Int J Clin Exp Pathol 2016;9(3):3161-3169
www.ijcep.com /ISSN:1936-2625/IJCEP0020782

Original Article

HtrA2/Omi protease in epithelial ovarian carcinoma: clinical significance and antineoplastic efficacy of its overexpression on subcutaneous transplanted tumor in nude mice model

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Received November 29, 2015; Accepted January 31, 2016; Epub March 1, 2016; Published March 15, 2016

Abstract: The serine protease HtrA2/Omi, a regulator of apoptosis, is localized in mitochondria and is released into the cytosol after apoptotic stimuli. Previous studies demonstrate that Omi/HtrA2 is associated with a variety of malignancies. In the present study, we analyzed the expression of Omi/HtrA2 in 52 epithelial ovarian carcinoma (EOC) tissues and paired corresponding adjacent non-tumor tissues to elaborate the clinical significance and antineoplastic efficacy of Omi/HtrA2 on subcutaneous transplanted tumor in a nude mice model. The results showed that the mRNA and protein expression of Omi/HtrA2 in the EOC tissues was dramatically higher than that in the adjacent non-tumor tissues, and Omi/HtrA2 was highly correlated with clinicopathological parameters, such as clinical stage and distant metastasis. Moreover, HtrA2/Omi gain-of function acute and HtrA2/Omi loss-of function blunted apoptosis in A2780 and OVCAR-3 cell lines. In vivo, HtrA2/Omi gain-of function could attenuate A2780- and OVCAR-3-engrafted tumors growth and increase the protein expression of caspase3 and caspase9 in engrafted tumors mice as compared to the control group. In conclusion, these results suggested that overexpressed HtrA2/Omi could inhibit EOC cell growth in vitro and in vivo, and the underlying mechanism was mediated, at least partially, through the activation of caspase3 signaling pathway.

Keywords: Epithelial ovarian carcinoma, Omi/HtrA2, serine protease, caspase3, apoptosis

Introduction

Ovarian cancer is the second most common and is the most lethal gynecological malignancy in women [1, 2]. Nearly 90% of all ovarian cancer cases are epithelial ovarian carcinoma (EOC), which is especially prone to recurrence and metastasis, and the estimated 5-year survival rate for all stages is less than 40% [3, 4]. In 2014, there are 21,980 new cases and 14,270 deaths as a consequence of EOC [5]. Due to the high mortality, exploring the related molecular mechanisms and identifying the major factors of invasion and metastasis of EOC will be of great significance for improving the survival of EOC patients.

HtrA2/Omi belongs to the family of high temperature requirement protein A (Hart) serine proteases conserved from bacteria to humans and has been described both as a chaperone protein and as a serine protease responsible for cleavage of denatured proteins at elevated temperatures [6]. The mature HtrA2/Omi is localized to the mitochondrial intermembrane space (MIS), where it remains until subsequent translocation to the cytosol and participates in both caspase-dependent as well as caspase-independent cell death [7, 8]. In human colon cancer HCT116 cells, HtrA2/Omi promotes caspase activation by direct binding to and inhibiting the IAP family caspase inhibitors [9]. Moreover, several studies have demonstrated that HtrA2/Omi mRNA expresses in cancer cell lines and over-expresses in prostate cancer and correlates with prostate cancer differentiation [10]. Similar to the results in prostate cancer, stomach cancer cells in vivo may need HtrA2/Omi expression for apoptosis, and HtrA2/Omi expression might be involved in

stomach cancer development [11]. However, the molecular mechanisms of HtrA2/Omi contribute to the induction of EOC remains to be determined.

In the present study, we compiled data from patients diagnosed with EOC to elaborate the clinical significance of HtrA2/Omi, and the antineoplastic efficacy of HtrA2/Omi overexpression on subcutaneous transplanted tumor in nude mice model was elaborated.

Materials and methods

Patients' samples

Fifty-two human epithelial ovarian carcinoma (EOC) tissues and corresponding non-tumourous tissues were collected from the Department of Obstetrics and Gynecology, the Affiliated Hospital of Qingdao University between June 2010 and June 2015. All collected tissue samples were immediately stored at liquid nitrogen until use. Human samples were obtained with written informed consent from all patients. The study was approved by the Ethics Committee of the Qingdao University, China.

Cell culture and animal model

Normal ovarian epithelial cells (NOECs) and EOC cell lines were purchased from Chinese Academy of Sciences Cell Bank (Shanghai, China) and cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco Life Technologies) that contained 10% fetal calf serum (Gibco Life Technologies), 10% L-glutamine, 0.5% penicillin/streptomycin, 10% nonessential amino acids and 10% pyruvate, in a 5% CO_2 atmosphere and incubated at 37°C.

For xenograft experiment, 5×10^6 A2780 and OVCAR-3 cells in 120 μL mixed solution (PBS: Matrigel (BD falcon) = 5:1) were injected subcutaneously into 6 weeks-old BALB/c nu/nu female mice. Tumor growth was monitored by measuring tumor diameters. Tumor volume was calculated according to the formula TV (cm^3) = a × b2 × π/6, where "a" is the longest diameter, and "b" is the shortest diameter. Mice were euthanized when a tumor reached 1 cm in diameter. All animal experiments were performed with the approval of Qingdao University Animal Care and Use Committee.

Short interfering RNA (siRNA) preparation and gene overexpression

Omi/HtrA2 was silenced using commercialized shRNA kits (Santa Cruz Biotechnology). Transfection was performed by electroporation using a pipette-type microporator (Microporator system; Digital Bio Technology, Suwon, Korea). After they had been transfected, the cells were incubated for 48 h in DMEM supplemented with 10% fetal bovine serum at 37°C, before stimulation. A nonspecific scrambled shRNA was the negative control.

For the transfection of A2780 and OVCAR-3, lentiviral vectors harboring HtrA2/Omi was constructed, and the A2780 and OVCAR-3 were infected. Briefly, A2780 and OVCAR-3 were cultured in McCoy's 5α medium containing 10% FBS and when they reached the exponential growth phase, 1.0×10^5 cells per well were plated in 96 plates. Next, 300 μl complete culture medium, containing recombinant lentiviruses, control lentiviruses or McCoy's 5α medium (all containing 6 μg/ml polybrene; Sigma) was added into the plates when the cells reached 50-60% confluence. Two days later, the virus-containing medium was replaced with fresh complete medium.

Immunohistochemistry

Paraffin embedded EOC tissues were cut into about 4 μm section, mounted on glass slides and stained using indirect immunoperoxidase. The paraffin sections were baked in oven at 65°C for 24 h, then dewaxing to water, rinsed with PBS three times (5 min per time). Well washed section was placed in the EDTA buffer for microwave antigen retrieval, the fire to boil, then low heat to boil after an interval of 10 min. After natural cooling, the sections were washed with PBS 3 times. The sections were put into 3% hydrogen peroxide solution and incubated at room temperature for 10 min, which was to block endogenous peroxidase, then washed with PBS 3 times, closed with 5% bovine serum albumin (BSA) for 20 min after drying (close charge). After removal of BSA liquid, each section was added with 50 μl diluted primary antibody overnight at 4°C, then washed with PBS 3 times. After the removal of PBS liquid, each slice which added with 50-100 μl secondary antibody was incubated at 4°C for 50 min, then washed with PBS 3 times, each slice was added

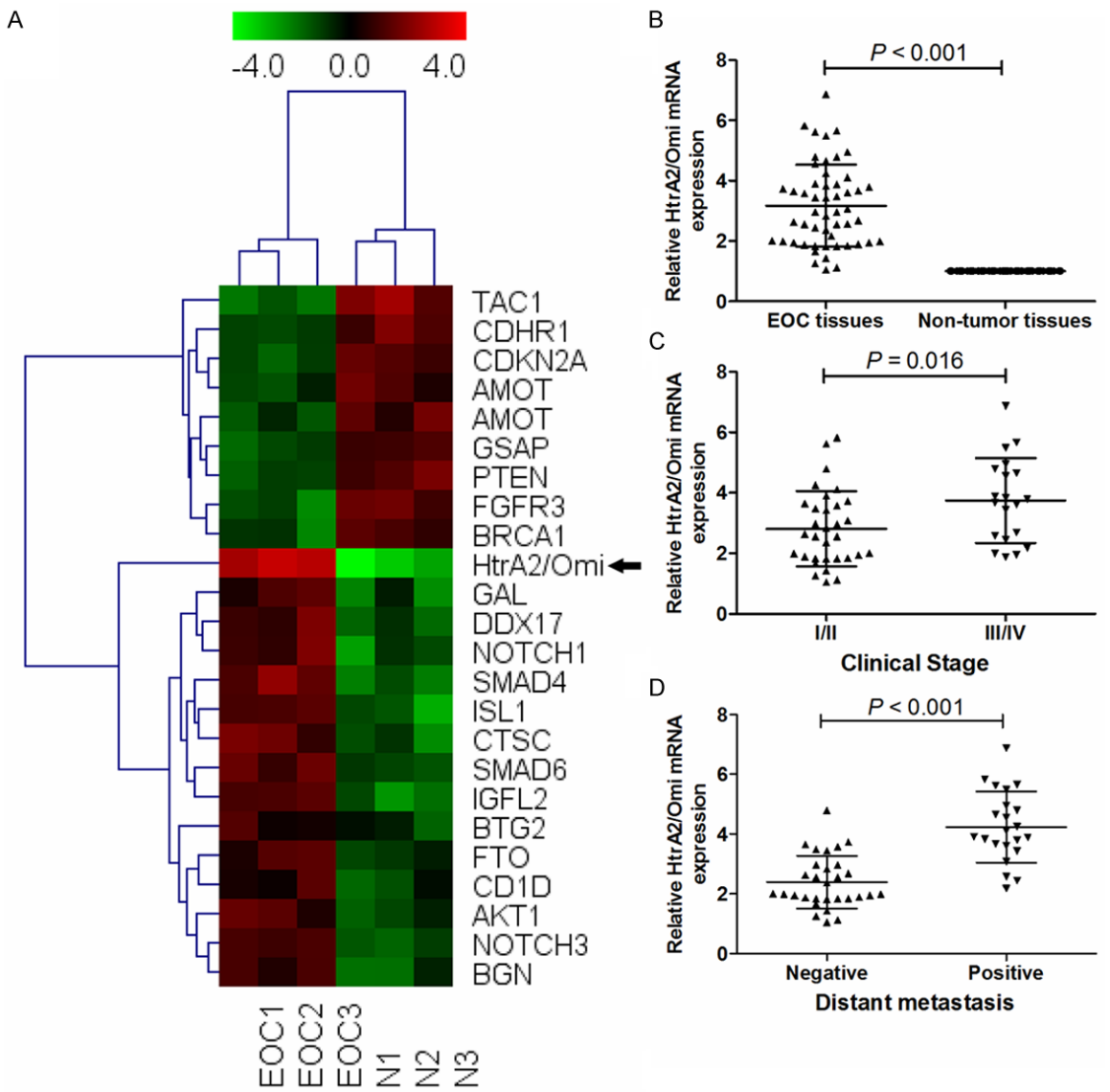

Figure 1. Microarray and hierarchical cluster analysis of the differentially expressed genes in tumor tissues of EOC patients and corresponding non-tumourous tissues (A). Relative HtrA2/Omi mRNA expression in EOC tumor tissues and in paired adjacent non-tumourous tissues (B, n = 52). Relative HtrA2/Omi mRNA expression was measured in different clinical stage (C) and whether distant metastasis (D).

with 50-100 μl freshly prepared DAB solution with the help of microscope for controlling color. After being washed, sections were counterstained with hematoxylin, rinsed with tap water, dehydrated and mounted, and visualized under a microscope (Leica DM 2500).

Real-time polymerase chain reaction

RNA extraction was performed according to the TRIzol manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). Synthesis of cDNAs was performed by reverse transcription reactions with 2 μg of total RNA using moloney murine leukemia virus reverse transcriptase (Invitrogen) with oligo dT (15) primers (Fermentas) as described by the manufacturer. PCR with the following primers: GAPDH, Forward 5'-GACCGGCACCCTTTCTTG-3' and Reverse 5'-CCCCCACTGGTTCATTT-3'; GAPDH, Forward 5'-GGATTTGGTCGTATTGGG-3' and Reverse 5'-GGAAGATGGTGATGGGATT-3'.

Western blotting

EOC tissues and cells were homogenized and extracted in NP-40 buffer, followed by 5-10 min boiling and centrifugation to obtain the super-

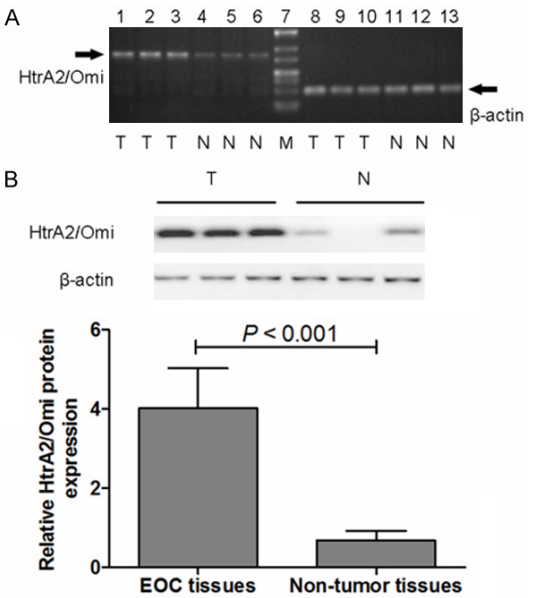

Figure 2. HtrA2/Omi mRNA (A) and protein (B) expression in EOC tumor tissues and in paired adjacent non-tumourous tissues were measured by RT-PCR and western blotting respectively. T, EOC tumor tissues; N, paired adjacent non-tumourous tissues; M, DNA Marker.

natant. Samples containing 50 μg of protein were separated on 10% SDS-PAGE gel, transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA). After saturation with 5% (w/v) non-fat dry milk in TBS and 0.1% (w/v) Tween 20 (TBST), the membranes were incubated with primary antibodies (Santa Cruz Biotechnology, CA, USA). After three washes with TBST, The membranes were next incubated with the appropriate HRP (horseradish peroxidase)-conjugated antibody visualized with chemiluminescence (Thermo, USA).

Statistical analysis

The data from these experiments were reported as mean ± standard deviation (SD) for each group. All statistical analyses were performed by using PRISM version 5.0 (GraphPad). Intergroup differences were analyzed by one-way ANOVA. Differences with P value of < 0.05 were considered statistically significant.

Results

HtrA2/Omi expression in patients with epithelial ovarian carcinoma (EOC)

The microarray data of differentially expressed genes related to EOC were measured in 3 EOC tissues and paired corresponding adjacent non-tumor tissues. Fold change greater than 4 and P value less than 0.05 between EOC tissues and adjacent non-tumor tissues were set as the criteria in filtering differently expressed tumor-related genes. After the removal of redundant and unannotated genes, 9 tumor-related genes were found to be significantly down-regulated and 15 genes to be significantly up-regulated in the EOC tissues by real-time PCR, and we finally focused on HtrA2/Omi in our study (**Figure 1A**). To further study whether HtrA2/Omi was differentially expressed in EOC tissues, a total of 52 paired tumor tissues and adjacent non-tumor tissues were analyzed for HtrA2/Omi expression using real-time PCR. The results showed that HtrA2/Omi expression in EOC tissues was significantly increased compared with adjacent non-tumor tissues (**Figure 1B**). Next, to validate the clinical significance of HtrA2/Omi, we assessed the expression of HtrA2/Omi with clinicopathological parameters, such as tumor size, clinical stage and metastasis. As shown in **Figure 1C** and **1D**, the HtrA2/Omi expression levels were markedly higher in III/IV stage than I/II stage in patients with EOC (**Figure 1C**). Moreover, HtrA2/Omi levels were increased in distant metastasis patients as compared to that of patients without distant metastasis (**Figure 1D**). However, HtrA2/Omi was no obvious difference with tumor size in EOC patients (data not shown). Furthermore, the semiquantitative RT-PCR and western blotting assay showed that the expression of Omi/HtrA2 in the EOC tissues was dramatically higher than that in the adjacent non-tumor tissues (**Figure 2A** and **2B**). Immunohistochemistry staining results showed that HtrA2/Omi levels were significantly higher in EOC tumor tissues than paired adjacent non-tumourous tissues (**Figure 3A-D**).

HtrA2/Omi regulates EOC cell viability and death

To further characterize the expression of HtrA2/Omi in EOC, we performed real-time RT-PCR analyses and found that HtrA2/Omi was markedly up-regulated to various levels in four ovarian cancer cell lines examined compared with NOEC (**Figure 4A**). Meanwhile, the protein expression of HtrA2/Omi was significantly increased in all four ovarian cancer cell lines as compared to NOEC, especially A2780 and OVCAR-3 cell lines (**Figure 4B**). Therefore, we finally focused on A2780 and OVCAR-3 cell

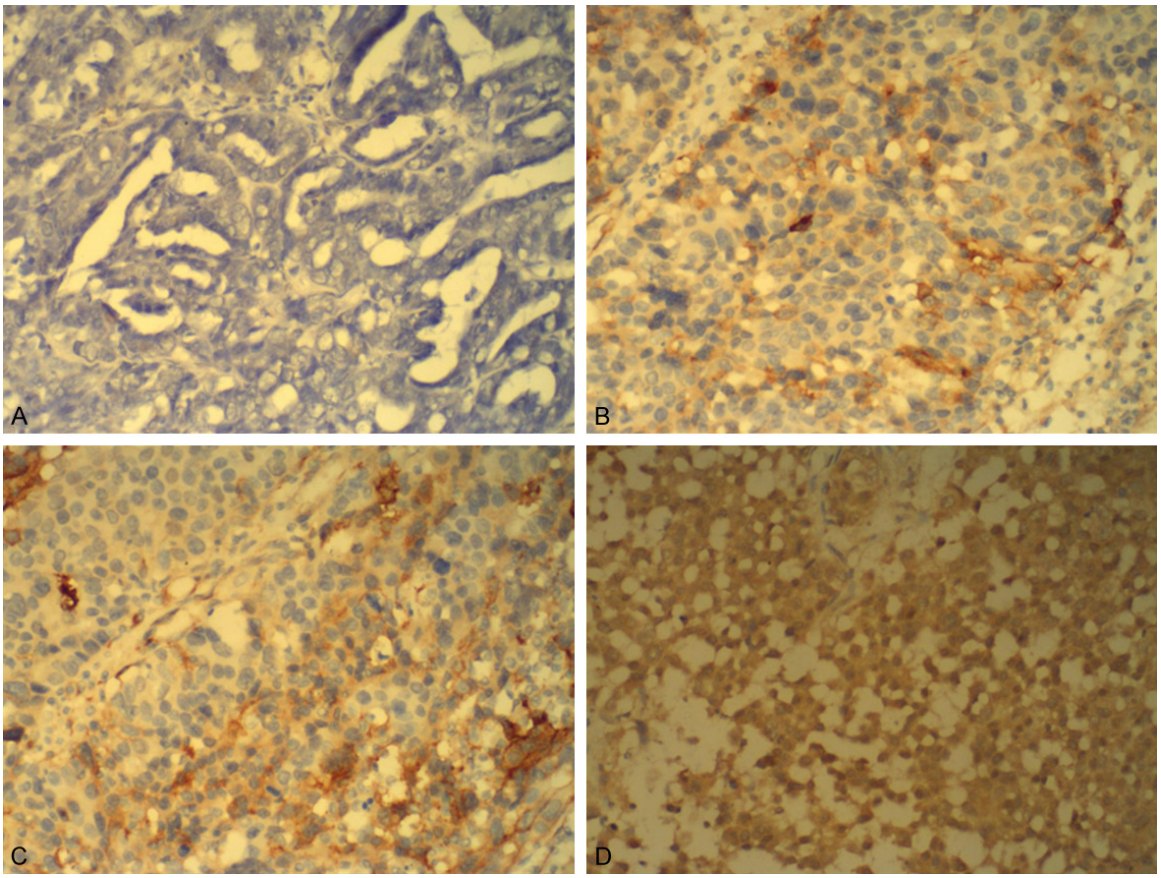

Figure 3. Immunochemical staining of HtrA2/Omi in paired adjacent non-tumourous tissues (A) and EOC tumor tissues (B: Weak expression, C: Moderate expression, D: Strong expression).

lines in the next experiment. Then we infected EOC cells with retrovirus carrying sh-HtrA2/Omi and evaluated the expression of HtrA2/Omi on EOC cell. We found that cell proliferation was markedly increased when HtrA2/Omi was knocked down in A2780 and OVCAR-3 cell lines. However, HtrA2/Omi gain-of function could inhibit the cell proliferation in A2780 and OVCAR-3 cell lines as compared to control group (**Figure 4C**). Next, we examined whether HtrA2/Omi regulates cell death in A2780 and OVCAR-3 cell lines through an apoptotic mechanism. Caspase-3 activity assay and triphosphate nick-end labeling (TUNEL) staining were measured after A2780 and OVCAR-3 cell with HtrA2/Omi gain-of function or loss-of function. The results indicated that A2780 and OVCAR-3 cell with HtrA2/Omi gain-of function showed significant cell apoptosis as compared to that of the control group, and the caspase-3 activity in A2780 and OVCAR-3 cell with HtrA2/Omi gain-of function was significantly higher than that of the control group (**Figure 4D** and **4E**). Intriguingly, HtrA2/Omi gain-of function increased and HtrA2/Omi loss-of function decreased the protein expression of casapse3 in A2780 and OVCAR-3 cell lines (**Figure 4F**).

HtrA2/Omi gain-of function inhibited the growth of A2780- and OVCAR-3-engrafted tumors

We next explored whether HtrA2/Omi gain-of function could regulate EOC cell growth in vivo, thus we performed tumor xenograft experiment. Significantly, HtrA2/Omi gain-of function could attenuate A2780- and OVCAR-3-engrafted tumors growth in vivo (**Figure 5A** and **5B**). Moreover, HtrA2/Omi gain-of function could increase the protein expression of caspase3 and caspase9 in engrafted tumors mice as compared to the control group (**Figure 5C**). In summary, these results suggested that HtrA2/Omi gain-of function could inhibit EOC cell growth in vitro and in vivo, and the underlying mechanism was mediated, at least partially,

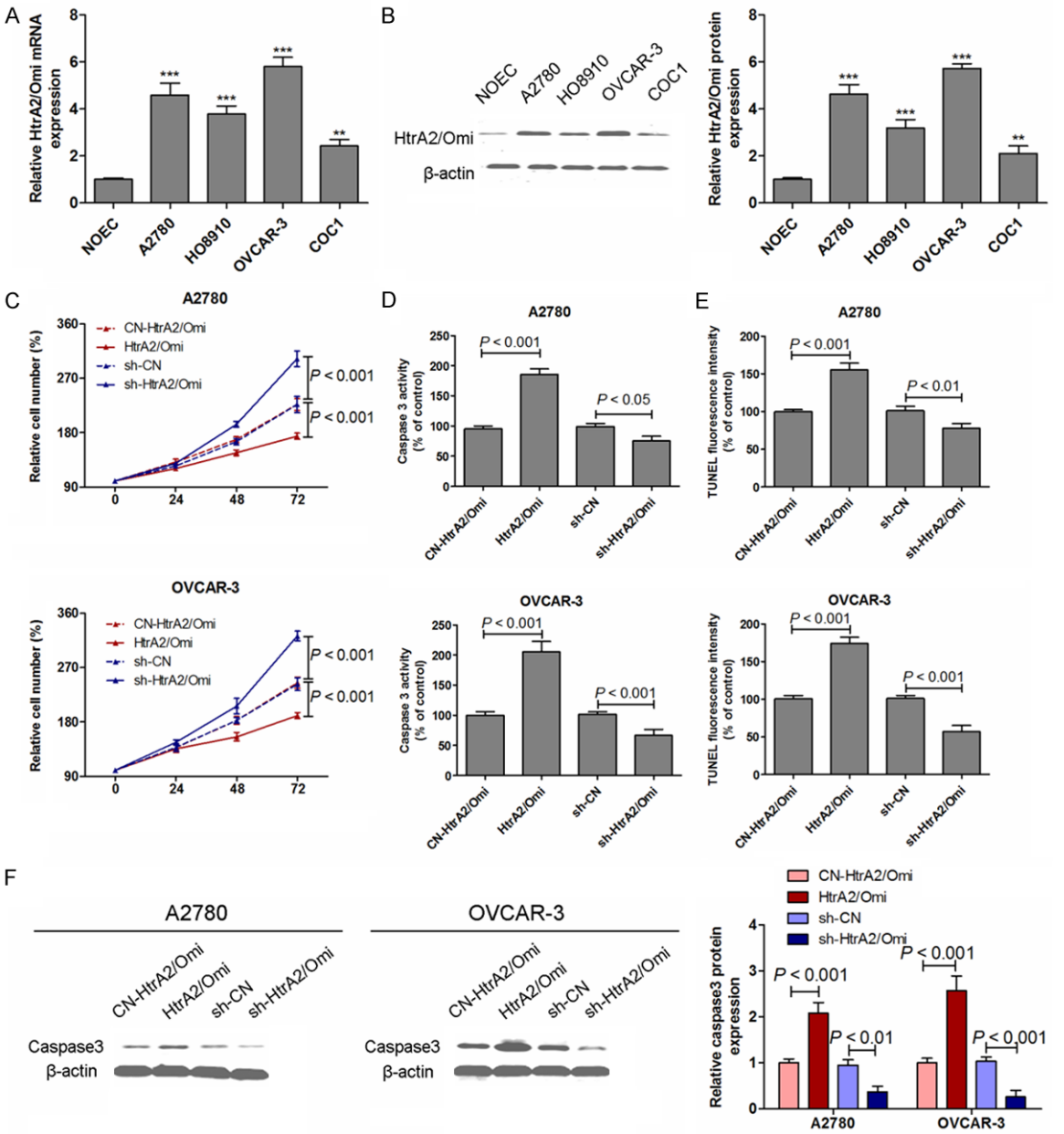

Figure 4. HtrA2/Omi mRNA (A) and protein (B) expression in EOC cell lines, NOEC, normal ovarian epithelial cells. Growth of A2780 and OVCAR-3 cell lines (C), caspase3 activity (D), TUNEL staining (E) in response to HtrA2/Omi gain-of function or loss-of function. Caspase3 protein expression in A2780 and OVCAR-3 cell lines was measured by western blotting in response to HtrA2/Omi gain-of function or loss-of function (F).

through the activation of caspase3 signaling pathway.

Discussion

HtrA2/Omi serine protease is a nuclear-encoded mitochondrial protein, which can promote cell death by two different mechanisms: (1) HtrA2/Omi binds to an inhibitor of apoptosis proteins (IAPs) via its amino terminal, reaper-related motif, and induces caspase activity; (2) HtrA2/Omi can mediate caspase-independent death through its own protease activity [7, 10-12]. In retinal pigment epithelial cells (RPECs), oxidative stress-induced apoptosis is mediated, at least partially, by HtrA2/Omi, which translocates from the mitochondria to the cytosol and subsequently induces apoptosis [7, 13]. In the present study, we demonstrated that the mRNA and protein expression of

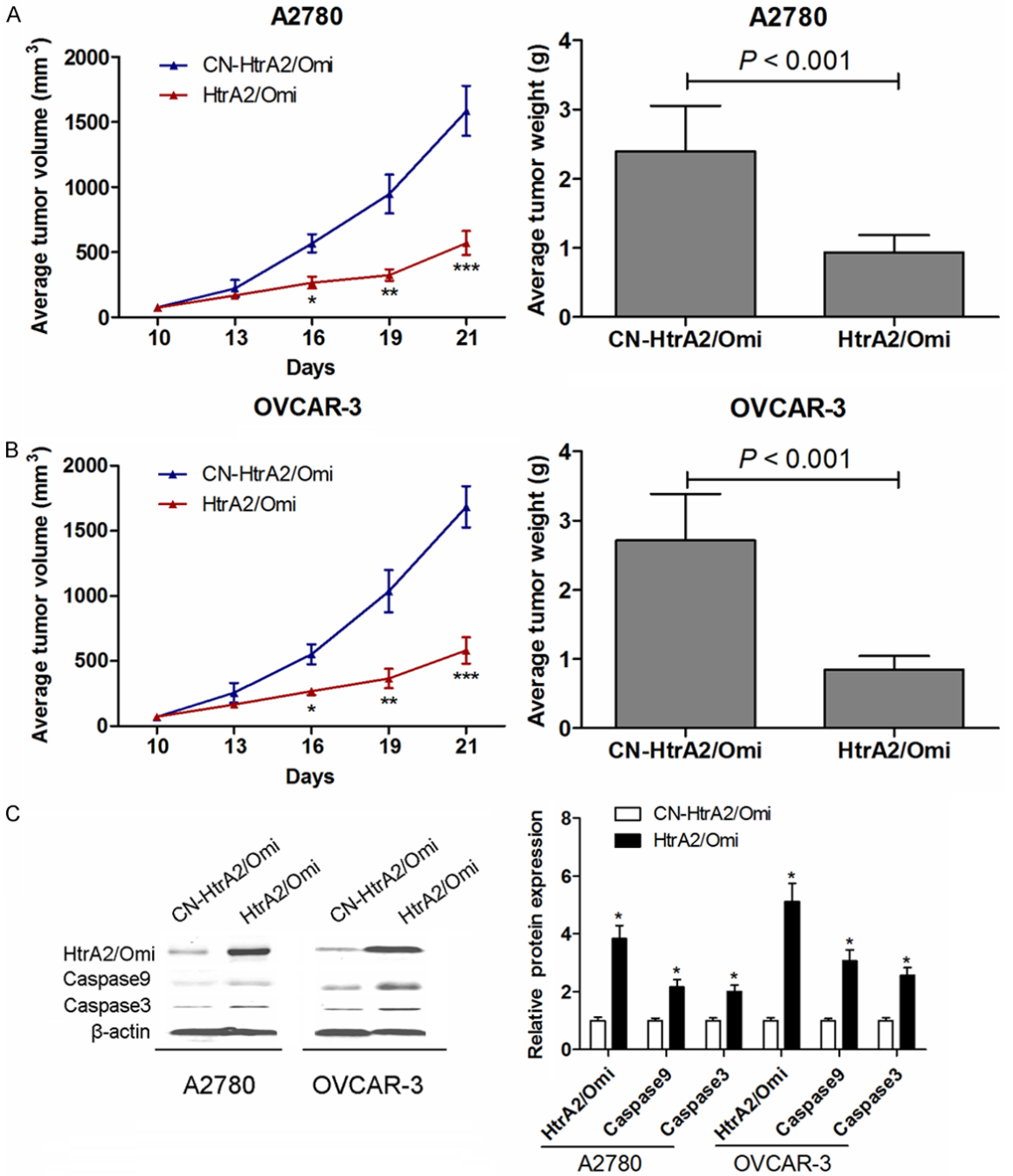

Figure 5. Average tumor volume and weight of A2780 (A) and OVCAR-3 (B) xenografted tumor mice model were evaluated during the experiment, n = 12 in each group. HtrA2/Omi, caspase3 and caspase9 protein expression were measured by western blotting in A2780 and OVCAR-3 xenografted solid tumor (C). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus control group.

Omi/HtrA2 in the EOC tissues was dramatically higher than that in the adjacent non-tumor tissues, and it was highly correlated with clinicopathological parameters. HtrA2/Omi gain-of function acuted and HtrA2/Omi loss-of function blunted apoptosis in A2780 and OVCAR-3 cell lines. In vivo, HtrA2/Omi gain-of function could attenuate A2780- and OVCAR-3-engrafted tumors growth and increase the protein expression of caspase3 and caspase9 in engrafted tumors mice. These results suggested that overexpressed HtrA2/Omi could inhibit EOC cell

growth in vitro and in vivo, and the underlying mechanism was mediated, at least partially, through the activation of caspase3 signaling pathway.

Previous studies report that HtrA2/Omi is widely expressed in cancer cell lines, and the intensity of its expression is cell-type specific [10, 11, 14]. In prostate cancer cells and gastric cancer cells, HtrA2/Omi expression is an essential part of apoptosis, and its expression may be involved in prostate cancer and gastric cancer progression [10, 11]. In ovarian cancers, HtrA2 expression is a predictor for sensitivity to chemotherapy and can be a candidate of molecular target in the treatment of high-grade serous ovarian cancers [15]. However, for all we know, no literature has been reported that the HtrA2/Omi expression is associated with EOC patients. In our study, HtrA2/Omi expression in EOC tissues was significantly increased compared with adjacent non-tumor tissues, and the HtrA2/Omi expression levels were markedly higher in III/IV stage than I/II stage in patients with EOC. Moreover, HtrA2/Omi levels were increased in distant metastasis patients as compared to that of patients without distant metastasis. However, HtrA2/Omi was no obvious difference with tumor size in EOC patients. Interestingly, our study is the first to report that the HtrA2/Omi is differentially expressed in EOC tissues and is associated with clinicopathological parameters in EOC patients.

Previous studies using ovarian cancer cells suggest that cytosolic HtrA2/Omi level was an important marker in cisplatin-induced apoptosis [16, 17]. Cisplatin decreases Xiap content and increases cytosolic HtrA2/Omi content and caspase-3 activity in cisplatin-sensitive ovarian cancer cell lines (A2780 and COC1). Downregulation of Xiap by antisense Xiap oligonucleotides increased caspase-3 activity and sensitized cisplatin-resistant cells to cisplatin treatment. Cytosolic HtrA2/Omi level increased while Xiap was downregulated in cisplatin-resistant ovarian cancer cells. These results indicate that cytosolic HtrA2/Omi level can regulate ovarian cancer cells death [16]. Three ovarian serous cancer cell lines tested clearly showed an inverse correlation of HtrA2/Omi expression and sensitivity to cisplatin, and HtrA2 loss-of function by siRNA is related with lower sensitivity to cisplatin in ovarian cancer cell lines [15, 18]. In our study, we infected EOC cells with retrovirus carrying sh-HtrA2/Omi and evaluated the expression of HtrA2/Omi on EOC cell. We found that cell proliferation was markedly increased when HtrA2/Omi was knocked down in A2780 and OVCAR-3 cell lines. On the contrary, HtrA2/Omi gain-of function could inhibit the cell proliferation in A2780 and OVCAR-3 cell lines as compared to control group. Moreover, HtrA2/Omi gain-of function increased and HtrA2/Omi loss-of function decreased the protein expression of casapse3 in A2780 and OVCAR-3 cell lines. In vivo, we found that HtrA2/Omi gain-of function could attenuate A2780- and OVCAR-3-engrafted tumors growth in vivo and could increase the protein expression of caspase3 and caspase9 in engrafted tumors mice as compared to the control group.

Collectively, the present study demonstrated that overexpressed HtrA2/Omi was found in EOC tissues and cell lines, which was attributed to suppress EOC cell growth in vitro and in vivo. This antineoplastic efficacy was mediated, at least partially, through the activation of caspase3 signaling pathway. However, the molecular changes of HtrA2/Omi-caspase3 signaling during the development of malignancies still need to be further investigated.

Disclosure of conflict of interest

None.

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