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HtrA1 Downregulation Induces Cisplatin Resistance in Lung Adenocarcinoma by Promoting Cancer Stem Cell-Like Properties

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ABSTRACT

Cisplatin (CDDP) resistance usually develops during lung adenocarcinoma (LAC) therapy. However, the comprehensive mechanisms remain largely unclear. In this study, we first established a CDDP-resistant LAC cell line-A549/CDDP from its parental cell line-A549. The results showed that CDDP resistance in A549/CDDP cells correlates with acquirement of cancer stem cell-like properties (increased percentage of CD133-expressing subpopulation, sphere formation and levels of some pluripotency-associated markers). HtrA1 expression at both mRNA and protein levels was reduced in CDDP-resistant A549/CDDP cells compared with that in A549 cells. Ectopic expression of HtrA1 in A549/CDDP cells reversed cancer stem cell-like properties and CDDP resistance. In A549 cells, stable knockdown of HtrA1 expression promoted cancer stem cell-like properties and CDDP insensitivity, however, these effects were blocked by inhibition of PI3K/Akt pathway using LY294002. Furthermore, HtrA1 knockdown could significantly stimulate PI3K/Akt signaling in A549 cells. In vivo studies, HtrA1 knockdown promoted tumorigenesis and conferred CDDP resistance in xenograft A549 tumors, which were reversed by intraperitoneal injection of LY294002. In conclusion, these results indicate that HtrA1 downregulation confers CDDP resistance by inducing cancer stem cell-like properties via PI3K/Akt-dependent pathway in A549 cells. Therefore, HtrA1 may be a potential target for overcoming CDDP resistance in LAC. J. Cell. Biochem. 115: 1112–1121, 2014.

KEY WORDS: CISPLATIN; HtrA1; PI3K/Akt; CANCER STEM CELL; LUNG ADENOCARCINOMA

Lung cancer is the most prevalent form of cancer worldwide and is one of the leading causes of cancer death worldwide [Spira and Ettinger, 2004]. Particularly, lung adenocarcinoma (LAC) is the most common histologic type of lung cancers. Cisplatin (CDDP) is one of the most effective and widely used anticancer drugs against various human cancers including lung cancer [Siddik, 2003]. However, CDDP resistance usually develops during LAC therapy and becomes a major obstacle for the effective treatment of LAC.

The cancer stem cell theory has been well accepted to explain the tumor heterogeneity and the carcinogenesis process [Reya et al., 2001; Pardal et al., 2003]. Specifically, it has been demonstrated that cancer stem cells, which are important in carcinogenesis, tumor progression and metastasis, are also responsible for chemoresistance [Spillane and Henderson, 2007]. For example, Fillmore and Kuperwasser [2008] reported that stem-like cells from breast cancer cell lines are less sensitive to paclitaxel and 5-fluorouracil in vitro. Yang et al. [2011] showed the resistance to doxorubicin of a side population of cells in hepatocellular carcinoma.

HtrA1, belonging to the HtrA family of serine proteases, is well conserved from bacteria to humans [Pallen and Wren, 1997]. The chief cellular function of HtrA1 is to recognize and degrade misfolded proteins in the periplasm, combining the activity of both chaperone and protease [Spiess et al., 1999]. Increasing studies have confirmed that HtrA1 is downregulated in various types of cancer including

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melanoma [Baldi et al., 2002], ovarian cancer [Chien et al., 2004], and endometrial cancer [Mullany et al., 2011]. HtrA1 downregulation is closely correlated with carcinogenesis and tumor progression in these cancer cells. Specially for lung cancer, HtrA1 is clearly downregulated in the lymph node metastases and could be involved in lung cancer progression [Esposito et al., 2006]. Interestingly, several reports show that HtrA1 also contributes to cancer cell response to chemotherapy, for example, HtrA1 mediates paclitaxel- and cisplatin-induced cytotoxicity and loss of HtrA1 in ovarian and gastric cancers may contribute to chemoresistance [Chien et al., 2006]. Nevertheless, the exact mechanisms underlying HtrA1 downregulation-mediated chemoresistance remains unclear, and no studies have addressed the potential role of HtrA1 in CDDP resistance development in LAC.

MATERIALS AND METHODS

MATERIALS

CDDP was obtained from Sigma (St. Louis, MO). LY294002 (PI3K inhibitor) was obtained from Merck. Cell culture reagents were from Invitrogen. The HtrA1, Akt, p-Akt, and horseradish peroxidase (HRP)-labeled anti-rabbit secondary antibodies were purchased from Cell Signaling Technology (Boston, MA). All other reagents were from Sigma unless stated otherwise.

CELL LINES AND CELL CULTURE

Human non-small-cell lung cancer A549 cell line was from American Tissue Culture Collection (ATCC, USA) and were cultured in RPMI 1640 containing 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin G, and 100 mg/ml streptomycin in a 37°C CO_2 incubator. The CDDP-resistant cell subline (A549/CDDP) was developed by continuous exposure to CDDP starting at 0.1 μg/ml and increasing in a stepwise manner to 5 μg/ml according to another study [Liu et al., 2007]. Then, the cells were maintained in the medium containing 5 μg/ml CDDP for 6 months and cultured without CDDP for 1 month before the analysis was performed.

MTT ASSAY

The protocols and reagents used of MTT assay (detection of cell proliferation/viability) were strictly according to another study [Chen et al., 2012].

FLOW CYTOMETRY

For analysis of the proportion of $CD133^+$ cells, we performed flow cytometry assay. Cells were detached with PBS without Ca^{2+}/Mg^{2+}, containing 1 mM EDTA at 37°C. The cells were incubated with phycoerythin (PE)-conjugated CD133 or with the corresponding isotype controls (both Miltenyi Biotech). Then the data were analyzed using a FACSCalibur cell analyzer (Becton Dickinson) and further processed using WinMDI 2.9 software.

SPHERE FORMATION ASSAY

Five thousand cells in 1 ml of serum-free Dulbecco's modified Eagle's medium (DMEM; Sigma) were plated in 24-well plates (Falcon; BD), supplemented with N2 plus media supplement (Invitrogen), 20 ng/ml of EGF, 20 ng/ml of bFGF (Invitrogen), and 4 mg/ml of heparin (Sigma). Cells were further cultured for 12 days, and the number of spheres was counted under microscope.

REAL-TIME PCR

Total RNA was extracted from cells using Paradise Sample Quality Assessment Kit (Molecular Devices). The isolated RNA was used for cDNA synthesis using reverse transcription reaction with Superscript II RNase H^- Reverse Transcriptase (Invitrogen) and random hexamers (Promega). Real-time PCR was performed using a Stratagene Mx3000 Real-Time PCR System and Brilliant SYBR Green QPCR Master Mix (Stratagene). The primers were following: HtrA1, 5′-tggaatctcctttg-caatcc-3′ and 5′-ttcttggtgatggcttttcc-3′; ABCG2, 5′-agctccgatg-gattgccag-3′ and 5′-gagggttcccgagcaagttt-3′; SOX2, 5′-atggag-aaaacccggtacgc-3′ and 5′-ttttgcgtgagtgtggatgg-3′; OCT4, 5′-tcccatg-cattcaaactgagg-3′ and 5′-cctttgtgttcccaattccttcc-3′; NANOG, 5′-ggacactggctgaatccttcc-3′ and 5′-tcccatgcattcaaactgagg-3′. As an internal control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was quantified using LightCycler Primer Set (Search LC GmbH). The real-time PCR cycling conditions were as follows: 95°C for 10 min, 45 cycles for 30 s at 95°C, 60 s at 55°C, and 60 s at 72°C followed by fluorescence measurement. The Second Derivative Maximum method was performed to determine the crossing point (CP) for each transcript using LightCycler software version 3.5 (Roche Diagnostic).

ShRNA TRANSFECTION

HtrA1 targeting shRNA and non-targeting shRNA (NT) were purchased from Sigma-Aldrich. The oligonucleotides were subcloned into the pSuper retroviral vector. Packing of the retrovirus using 293T cells and transduction of A549 cells with the virus followed a standard protocol [Liu et al., 2007]. Cells were harvested after 48 h and analyzed for the expression of HtrA1 by Western blotting. Controls were transfected with NT and grown under similar conditions.

CELL TRANSFECTION

A549/CDDP cells were plated at 1×10^5 cells per well on uncoated six-well plastic dishes. Full-length HtrA1 cDNA was cloned into a pcDNA 3.1 plasmid with cytomegalovirus promoter (Clontech). The recombinant plasmid (pcDNA3.1–HtrA1) or vector alone (pcDNA3.1) was transfected into A549/CDDP cells for 48 h using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions, followed by screening with G418 (Life Technologies). Overexpression of HtrA1 was confirmed by Western blotting.

WESTERN BLOTTING

Total cell lysates were separated by SDS/PAGE in 10% Tris–glycine gels (Invitrogen) and transferred to an NC membrane. The blotted membrane was then blocked with 5% nonfat dry milk in TBST for 1 h at room temperature. For Western blotting analysis of Akt, pAkt, and HtrA1, blots were probed with their specific antibodies (diluted with 5% BSA to 1:1,000), respectively. Then, blots were incubated at 4°C with these primary antibodies. After overnight incubation, membranes were probed with horseradish peroxidase (HRP)-labeled anti-mouse secondary antibody (Cell Signaling Technology). The bolted membrane was detected by enhanced chemiluminescence detection

kit (ECL) (Amersham) and captured on X-ray films. The densitometry was quantified using Bio-Rad Quantity One software.

IMMUNOSTAINING ASSAY

For determining the levels of HtrA1 in vivo, HtrA1 shRNA-transfected A549 cells and their parental A549 cells (both approximately 1×10^7 cells) were subcutaneously inoculated into the inguinal folds of nude mice. When xenograft tumors grew up to 80–100 mm^3, mice were sacrificed and tissues harvested from A549 and HtrA1 shRNA-transfected A549 tumors were subjected to immunostaining assay. Immunostaining analysis was carried out as previously described previously [Chen et al., 2013]. Briefly, paraffin sections were deparaffinized, rehydrated, and probed with anti-HtrA1 (Abcam), followed by incubation with secondary antibodies conjugated with HRP. The expression of HtrA1 was visualized by DAB staining. Slides were counterstained with hematoxylin, and then examined under a microscope.

XENOGRAFT STUDIES

For determining the tumorigenesis of A549 and HtrA1 shRNA-transfected A549 cells in vivo, the nude mice were randomly divided into nine groups (n = 5 per group). Indicated cells of three dosages (1×10^5, 1×10^4, 1×10^3) were subcutaneously inoculated with 25% Matrigel into the inguinal folds of nude mice. Mice were examined by palpation for tumor formation up to 60 days. After tumors were detected, mice were scarified and the presence of tumors was confirmed by necropsy.

For determining the role of HtrA1 downregulation in CDDP efficacy in LAC in vivo, HtrA1 shRNA-transfected A549 cells and their parental A549 cells (both approximately 1×10^7 cells) were subcutaneously inoculated into the inguinal folds of nude mice. Treatments were initiated when tumors grew up to 80–100 mm^3. The mice were treated with CDDP (5 mg/kg, two times/week, i.p.) or CDDP plus LY294002 group (CDDP, 5 mg/kg, two times/week, i.p.; LY294002, 100 mg/kg, two times/week, i.p.). Mice were randomized to receive above treatments. Tumor volumes were measured every week and calculated with the formula: $(mm^3) = (L \times W^2) \times 0.5$. The tumor growth-inhibitory effects were examined for about 4 weeks. For evaluating the effect of CDDP or CDDP plus LY294002 on the survival rate of tumor-beared mice, we further compared the survival rate among different treatment groups in a cycle approximately including 70 days.

STATISTICAL ANALYSIS

Data were statistically analyzed with unpaired Student's *t*-test using Sigma Plot software (Jandel Scientific) and presented as means ± SD. Differences between values were considered as a significance level $^*P$-value of <0.05 and $^{**}P$-value of <0.01.

RESULTS

CDDP RESISTANCE IN A549/CDDP CELLS CORRELATES WITH CANCER STEM-LIKE PROPERTY IN LAC CELLS

We first established a CDDP-resistant LAC cell line-A549/CDDP by continuous exposure to CDDP starting at 0.1 μg/ml and increasing in a stepwise manner to 5 μg/ml. Figure 1A shows that parental A549 cells were sensitive to CDDP (1–10 μM), while established A549/CDDP cells were resistant to clinically relevant doses of CDDP (1–5 μM). Flow cytometry analysis showed that the level of cancer stem-like cell marker, CD133, was significantly higher in A549/CDDP cells than that in their parental A549 cells (Fig. 1B). Sphere formation is another important measurement used to define cancer stem-like cells. As shown in Figure 1C, A549/CDDP cells formed approximately two- to threefold more spheres compared with the spheres formed by A549 cells. The expression levels of pluripotency-associated markers, including ABCG2, SOX2, OCT4, and NANOG, were also substantially elevated in A549/CDDP cells as compared with those in A549 cells (Fig. 1D). These results indicate that CDDP resistance development correlates cancer stem-like phenotype in LAC cells.

HtrA1 PROMOTES CANCER STEM-LIKE PHENOTYPE AND CDDP INSENSITIVITY IN LAC CELLS

To explore the effect of HtrA1 on CDDP resistance in LAC cells, we first compared the expression levels of HtrA1 in A549 cells with those in A549/CDDP cells at both mRNA and protein levels. The results showed that both the mRNA (Fig. 2A) and protein levels (Fig. 2B) of HtrA1 in A549/CDDP cells were significantly lower than those in their parental cells. These results suggest a potential role of HtrA1 in CDDP resistance development.

To confirm the role of HtrA1 in CDDP resistance in LAC cells, we used the specific stable HtrA1 shRNA to inhibit HtrA1 expression in A549 cells. As shown in Figure 2C, the HtrA1 shRNA (50 nM) substantially reduced HtrA1 expression in A549 cells approximately by ~87% compared with scramble control. Knockdown of HtrA1 expression in A549 cells resulted in decreased CDDP efficacy (Fig. 2D), accompanied by increased proportion of $CD133^+$ cells (Fig. 2E), sphere formation (Fig. 2F), and expression levels of ABCG2, SOX2, OCT4, and NANOG (Fig. 2G). Next, we generated stable cell lines (A549/CDDP-HtrA1) expressing HtrA1 from A549/CDDP cells using lentiviral infection system with plasmid vectors encoding HtrA1 (Fig. 3A). An empty vector-transfected control (A549/CDDP-vector) was produced simultaneously. Figure 3B shows that ectopic expression of HtrA1 in A549/CDDP cells overcome CDDP resistance. As shown in Figure 3C–E, HtrA1 overexpression significantly reduced the proportion of $CD133^+$ cells, sphere formation and expression levels of ABCG2, SOX2, OCT4, and NANOG in A549/CDDP cells, respectively. Collectively, these data indicate that HtrA1 down-regulation induces CDDP resistance in LAC cells probably through promotion of cancer stem-like phenotype in these cells.

PI3K/Akt SIGNALING IS REQUIRED FOR THE CANCER STEM CELL-PROMOTING EFFECT OF HtrA1 DOWNREGULATION

To explore the mechanism underlying HtrA1 downregulation-induced cancer stem-like phenotype and CDDP resistance in A549 cells, the PI3K/Akt pathway inhibitor LY294002 was used. As mentioned above, HtrA1 downregulation decreased CDDP efficacy, accompanied by increased proportion of $CD133^+$ cells, sphere formation and expression levels of ABCG2, SOX2, OCT4, and NANOG in A549 cells. However, these effects were reversed or blocked by additional LY294002 (30 μM) treatment after 48-h incubation (Fig. 4A–D, respectively). Furthermore, Figure 4E shows

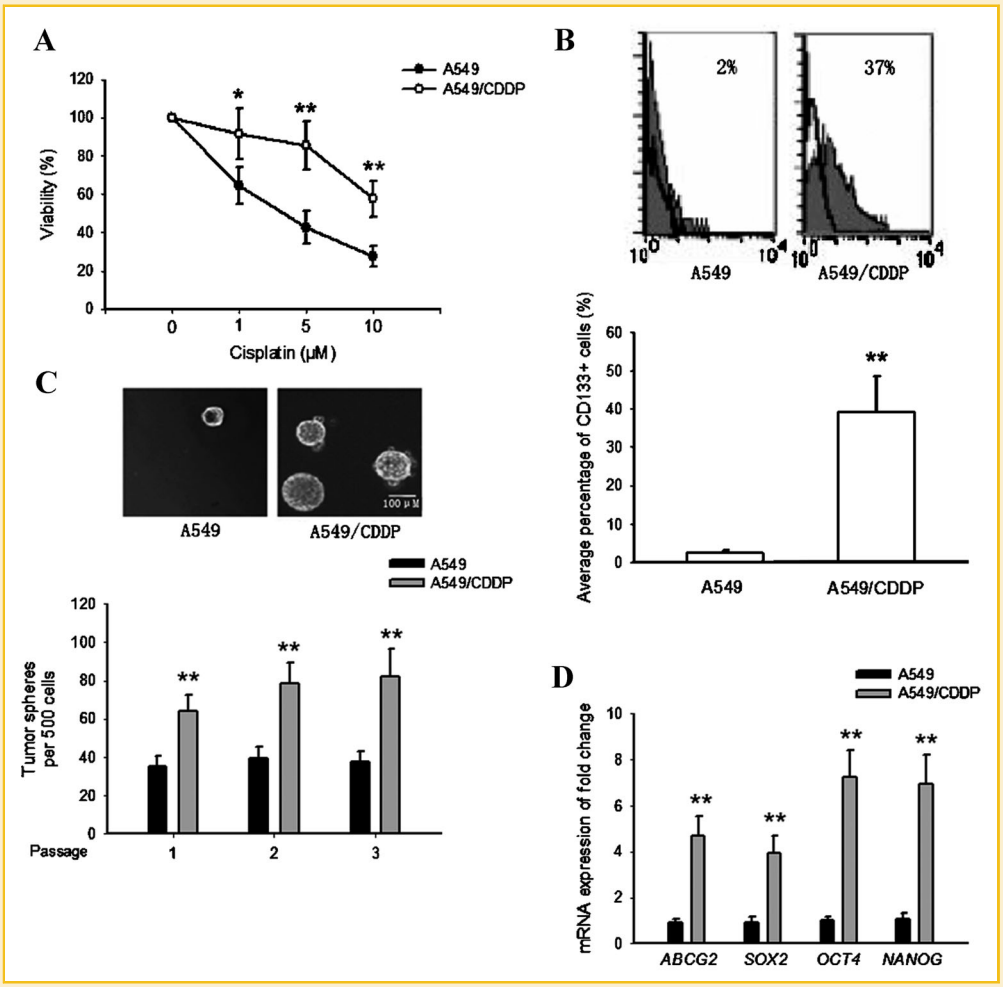

Fig. 1. CDDP resistance correlates cellular cancer stem cell-like phenotypes in A549/CDDP cells. A: A549 and A549/CDDP cells were seeded into 96-well plates and treated with CDDP for 48 h. Cell survival was determined by MTT assay. Bars are mean ± SD from four independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus DMSO control. B: A549 clones or A549/CDDP were subjected to flow cytometry to analyze the population of $CD133^{+}$ cells. Bars are mean ± SD from five independent experiments. $^{**}P < 0.01$ versus A549 group. C: Representative images of spheres formed by the indicated cells (top panel). Histograms showing the mean number of spheres formed by the indicated cells (low panel). Bars are mean ± SD from four independent experiments. $^{**}P < 0.01$ versus A549 group. D: Real-time PCR analysis of the expression of pluripotency-associated markers in the indicated cells. Bars are mean ± SD from three independent experiments. $^{**}P < 0.01$ versus A549 group.

that HtrA1 knockdown could significantly stimulate PI3K/Akt signaling as expressed by enhanced levels of p-Akt in A549 cells. Combined with that Akt activity in A549/CDDP cells was much higher than that in A549 cells (Fig. 4F), the above data indicate that HtrA1 downregulation induces cancer stem-like phenotype and CDDP resistance via PI3K/Akt-dependent pathway in A549 cells.

HtrA1 KNOCKDOWN PROMOTES LAC TUMORIGENESIS AND REDUCES CDDP SENSITIVITY IN A549 XENOGRAFT TUMOR-HARBORED NUDE MICE

It was critical to determine whether HtrA1 downregulation could promote tumorigenesis and reduce CDDP sensitivity in vivo. We subcutaneously inoculated different numbers of cells mixed with Matrigel into the inguinal folds of nude mice. As shown in Table I, A549-HtrA1 shRNA cells presented higher rates of tumorigenesis than A549-vector cells. Only A549-HtrA1 shRNA cells formed tumors when 1×10^3 cells were implanted. Intraperitoneal injection of LY294002 (100 mg/kg) into mice harboring A549-HtrA1 shRNA tumor reversed the effect of HtrA1 downregulation on tumorigenesis in vivo. The inhibitory effect LY294002 (100 mg/kg) on Akt activity in vivo was confirmed by ELISA (data not shown).

To determine the effect of HtrA1 expression on CDDP sensitivity in vivo, HtrA1 shRNA-transfected A549 cells and their parental A549 cells (both approximately 1×10^7 cells) were subcutaneously inoculated into the inguinal folds of nude mice. When xenograft tumors grew up to 80–100 mm^3, mice were sacrificed and tissues harvested from A549 and HtrA1 shRNA-transfected A549 tumors were subjected to immunostaining assay to determine the real expression level of HtrA1 in vivo before drug administration. As shown in Figure 5A, A549 tumor tissues stained positively for HtrA1, while A549-HtrA1 shRNA tumor tissues showed few signals for HtrA1. Figure 5B shows that throughout the animal experiment, A549-HtrA1

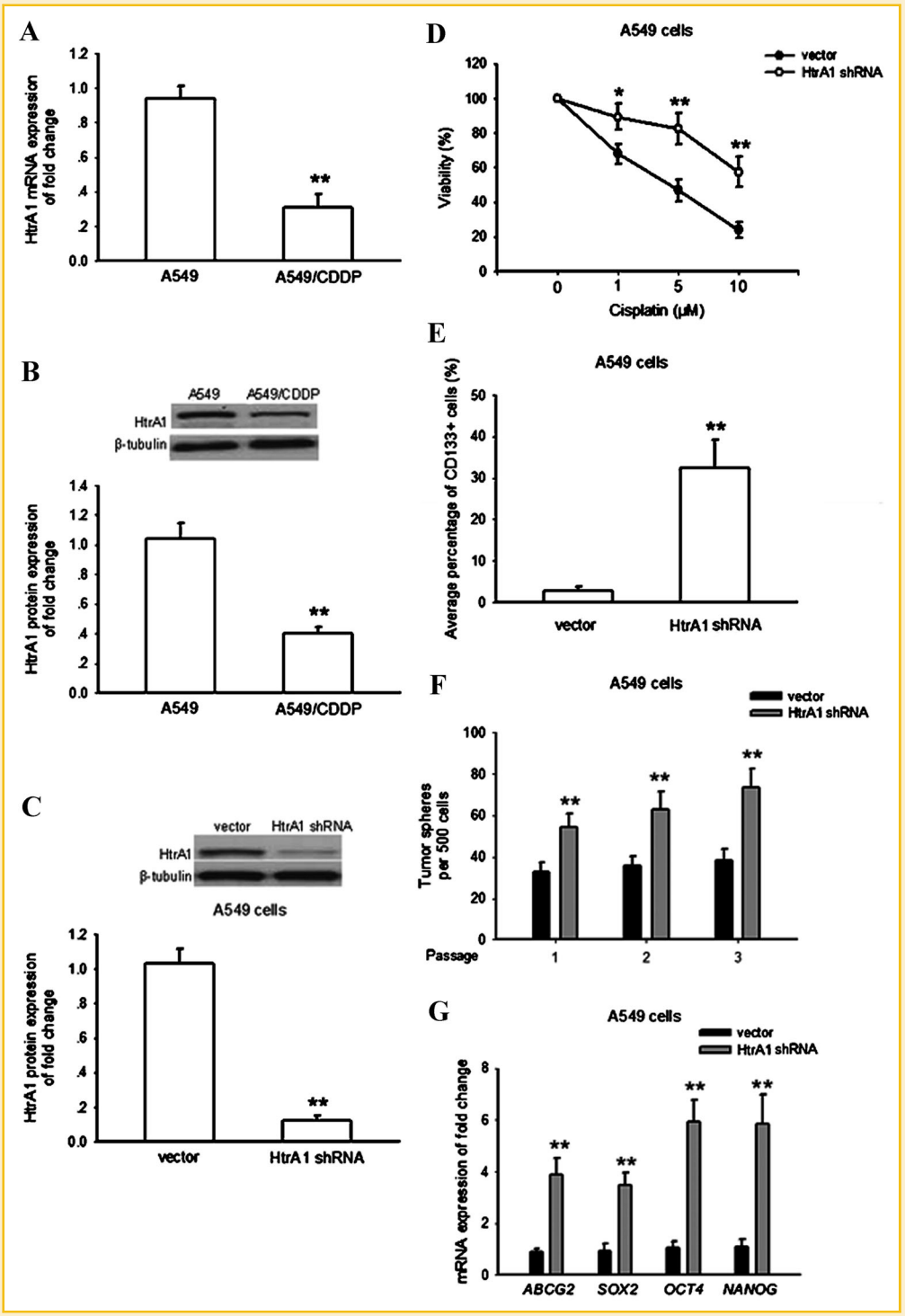

Fig. 2. HtrA1 knockdown promotes cancer stem cell-like properties and reduces CDDP sensitivity in A549 cells. A: The differential expression pattern of HtrA1 in A549/CDDP cells and their parental A549 cells at both mRNA levels (A) and protein levels (B) was determined by real-time RT-PCR and Western blotting, respectively. A: Bars are mean ± SD from four independent experiments. $^{**}P < 0.01$ versus A549 group. B: Bar graphs are derived from densitometric scanning of the blots. Bars are mean ± SD from three independent experiments. $^{**}P < 0.01$ versus A549 group. C: Effect of HtrA1 shRNA on HtrA1 expression in A549 cells determined by Western blotting after 48-h transfection. Bar graphs are derived from densitometric scanning of the blots. Bars are mean ± SD from three independent experiments. $^{**}P < 0.01$ versus vector group. D: Effect of HtrA1 knockdown on CDDP sensitivity after 48-h treatment of CDDP in A549 cells. E–G: Effect of HtrA1 knockdown on the population of $CD133^{+}$ cells, sphere formation and the expression of pluripotency-associated markers in A549 cells, respectively. All experiments were repeated thrice. Bars are mean ± SD. $^{**}P < 0.01$ versus vector group.

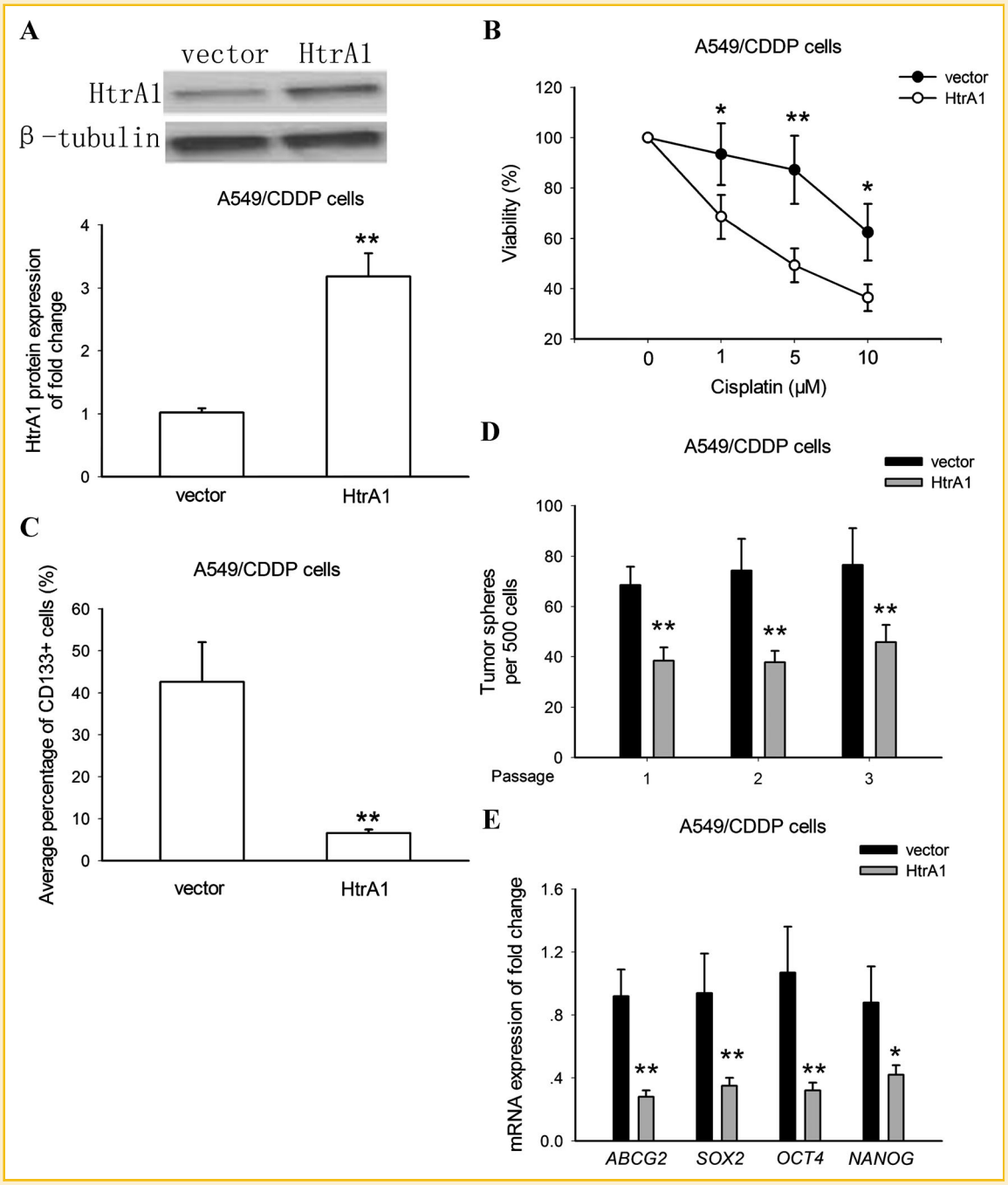

Fig. 3. Ectopic expression of HtrA1 reverses cancer stem cell-like properties and CDDP resistance in A549/CDDP cells. A: Western blotting data indicated the success of HtrA1 transfection. Bar graphs are derived from densitometric scanning of the blots. Bars are mean ± SD from three independent experiments. $^{**}P < 0.01$ versus vector group. B: Effect of HtrA1 overexpression on CDDP sensitivity after 48-h treatment of CDDP in A549/CDDP cells. C–E: Effect of HtrA1 overexpression on the population of $CD133^+$ cells, sphere formation, and the expression of pluripotency-associated markers in A549/CDDP cells, respectively. All experiments were repeated thrice. Bars are mean ± SD. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus vector group.

shRNA tumors were resistant to CDDP treatment. However, the reduced effect of HtrA1 knockdown on CDDP efficacy was reversed by LY294002 (100 mg/kg) treatment. We further compared the survival rate among different treatment group in a cycle approximately including 70 days (Fig. 5C). Our results showed that A549 tumors were sensitive to CDDP as the survival rate of A549-vector tumor-beared nude mice treated with CDDP was extended from 45 to 68 days compared with control. However, the survival rate of HtrA1 shRNA-transfected A549 tumor-beared nude mice treated with CDDP alone was not different from, but a little lower than that of control

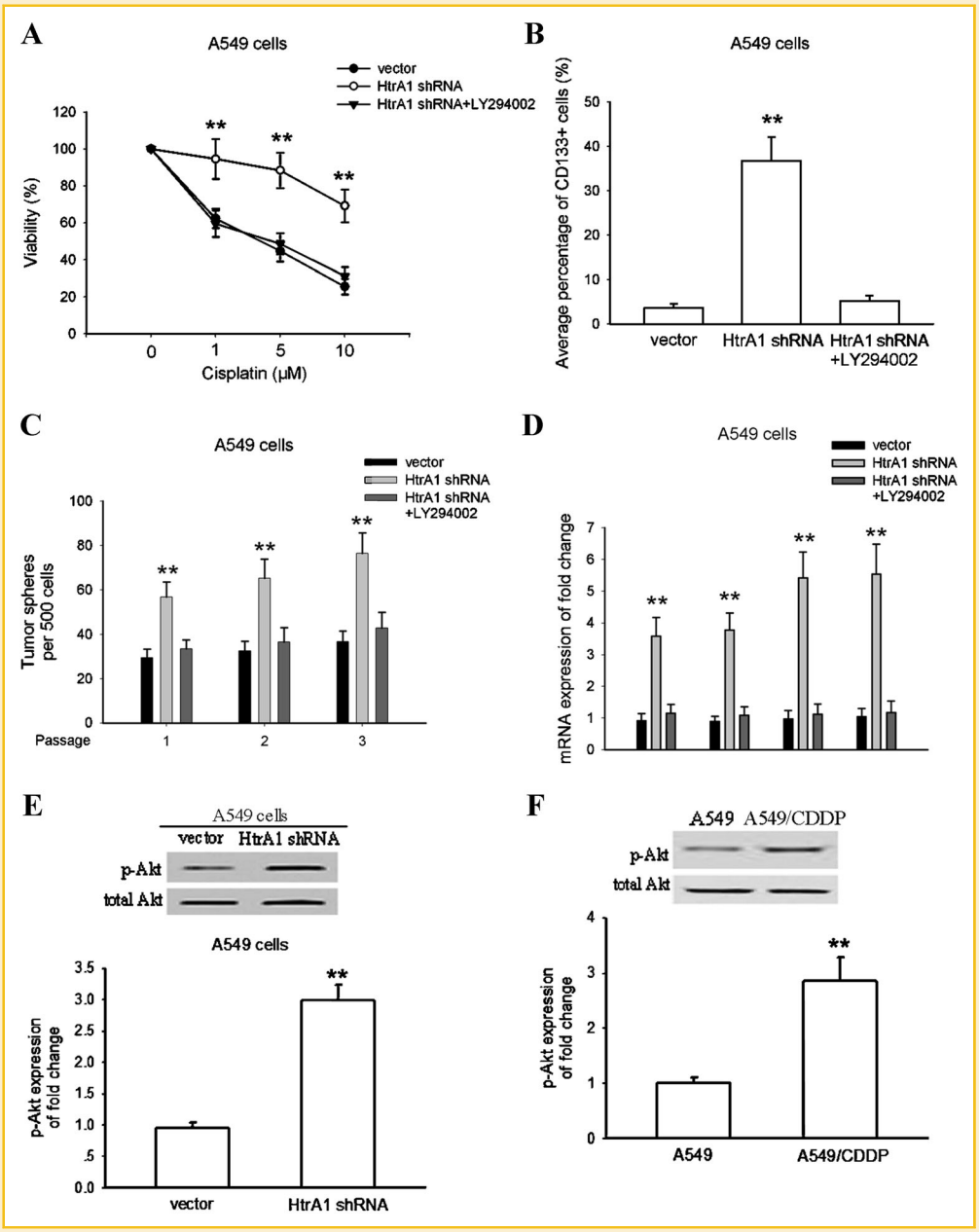

Fig. 4. HtrA1 downregulation promotes cancer stem cell-like properties and CDDP resistance in A549 cells via PI3K/Akt-dependent pathway. A: Effect of inhibition of PI3K/Akt pathway by LY294002 on HtrA1 shRNA-induced CDDP resistance in A549 cells. Bars are mean ± SD from three independent experiments. $^{**}P < 0.01$ versus vector group. B–D: Effects of LY294002 on HtrA1 shRNA-mediated increased population of $CD133^+$ cells, sphere formation, and the expression of pluripotency-associated markers in A549/CDDP cells, respectively. All experiments were repeated thrice. Bars are mean ± SD. $^{**}P < 0.01$ versus vector group. E: Effect of HtrA1 shRNA on Akt activity determined by Western blotting. Bar graphs are derived from densitometric scanning of the blots. Bars are mean ± SD from three independent experiments. $^{**}P < 0.01$ versus vector group. F: The differential activity pattern of Akt in A549/CDDP cells and their parental A549 cells determined by Western blotting. Bar graphs are derived from densitometric scanning of the blots. Bars are mean ± SD from three independent experiments. $^{**}P < 0.01$ versus A549 group.

group. Intraperitoneal injection of LY294002 (100 mg/kg, two times/week) significantly enhanced the survival rate compared with CDDP treatment alone in HtrA1 shRNA-transfected A549 tumor-beared nude mice. Taken together, these results demonstrated that HtrA1 downregulation reduces CDDP efficacy in vivo, and inhibition of PI3K/Akt signaling blocks such effect.

DISCUSSION

Intense researches concerning mechanisms that account for the cisplatin-resistant have been conducted during the past 30 years. These mechanisms are classified to four categories, which are (1) steps preceding the binding of cisplatin to DNA (pre-target resistance),

TABLE I. Effect of HtrA1 Silencing on A549 Tumor Formation In Vivo

	Number of cells inoculated		
Inoculated cells	1×10^3	1×10^4	1×10^5
A549-vector	0 of 5	0 of 5	2 of 5
A549-HtrAl shRNA	4 of 5	5 of 5	5 of 5
A549-HtrAl shRNA[a]	0 of 5	1 of 5	2 of 5

[a]Additional LY294002 treatment (100 mg/kg, two times/week) by intraperitoneal injection.

(2) forming DNA-cisplatin adducts (on-target resistance), (3) concerning the lethal signaling pathways elicited by cisplatin-mediated DNA damage (post-target resistance), and (4) molecular circuitries interference that does not present obvious links with cisplatin-elicited signals (off-target resistance) [Galluzzi et al., 2012]. In recent years, increasing evidences have shown that cancer stem cells contribute to cancer chemoresistance in several tumor types. For example, Shafee et al. [2008] reported that cancer stem cells contribute to cisplatin resistance in Brca1/p53-mediated mouse mammary tumors. On the other hand, consistent with other reports [Levina et al., 2008; Xu et al., 2012], our data also suggest that chemotherapy drugs can selectively kill non-stem-like cells, resulting in enrichment of stem-like cells. Furthermore, targeting cancer stem cells was shown effective to enhance chemosensitivity in cancer treatment [Yu et al., 2009; Hossain et al., 2012]. These results indicate the close interconnection between chemoresistance and cancer stem cell-like properties in cancer cells. However, what are the molecular events or factors that induce cancer stem cell-like properties and resultant chemoresistance in cancer cells remain largely unknown.

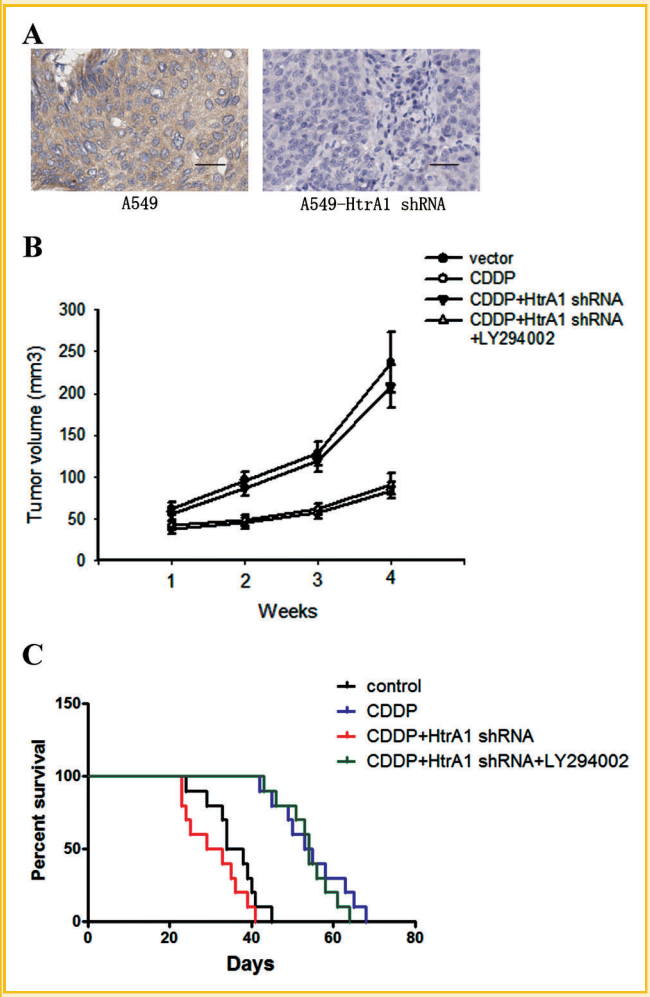

Fig. 5. Effects of HtrA1 shRNA and combination treatment with LY294002 on antitumor activity of CDDP in nude mice bearing A549 or HtrA1 shRNA-transfected A549 tumor xenografts. A: Immunostaining of HtrA1 expression in A549 and A549-HtrA1 shRNA xenograft tumors. B: Treatments were initiated when tumors reached 80–100 mm^3. Tumor size was measured every 1 week for the indicated period. Ten mice per group, mean ± SD. C: Kaplan–Meier curves for illustration of the survival periods of tumor xenograft-bearing mice. Control mice received the vehicle. Ten mice per group.

Specifically, increasing evidences have shown a potential role of HtrA1 downregulation in cancer cells with chemoresistance. For example, the report by Catalano et al. [2011] showed that human gastric tumors with high and medium HtrA1 expression show a better response to a CDDP-based combination chemotherapy than tumors with a low HtrA1 value. Besides, the study also indicated a close correlation of reduced HtrA1 expression with gastric cancer progression. Consistent with such study, our work also demonstrates that HtrA1 downregulation induces malignant phenotype and CDDP resistance in A549 cells. Furthermore, we found that HtrA1 downregulation induces CDDP resistance probably through promoting cancer stem cell-like properties, thus revealing a novel role of HtrA1 in tumor biology. However, as HtrA1 downregulation exists in multiple lines of cancer cells, whether such effect appears non-specific to various cancer cells such gastric cancer cells needs further explored.

Growing evidences have shown the close correlation of HtrA1 and cancer malignant progression. Baldi et al. [2002] found that HtrA1 is downregulated during human melanoma progression and represses melanoma metastasis. Loss of HtrA1 was also shown to contribute to the aggressiveness and metastatic ability of endometrial tumors [Mullany et al., 2011]. However, the mechanisms responsible for HtrA1 downregulation-mediated cancer progression remain elusive. A previous study showed that downregulation of HtrA1 activates the epithelial–mesenchymal transition (EMT) in breast cancer cells [Wang et al., 2012]. EMT is well thought to promote malignant tumor progression [Thiery, 2002]. In this study, we found that stable HtrA1 downregulation promotes cancer stem cell-like properties in LAC cells. As the ability of a tumor to metastasize is considered as an inherent property of a subset of cancer stem cells [Li et al., 2007], and there exists a link between EMT and cancer stem cells during tumor malignant progression [Biddle and Mackenzie, 2012], our data combined with Wang et al.'s [2012] suggest that HtrA1 downregulation contributes to cancer metastasis probably through induction of EMT and cancer stem cell-like phenotypes.

PI3K/Akt signaling is correlated with cancer chemoresistance. For example, activation of PI3K/Akt pathway mediates ADAM17-induced drug resistance in hepatocellular carcinoma cells [Wang et al., 2013]. Activation of PI3K/Akt pathway also contributes to CDDP resistance in ovarian cancer cells [Lee et al., 2005] and lung cancer cells [Liu et al., 2007]. PI3K/Akt pathway has also been

confirmed to regulate cancer stem cell-like phenotypes in various types of cancer [Bleau et al., 2009; Dubrovska et al., 2009]. In our study, after inhibition of PI3K/Akt by LY294002, knockdown of HtrA1 expression-induced cancer stem cell-like properties and CDDP resistance were reversed in A549/CDDP cells. These results indicate that HtrA1 downregulation induces cancer stem cell-like properties and CDDP resistance via PI3K/Akt pathway. Our data also suggest a critical role of PI3K/Akt-mediated cancer stem cell-like properties in CDDP resistance development in LAC.

Our data show that HtrA1 knockdown can significantly stimulate PI3K/Akt signaling in LAC cells. The possible mechanism for HtrA1 downregulation-mediated regulation of PI3K/Akt signaling may be that as in ovarian cancer cells [He et al., 2010], HtrA1 inhibits EGFR activity via direct interaction with EGFR, and in turn suppresses PI3K/Akt signaling. Constitutive activation of PI3K/Akt signaling has been found in multiple types of cancer [Vivanco and Sawyers, 2002]. Many studies have confirmed that mutations in PTEN [Osaki et al., 2004] as well as overexpression or constitutive activation of RTKs [Blume-Jensen and Hunter, 2001] account partly for such constitutive activation. In this study, our results that HtrA1 knockdown stimulates PI3K/Akt signaling suggest that HtrA1 downregulation during cancer development and progression may contribute to constructive PI3K/Akt activation in human cancers.

It remains to be investigated whether the effect of HtrA1 downregulation on cancer stem cell-like properties is unspecific to certain cancer cell lines. Notwithstanding this limitation, our study does indicate that HtrA1 downregulation confers CDDP resistance in LAC cells probably via PI3K/Akt pathway-dependent promotion of cancer stem cell-like properties. In conclusion, HtrA1 may be a potential target for overcoming CDDP resistance in LAC therapy.

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