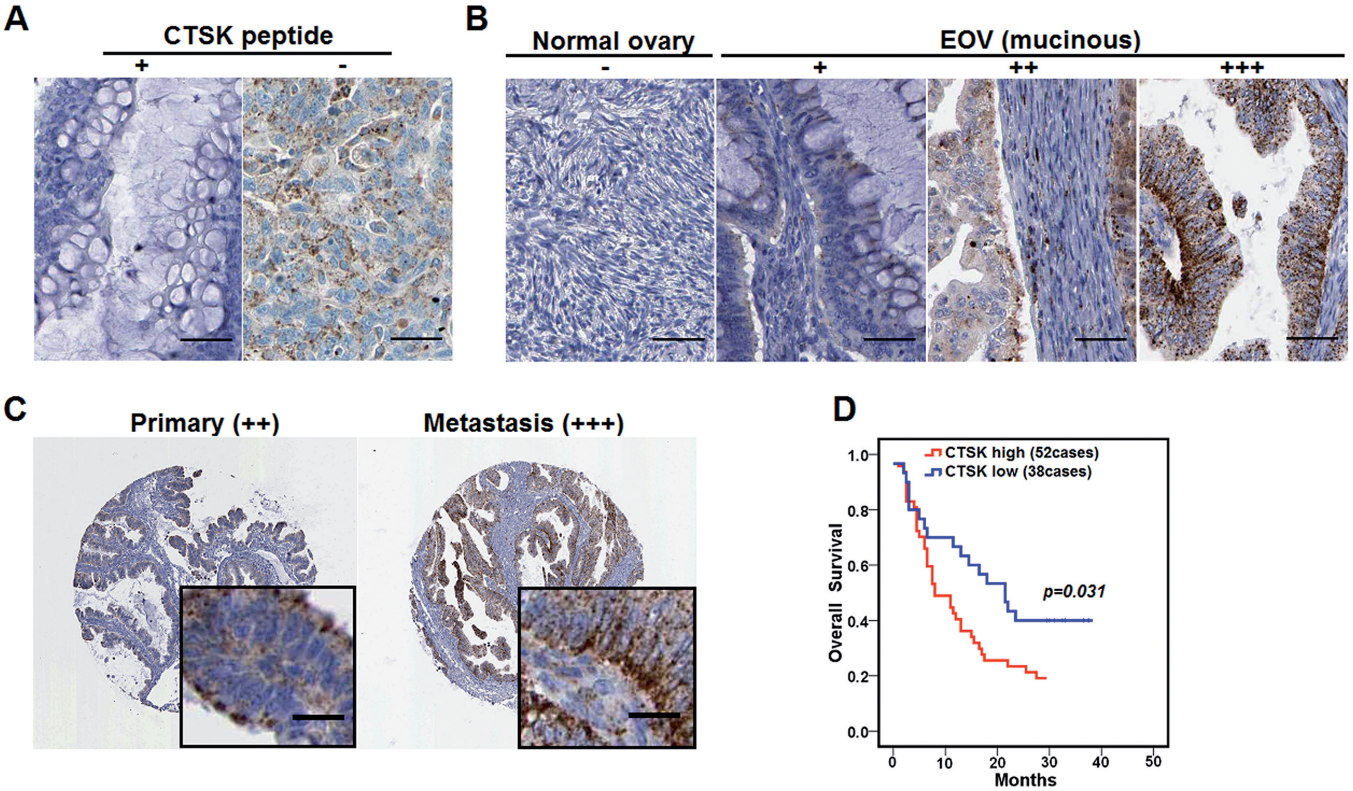

Fig. 1. CTSK was shown to be markedly up-regulated in EOC tissues and up-regulation of CTSK was significantly associated with poor overall prognosis. **A.** Antigen pre-adsorption test was carried out to pre-test the specificity and correctness of the primary antibody to human CTSK. Recombinant CTSK peptides were commercially obtained from CUSABIO and CusAb company (Catalogue number: CSB-YP006192HU, CUSABIO and CusAb company, MD, USA). 20 ug/ml recombinant CTSK peptide was incubated with primary antibody to CTSK, followed by incubation with sections. The following procedures were routines as IHC was performed. **B.** CTSK expression was evaluated in normal ovary and EOV tissues using IHC method. In terms of immunostaining of CTSK, -, means negative immunostaining; +, denotes weak; ++, represents medium; +++, stands for strong positive immunostaining. Here shown ovarian cancer tissues were all mucinous subtype of EOV. **C.** Shown was CTSK expression in primary and metastasis tissues of EOC. **D.** Overall prognosis was analyzed using Kaplan-Meier survival curve. p=0.031, using Log-Rank test method. Scale bars: 100 μm.

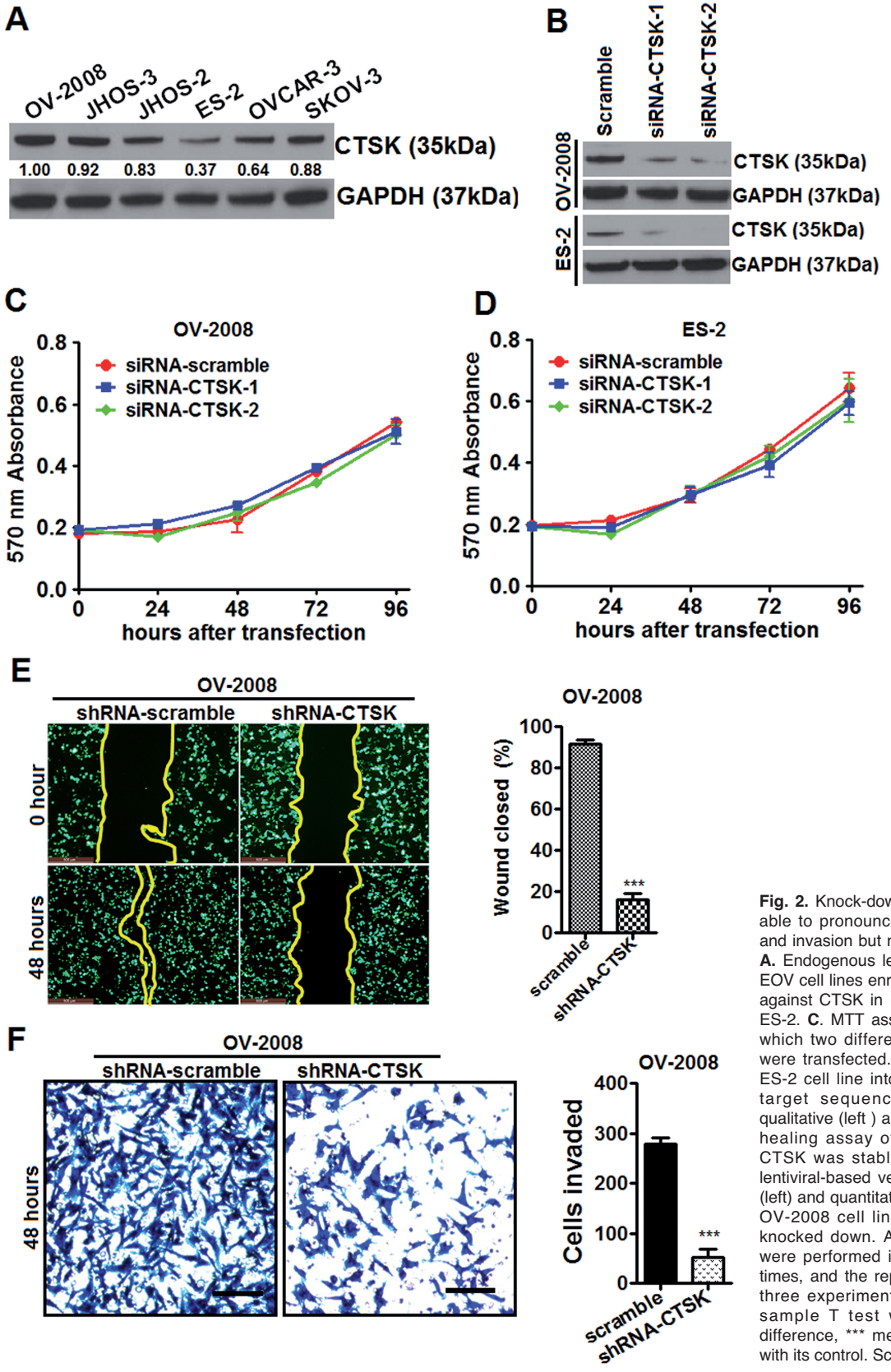

Fig. 2. Knock-down of CTSK was found to be able to pronouncedly suppress the migration and invasion but not proliferation of EOC cells. **A.** Endogenous level of CTSK in the panel of EOV cell lines enrolled. **B.** Evaluation of siRNA against CTSK in EOC cell lines OV-2008 and ES-2. **C**. MTT assay of OV-2008 cell line into which two different siRNA target sequences were transfected. **D.** Likewise, MTT assay of ES-2 cell line into which two different siRNA target sequences were transfected. **E.** qualitative (left) and quantitative (right) wound-healing assay of OV-2008 cell line whose CTSK was stably knocked down based on lentiviral-based vector. **F.** Similarly, qualitative (left) and quantitative (right) Transwell assay of OV-2008 cell line whose CTSK was stably knocked down. All the experiments involved were performed independently at least three times, and the representative figures from the three experiments are shown. Independent sample T test was used to analyze the difference, *** means $p<0.001$ in comparison with its control. Scale bars: 50 μm.

CTSK was detected using immunoblotting (Fig. 2A). Among these cell lines, two extremes, that is, OV2008 and ES2 cells were selected for the following functional analysis of CTSK. To observe the phenotypic variation mediated by CTSK, CTSK was transiently knocked down using transfection with siRNA into OV2008 and ES2 cells. As shown in Fig. 2B, both of the two interference sequences, siRNA-CTSK-1 and siRNA-CTSK-2 can effectively and markedly silence CTSK expression compared with control group. Next, based on the two siRNA target sequences that were adequate to silence, we constructed the lentiviral-based shRNA vectors. To understand whether stable knock-down of CTSK could have a significant effect on the variation of proliferation, migration and invasion of OV2008 and ES2 cells, both MTT, wound-healing and Transwell assays were conducted in OV2008 and ES2 cells after their CSTK being stably knocked down. It was shown that knock-down of CTSK makes little difference on the proliferation of OV2008 and ES2 cells (Fig. 2C). However, silence of CTSK was shown to be pronouncedly capable of suppressing the migration (Fig. 2D) and invasion (Fig. 2E) of OV2008 cells compared with control. Taken as a whole, these results showed that knock-down of CSTK can prevent the migration and invasion but not proliferation of EOC cells, supporting that CTSD promotes metastasis of EOC cells.

Discussion

In the present investigation, CTSK was found to be markedly up-regulated in EOC tissues compared with paired normal control and was significantly associated with metastases and poor overall survival of patients with EOC. *In vitro*, knock-down of CTSK was shown to be remarkably able to suppress the migration and invasion but not proliferation of EOC cells, supporting the metastasis-promoting role of CTSK in EOC.

CTSK belongs to the Cathepsins super family (Rood et al., 1997). Original extensive functional studies regarding CTSK were from bone research, which was thought to perform a major role in osteoclast-mediated bone resorption (Bossard et al., 1996; Dodds et al., 2001). Then it was extended to the realm of cancer research where CTSK was observed to mediate the metastases in various types of cancer, including breast cancer (Littlewood-Evans et al., 1997; Le Gall et al., 2007), prostate cancer (Brubaker et al., 2003; Corey et al., 2003; Podgorski et al., 2007), lung cancer (Rapa et al., 2006; Cordes et al., 2009), glioblastoma (Verbovsek et al., 2014), melanoma (Raimondi et al., 2015), osteosarcomas (Husmann et al., 2008) and ovarian carcinoma (Xu et al., 2016). Information from these studies showed that CTSK level has been linked to bone metastasis (Le Gall et al., 2007), which leads to the proposal that targeting against CTSK could be a potentially important and novel approach for bone metastasis (Le Gall et al., 2008). Nevertheless, the direct evidence for CTSK hasn't been established in the setting of ovarian cancer although Xu H and co-workers had identified using microarray that CTSK was involved in the peritoneal metastasis of ovarian cancer (Xu et al., 2016). Therefore, the state and its clinical implications of CTSK remain unknown. In our investigation, CTSK was expectedly over-expressed in EOC relative to paired normal control, which was in total agreement with its status in other types of cancers as previously reported (Brubaker et al., 2003; Verbovsek et al., 2014). Statistical analysis showed that CTSK expression was significantly associated with metastasis and poor overall prognosis, which was supported by the similar finding of CTSK in prognosis of lung cancer (Cordes et al., 2009). To verify the phenotype we observed in clinical samples, we extended to EOC cells *in vitro*. It was found that knock-down of CTSK can remarkably suppress the migration and invasion but not proliferation of EOC cells, which was fundamentally in line with Le Gall et al. (Le Gall et al., 2007)'s finding that use of CTSK inhibitor was shown to be significantly able to impair the bone metastasis but not to block the growth of breast cancer cells in xenograft nude mice. It may be that CTSK might have diverse functions or influence numerous pathways that might account for knock down of only CTSK would be insufficient or fail to have an apparent effect on growth of cancer cells. Given this, knock-down of CTSK only was unlikely to be expected to suppress the growth of EOC cells. Consequently, targeting CTSK with other relevant oncogenic molecules could be an alternative (Gondi and Rao, 2013). As for the underlying mechanism by which CTSK mediates metastasis in EOC, it remains to be further investigated. One possible mechanism may be that CTSK can cleave and activate MMP-9 (Christensen and Shastri, 2015), whose main function is to degrade and remodel the extracellular matrix in acidic environments such as seen in tumors and during bone resorption. Furthermore, among cathepsin family members, several studies have reported that Cathepsin L was found to be able to confer chemoresistance in tumor cells. However, little has been known regarding whether CTSK could be involved in the chemoresistance. In our setting, there has been lack of corresponding clinical information with respect to chemoresistance, we thus can't arrive at any relevant conclusion concerning whether CTSK could be involved in chemoresistance. Nor did we perform the *in vitro* analysis regarding the possible involvement of CTSK in chemoresistance. The further study therefore is required in this respect in the following.

There were several limitations that deserve to be acknowledged. For one thing, the relevant working mechanism of CTSK in EOC hasn't been touched upon. Further basic and clinical investigation therefore may be warranted in the following; Secondly, only expression status of CTSK was evaluated in the study, the activity of CTSK in EOC still needs to be further assayed;

thirdly, given the other Cathepsins parallel to CSTK, including Cathepsin B (CTSB) (Giusti et al., 2008), Cathepsin D (CTSD) (Pranjol et al., 2015), have also been reported to be implicated in the mediation of EOC metastases, understanding of the expression and significance therefore should have been extended to them.

In conclusion, in the current investigation, we showed that CTSK was markedly up-regulated in EOC tissues in comparison with paired normal controls and that up-regulation of CTSK was significantly associated with metastasis and poor prognosis of patients with EOC. *In vitro*, Silence of CTSK was shown to be able to inhibit migration and invasion but not proliferation of EOC cells. Our results support that CTSK promotes metastasis in EOC.

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Conflict of interest. The authors declare that there was no conflict of interest.

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