Loss of MKP3 mediated by oxidative stress enhances tumorigenicity and chemoresistance of ovarian cancer cells

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The RAS-RAF-MEK-extracellular signal-regulated kinase (ERK) pathway plays a pivotal role in various cellular responses, including cellular growth, differentiation, survival and motility. Constitutive activation of the ERK pathway has been linked to the development and progression of human cancers. Here, we reported that mitogen-activated protein kinase phosphatase (MKP)-3, a negative regulator of ERK1/2, lost its expression particularly in the protein level, was significantly correlated with high ERK1/2 activity in primary human ovarian cancer cells using quantitative reverse transcription–polymerase chain reaction and western blot analyses. Intriguingly, the loss of MKP3 protein was associated with ubiquitination/proteosome degradation mediated by high intracellular reactive oxygen species (ROS) accumulation such as hydrogen peroxide in ovarian cancer cells. Functionally, short hairpin RNA knock down of endogenous MKP3 resulted in increased ERK1/2 activity, cell proliferation rate, anchorage-independent growth ability and resistance to cisplatin in ovarian cancer cells. Conversely, enforced expression of MKP3 in MKP3-deficient ovarian cancer cells significantly reduced ERK1/2 activity and inhibited cell proliferation, anchorage-independent growth ability and tumor development in nude mice. Furthermore, the enforced expression of MKP3 succeeded to sensitize ovarian cancer cells to cisplatin-induced apoptosis in vitro and in vivo. These results suggest a molecular mechanism by which the accumulation of ROS during ovarian cancer progression may cause the degradation of MKP3, which in turn leads to aberrant ERK1/2 activation and contributes to tumorigenicity and chemoresistance of human ovarian cancer cells.

Introduction

The extracellular signal-regulated kinase (ERK)-signaling pathway plays a crucial role in various cellular responses including cell proliferation, growth, differentiation and survival by relaying extracellular signals from ligand-bound cell surface tyrosine kinase receptors to gene transcription in nucleus via a cascade of specific phosphorylation events (1). Aberrant activation of this pathway due to increased exposure to growth factors, overexpression or mutation of receptor tyrosine kinases, Ras and Raf, has been implicated in many aspects of tumorigenesis including proliferation, differentiation, invasion, angiogenesis and apoptosis (2,3). Recent studies have identified several negative regulators such as mitogen-activated protein kinase phosphatases (MKPs), Spred and Sef that can modulate duration, magnitude and subcellular compartmentalization of ERK1/2 activity, suggesting that these inhibitor proteins also play important roles in tumorigenesis (4–6). In ovarian cancer, constitutive activation of ERK activity has been associated with high tumorigenicity and chemoresistance (7,8). Therefore, the ERK pathway is a crucial target for therapeutic intervention in ovarian cancer.

MKP3 is a member of the subfamily of protein tyrosine phosphatases known as dual-specificity phosphatases (MKPs) (9,10). Biochemical and structural studies have shown that MKP3 is capable of dephosphorylating ERK1/2 by protein–protein interactions via mitogen-activated protein kinase interaction motif within the N-terminal ERK1/2-binding domain (11). Moreover, MKP3 negatively modulates ERK1/2 but not p38 and c-jun N-terminal kinase activities (12,13). Recent studies have shown that MKP3 is underexpressed in pancreatic cancer due to promoter hypermethylation and frequent loss of heterozygosity (14,15). Overexpression of MKP3 has shown to have apparent tumor-suppressive effects through reduction of phosphorylated ERK1/2 in a dose-dependent manner in pancreatic cancer cells (16,17). However, there is lack of similar reports in other human cancers so far.

In the present study, we analyzed the expression levels of MKP3 ovarian cancer samples and cell lines. We provide here the mechanistic correlation between oxidative stress and ubiquitination/proteosome degradation of MKP3 protein in ovarian cancer cells. Functional analyses employing MKP3 knockdown and ectopically overexpression revealed the role of MKP3 in negatively regulating ERK1/2 activity and inhibiting tumorigenicity and chemoresistance in vitro and in vivo.

Materials and methods

Clinical samples and cell lines

Seventy-seven tissue samples of surgically resected primary ovarian cancer and 41 normal ovary samples from women who had surgery for benign diseases such as uterine fibroids were randomly selected for this study. All the clinical specimens used in the present study were approved by the local institutional ethics committee (UW 05-143 T/806). Six immortalized human ovarian surface epithelial (HOSE) cells, HOSE 6-3, HOSE 10-2, HOSE 11-12, HOSE 17-1, HOSE 29-3 and HOSE 21-3, and ovarian cancer cell lines, OV208, C13, A2780s, A2780cp, DOV13-5, OVCAR3, SKOV3, OV420, OV429 and OV433 (American Type Culture Collection, Rockville, MD), were used in this study.

Plasmids and RNA interference

The MKP3-expressing construct, pcDNA3.1/V5-His-DUSP6/MKP3, and the HA-tagged ubiquitin-expressing construct, pcDNA-HA/Ub, had been used previously (16,18). The MKP3 mutant-expressing construct, pcDNA3.1/V5-His-DUSP6/mut-MKP3, was generated by creating point mutations at serines 159 and 197. The short hairpin RNA interference targeting MKP3 (target sequence: TACAGAGGCGGTCTAGATGG) was ligated into pcDNA™ 6.2-GW/EMGFP-miR plasmid (Invitrogen Life Technology, Carlsbad, CA). Three synthetic small interfering RNAs specific for human MKP3 were provided and synthesized by Ambion (Ambion K.K., Tokyo, Japan).

Quantitative and semiquantitative reverse transcription–polymerase chain reaction

Total RNA from each cell line was prepared by TRIzol reagent (Invitrogen Life Technology). First-strand complementary DNA was synthesized by random hexamers and Taqman reverse transcription reagent kit (Applied Biosystems, Foster city, CA). For real-time quantitative reverse transcription–polymerase chain reaction, the amount of MKP3 messenger RNA (mRNA) was quantified by TaqMan Gene Expression Assays and in an ABI 7700 system (Applied Biosystems). The MKP3 primers and probe were obtained from Applied Biosystems (Assay ID: Hs00169257_m1). Each sample was performed in triplicate and normalized with human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Assay ID: Hs99999905_m1; Applied Biosystem). For semiquantitative reverse transcription–polymerase chain reaction, the MKP3 mRNA level was evaluated by a pair of primers—MKP3-sense 5′-CTACCCTGG-AAAGGTTGGCTCA-3′ and MKP3 antisense 5′-GTGGAGACGGGCAC-TACCACT-3′—with the following conditions for 30 cycles: denaturation at
DUSP6/MKP3 and pcDNA-HA(Ub)8, were cotransfected into A2780cp cells, were seeded onto 100 mm culture plates. The plasmids, pcDNA3.1/V5-His-conjugated to horseradish peroxidase (Amersham Pharmacia, Cleveland, OH) and anti-V5 (Invitrogen), respectively. Blots were then incubated with goat anti-rabbit or anti-mouse secondary antibody.

**Western blot analysis**

Harvested cells were lysed by Cell Lysis Buffer (Cell Signaling Technology, Beverly, MA) supplemented with Protease Inhibitor Cocktail (Sigma Chemical Co., St Louis, MO). For western blot analyses, samples containing equal amounts of protein were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electroblotted onto Immobilon-P Transfer Membrane (Millipore Corporation, Bedford, MA). Blots were blocked with 5% skim milk, followed by incubation with antibodies specific for anti-phospho p44/42 ERK1/2 (Thr202/Tyr204, Cell Signaling Technology), anti-ERK1/2 (Chemicon International, Temecula, CA), anti-MKP3 (C-20, Santa Cruz Biotechnology, Santa Cruz, CA), anti-β-actin (AC-74, Sigma Chemical Co), anti-HA (12CA5, Roche Diagnostics Corp, Indianapolis, IN) and anti-V5 (Invitrogen), respectively.

In vivo ubiquitination assay

This assay was done as described by Chan et al. (18). Briefly, A2780cp cells were seeded onto 100 mm culture plates. The plasmids, pcDNA3.1/V5-His-DUSP6/MKP3 and pcDNA-HA(Ub)8, were cotransfected into A2780cp cells, respectively, using FuGENETM 6 transfection kit (Roche Diagnostics Corp). Cells were then harvested in NET lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl and 5 mM ethylenediaminetetraacetic acid) with 1% NP40, pH 8.0, 0.1 mM phenylmethylsulfonyl fluoride and 1 mM Complete TM protease inhibitor cocktail (Roche). One milligram of cell lysate was incubated with 1 µg of mouse anti-IgG and anti-V5 antibodies at 4°C. After incubation, 40 µl of Protein A/G Plus-Agarose beads (Santa Cruz Biotechnology) was added to each sample and the mixture was rotated overnight at 4°C. After centrifugation, the beads were washed four times with NET lysis buffer, followed by addition of 30 µl of 2 × sample buffer containing β-mercaptoethanol and boiled for 10 min prior to electrophoresis. The proteasome inhibitor, MG132, was obtained from Calbiochem (La Jolla, CA).

**Determination of cellular hydrogen peroxide**

To evaluate the production of cellular hydrogen peroxide (H2O2), cells were seeded in 96-well plates and cultured in serum-free medium for 2 days. Intracellular generation of H2O2 from these cells was detected by using fluorogenic substrate 2′,7′-dichlorodihydrofluorescein (DCF) diacetate (DCFH-DA, Calbiochem) and measured by a Tecan optical-fluorescence Plate Reader (485–530 nm, Tecan Group Ltd, Männedorf, Switzerland) (19). The readings were normalized by cell numbers detected by Cell Proliferation Kit II (XTT) (Roche Diagnostics, Mannheim, Germany). This assay was performed in at least three independent experiments. The antioxidant, N-acetyl-L-cysteine (NAC), was used for the removal of intracellular reactive oxygen species (ROS) (Calbiochem).

**Cell viability analysis**

Cell viability was measured by Cell Proliferation Kit II (XTT) according to the manufacturer’s instructions (Roche). The experiment was performed in triplicate for each time point. Cisplatin (CDDP) and mitogen-activated protein kinase/ERK kinase 1/2 (MEK1/2) inhibitor PD98059 were obtained from Calbiochem.

**Anchorage-independent growth assay in soft agar**

A total of 5 × 10³ cells were trypsinized and suspended in 2 ml of full medium plus 0.3% agar (Sigma). The agar–cell mixture was plated on top of a bottom...
layer with 1% full medium agar mixture. The experiment was performed in triplicate for each cell line. After 10–20 days, viable colonies of 20 cells or more were scored.

**In vivo tumorigenicity assay**

This assay was done as described by Chan et al. (20). To investigate the cisplatin resistance of MKP3 stably transfected A2780cp cells in vivo, 5 mg/ body wt (kg)/day of cisplatin (DDP) (EBEWE Pharma Unterach, Austria) was administered intraperitoneally once for every 6 days with total of three injections into five nude mice when the size of every tumor became ~3 mm in diameter. As a control group, physiological saline alone was used. All the animal experiments were approved by the University of Hong Kong Committee on the Use of Live Animals in Teaching and Research (CULATR No.1391-06).

**Statistical analysis**

Student’s t-test (for parametric data) and the Mann–Whitney test (for non-parametric data) were used. A P-value was considered significant when <0.05.

**Results**

MKP3 is underexpressed in ovarian cancer

Real-time quantitative reverse transcription–polymerase chain reaction analysis showed that SKOV3, OVCA429, OVCA433 and A2780cp expressed relatively lower levels of MKP3 mRNA (~2- to 10-fold reductions) as compared with HOSE cell lines (Figure 1A). But western blot analysis revealed that more ovarian cancer cell lines (OVCAR3, SKOV3, OVCA420, OVCA429, OVCA433, A2780cp)
Intracellular H₂O₂ induces MKP3 ubiquitination/proteasome degradation

It has been shown that accumulation of ROS induces protein ubiquitination/proteasome degradation (21,22). By measuring the intracellular H₂O₂ using fluorogenic substrate, H2DCFDA, we found that both MKP3-deficient cell lines, A2780cp and C13³, had higher intracellular H₂O₂ content than their counterparts (A2780cp, P = 0.01 and C13³, *P = 0.001) (Figure 2A). Upon treatment of H₂O₂ on OV2008 and A2780s cells with different concentrations (0.1, 0.3, 0.5, 1.0, 3.0, 5.0 and 10.0 mM) for 6 h, the endogenous MKP3 was remarkably diminished in a dose-dependent manner at 1.0 and 3.0 mM of H₂O₂ in OV2008 and A2780s cells, respectively (Figure 2C). On the contrary, the degradation of MKP3 in both A2780cp and C13³ ovarian cancer cells was significantly inhibited by an antioxidant, NAC, in a dose-dependent manner and was further enhanced when cotreatment with MG132 (10 μM) (Figure 2D). However, unchanged levels of MKP3 mRNA were observed (data not shown). The restoration of MKP3 by treatment of NAC was further confirmed by in vivo ubiquitination assay using A2780cp cell model (supplementary Figure S1 is available at Carcinogenesis Online).

To further ascertain the role of H₂O₂ in promoting MKP3 proteosome-mediated proteolysis, we performed the in vivo ubiquitination assay in HEK293T and A2780cp cells. Western blot analysis showed that the levels of ubiquitinated MKP3 were increased when treated with MG132 (21,22). Such ubiquitinated MKP3 protein levels were further enhanced when treated by H₂O₂ (1 mM) and/or MG132 (Figure 2E). However, high concentration of H₂O₂ (10 mM) could not degrade MKP3 via ubiquitination/proteasome pathway but lead to extensive degradation via oxidative breakage on MKP3 protein (23) (Figure 2E). To test whether phosphorylation of MKP3 is the possible mechanism leading to proteasomal degradation of MKP3, the mut-MKP3-expressing plasmid (point mutations at serines 159 and 197 MKP3 phosphorylation sites) was transfected into HEK293T cells and treated with H₂O₂. Western blot analysis showed that both MKP3 and mut-MKP3 proteins were still degraded by H₂O₂ treatment. However, the reduced level of mut-MKP3 was much less as compared with MKP3 (supplementary Figure S2 is available at Carcinogenesis Online). In vivo ubiquitination assay also revealed that the level of ubiquitinated mut-MKP3 was obviously reduced compared with ubiquitinated MKP3 under treatment of H₂O₂ and/or MG132 (supplementary Figure S3 is available at Carcinogenesis Online). This indicates that the phosphorylation of MKP3 is associated with proteasomal degradation of MKP3. Taken together, these findings suggest that the loss of MKP3 is due to ROS-mediated degradation via the proteasome/ubiquitination pathway in ovarian cancer cells and phosphorylation of MKP3 is the possible mechanism leading to ubiquitination/degradation.

MKP3 is a negative regulator to ERK1/2 in ovarian cancer cells

Previous studies have demonstrated that MKP3 acts as a negative regulator on ERK1/2-signaling pathway in pancreatic cancer
(16,17). In this study, our ovarian cancer cell lines and samples expressed low MKP3 but high phospho-ERK1/2 expression levels (Figure 1B and D). This similar scenario was observed in cisplatin-sensitive ovarian cancer cells (A2780s and OV2008) and vice versa for their cisplatin-resistant variants (A2780cp and C13*) (Figure 3A).

To investigate the regulatory mechanism of MKP3 on ERK1/2 activity, we employed vector-based RNAi (shMKP3) and was successfully depleted >70% endogenous MKP3 in A2780s cells (shMKP3 C4 and shMKP3 C6) (Figure 3B). We also used short interfering RNA (siMKP3#1 and siMKP3#3) to knock down >55 and >62% endogenous MKP3 in A2780s cells and OVCA433 cells, respectively (Figure 3C). As expected, the depletion of MKP3 was associated with the increased phospho-ERK1/2 levels in A2780s cells (Figure 3B and C). Conversely, ectopic expression of V5-tagged MKP3 (V5/MKP3) in A2780cp (C7, C13 and C19) and SKOV3 cells remarkably reduced the expression levels of phospho-ERK1/2 (Figure 3D). These data suggest that MKP3 negatively regulates ERK1/2 activity in ovarian cancer cells.

**MKP3 inhibits tumorigenicity of ovarian cancer cells**

Constitutive activation of ERK1/2 pathway has been shown to be associated with tumorigenicity in many cancers (24). To determine the involvement of MKP3 in suppressing the tumorigenicity of ovarian cancer, we assessed both cell proliferation rate and anchorage-independent growth ability of MKP3-deficient ovarian cancer cell lines, C13* and A2780cp. With XTT assay, both cisplatin-resistant cell lines, C13* and A2780cp, showed higher cell proliferation rate (Figure 4A). Besides, A2780cp cells showed higher anchorage-independent growth ability by soft agar assay (Figure 4B).

On the other hand, depletion of MKP3 significantly increased cell proliferation rate and anchorage-independent growth ability in MKP3-deficient ovarian cancer cell lines, C13* and A2780cp. With XTT assay, both cisplatin-resistant cell lines, C13* and A2780cp, showed higher cell proliferation rate (Figure 4A). Besides, A2780cp cells showed higher anchorage-independent growth ability by soft agar assay (Figure 4B).

To further examine whether MKP3 could suppress in vivo tumorigenic activity of ovarian cancer cells, two MKP3 transfectants (C13 and C19) and the vector control of A2780cp were inoculated into nude mice. The tumor growth rates for both MKP3 transfectants (C13 and C19) were ~2-fold slower compared with the vector control (Figure 5C). This could be confirmed by their tumor sizes on day 26 that the tumor size of vector control was 2111 ± 296 mm³, whereas C13 and C19 were 538 ± 79 mm³ and 836 ± 93 mm³, respectively ($P < 0.005$ for C13 and $P < 0.05$ for C19) (Figure 5C). These data highlight the inhibitory functions of MKP3 on tumorigenicity in vitro and in vivo.

**MKP3 reduces cisplatin resistance of ovarian cancer cells**

It has been demonstrated that high ERK1/2 activity contributes to cisplatin resistance of ovarian cancer cells (25). Two pairs of cell models including cisplatin-sensitive ovarian cancer cell lines (A2780s and OV2008) and their cisplatin-resistant counterparts (A2780cp and C13*) were examined (26). Upon treatment with cisplatin (0, 5 and 10 μM) for 24 h, both cisplatin-sensitive cell lines, A2780s and OV2008, showed >2-fold reduction of cell viability when compared with their cisplatin-resistant counterparts (Figure 6A). Previous studies have demonstrated that pretreatment of cisplatin-resistant ovarian cancer cells with MEK1/2 inhibitor, PD98059, may sensitize cells to cisplatin-induced apoptosis (8). Consistent with this finding, we found that treatment of PD98059 (20 μM) could moderately sensitize both cisplatin-resistant cell lines, A2780cp and C13*, to cisplatin-induced apoptosis by 0.4- to 1-fold (Figure 6B).

On the contrary, under the same condition of cisplatin treatment, knockdown of MKP3 in A2780s cells (shMKP3 C4 and shMKP3 C6) significantly enhanced cisplatin resistance (Figure 6C), whereas enforced expression of MKP3 in A2780cp (C13 and C19) showed significant reduction of cisplatin resistance by 1- to 2.5-fold when compared with their vector controls (Figure 6D).

Next, we examined whether the above functions of MKP3 were associated with in vivo tumor growth in nude mice. We found that there were no differences in relative tumor sizes among any of the cisplatin treated or saline treated for the vector control groups (Figure 6E). However, for mice injected with MKP3 stably transfected A2780cp cells (C19), the relative tumor growth rate for cisplatin-treated group was significantly lower than that of saline-treated group (Figure 6E).

**Discussion**

In the present study, we have shown that the loss of MKP3 protein predominated in ovarian cancer and was inversely correlated with the ERK1/2 activity. Loss of MKP3 and high ERK1/2 activity was found to be associated with high intracellular H₂O₂ accumulation, whereas mild treatment of ovarian cancer cells by H₂O₂ resulted in MKP3 degradation through proteasome/ubiquitination pathway. We have investigated the molecular mechanism by which intracellular H₂O₂ regulates MKP3 in ovarian cancer cells. Moreover, the decrease or increase of phospho-ERK1/2 levels due to ectopic overexpression of MKP3- or RNAi-mediated MKP3 knockdown indicates that ERK1/2

![Fig. 4. Higher tumorigenicity in cisplatin-resistant ovarian cancer cells.](image)
activity is regulated by MKP3 in ovarian cancer cells. Interestingly, the reduction of ERK1/2 activity by restoration of MKP3 sensitized MKP3-deficient ovarian cancer cells to cisplatin-induced apoptosis and inhibited tumorigenicity of ovarian cancer cells in vitro and in vivo, suggesting that MKP3 possesses tumor-suppressive functions in ovarian cancer. To the best of our knowledge, this is the first study reporting such a regulatory mechanism between ROS and MKP3 expression in human cancers.

Underexpression of MKP3 gene has been reported in pancreatic cancer due to promoter hypermethylation and allelic loss (15). Our study also confirmed that MKP3 gene was underexpressed in some ovarian cancer cell lines. However, as we did not extensively investigate the mechanisms on silencing MKP3 genetically or epigenetically, we do not rule out that there may be other mechanisms causing silencing of expression of MKP3 in ovarian cancer. But the high discrepancy between the MKP3 protein and mRNA levels in ovarian cancer cell lines and cancer tissues indicates that the loss of MKP3 protein relatively predominates and may involve posttranscriptional modifications for MKP3 protein in ovarian cancer cells.

Increased ROS stress is common in cancer cells, which is attributed to the active metabolism from the effect of oncogenic signaling and/or mitochondrial malfunction during development and progression (27–29). Previous studies have shown that the activity of MKP3 is inactivated by low concentration H2O2 in cells (30,31). It is of interest to know whether the frequent loss of MKP3 is due to ROS-mediated degradation in ovarian cancer cells. Our data presented here clearly

Fig. 5. The expression levels of MKP3 determine the tumorigenicity of ovarian cancer cells. (A) Left, XTT cell proliferation assay showed the knockdown of MKP3 in A2780s ovarian cancer cells (shMKP3 C4 and shMKP3 C6), increased cell proliferation rate as compared with their vector control. *P < 0.001. Right, the soft agar assay showed that knockdown of MKP3 in A2780s cells (shMKP3 C4 and shMKP3 C6) had higher anchorage-independent growth ability as compared with vector control. "P < 0.01. (B) Left, XTT cell proliferation assay showed overexpression of MKP3 in A2780cp cells (C13 and C19) had reduction in cell proliferation rate as compared with vector control. "P < 0.01. Right, the soft agar assay showed both MKP3 transfectants (C13 and C19) showed reduction of anchorage-independent growth ability as compared with vector control. "P < 0.001. (C) Tumor formation assay in nude mice. Two MKP3 transfectants (C13 and C19) and vector control cells were inoculated subcutaneously into nude mice. Tumor formation in each nude mouse was monitored every 2 days for 26 days. The tumor size was calculated and depicted as mean tumor volume ± SE of five mice for each group. Both MKP3 transfectants (C13 and C19) had significant tumor growth rate (*P < 0.05 and **P < 0.005, respectively) as compared with vector control group.
demonstrated that the intracellular H\textsubscript{2}O\textsubscript{2} content was higher in MKP3-deficient ovarian cancer cells, A2780cp and C13\textsuperscript{+}, and this finding is consistent with other studies (32). As expected, treatment of these cells by an antioxidant, NAC, could restore MKP3. Conversely, treatment of H\textsubscript{2}O\textsubscript{2} on MKP3-sufficient ovarian cancer cells, A2780s and OV2008, could degrade MKP3. As H\textsubscript{2}O\textsubscript{2} is capable of inducing protein ubiquitination/degradation (22,33), we have thus proved the degradation of MKP3 by low concentration of H\textsubscript{2}O\textsubscript{2} was mediated by the ubiquitin–proteasome system using proteasome inhibitor, MG132, and in vivo ubiquitination assay. Although we did not clarify the precise mechanism on MKP3 degradation via ubiquitination/degradation pathways, increasing evidences have demonstrated that phosphorylation of MKP3 is the possible mechanism leading to proteasomal degradation of MKP3 (34,35). Indeed, numerous studies have shown that ROS is able to regulate the phosphorylation and ubiquitination of proteins such as Bcl-2 family proteins (36), p21\textsuperscript{Waf1/Cip1} (37) and Arg tyrosine kinase (22). We therefore examined whether the phosphorylation of MKP3 is associated with proteasomal degradation by generating point mutations at serines 159 and 197 of MKP3 according to recent reports (34,35). The loss-of-function mutation on these two phosphorylation sites of MKP3 could reduce the level of proteasomal degradation. However, certain level of proteasomal degradation on this mut-MKP3 was still observed, suggesting that there are other phosphorylation sites of MKP3 or interacting partners responsible for MKP3 proteasomal degradation. Recent studies have reported that casein kinase 2-alpha could interact and phosphorylate MKP3 (34,38). Protein casein kinase 2 is a ubiquitous Ser/Thr kinase and plays as anchoring elements and/or as a docking platform for proteins involved in ubiquitination such as the E2 ubiquitin-conjugating enzyme UBC3B (39,40). Therefore, further investigations are needed to investigate the relationship between casein kinase 2-alpha and the phosphorylation and degradation of MKP3. However, the present Fig. 6. The expression levels of MKP3 control cisplatin resistance of ovarian cancer cells. (A) Cisplatin-sensitive (A2780s and OV2008) and cisplatin-resistant counterparts (A2780cp and C13\textsuperscript{+}) were treated with different concentration of cisplatin (5 and 10 \textmu M) for 24 h. (B) Pretreatment of cisplatin-resistant cell lines, A2780cp and C13\textsuperscript{+}, by PD98059 (20 \textmu M) for 3 h significantly reduced cell viability under treatment of cisplatin (5 and 10 \textmu M). *P = 0.005 and **P < 0.001. (C) Knockdown of MKP3 in A2780s ovarian cancer cells (shMKP3 C4 and shMKP3 C6) significantly enhanced cisplatin resistance. *P < 0.005. (D) MKP3 transfectants of A2780cp were treated by cisplatin with similar condition. Both C13 and C19 MKP3 transfectants with relatively high level of V5/MKP3 showed significant increased sensitivity to cisplatin treatment for A2780cp cells. *P < 0.001. The percentage of cell viability was analyzed by XTT proliferation assay. (E) The cisplatin resistance of MKP3 stably transfected A2780cp cells in vivo. Left, the vector control and Right, C19 MKP3 transfectant, of A2780cp cells were investigated the cisplatin resistance in nude mice. Mice were divided into two groups of five and treated with either saline as control or cisplatin (5 mg/kg CDDP). Arrows represent the injection. The relative tumor size was calculated relative to those of the first day of treatment (day 0) and represented as relative mean size (%) ± SE for each group. *P < 0.05, significantly different from saline control group.
findings have clearly demonstrated that the frequent loss of MKP3 protein particularly in cisplatin-resistant and highly tumorigenic ovarian cancer cells is due to the accumulation of ROS during the development and progression of ovarian cancer.

It is well known that constitutive activation of ERK-signaling pathway is crucially involved in the development of many cancers, including ovarian cancer. The overexpression of growth factor tyrosine kinase receptors or mutations in the Ras and BRAF gene families has been linked to aberrant activation of ERK pathway (2.41). This has attracted the exploration of anticancer agents targeting these receptors and kinases such as the Raf kinase inhibitor, BAY 43-9006, for the past decade (2). However, there is still a lack of studies on the anti-tumorigenic functions of the negative regulators of ERK-signaling pathway. Recent studies have identified that MKPs negatively regulate the mitogen-activated protein kinase kinases by dephosphorylation and inactivation of the mitogen-activated protein kinase (9,10).

MKP3, one of the MKPs members, was found to specifically bind to and inactivate ERK1/2 in mammalian cells (11–13,42). This means that MKP3 plays an important role in modulating the duration, magnitude and subcellular compartmentalization of ERK1/2 activity through a negative feedback mechanism (43). Therefore, low MKP3 expression may compromise its ability to control ERK1/2 function activated by upstream factors such as tyrosine kinase receptors, mutated Ras or BRAF. Indeed, the loss of MKP3 has been found in pancreatic cancer (14–17). Consistent with these findings, we found that MKP3 was downregulated in ovarian cancer and this led to high levels of ERK1/2 activity. In fact, we have proved in this study that knockdown of endogenous MKP3 increased ERK1/2 activity which in turn enhanced cell proliferation and anchorage-independent growth ability of A2780s ovarian cancer cell line. On the contrary, enforced expression of MKP3 in MKP3-deficient ovarian cancer cells inhibited their cell proliferation rate, anchorage-independent growth ability and tumor growth in nude mice.

Cisplatin is one of the most potent antimutator agents commonly used in ovarian cancer (44). However, the major limitation of using this drug is the acquisition of resistance to initially responsive tumors (45). Many studies have documented that constitutive activation of ERK1/2 is involved in cisplatin resistance in ovarian cancer, whereas inhibition of ERK1/2 activity by PD98059 can increase sensitivity to cisplatin-induced apoptosis (8,46). Similar to these findings, we demonstrated that inhibition of ERK1/2 activity by MKP3 could increase sensitivity of cisplatin-resistant ovarian cancer cells to cisplatin-induced apoptosis and could inhibit tumor growth in nude mice. Interestingly, the enhanced sensitivity by MKP3 overexpression was much more obvious than PD98059 treatment. This suggests that constitutive expression of MKP3 in MKP3 transfectants has a stronger influence on the suppression of ERK1/2 activity and cisplatin resistance.

In conclusion, our results suggest that the loss of MKP3 is a crucial factor in the aberrant activation of ERK1/2 in ovarian cancer. Importantly, our findings indicate the accumulation of intracellular ROS may induce ubiquitination/degradation of MKP3 during the development and progression of ovarian cancer. The understanding of this molecular mechanism may assist the development of new therapeutic interventions for this disease.

Supplementary material

Supplementary Figures S1–S3 can be found at http://carcin.oxfordjournals.org/

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References


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