Human Cancer Biology

HDM2 Regulation by AURKA Promotes Cell Survival in Gastric Cancer

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Abstract

Purpose: Suppression of P53 (tumor protein 53) transcriptional function mediates poor therapeutic response in patients with cancer. Aurora kinase A (AURKA) and human double minute 2 (HDM2) are negative regulators of P53. Herein, we examined the role of AURKA in regulating HDM2 and its subsequent effects on P53 apoptotic function in gastric cancer.

Experimental Design: Primary tumors and in vitro gastric cancer cell models with overexpression or knockdown of AURKA were used. The role of AURKA in regulating HDM2 and cell survival coupled with P53 expression and activity were investigated.

Results: Overexpression of AURKA enhanced the HDM2 protein level; conversely, knockdown of endogenous AURKA decreased expression of HDM2 in AGS and SNU-1 cells. Dual co-immunoprecipitation assay data indicated that AURKA was associated with HDM2 in a protein complex. The in vitro kinase assay using recombinant AURKA and HDM2 proteins followed by co-immunoprecipitation revealed that AURKA directly interacts and phosphorylates HDM2 protein in vitro. The activation of HDM2 by AURKA led to induction of P53 ubiquitination and attenuation of cisplatin-induced activation of P53 in gastric cancer cells. Inhibition of AURKA using an investigational small-molecule specific inhibitor, alisertib, decreased the HDM2 protein level and induced P53 transcriptional activity. These effects markedly decreased cell survival in vitro and xenograft tumor growth in vivo. Notably, analysis of immunohistochemistry on tissue microarrays revealed significant overexpression of AURKA and HDM2 in human gastric cancer samples (P < 0.05).

Conclusion: Collectively, our novel findings indicate that AURKA promotes tumor growth and cell survival through regulation of HDM2-induced ubiquitination and inhibition of P53. Clin Cancer Res; 1–11. ©2013 AACR.

Introduction

Gastric cancer exhibits poor patient survival rates due to intrinsic resistance to chemotherapeutic drugs (1, 2). According to current estimates, gastric cancer is a frequently diagnosed disease worldwide with an estimated incidence rate of approximately one million cases and mortality rate of 740,000 cases, respectively (3, 4). Multiple oncogenic signaling mechanisms have been shown to mediate cancer cell survival and drug resistance against several chemotherapeutics in gastric cancers (5–9). Unfortunately, the survival rate for patients with gastric cancer has shown only marginal improvement (10). Therefore, investigations specifically aimed at further understanding of the mechanisms regulating cell death and drug resistance in gastric cancer are essential for the development of novel effective anticancer therapeutic regimens.

Aurora kinase A (AURKA), a serine/threonine cell-cycle kinase, has been mapped to the 20q13 chromosomal region, which is frequently amplified in gastric cancers (11). Frequent amplification and/or overexpression of AURKA have been reported in breast, colon, esophageal, gastric, liver, ovarian, and pancreatic cancers (8, 12–18). AURKA plays an important role in facilitating mitosis and its expression is tightly regulated in normal cells. However, deregulated overexpression of AURKA causes genetic instability, dedifferentiated morphology, and oncogenic transformation (6, 13). Overexpression of AURKA promotes progrowth and anti-apoptotic signaling pathways resulting in cancer cell proliferation, drug resistance, and poor patient prognosis in gastric cancers (13, 19). The potent

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Translational Relevance
Gastric cancer is characterized by poor patient survival and resistance to chemotherapy. Several chemotherapeutic agents used in the treatment of gastric cancer induce DNA damage and activate P53 (tumor protein 53) pro-apoptotic functions. Conversely, Aurora kinase A (AURKA) and human double minute 2 (HDM2) suppress P53 protein expression and activity. Herein, we report, for the first time, that AURKA and HDM2 proteins are frequently co-overexpressed in gastric cancer tissues and cell lines. Our data indicate that AURKA regulates the expression and phosphorylation levels of HDM2, which could be a major mechanism attenuating the P53 protein function in gastric cancer cells. The use of AURKA inhibitor, alisertib, reversed these effects, leading to significant suppression of tumor cell growth in vitro and in vivo. The fact that AURKA activates HDM2 and is frequently overexpressed in gastric cancer strongly justifies the use of AURKA inhibitors in the treatment of gastric cancer.

docetaxel, cisplatin, and 5-fluorouracil (DCF) chemotherapy regimen is frequently used against gastric cancers (20, 21). Nonetheless, AURKA overexpression has been shown to mediate resistance against taxol and cisplatin and can thereby undermine the therapeutic outcome of the DCF regimen in gastric cancers (22, 23). The efficacy of chemotherapeutic drugs is dependent on induction of apoptosis by the P53 (tumor protein 53) family of pro-apoptotic proteins. We and others have previously reported that overexpression of AURKA can attenuate both P53 and P73 (tumor protein 73) protein expression and function (24, 25). These findings indicate that constitutive overexpression of AURKA can mediate poor response to chemotherapy in gastric cancers. Therefore, further investigation of mechanism(s) by which AURKA suppresses P53 expression and function can aid in the development of novel and effective therapeutic strategies against gastric cancers.

Human double minute 2 (HDM2), an E3-ubiquitin ligase, is one of the critical negative regulators of P53 protein function and expression. HDM2 has been shown to inhibit P53 by blocking P53 transcriptional activity, promoting cytosolic translocation of P53 from the nucleus, and tagging it with ubiquitin for proteasomal degradation (26). HDM2 gene amplification has been reported in various cancers and when overexpressed HDM2 becomes a bona fide oncogene that can promote malignant transformation and drug resistance (27, 28). Given the fact that HDM2 plays a critical role in regulating P53, a function shared with AURKA, we postulated that activation of HDM2 by AURKA regulates P53 in gastric cancer cells. In this study, we investigated the role of AURKA in regulating HDM2-mediated P53 inhibition affecting gastric tumor growth and cell survival. We also examined the role of an investigational small molecule–specific inhibitor of AURKA, alisertib, in suppressing HDM2 and promoting cell death in vitro and in vivo.

Materials and Methods
Cell culture and pharmacologic reagents
The gastric adenocarcinoma cell lines (AGS, SNU-1, SNU-16, MKN28, MKN45, MKN75, Kato III, and RF-1) were obtained from American Type Culture Collection (ATCC) and RIKEN BioResource Center. The cell lines were maintained in F-12 or Dulbecco’s Modified Eagle Medium (DMEM; Gibco) supplemented with 10% (v/v) FBS (Gibco; ref. 29). All cell lines were ascertained to conform to in vitro morphologic characteristics.

Alisertib (Millennium Pharmaceuticals, Inc.) solutions for in vitro and in vivo studies were prepared as described previously (8). Nutlin3A (Cayman Chemicals) solution was prepared in dimethyl sulfoxide (DMSO; Sigma-Aldrich). Cisplatin (CDDP; APP Pharmaceuticals, LLC.) solution (3.3 mmol/L) was prepared in sterile water. AKT, p-AKT (Ser473), p-AURKA (Thr288), AURKA, HDM2, p-HDM2 (Ser166), P53, P21, and β-actin primary antibodies were obtained from Cell Signaling Technology.

AURKA and HDM2 expression and plasmids
The AURKA expression plasmid was generated as described previously (30). The HDM2 expression plasmid was purchased from Addgene. Transient transfection of gastric cancer cells was performed using X-tremeGENE HP (Roche Applied Sciences). The recombinant adenovirus expressing AURKA or control was generated as described previously (31).

Clonogenic cell survival assay
Gastric cancer cells were seeded at 5,000 cells per well in a six-well plate and treated with vehicle (DMSO) or alisertib (0.25–5.0 μmol/L) for 24 hours. Next, cells were cultured for 10 days and colonies were stained and quantified as described previously (8).

Western blot analysis
Cells were lysed in lysis buffer (50 mmol/L Tris-HCl buffer, pH 7.4, 150 mmol/L NaCl, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS) supplemented with 1× Halt Protease Inhibitor Cocktail (Pierce). Proteins were analyzed by Western blot as described previously (32).

Dual immunofluorescence
Gastric cancer cells plated in eight-chamber slides (BD Falcon) were permeabilized and fixed in 2% paraformaldehyde. Cells were then incubated in a mixture of rabbit AURKA (1:100) and mouse HDM2 (1:100) primary antibodies for 3 hours. After washing with PBS, cells were stained with Alexa Fluor 488 anti-rabbit and Alexa Fluor 568 anti-mouse secondary antibodies. The cells were washed and mounted with 4’,6-diamidino-2-phenylindole (DAPI) and examined by fluorescence microscopy (Olympus America Inc.).
Immunoprecipitation

Immunoprecipitation was performed as described previously (33). Briefly, cells were lysed in lysis buffer and proteins were immunoprecipitated at room temperature with primary antibodies previously bound to 50 μL Dynabeads Protein G (Invitrogen).

In vitro kinase activity assay

Active human recombinant AURKA (Cell Sciences) and HDM2 (Thermo Scientific) proteins were used for an in vitro kinase assay. Briefly, increasing concentrations of AURKA (0.2–1.0 μg) were added to a fixed concentration of HDM2 (0.5 μg) in the assay buffer. The reaction mixtures were incubated at 37°C for 30 minutes to initiate kinase activity, and the protein samples were subjected to Western blot analysis.

In vivo tumor xenograft

AGS cells (4 × 10^6) suspended in 200 μL of DMEM Matrigel mixture (50% DMEM supplemented with 10% FBS and 50% Matrigel) were injected into the flank regions of female athymic nude-Foxn1 nu/nu mice (Harlan Laboratories Inc.). The tumors were allowed to grow until 200 mm^3 in size before starting the treatment with daily alisertib (30 mg/kg, orally) for 21 days. Tumor xenografts were measured every 4 days and tumor size was calculated according to the following formula: \[ T_{\text{vol}} = L \times W^2 \times 0.5 \], in which \( T_{\text{vol}} \) is tumor volume, \( L \) is tumor length and \( W \) is tumor width (34). At the end of treatment, the xenograft tumors were collected and processed for quantitative real-time PCR (qRT-PCR; PUMA, NOXA, P21, and BAX) or immunohistochemistry (IHC; P53, SC-126, and HDM2; SMPI4; Santa Cruz Biotechnology, Inc.) antibodies as described previously (31).

Tissue microarray and IHC

IHC was performed on tissue microarrays (TMA) containing 94 deidentified archival cases of gastric cancer and 113 cases of esophageal adenocarcinomas (EAC) along with 71 normal gastric epithelial tissue samples and 26 normal esophageal tissue samples. All tissue samples were obtained in accordance with the Institutional Review Board (IRB)-approved protocols at Vanderbilt University. Five-micrometer thick TMA sections of normal and tumor tissue samples were used for IHC staining of AURKA and HDM2 proteins. The intensity and frequency of staining was scored as described previously (35).

AKT and HDM2 silencing by siRNA

AGS cells overexpressing AURKA or control vector were transfected with AKT siRNA, HDM2 siRNA, or control siRNA for 48 hours, purchased from Cell Signaling Technology and Integrated DNA technologies Inc., respectively. The cell lysates were analyzed by Western blot.

Statistical analysis

Data are presented as means ± SEM. All in vitro experiments were performed in triplicates. ANOVA with Tukey post hoc analysis was used to evaluate statistical difference between groups. Statistical analyses were carried out using GraphPad Prism 5 software (GraphPad Software Inc.). The correlation between two parameters was determined by the Spearman correlation and \( \chi^2 \) test. The \( P \) value of 0.05 or less was considered statistically significant.

Results

AURKA regulates HDM2 and cell survival in gastric adenocarcinoma cells

We examined AURKA and HDM2 protein expression in gastric adenocarcinoma (gastric cancer) cell lines. The Western blot analysis data indicated frequent concomitant overexpression of AURKA and HDM2 proteins in 5/8 gastric cancer cell lines with the RF-1 cell line exhibiting relatively low expression of AURKA and HDM2 (Fig. 1A). Similarly, the data showed that 4/5 EAC cell lines concomitantly overexpressed AURKA and HDM2 proteins, and 3/3 untransformed immortalized esophageal cells were negative for AURKA and HDM2 expression (Supplementary Fig. S1). We have previously reported that AURKA suppresses P53 protein function in gastric cancer cells (36). HDM2 is an E3-ubiquitin ligase closely involved in regulating P53 protein expression and stability. Because AURKA and HDM2 are frequently overexpressed in gastric cancer cell lines, we hypothesized that AURKA regulates HDM2 expression in gastric cancer. To test this hypothesis, we used P53 wild-type AGS and SNU-1 gastric cancer cell lines in vitro models. The Western blot analysis indicated that transient overexpression of AURKA using adenovirus system enhanced HDM2 protein levels in AGS and SNU-1 cells (Fig. 1B). Following AURKA overexpression, P53 and its downstream targets (P21 and BAX) were downregulated in AGS cells. Conversely, siRNA-mediated knockdown of endogenous AURKA increased P53, P21, and BAX expression in AGS cells. AURKA overexpression and knockdown exhibited similar effects on HDM2, P53, and P21 in SNU-1 cells (Fig. 1B). However, unlike AGS cells, we did not observe a change in BAX protein expression in SNU-1 cells. To investigate whether AURKA-mediated increase in the HDM2 protein level was dependent on its serine/threonine kinase activity, we used kinase-dead mutant AURKA (D274A). The Western blot analysis revealed that, unlike wild-type AURKA, kinase-dead mutant AURKA (D274A) did not significantly affect HDM2 expression in the AGS cells (Fig. 1C). To validate the role of AURKA in regulating HDM2 and examine its impact on cell survival, we pharmacologically inhibited AURKA with alisertib, an investigational AURKA-specific inhibitor, in AGS and SNU-1 cells. The Western blot analysis data confirmed that inhibition of AURKA led to a significant decrease in the HDM2 protein level coupled with upregulation of P53 and P21 expression in both cell lines (Fig. 1D). The clonogenic survival assay data indicated that alisertib-mediated inhibition of AURKA activity significantly suppressed AGS (\( P < 0.01 \)) and SNU-1 (\( P < 0.01 \)) cell survival (Fig. 1E).
AURKA directly interacts and phosphorylates HDM2 in gastric cancer

HDM2 localizes to the nucleus, inducing ubiquitination and degradation of the P53 protein in the proteasome (26). The dual-immunofluorescence analyses results indicated that transient overexpression of AURKA significantly enhanced HDM2 protein expression, confirming the Western blot analysis data (Fig. 1B). The immunofluorescence data showed an overlap of AURKA and HDM2 signals in the merged image, suggesting colocalization of AURKA and HDM2 in both AGS and SNU-1 cells (Fig. 2A and B). We have previously reported that AURKA phosphorylates AKT at Ser473 amino acid residue and active AKT has been shown to phosphorylate HDM2 at the Ser166 site (36, 37). Therefore, it is plausible that AURKA-mediated increase in HDM2 protein expression could be mediated by an AKT-dependent mechanism. To examine this hypothesis, we transiently overexpressed AURKA in AGS cells and evaluated HDM2 protein levels after treatment with an AKT inhibitor (AKTI) or genetic knockdown of AKT with siRNA. Overall, the Western blot analysis data showed that HDM2 expression levels were not significantly affected by AKT inhibition or knockdown in AURKA-overexpressing cells (Supplementary Fig. S2). Collectively, the data suggested
Together (MERGE), the data showed that overexpression of AURKA leads to ubiquitination and degradation of the P53 protein. Western blot analysis data indicated that AURKA-mediated down-regulation of P53 and its downstream target P21 was attenuated by Nutlin3A in AGS cells (Fig. 3C). As a confirmation of the effect of Nutlin3A, the data showed that exogenous HDM2-mediated decrease in P53 and P21 expression was partially blocked by Nutlin3A (Fig. 3C). Similar results were obtained in SNU-1 cells (Supplementary Fig. S3B). Genetic knockdown of HDM2 by siRNA abrogated AURKA-induced suppression of P53 and P21 in AGS cells, confirming the results obtained by Nutlin3A (Supplementary Fig. S3C). Together, these results indicated that AURKA-induced downregulation of P53 is dependent on HDM2. In addition, we evaluated resistance to cytotoxic effects of CDDP in AGS cells overexpressing AURKA. The survival data indicate that transient overexpression of AURKA promotes resistance to CDDP in AGS cells (Supplementary Fig. S4).

To ascertain if AURKA interacts with HDM2, we performed dual co-immunoprecipitation (Co-IP) following overexpression of AURKA in AGS cells. The dual Co-IP data indicated the presence of endogenous HDM2 protein in an immunocomplex with exogenous AURKA protein (Supplementary Fig. S5). To investigate if AURKA directly interacted and phosphorylated HDM2 in the presence or absence of alisertib, we carried out an in vitro kinase assay using recombinant AURKA and HDM2 proteins followed by Co-IP assay. The data indicated that AURKA directly interacts and phosphorylates HDM2; these effects were abrogated upon inhibition of AURKA with alisertib (Fig. 4A). To further confirm that AURKA directly phosphorylates HDM2, we performed the in vitro kinase assay with HDM2 and increasing concentrations of AURKA. The data indicated that AURKA protein can directly phosphorylate the HDM2 protein at the Ser166 residue in a concentration-dependent manner (Fig. 4B). Collectively, our data clearly indicated that AURKA can directly interact and phosphorylate HDM2.

**P53 suppression by AURKA is dependent on regulation of HDM2 in gastric cancer**

On the basis of our results showing that AURKA upregulates HDM2 expression, we postulated that AURKA can regulate P53 protein expression and function through modulation of HDM2 in response to DNA damage in gastric cancer cells. To test this hypothesis, we transiently overexpressed AURKA in AGS and SNU-1 cells, and treated them with vehicle or cisplatin (CDDP). The Western blot analysis data showed that AURKA overexpression attenuated CDDP-induced upregulation of P53 and P21 expression in AGS and SNU-1 cells (Fig. 3A and Supplemental Figure 3A). Because HDM2 regulates P53 through ubiquitination and proteasomal degradation, we hypothesized that AURKA can enhance P53 ubiquitination. To examine this hypothesis, we assessed the effect of AURKA and/or HDM2 expression on P53 ubiquitination in AGS cells. The immunoprecipitation data indicated that transient overexpression of AURKA or HDM2 significantly enhanced P53 ubiquitination (Fig. 3B). To confirm that inhibition of P53 by AURKA is dependent on HDM2, we transiently overexpressed AURKA or HDM2 and treated with vehicle or Nutlin3A in AGS and SNU-1 cells. Nutlin3A is a HDM2 inhibitor that prevents HDM2 from binding to P53, thereby preventing ubiquitination and degradation of the P53 protein. Western blot analysis data indicated that AURKA-mediated down-regulation of P53 and its downstream target P21 was attenuated by Nutlin3A in AGS cells (Fig. 3C). As a confirmation of the effect of Nutlin3A, the data showed that exogenous HDM2-mediated decrease in P53 and P21 expression was partially blocked by Nutlin3A (Fig. 3C). Similar results were obtained in SNU-1 cells (Supplementary Fig. S3B). Genetic knockdown of HDM2 by siRNA abrogated AURKA-induced suppression of P53 and P21 in AGS cells, confirming the results obtained by Nutlin3A (Supplementary Fig. S3C). Together, these results indicated that AURKA-induced downregulation of P53 is dependent on HDM2. In addition, we evaluated resistance to cytotoxic effects of CDDP in AGS cells overexpressing AURKA. The survival data indicate that transient overexpression of AURKA promotes resistance to CDDP in AGS cells (Supplementary Fig. S4).

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**Pharmacologic inhibition of AURKA induces P53 through downregulation of HDM2 in vivo**

To establish the role of AURKA in regulating HDM2 and thereby modulating P53 expression and function in vivo, we treated AGS mouse xenografts with alisertib and examined the tumor growth and expression levels of HDM2 and P53. In addition, we assessed P53 transcriptional activity by measuring the mRNA levels of P53 downstream targets, PUMA, NOXA, P21, and BAX. The treatments were initiated after the tumor xenografts reached 200 mm³ in size. The endpoint data for day 21 indicated that treatment with alisertib significantly reduced tumor volume by approximately 55-fold relative to control (P < 0.01; Fig. 5A). The IHC data revealed that alisertib-mediated inhibition of AURKA significantly suppressed HDM2 expression (P = 0.01) and induced P53 expression (P < 0.01) in the AGS xenografts (Fig. 5B and C). The qRT-PCR data showed a significantly higher induction of P53 downstream targets, P21 (P < 0.01), BAX (P < 0.05), NOXA (P < 0.05), and PUMA (P = 0.01) in alisertib-treated AGS xenografts than control (Fig. 5D). In accordance with the in vitro data, the xenograft...
results clearly show that AURKA inhibition induces P53 through downregulation of HDM2 in vivo.

Frequent overexpression of AURKA and HDM2 are directly correlated in human gastric cancer

We examined AURKA and HDM2 protein expression in human gastric TMA containing 94 gastric adenocarcinoma (GC) and 71 normal gastric (NT) tissue samples by IHC. The IHC data showed that both AURKA and HDM2 expression levels were highly overexpressed in gastric adenocarcinoma tissue samples as compared with normal tissue samples (Fig. 6A and B). The data indicated that AURKA was localized in both the nucleus and cytoplasm, whereas HDM2 was mainly localized in the nucleus (Fig. 6A and B). The IHC analyses revealed that HDM2 was overexpressed in approximately 56% (53/94) of gastric adenocarcinoma tissue samples (Fig. 6D). In addition, AURKA was similarly overexpressed in 70.4% (50/73) of gastric adenocarcinoma tissues (Fig. 6C). The data also indicated a significant direct correlation between AURKA and HDM2 protein expression in gastric adenocarcinoma tissues ($r = 0.41; P = 0.04$; Fig. 6E). Similar results with regard to AURKA and HDM2 overexpression and correlation were obtained in human esophageal TMA containing 113 EAC and 26 normal esophageal (NT) tissue samples by IHC analysis (Supplementary Fig. S6). Together, these results demonstrate that frequent overexpression of AURKA and HDM2 is directly correlated in human gastric and esophageal adenocarcinoma.

Discussion

Gastric cancer is the second most frequently diagnosed form of cancer accounting for 10% of all cancer-related deaths worldwide (4). Despite improvement in chemotherapy, the 5-year survival rate for patients with gastric cancer is a dismal 22% (38, 39). Development of resistance to chemotherapeutic drugs is a major cause for disease recurrence and low patient survival rates (40–42). Therefore, identification of molecular mechanism(s) mediating chemotherapeutic drug resistance is vital for developing more effective therapeutic strategies against gastric cancer. In this
study, we examined the role of AURKA in regulating cell survival and apoptosis in gastric cancer. Herein, we report for the first time that HDM2 and AURKA are frequently overexpressed in gastric cancer cell lines and in approximately half of primary gastric cancer tumors. Similar results were obtained from EAC tumors and cell lines, suggesting that overexpression of AURKA and HDM2 is a common molecular event in upper gastrointestinal cancers.

We have previously reported that AURKA suppresses P53 transcriptional activity in gastric cancer (36). P53 is a vital transcriptional factor that induces expression of pro-apoptotic proteins in response to DNA-damaging chemotherapeutic agents. AURKA has been reported to inhibit P53 function by directly phosphorylating P53 at Ser315 and thereby inducing its proteolytic degradation in H1299 human non–small cell lung carcinoma cells (43). In addition, AURKA-mediated phosphorylation of P53 at Ser215 inhibits P53 transcriptional activity and suppresses expression of its downstream targets (24). Although we have previously shown that overexpression of AURKA inhibits P53 expression and activity (36), we did not observe AURKA/P53 interaction in gastric cancer cell models (data not shown). These results indicate that this protein interaction may be cell-line dependent, and suggest that AURKA-induced suppression of P53 is mediated by other mechanisms. HDM2, a critical negative regulator of P53, blocks P53 function by inhibiting its transcriptional activity through nuclear export into the cytoplasm and ubiquitin-mediated proteasomal degradation (26). Our results showed that overexpression of AURKA significantly increased the HDM2 protein level coupled with downregulation of P53 and P21 expression. Conversely, knockdown of endogenous AURKA or pharmacologic inhibition with alisertib attenuated HDM2 protein levels and upregulated P53 and P21 expression. In addition, mutant AURKA failed to affect expression of HDM2, indicating that AURKA function is dependent on its kinase

![Figure 4. AURKA directly associates and phosphorylates HDM2 protein. A, in vitro kinase assay followed by a subsequent immunoprecipitation with AURKA antibody was done on recombinant AURKA and HDM2 proteins. Western blot analysis of the immunoprecipitates showed that AURKA-mediated interaction of HDM2 is dependent on AURKA kinase activity. B, Western blot analysis of in vitro kinase assay with recombinant AURKA, HDM2, AKT, and GPX7 proteins indicated that AURKA directly phosphorylates HDM2 in a concentration-dependent manner. AKT and GPX7 recombinant proteins were used as positive and negative controls, respectively. GPX7, glutathione peroxidase enzyme.](image)
activity. These results clearly showed that AURKA is a positive regulator of HDM2 in gastric cancer cells.

Phosphorylation of HDM2 (S166) by AKT is known to enhance HDM2-mediated P53 ubiquitination and degradation (26). Of note, we have previously shown that AURKA can phosphorylate AKT at Ser473 (36). Using immunofluorescence, we confirmed the AURKA mediates an increase in HDM2 protein levels. This is in accordance with previous reports showing that both AURKA and HDM2 localize to the nucleus, thereby promoting mitosis and suppressing P53 function, respectively (44, 45). The co-IP data confirmed that AURKA and HDM2 were present in the same immunoprotein complex in gastric cancer cells. The co-IP and \textit{in vitro} kinase assay data indicated, for the first time, that recombinant AURKA directly interacts with and phosphorylates HDM2 recombinant protein. We further validated this novel finding and demonstrated that inhibition of AURKA with alisertib blocks AURKA/HDM2 interaction \textit{in vitro}.

Because HDM2 is a major negative regulator of P53 (46), we examined the effects of AURKA on HDM2-mediated ubiquitination of P53. The immunoprecipitation data indicated that AURKA overexpression substantially enhanced HDM2-mediated ubiquitination of P53. To examine if AURKA-mediated suppression of P53 is dependent on HDM2/P53 interaction, we used Nutlin3A, an investigational HDM2-specific inhibitor with antitumor activity (47), to disrupt this protein interaction. Indeed, the data showed that Nutlin3A treatment can abrogate AURKA-mediated inhibition of P53, confirming that HDM2/P53 interaction is critical for AURKA-dependent suppression of P53.

Our aforementioned results confirm that AURKA activity is essential for enhancing HDM2-induced ubiquitination and degradation of P53. This could be an important...
mechanism that mediates resistance to chemotherapeutic drugs whereby activation of HDM2 prevents apoptosis by downregulating P53 expression. Accordingly, our data indicated that AURKA overexpression significantly attenuated CDDP-mediated induction of P53 and P21 protein expression. This finding provides further evidence that AURKA can mediate resistance to CDDP through regulation of HDM2/P53 signaling. Conversely, the inhibition of AURKA with alisertib attenuated cell survival in a dose-dependent manner, and was associated with decreased expression of HDM2 coupled with increased P53 and P21 protein levels in gastric cancer cells. In line with the in vitro data, the tumor xenograft data and molecular analysis indicated that inhibition of AURKA with alisertib significantly reduced tumor growth coupled with downregulation of HDM2 and induction of P53 and its downstream transcriptional targets. This underscores the importance of AURKA as an effective therapeutic target upstream of HDM2 in gastric cancer. Our data further support the ongoing clinical trials with AURKA-specific small-molecule pharmacologic inhibitors (clinicaltrials.gov). In addition, we have previously reported that AURKA regulates cell death in P53-deficient cancer cells by inhibiting P73 apoptotic protein expression and activity (8, 25). HDM2 has been shown to attenuate P73 protein function by suppressing P73 transcriptional activity (48). Therefore, in addition to AURKA-mediated direct regulation of p73 protein expression, AURKA–HDM2 axis could be an alternative mechanism that regulates cell death in P53-deficient cancer cells.

In summary, we demonstrated that AURKA and HDM2 are overexpressed in gastric cancer. Upregulation of AURKA enhanced expression and phosphorylation of HDM2 coupled with suppression of P53 and its downstream targets. Therefore, targeting AURKA–HDM2 axis with AURKA inhibitors could be an effective therapeutic approach in gastric cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Figure 6. Frequent overexpression and direct correlation between AURKA and HDM2 in human gastric cancer tissues. A and B, representative images of AURKA and HDM2 IHC staining in normal gastric (NT) and gastric cancer (GC) tissue samples are shown at ×40 magnification. The data showed that AURKA and HDM2 protein expression levels are significantly higher in GC than NT. C and D, IHC analysis of human gastric tissue array exhibited significantly high expression scores for AURKA and HDM2 proteins in GC than NT (P < 0.05). E, AURKA and HDM2 protein expression correlation analysis in human gastric tissue array indicated strong direct correlation between AURKA and HDM2 expression (P = 0.04; r = 0.41; n = 24) in GC.
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