iASPP and Chemoresistance in Ovarian Cancers – Effects on Paclitaxel-Mediated Mitotic Catastrophe

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Translational relevance

Drug resistance and disease recurrence are important concerns for the treatment of ovarian cancer. The mechanisms underlying chemosensitivity of ovarian cancer cells remain elusive. Relapsed ovarian cancer is incurable, and there is no universal accepted therapeutic regime for patients. In this study, we demonstrate that iASPP is overexpressed in ovarian cancers, highlighting its relationship with chemoresistance and prognosis. The data presented in this study supports iASPP as a potent predictor for paclitaxel resistance. Importantly, the association of iASPP with mitotic catastrophe provides a more rational therapeutic target, which could allow the future development of cancer intervention, particularly for recurrent patients.
Abstract

Purpose: iASPP is a specific regulator of p53-mediated apoptosis. Herein, we provided the first report on the expression profile of iASPP in ovarian epithelial tumor and its effect on paclitaxel chemosensitivity.

Experimental design: Expression and amplification status of iASPP was examined in 203 clinical samples and 17 cell lines using immunohistochemistry, quantitative real-time PCR and immunoblotting, and correlated with clinicopathological parameters. Changes in proliferation, mitotic catastrophe, apoptosis and underlying mechanism in ovarian cancer cells of different p53 status following paclitaxel exposure were also analyzed.

Results: The protein and mRNA expression of iASPP was found to be significantly increased in ovarian cancer samples and cell lines. High iASPP expression was significantly associated with clear cell carcinoma subtype (P=0.003), carboplatin and paclitaxel chemoresistance (P=0.04), shorter overall (P=0.003) and disease-free (P=0.001) survival. Multivariate analysis confirmed iASPP expression as an independent prognostic factor. Increased iASPP mRNA expression was significantly correlated with gene amplification (P=0.023). iASPP overexpression in ovarian cancer cells conferred resistance to paclitaxel by reducing mitotic catastrophe in a p53-independent manner via activation of separase, whereas knockdown of iASPP enhanced paclitaxel-mediated mitotic catastrophe through inactivating separase. Both securin and cyclin B1/CDK1 complex were involved in regulating separase by iASPP. Conversely, overexpressed iASPP inhibited apoptosis in a p53-dependent mode.
Conclusions: Our data demonstrate an association of iASPP overexpression with gene amplification in ovarian cancer and suggest a role of iASPP in poor patient outcome and chemoresistance, through blocking mitotic catastrophe. iASPP should be explored further as a potential prognostic marker and target for chemotherapy.

Introduction

Ovarian cancer is the most lethal gynecologic cancer worldwide because it is frequently diagnosed at advanced stage and demonstrates high rates of chemoresistance especially in patients with recurrent diseases (1-3). For decades, a combination of paclitaxel and carboplatin has been used as the first-line chemotherapy for ovarian cancer. Although apoptosis is usually regarded as the major mechanism of cell death in response to paclitaxel (4-7), another form of cell death known as mitotic catastrophe has been observed in a wide range of human cancers treated with paclitaxel(5-9). Mitotic catastrophe is a distinctive form of cell death related to abnormal mitosis, but a generally accepted definition is lacking. The morphology of cells undergoing mitotic catastrophe is initially characterized by asymmetric chromosomal condensation and missegregation, followed by formation of nuclear envelopes and multi-nucleated giant cells in later stage (10-14). On the other hand, cytoplasmic condensation and nuclear pyknosis are hallmarks of apoptosis (12). Previous reports have provided several hypotheses on paclitaxel resistance, but most of these studies focused on apoptosis caused by paclitaxel (15, 16). The relationship between paclitaxel resistance and mitotic catastrophe in cancers is still not fully
Members of the ASPP (the ankyrin repeat, SH3 domain and proline-rich region containing protein) family have been recently identified as specific regulators of p53-mediated apoptosis (17-19). Among them, inhibitory member of the ASPP family (iASPP, encoded by PPP1R13L in human) is considered to be an oncoprotein and is overexpressed in breast cancer (20) and acute leukemias (21). Moreover, increased iASPP confers resistance to apoptosis induced by UV radiation or exposure to cisplatin in human osteosarcoma cells and breast cancer cells (20). The mechanism by which iASPP is overexpressed is unclear but is unlikely to be directly due to p53 inhibition (22).

In the present study, we provided the first experimental evidence that iASPP is upregulated in ovarian cancers in relation to gene amplification and clinicopathological parameters. This study also highlighted the role of iASPP in chemoresistance to paclitaxel via its effect on mitotic catastrophe.

**Material and Methods**

**Clinical samples and cell lines.** One hundred and forty cases of archival formalin-fixed paraffin-embedded tissues, including 10 ovarian epithelial benign tumors (ages, 20~77 years; mean age, 32 years), 19 ovarian epithelial borderline tumors (ages, 20~46 years; mean age, 30 years), 111 epithelial ovarian cancers (ages, 23~78 years; average age, 50 years) and 63 corresponding metastatic foci of ovarian cancers (supplementary Table 1), were selected for immunohistochemistry from
Queen Mary Hospital, the University of Hong Kong. Among the patients with cancer, 96 have been treated with chemotherapy. Patients were considered chemoresistant if they did not achieve complete response or recurred within 6 months from the last chemotherapy regimen. Patients were considered chemosensitive if they remain in remission or recurred more than 6 months after the last therapy (23). Paired snap frozen samples of ovarian cancers and their corresponding normal tissues from another 36 patients with ovarian cancers were also collected for total RNA and genomic DNA extractions. These frozen blocks have been histologically reviewed and microdissected to ensure that the carcinoma constitutes to at least 80% of the samples. The normal tissues were mainly obtained from the ampulla and fimbria of the Fallopian tubes of the corresponding patients and have also been histologically examined to ensure absence of invasive or in situ carcinoma. The collection of such tissue was approved by the Institutional Ethics Board.

Two immortalized normal human ovarian surface epithelium cell lines (HOSE6-3 and HOSE11-12) and 20 ovarian cancer cell lines were used. Ovarian cancer cell lines, SKOV-3 and OVCAR3 were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). The two HOSE cell lines and OVCA 420 were kindly provided by Prof. G.S.W. Tsao (Department of Anatomy, the University of Hong Kong). OC316, DOV13, ES2, PA-1, TOV21G, TOV112D and SKOV-TR were generous gifts from Dr. Lawrence XF Le (Division of Cancer Medicine, University of Texas M. D. Anderson Cancer Center, Houston, Texas, USA). 2008 (CDDP-sensitive) and 2008/C13 (CDDP-resistant) cells were provided by Drs.
S. B. Howell (University of California at San Diego, La Jolla, CA), S. G. Chaney (University of North Carolina, Chapel Hill, NC), and Z. H. Siddik (M. D. Anderson Cancer Center) (24), respectively. 2780S and 2780CP were provided by Prof. Benjamin B.K. Tsang (Department of Obstetrics and Gynecology, University of Ottawa, Canada). The PEO1, PEO4, PEO14, PEO23, PEA1 and PEA2 cells were acquired from the Cell Culture Service, Cancer Research UK (London, UK), where they were tested and authenticated.

**Immunohistochemistry, immunoblotting and immunofluorescence.**

Immunohistochemistry was performed using EnVision_Dual Link System (K4061; Dako, Carpinteria, CA) with standard procedures as previously described (25, 26). Immunoblotting and immunofluorescence were performed as previously described (27). Antibodies used in this study were summarized in supplementary Table 2. The specificity of the anti-iASPP antibody used for this study was illustrated by using immunoblot in clinical samples (supplementary Figure 1B). Omission or replacement of the primary antibody with PBS was used as a negative control. For immunoreactivity evaluation, both intensity and percentage of immunoreactivity of each section were semi-quantitated. Briefly, the level of intensity was recorded as 0 (negative), 1 (weak), 2 (mild), 3 (moderate), and 4 (strong). The percentage of positive staining was rated as 0 (<5%), 1 (6% to 25%), 2 (26% to 50%), 3 (51% to 75%), and 4 (>75%). The final immunoreactivity score was determined as a product of the two parameters, giving the range of 0 to 16.
**RNA and DNA Preparation, Quantitative Real-Time PCR (qPCR).** Total RNA was isolated from frozen tissues using Trizol reagent (Invitrogen) according to the manufacturer’s instruction. First-strand cDNA was synthesized with oligo-dT primer and SuperScript III Reverse Transcriptase kit (Invitrogen). Genomic DNA was extracted using phenol/chloroform. ABI PRISM 7900 Sequence Detection System and SYBR-green PCR master mix (Applied Biosystems) were used for qPCR. All primers used in this study are listed in supplementary Table 3. The mRNA expression was normalized with respect to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and determined by $2^{-\Delta\Delta C_T}$ method after normalization. The primers to genomic sequences of iASPP were designed using Primer3 (v.0.4.0) software based on the sequence of intron 2 of iASPP (Ensemble database). LINE-1 was applied as reference gene for the evaluation of iASPP copy number (28). Each sample was verified in duplicate. Ovarian cancer samples scoring ≥ two-fold difference from the corresponding normal counterpart were considered as positive for iASPP gene amplification.

**Plasmids, transfections and paclitaxel treatment.** pcDNA3.1/V5-His-iASPP expression plasmid containing human full-length cDNA of iASPP (encoded by PP1R13L) and pSuper plasmid containing siRNA specific to iASPP were used as described previously(18). Additional siRNAs specific for iASPP and separase were obtained from Ambion (USA). OVCA 420 and SKOV-3 cells stably expressing iASPP
were generated and selected using G418 (8mg/ml). Transient transfections were performed using LipofectamineTM 2000 (Invitrogen) according to manufacturer’s recommendation. Twenty-four hours after transfection, cells were incubated in 1.7\mu M paclitaxel (a dose corresponding to the lower area under the curve obtained in pharmacokinetic studies (9, 29)) for 36 hours (knockdown iASPP) or 48 hours (overexpressed iASPP).

**TdT-mediated dUTP nick end labeling (TUNEL) assay and evaluation of apoptotic and mitotic catastrophe indices.** After transfections and paclitaxel treatments, TUNEL assay was performed using an In Situ Cell Death Detection kit (Roche Biochemical, Indianapolis, IN) following the manufacturer’s protocol (30). Apoptotic and mitotic catastrophe figures were also assessed under fluorescence microscopy. Apoptotic figures were shown by green fluorescein following TUNEL assay. Mitotic catastrophe figures were observed by the morphological changes in nuclei (stained with DAPI) as mentioned above (10-14). More than 1,000 viable cells in each experiment were examined and the apoptotic and mitotic catastrophe indices were evaluated as percentages of the viable cells counted. Every assay was run in triplicate.

**Statistical analysis.** Statistical Package for Social Science 15.0 for windows (SPSS Inc., Chicago, IL, USA) software was used for statistical analysis as described (25). Mann-Whitney U and Spearman’s test were used to evaluate the difference and correlation of different groups. Kaplan-Meier method and Cox’s regression model
were served to estimate the survival probability and multivariate survival analysis. P value <0.05 was considered as critical significant.

Results

Overexpression of iASPP in ovarian cancers correlates with poor survival. The expression of iASPP in ovarian benign and borderline tumors as well as invasive cancers were evaluated (supplementary Table 1). iASPP protein was predominantly expressed in the cytoplasm, though focal immunoreactivity could be detected in the nucleus of six borderline tumors and 21 cancers (Figure 1, A). The subcellular localization of iASPP was confirmed by immunofluorescence in OVCA 420 cell line (supplementary Figure 1A).

iASPP staining was diffuse and strong in ovarian cancers, in contrast to the patchy and moderate staining in borderline tumors and focal and weak staining in benign tumors. There was statistically significant higher expression of iASPP in borderline tumors (P<0.001) and ovarian cancers (P<0.001) when compared with benign tumors. In addition, expression of iASPP in ovarian cancers was also significantly higher than that in borderline tumors (P=0.009). However, there was no significant difference between the primary carcinomas and their metastatic foci.

Among the four major histological types of ovarian cancers (serous, endometrioid, clear cell, and mucinous), high iASPP expression was significantly associated with clear cell subtype when compared with the other histological types combined (P=0.023) (Figure 1, B). High iASPP expression (immunoreactivity score ≥
9) was also found to be correlated with shorter overall survival (P=0.003) and
disease-free survival (P=0.001) (Figure 1, C) (supplementary Table 1). There was no
significant correlation between iASPP immunoactivity and histological grade
(P=0.110) or clinical stage of the cancers (P=0.174).

**Gene amplification of iASPP contributes to iASPP overexpression.** In a panel of 36
paired clinical frozen ovarian cancers specimens, the mRNA levels of iASPP were
found to be significantly up-regulated in cancers when compared with the
corresponding non-tumor counterparts (P<0.001) by qPCR (Figure 1, D upper left
panel).

Six out of the 12 (50%) ovarian cancer cell lines also showed an up-regulation
of iASPP at mRNA level when compared with HOSE6-3 and HOSE11-12 (Figure 2,
A). iASPP has at least two isoforms, the full-length 828 amino acids (aa) isoform and
the iASPP/RAI (351 aa) isoform, both are effective in inhibiting p53-mediated
apoptosis (20). By immunoblotting, increased expression of at least one of the two
iASPP isoforms was observed in nine out of the 13 (69%) ovarian cancer cell lines
(OVCA 420, OV316, DOV13, SKOV-3, PA-1, 2780S, 2780CP, 2008, and 2008/C13)
(Figure 2, B).

To evaluate the mechanisms underlying up-regulation of iASPP, iASPP DNA
copy number was further evaluated by real time PCR (Figure 1, D, upper right panel).
Indeed, 18 out of 36 (50%) cancer samples showed gene amplification of iASPP when
compared with the corresponding non-tumor counterparts. More than 80% of the
amplified cases displayed elevated mRNA (Figure 1, D lower panel). Spearman’s rho analysis demonstrated significant correlation between iASPP amplification and its mRNA expression (P=0.023).

**Elevated iASPP is associated with chemoresistance.** Among the 111 patients with ovarian cancers, 96 received chemotherapy after surgical operation. High levels of iASPP expression were significantly associated with carboplatin and paclitaxel chemoresistance (P=0.040) (Figure 2, C) (supplementary Table 1). Multivariable analysis showed high expression of iASPP, chemoresistance, high histological grade (poor differentiation) and advanced cancer stage were independent predictors of short overall survival (95% CI=1.046-4.336, P=0.037; 95% CI=6.797-46.154, P<0.001; 95% CI=1.096-3.066, P=0.021; 95% CI=1.182-2.474, P=0.004, respectively) and disease-free survival (95% CI=1.001-4.059, P=0.050; 95% CI=2.309-11.932, P<0.001; 95% CI=0.957-2.493, P=0.075; 95% CI=1.170-2.473, P=0.005, respectively).

To further investigate the effect of iASPP on chemotherapy, four pairs of chemo-sensitive and chemo-resistant ovarian cancer cell lines were examined for iASPP protein level by immunoblotting. SKOV3-TR is a paclitaxel-resistant derivative of SKOV3, whereas PEO4, PEO23 and PEA2 are cisplatin-resistant derivatives of PEO1, PEO14 and PEA1, respectively (31, 32). Three of the chemo-resistant ovarian cancer cell lines, SKOV3-TR, PEA2 and PEO23, showed higher protein expression of iASPP as compared with their chemo-sensitive counterparts (Figure 2, D).
**Mitotic catastrophe is the dominant mode of cell death induced by paclitaxel in OVCA 420 and SKOV-3 cells.** By analysis of nuclear morphology, both OVCA 420 and SKOV-3 cells were found to be sensitive to paclitaxel treatment, and cell death was mainly in the form of mitotic catastrophe with a time-dependent manner (Figure 3,A). At as early as 6 hours after drug treatment, swelling and condensation of scattered nuclei occurred. After 24 hours, diffuse asymmetric DNA condensation appeared. At 48 hours, multi-nucleated giant nuclei with chromosome vesicles were observed and some of the cells started to detach from the coverslips, indicative of necrosis-like cytolysis. Meanwhile, parallel increased expression of iASPP was observed (Figure 3, B). The demonstration of mitotic catastrophe as the dominant mode of cell death was supported by the concurrent up-regulation of cyclin B1 (Figures 5, 6), which was suggested to be a marker of mitotic catastrophe but not apoptosis (33).

**iASPP is involved in normal cell mitosis and paclitaxel-induced mitotic catastrophe.** To visualize the relationship between iASPP and mitotic process, OVCA 420 (Figure 4, A) and SKOV-3 (Figure 4, B) cells were probed with antibodies against iASPP and β-tubulin simultaneously. Without paclitaxel treatment (Figure 4, first column), iASPP was observed to be more commonly expressed in mitotic cells than interphase cells in both cell lines, suggesting involvement of iASPP in mitosis. After paclitaxel exposure, intense expression of iASPP was observed in cell undergoing mitotic catastrophe.
(Figure 4, second column, arrowhead), which further confirmed that iASPP may play a critical role in mitotic catastrophe type of cell death induced by paclitaxel. Moreover, among paclitaxel treated cells expressing exogenous iASPP (Figure 4, third column), many more interphase cells were found to express iASPP (arrow) when compared with cells in mitotic catastrophe (arrowhead). In contrast, after iASPP knockdown, paclitaxel treated cells showed universally low expression of iASPP in both interphase and mitotic catastrophe cells accompanied by an increased frequency of mitotic catastrophe index (Figure 4, fourth column, arrowhead). Overexpression of iASPP seems to help cells escaping mitotic catastrophe by favoring interphase state of cancer cells. In contrast, knockdown iASPP apparently enhances mitotic catastrophe in both cell lines.

Overexpressed iASPP prevents paclitaxel-induced mitotic catastrophe in a p53-independent manner and inhibits apoptosis in p53-dependent mode. To further elucidate the mechanisms by which iASPP affect response of ovarian cancer cells to paclitaxel, ovarian cancer cell lines with different p53 status were compared using TUNEL assay. In OVCA 420, an ovarian cancer cell line known to harbor wild-type p53, although paclitaxel treatment per se under the dose used in our experiments did not cause significant increase of apoptosis (Figure 5, A), overexpression of iASPP led to a reduction of apoptotic index in both DMSO (P=0.046) and paclitaxel-treated groups (P=0.05). More importantly, increased iASPP expression was significantly associated with reduction of paclitaxel-mediated mitotic catastrophe index (P=0.03).
The same experiments were performed in two other ovarian cancer cell lines with dysfunctional p53, SKOV-3 and OVCAR3 (Figure 5, A). SKOV-3 cells possess a single nucleotide deletion at point 267 (codon 90) of TP53, which blocks p53 expression(34) whereas OVCAR3 cells express mutant p53 protein(35). Concurring with our findings in OVCA 420, significant reduction of paclitaxel-mediated mitotic catastrophe indices were found after iASPP overexpression in both cell lines (P=0.016 and 0.005, respectively). However, no significant effects on paclitaxel-mediated apoptosis were observed in these two cell lines. These results implied that up-regulated iASPP could prevent mitotic catastrophe induced by paclitaxel in a p53-independent manner, whilst functional p53 is required for paclitaxel-induced apoptosis in ovarian cancer.

In addition to ectopic expression studies, OVCA 420 and SKOV-3 cell lines were also knockdown of iASPP using pSuper plasmid and commercial siRNA silencing iASPP (Figure 5, B and supplementary Figure 2). Consistent with previous findings, there were increased levels of paclitaxel-induced mitotic catastrophe after iASPP silencing in both cell lines (P=0.048 and 0.003, respectively), whereas significantly enhanced apoptosis was only observed in OVCA 420 cells with or without paclitaxel treatment (P=0.05 and 0.045, respectively).

**iASPP affected the securin/cyclin B1/separase pathway.** Physiologically, cell mitosis is highly regulated by mitotic spindle checkpoint in which separase plays an essential role. Once activated, separase would cleave downstream target multisubunit complex
(cohesion) and allow the separation of sister chromatids, leading to mitosis. Under mitotic catastrophe, the normal function of separase may be abrogated resulting in a failure of division and multinucleation. By immunoblotting, induction of iASPP followed by paclitaxel exposure in OVCA 420 cells was associated with up-regulation of separase auto-cleavage (activation) and down-regulation of securin, CDK1 and cyclin B1 (Figure 5, C). On the other hand, knockdown of iASPP abrogated the auto-cleavage of separase and enhanced the protein expression of securin, CDK1 and cyclin B1 upon paclitaxel treatment (Figure 5, C).

As an attempt to clarify the role of the securin/cyclin B1/separase pathway in iASPP mediated mitotic catastrophe inhibition, siRNA specific to separase was transfected into OVCA 420 and SKOV-3 cells stably expressing iASPP (Figure 6, A). Consistent with previous findings (Figure 5, C), ectopic iASPP could elevate active form of separase (Figure 6, B). As evidence of a central role of separase in mitosis, our results showed that knockdown of separase could enhance the mitotic catastrophe irrespective of paclitaxel treatment. More importantly, knockdown of separase could block iASPP-mediated inhibition of mitotic catastrophe in both OVCA 420 and SKOV-3 cells, particularly with paclitaxel treatment. These results suggest that iASPP confers resistance to paclitaxel by reducing mitotic catastrophe via activation of separase. However, our results cannot rule out the involvement of other signal molecules in the inhibitory effect of iASPP on paclitaxel-mediated mitotic catastrophe.

The mRNA levels of five mitotic checkpoint molecular components, including Bub 1, Bub 3, BubR 1, Mad 1 and Mad 2, besides separase, securin and cyclin B1,
were also examined by qRT-PCR. None of them showed significant alterations in expression levels (data not shown). Taken together, our results suggest that iASPP might affect the expression of separase via securin and cyclin B1/CDK1 complex at the protein level.

**Discussion**

In this study, we demonstrated upregulation of iASPP expression in ovarian cancers in vivo and ovarian cancer cell lines at both mRNA and protein levels. Furthermore, high iASPP expression level was significantly correlated with chemoresistance, shorter overall survival and disease-free survival. Multivariate analysis indicated that high iASPP expression could serve as a prognostic indicator that was independent of clinical stage, grade, histological subtype and chemosensitivity in ovarian cancers. These observations suggest that iASPP may play a role in ovarian carcinogenesis, predict the patients’ outcome and are likely to exert an effect on chemosensitivity.

To investigate the mechanism of iASPP up-regulation, the relative copy number of iASPP (PPP1R13L, chromosome 19q13.3), a gene located within the previously reported 19q amplicon of ovarian cancers, was determined (36). Our data revealed that half of the cancers demonstrated gene amplification of iASPP, and increased mRNA expression was significantly correlated with its gene amplification. This is the first report on iASPP gene amplification in human cancers.

We next sought to investigate the effect of iASPP on chemotherapy in vitro. Concurring with previous reports on other human malignancies (36), we demonstrated
that paclitaxel could predominantly induce mitotic catastrophes and to a relatively lesser extent, apoptosis in ovarian cancer cells (OVCA 420 and SKOV-3). One of the most striking findings in the present study is that overexpressed iASPP could abrogate not only paclitaxel-mediated apoptosis, but also an alternative mode of cell death, paclitaxel-mediated mitotic catastrophe. Conversely, knockdown of iASPP could enhance the mitotic catastrophe.

Unlike apoptosis, the process of mitotic catastrophe is still controversial. Two mechanisms have been considered to be crucial for mitotic catastrophe, namely G2/M checkpoint and mitotic spindle checkpoint (38). For G2/M checkpoint, abolishment of genes such as p53, p21\textsuperscript{Cip1} and 14-3-3Sigma (39,40) were found to promote DNA damage-induced mitotic catastrophe. It was therefore logical to speculate that iASPP may prevent paclitaxel-mediated mitotic catastrophe via p53 (20, 41). But our findings on OVCAR3 and SKOV-3, two cell lines with dysfunction and deletion of p53 respectively, that iASPP could still inhibit mitotic catastrophe suggest otherwise. However, further investigation is needed to understand how this p53 independent mitotic catastrophe inhibition was mediated. Nevertheless, our work does provide the first experimental evidence that iASPP plays a pivotal role in mitotic catastrophe in a p53-independent manner.

Separase is a cysteine protease and regarded as a central downstream target of mitotic spindle checkpoint (42). The proteolytic activity of separase is regulated by at least two mechanisms (43). Securin has long been appreciated as a direct inhibitor of separase, and cell division cycle 2 (also known as CDK1)/cyclin B1 complex
constituted the second pathway via inhibitory phosphorylations. Our data demonstrated that increased iASPP could enhance the activity of separase via degradation of securin and cyclin B1/CDK1 complex. As a result, decreased frequency of paclitaxel-mediated mitotic catastrophe occurred. Conversely, iASPP suppression enhanced paclitaxel-mediated mitotic catastrophe index following elevated levels of securin and cyclin B1/CDK1 complex and inactive separase. Furthermore, siRNA silencing of separase was found to promote the mitotic catastrophe, which was overcome by overexpressed iASPP. However, we have only begun to understand the function of iASPP, further research on this versatile protein and its interaction with separase, securin and/or cyclin B1 is necessary.

On the other hand, recent studies demonstrated the contribution of drug transporters as important mechanisms of chemoresistance in ovarian cancer (44, 45). For example, as a primary efflux transporter, P-glycoprotein-mediated drug efflux could contribute to chemotherapy failure (45). To our best knowledge, there has been no report about the relationship between iASPP and drug transporters. Further delineation of the mechanisms would be examined in our future studies.

In summary, iASPP was found to exert a powerful effect on paclitaxel resistance in ovarian cancers, since it could inhibit both mitotic catastrophe and apoptosis irrespective of p53 function. Our findings highlight the great potential of iASPP to be regarded as a biomarker for paclitaxel chemosensitivity and clinical prognosis in ovarian cancer, as well as an effective chemotherapeutic target.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Legends

Figure 1. (A) Representative photomicrograph of iASPP immunoreactivity and (B) immunoscores of iASPP in benign (b), borderline (bl), and clear cell (c)/endometrioid (e)/mucinous (m)/serous (s) adenocarcinoma. Scale bar=100μm. Magnified images of overexpression of iASPP were added in insets. (C) Overall and disease-free survival curves with respect to iASPP expression. (D) iASPP mRNA expression in clinical samples (upper left panel) and gene amplification status (upper right panel) in relation to mRNA expression (lower panel).

Figure 2. (A) mRNA and (B) protein expression pattern of iASPP in HOSE cell lines and ovarian cancer cell lines (C) Higher iASPP expression was found in the chemoresistant patients than in the chemosensitive patients. (D) iASPP protein expression in four pairs of chemo-sensitive and resistant ovarian cancer cell lines were compared with higher expression demonstrated in chemoresistant cell lines (PEA2, PEO23). All immunoblot assays were done in duplicate.

Figure 3. Paclitaxel treatment induced mitotic catastrophe in OVCA 420 and SKOV-3 cells (A) and parallel increased iASPP (B). OVCA 420 and SKOV-3 treated with paclitaxel for indicated duration were stained with DAPI and their nuclear morphology was examined under a fluorescence microscope (arrowhead: asymmetric chromosomal condensation; arrow: multi-nucleated giant cells). Scale bar=50μm.
Figure 4. Representative pictures of double immunofluorescence images showing iASPP and β-tubulin in (A) OVCA 420 and (B) SKOV-3 cells (control, vector-transfected control cells; arrow, interphase cells; arrowhead, mitotic catastrophe). Scale bar=50μm.

Figure 5. Paclitaxel mediated mitotic catastrophe and apoptosis (TUNEL assay) after (A) ectopic expression of iASPP in OVCA 420, SKOV-3 and OVCAR3 cells and (B) silencing of iASPP in OVCA 420 and SKOV-3 cells (control, vector-transfected control cells; arrow, apoptosis; arrowhead, mitotic catastrophe). (C) Expression levels of iASPP and securin / cyclin B1 / separase pathway in OVCA 420 cells were illustrated. Scale bar=50μm.

Figure 6. (A) TUNEL assay showed the change of mitotic catastrophe and apoptosis after silencing separase in cells stably overexpressing iASPP (control, vector-transfected stable cells; siControl, negative control of siRNA; arrow, apoptosis; arrowhead, mitotic catastrophe). Scale bar=100μm. (B) Parallel protein expressions were shown by immunoblotting.

Supplementary Figure 1. (A) Confocal microscopic image of OVCA 420 cells stained with anti-iASPP antibody demonstrated that iASPP protein was predominantly expressed in cytoplasm, but small amount could be detected in the nucleus. Scale bar=50μm. (B) Immunoblot illustrated the specificity of the
anti-iASPP antibody used for this study in clinical samples.

**Supplementary Figure 2.** Consistent results were found with iASPP knockdown demonstrating that abrogating iASPP could enhance paclitaxel-mediated mitotic catastrophe in both ovarian cancer cell lines (control, vector-transfected control cells; arrow, apoptosis; arrowhead, mitotic catastrophe).

**Supplementary Table 1.** Association analysis between iASPP expression and the clinicopathological features of ovarian epithelial tumours.

**Supplementary Table 2.** Primary antibodies used for immunoblotting, immunofluorescence, and immunohistochemistry

**Supplementary Table 3.** Primers used for quantitative real-time PCR
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