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Original Paper

The Deubiquitinating Enzyme USP14 Regulates Leukemic Chemotherapy Drugs-Induced Cell Apoptosis by Suppressing Ubiquitination of Aurora Kinase B

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Key Words
Aurora B • USP14 • b-AP15 • Leukemia • Deubiquitinating enzyme

Abstract
Background/Aims: Aurora kinase B is a mitotic checkpoint kinase that plays a pivotal role in mitosis by ensuring correct chromosome segregation and normal progression through mitosis. Aurora B has been found to be amplified and overexpressed in several types of leukemia. The deubiquitinating enzyme USP14 is one of three proteasome-associated deubiquitinating enzymes and plays critical roles in diverse biological processes including cancer. However, whether USP14 has a role in leukemia cells remains elusive. ***Methods:*** Leukemic U937, NB4 and Jurkat cells were treated with diverse apoptosis-inducing drugs. The interaction between USP14 and Aurora B were determined by Western blot. The effect of USP14 in the regulation of Aurora B was detected by cycloheximide (CHX) and deubiquitination assays. FACS assay was used to determine the apoptosis ratio of cells after treatments. ***Results:*** We found that Aurora B was ubiquitinated and degraded during leukemic chemotherapy drugs-induced cell apoptosis. FBXW7 mediated Aurora B ubiquitination and degradation during chemotherapeutic drugs-induced apoptosis. USP14 associated with Aurora B and prevented Aurora B degradation. Functionally, overexpression of USP14 inhibits chemotherapeutic drugs-induced apoptosis in leukemia cells. On the contrary, administration of b-AP15, a specific inhibitor of USP14, significantly increased leukemia cells apoptosis in a dose-dependent manner. ***Conclusion:*** Thus, our data suggest that USP14 plays a novel critical role of in leukemia cells apoptosis through Aurora B stabilization and USP14 could be a potential therapeutic target for leukemia.

Introduction

The Aurora family of serine/threonine kinases consists of 3 members: Aurora A, B, and C, which share 67% to 76% amino acid sequence identity in catalytic domains, but various in NH2 terminus [1]. This family plays a critical role in chromosome alignment, segregation,

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and cytokinesis during mitosis [2]. Aurora B is part of the chromosomal passenger protein complex (CPC) and localizes at centromeres during prometaphase [3]. However, during anaphase and telophase, Aurora B relocates to midzone microtubules and midbodies and plays a role in chromosome alignment, kinetochore-microtubule biorientation, activation of the spindle assembly checkpoint, and cytokinesis [4]. Previous studies found that Aurora B was aberrantly expressed in hematologic malignant cells including those from acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL), and chronic myeloid leukemia (CML) [2, 5, 6]. In addition, AZD1152, a selective inhibitor of Aurora B kinases, effectively induced growth arrest and apoptosis of a variety of types of leukemia cells [6, 7].

The ubiquitin-mediated protein degradation pathway is important for controlling the abundance of proteins and plays a critical role cell cycle progress [8, 9]. Recently studies found the protein level of Aurora B is strictly controlled during each stage of mitosis, primarily through ubiquitination-mediated degradation [10]. Moreover, several E3 ligase complexes including FBXW7 and FBXL2 were reported to be able to target Aurora B for destruction [11, 12]. Although ubiquitination-mediated degradation of Aurora B has been studied, a detailed mechanistic regulatory pathway for Aurora B stability remains unclear.

In this study, we found that Aurora B is ubiquitinated during apoptosis in leukemia cells and a deubiquitinating enzyme USP14 interacted with Aurora B and was able to further indicate that USP14 positively regulates Aurora B via the deubiquitination pathway. Our data demonstrate the role of USP14 in controlling Aurora B upon chemotherapeutic drugs-induced apoptosis in leukemia cells and might be a potential therapeutic target for leukemia.

Material and Methods

Cell culture and reagents

Leukemic U937, NB4 and Jurkat cells were cultured in RPMI-1640 medium, supplemented with 10% fetal bovine serum (HyClone). Etoposide (VP16), paclitaxel (PTX), thapsigargin (TG), cycloheximide (CHX) and MG132 were purchased from Sigma. DMSO, b-AP15 and doxorubicin (DOX) were purchased from MedChemExpress (Princeton, USA).

Plasmids and transfection

Human USP14, FBXW7, FBXL2 and Aurora B cDNAs were amplified from the 293T cells by reverse transcription (RT)-PCR and subcloned into pMSCVpuro retroviral transfer vector (Clontech, Palo Alto, CA). The sequence of the cDNAs was verified by nucleotide sequencing.

Cycloheximide inhibition test

U937 cells were treated with etoposide in the presence or absence of 20 μg/ml cycloheximide (CHX, Sigma–Aldrich, USA) for the indicated times. The expression of Aurora B protein was measured by Western blot with β-actin as loading control.

Western blot

Leukemic cells were lysed in radioimmunoprecipitation (RIPA) buffer containing 50 mM Tris-HCl, 150 mM NaCl, 5 mM MgCl2, 2 mM EDTA, 1 mM NaF, 1% NP-40, and 0.1% sodium dodecyl sulfate. Western blots were performed using antibodies against FBXW7 and FBXL2 (Abcam, Cambridge, USA), Flag and HA (Sigma), cleaved caspase-3, Aurora B and USP14 (cell signaling, Beverly, MA), and β-actin (Calbiochem, Darmstadt, Germany).

Real-time PCR

Total RNA was isolated from leukemic cell lysates using the TRIzol method according to the manufacturer's instructions (Invitrogen, Shanghai, China). In order to quantify the transcripts of the genes of interest, quantitative real-time PCR was performed using a SYBR Green Premix Ex Taq (Takara, Japan) on Light Cycler 480 (Roche, Switzerland). Relative quantitation analysis of gene expression data was calculated according to the $2^{-\Delta\Delta Ct}$ method.

Apoptosis assay

Apoptosis was measured by the Annexin V-FITC Apoptosis detection kit (Merck biosciences, Darmstadt, Germany) following the manufacturer's instructions. Annexin V-positive and PI-negative cells were considered to be in the early apoptotic phase and those having positive staining both for Annexin V and PI were deemed to undergo late apoptosis or necrosis.

Statistical analysis

Student's t-test was used to evaluate the difference between two different groups. All experiments were repeated three times with similar results. $P < 0.05$ was considered statistically significant.

Results

Aurora B is down-regulated during apoptosis in leukemia cells

To investigate the function of Aurora B in leukemic cells, we first checked the endogenous protein level of Aurora B at the time when apoptosis was raised. Leukemic cell line U937 cells were treated with etoposide (VP16), a chemotherapeutic drug which induced the appearance of the active fragment of caspase 3 (Fig. 1A). We found that Aurora B was significantly decreased by VP16 treatment (Fig. 1A). To examine whether this effect is VP16-specific, U937 cells were treated with other apoptosis-inducing drugs including thapsigargin (TG), paclitaxel (PTX) as well as doxorubincin (DOX). All of these treatments led to down-regulation of Aurora B (Fig. 1B). In order to rule out cell type-specific effect, two additional leukemic cell lines NB4 and Jurkat were treated with VP16 for 12 hours. Like U937 cells, VP16 treatment also led to the reduction of Aurora B in these cells (Fig. 1C). Together, these data demonstrated that the down-regulation of Aurora B protein is a common event during chemotherapeutic drugs-induced apoptosis in leukemia cells

The ubiquitin–proteasome system mediates the down-regulation of Aurora B protein during chemotherapeutic drugs-induced apoptosis in leukemia cells

To investigate how Aurora B was down-regulated, we checked the mRNA level of Aurora B during VP16-induced apoptosis of U937 cells. As shown in Figure. 2A, Aurora B mRNA level remained unchanged in VP16-treated U937, NB4 and Jurkat cells. However, VP16 treatment could also decrease the expression of exogenous Aurora B, suggesting Aurora B might be

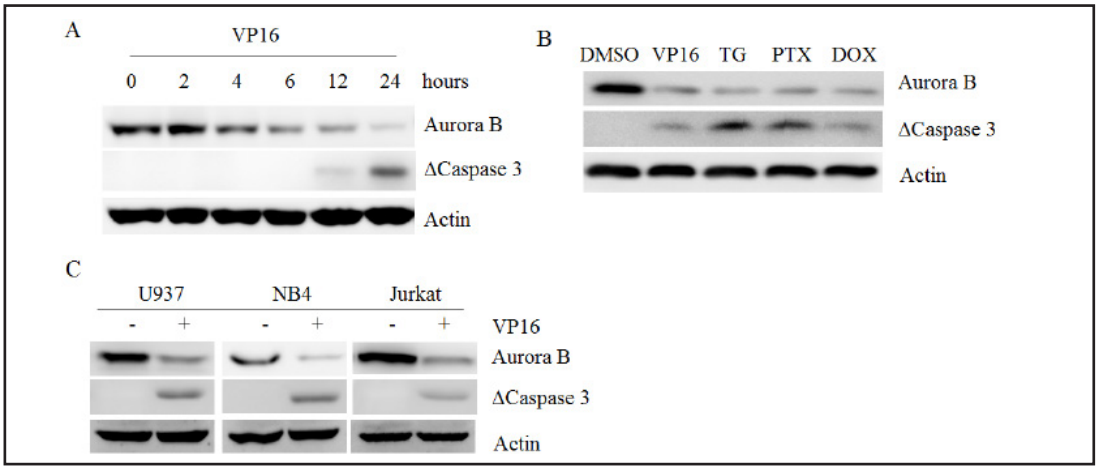

Fig. 1. Aurora B is down-regulated during apoptosis in leukemia cells. (A) U937 cells were treated with etoposide (VP16, 1 μM) for indicated times and the indicated proteins were detected by Western blot. (B) U937 cells were treated with thapsigargin (TG, 1 μM), paclitaxel (PTX, 5nM) as well as doxorubincin (DOX, 1 μM) for 12 hours and the indicated proteins were detected by Western blot. (C) U937, NB4 and Jurkat were treated with VP16 for 12 hours and the indicated proteins were detected by Western blot.

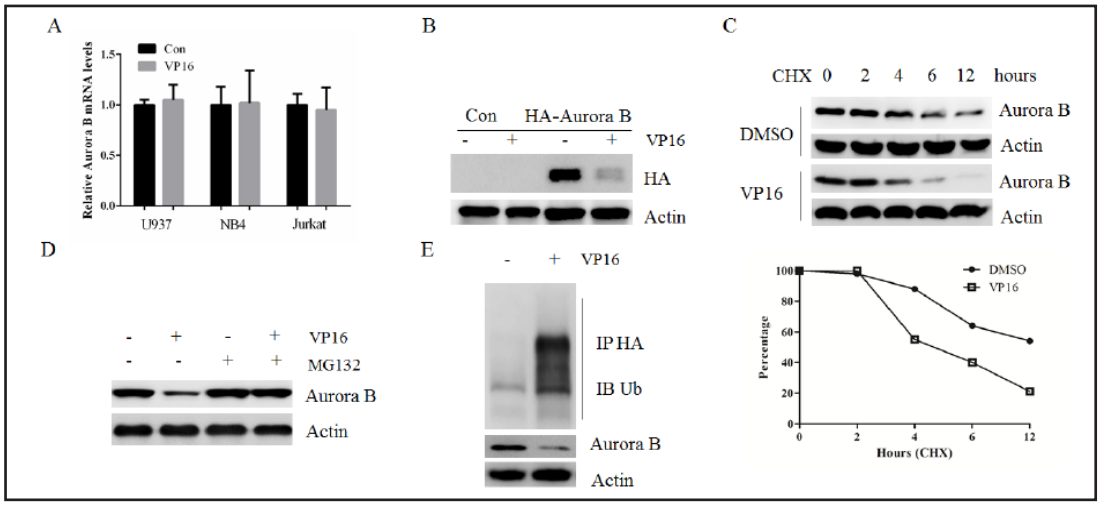

Fig. 2. The ubiquitin–proteasome system mediates the down-regulation of Aurora B protein during apoptosis. (A) U937 cells were treated with VP16 for 12 hours and the mRNA level of Aurora B was measured by real-time RT-PCR. (B) U937 cells transfected with or without HA-Aurora B were treated with VP16 for 12 hours. Cells were harvested and the indicated proteins were detected by Western blot. (C) U937 cells were treated with 20 μg/ml protein synthesis inhibitor cycloheximide (CHX) for 12 hours with or without VP16 treatment. Cells were harvested and the indicated proteins were detected by Western blot. (D) U937 cells were treated with VP16 for 12 hours and with or without 20μM MG132 for additional 4 hours. Cells were harvested and the indicated proteins were detected by Western blot. (E) U937 cells transfected with HA-Aurora B were treated with VP16 for 12 hours. Cells were harvested and subjected to Immunoprecipitation (IP) with anti-HA antibody, the indicated proteins were detected by Western blot.

regulated at the post-transcriptional level (Fig. 2B). To test this possibility, U937 cells were treated with 20 ug/ml protein synthesis inhibitor cycloheximide (CHX) with or without VP16 treatment for different time points. As depicted in Figure 2C, the half-life of Aurora B protein was shortened upon VP16 treatment. To identify which pathway is responsible for Aurora B protein degradation, U937 cells were treated with VP16 in the presence of proteasome inhibitor MG132. Our results showed that MG-132 could significantly block VP16-induced Aurora B degradation (Fig. 2D). Moreover, VP16 treatment rapidly increased the ubiquitinated Aurora B protein (Fig. 2E). All these data indicated Aurora B was rapidly ubiquitinated and degradated during apoptosis in leukemia cells.

FBXW7 is required for the degradation of Aurora B during apoptosis

Previous reports have shown that the ubiquitin E3 ligase complex SCF(FBXW7) and SCF(FBXL2) could mediated Aurora B ubiquitination and degradation [11, 12]. However, which E3 ligase is required for the degradation of Aurora B during apoptosis is still unclear. Thus, we silenced the expression of FBXW7 or FBXL2 by shRNAs, respectively. Although silencing the expression of both E3 ligases resulted in the increase of endogenous expression of Aurora B, VP16 treatment also decrease the expression of Aurora B in FBXL2-silenced U937 cells but not in FBXW7-silenced cells, indicating that FBXW7 is required for the degradation of Aurora B during apoptosis (Fig. 3A-B). However, VP16 treatment did not alter the expression of endogenous FBXW7 (Fig. 3B), suggesting that other mechanisms might be involved in the regulation of Aurora B during apoptosis.

USP14 interacts with and prevents apoptosis-induced Aurora B degradation through deubiquitination

By searching several large protein-protein interaction databases, we found that USP14 was one of the binding partners of Aurora B [13]. As USP14 is a deubiquitinating enzyme and has been reported to be associated with various signal transduction pathways

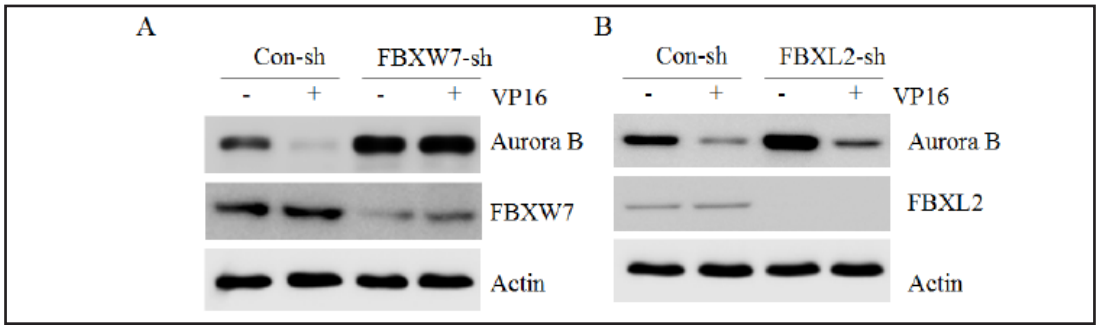

Fig. 3. FBXW7 is required for the degradation of Aurora B during apoptosis. (A) U937 cells were transfected with shRNAs against control or FBXW7 were treated with VP16 for 12 hours. Cells were harvested and the indicated proteins were detected by Western blot.(B) U937 cells were transfected with shRNAs against control or FBXL2 were treated with VP16 for 12 hours. Cells were harvested and the indicated proteins were detected by Western blot.

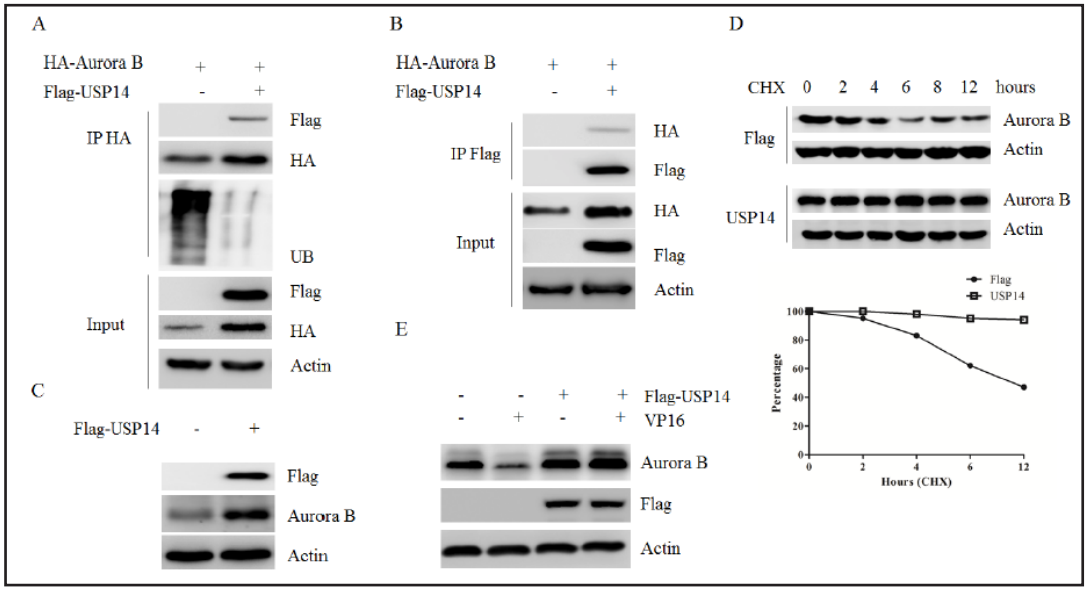

Fig. 4. Aurora B associates with USP14 and prevents apoptosis-induced Aurora B degradation. A-B. U937 cells were co-transfected with Flag-USP14 and HA-Aurora B for 48 hours, cells were harvested and subjected to IP procedure by using either Flag (A) or HA (B) antibodies. The binding martial was then eluted by SDS buffer and subjected to Western blot with indicated antibodies. (C) U937 cells were transfected with vector or Flag-USP14 for 48 hours. Cells were harvested and subjected to Western blot with indicated antibodies. (D) U937 cells were transfected with vector or Flag-USP14 for 24 hours and then treated with 20μg/ml CHX for different time points. Cells were harvested and the indicated proteins were detected by Western blot. (E) U937 cells were transfected with vector or Flag-USP14 for 24 hours and then treated with VP16 for additional 12 hours. Cells were harvested and subjected to Western blot with indicated antibodies.

and tumourigenesis [14], we then asked whether USP14 might regulate Aurora B during apoptosis. To this end, we first tested whether USP14 could interact with Aurora B. U937 cells were transfected with HA-Aurora B and/or Flag-USP14. Immunoprecipitation (IP) assay was then performed by using Flag M2 antibody and HA antibody, respectively. We found that exogenous USP14 was in the present of HA- Aurora B immunoprecipitate and vice versa. (Fig. 4A-B). The interaction between USP14 and Aurora B drove us to ask whether USP14 regulates the stability of Aurora B. Indeed, Overexpression of Flag-USP14 increased the protein level of HA-Aurora B (Fig. 4A-B). Moreover, endogenous Aurora B protein contents

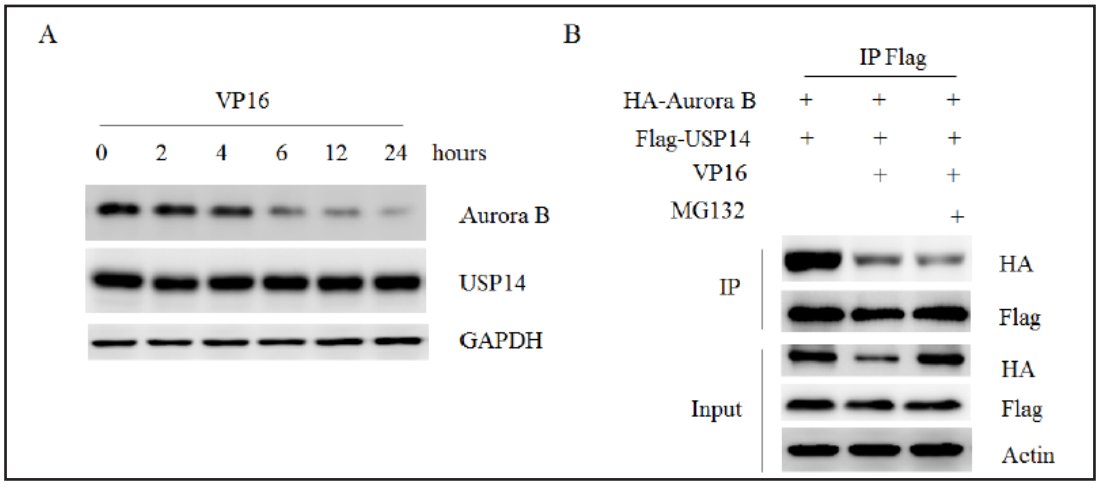

Fig. 5. Apoptosis-inducing reagents inhibit the interaction between USP14 and Aurora B. (A) U937 cells were treated with 1 μM VP16 for indicated times and the indicated proteins were detected by Western blot. (B) U937 cells were co-transfected with Flag-USP14 and HA-Aurora B for 24 hours and then treated with VP16 for additional 12 hours and with or without 20μM MG132 treatment for additional 4 hours. Cells were harvested and subjected to IP procedure by using Flag antibody. The binding martial was then eluted by SDS buffer and subjected to Western blot with indicated antibodies.

were dramatically increased in U937 cells infected with retrovirus expressing USP14 (Fig. 4C). Furthermore, by using CHX assay, overexpression of USP14 significantly increased the half-life of Aurora B (Fig. 4D). The ubiquitination of Aurora B was also decreased by ectopic expression of USP14 (Fig. 4A). Moreover, U937 cells were transfected with control vector or Flag-USP14 and treated with or without VP16. We found that overexpression of USP14 impaired VP16-induced decrease of Aurora B, supporting the notion that USP14 could regulate Aurora B stability and prevent its degradation (Fig. 4E). Taken together, these data demonstrates that USP14 deubiquitinates Aurora B to prevent apoptosis-induced Aurora B degradation.

Apoptosis-inducing reagents inhibit the interaction between USP14 and Aurora B

We next test whether the expression of USP14 was altered by apoptosis-inducing reagents treatment. Unexpectedly, the protein level of USP14 was unchanged in response to VP16 or Dox treatment (Fig. 5A and data not shown). We then asked whether the interaction between USP14 and Aurora B was altered in apoptosis. U937 cells were co-transfected with Flag-USP14 and HA-Aurora B and then treated with VP16 with or without MG132 treatment Cells were harvested and subjected to IP procedure by using Flag M2 antibody. From input, we could clearly observe that VP16 treatment decreased Aurora B protein level, while MG132 administration could rescue this phenomenon. However, the interaction between USP14 and Aurora B was significantly decreased in response to VP16 treatment even in the present of proteasome inhibitor MG132(Fig. 5B), suggesting that the interaction between Aurora B kinase and USP14 is decreased in response to VP16 treatment.

USP14 inhibits chemotherapeutic drugs-induced apoptosis in leukemia cells via Aurora B

To investigate the biological function of USP14-medated Aurora B stabilization, we overexpressed USP14 or empty vector in leukemia cells. Cells were then treated with 1um VP16. Upon VP16 treatment, significant decrease of apoptosis was observed in USP14 overexpressed U937 cells compared with that in empty vector transfection cells (Fig. 6A). The similar result was observed from NB4 cells with PTX treatment (Fig. 6B). More importantly, silencing the expression of Aurora B in USP14 overexpressing U937 or Jurkat cells largely restored the apoptosis sensitivity (Fig. 6C-D). On the contrary, U937 cells were

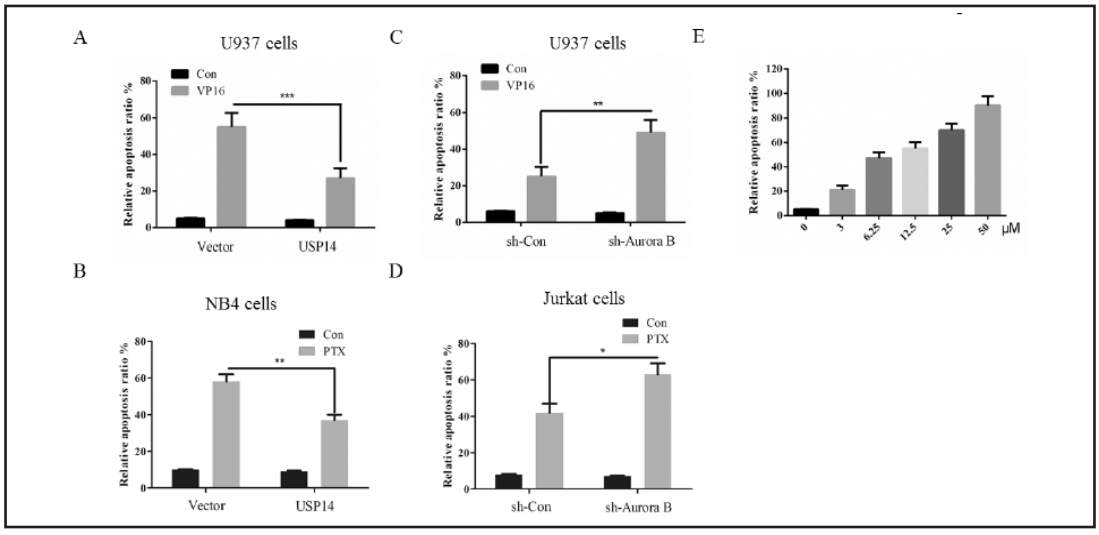

Fig. 6. USP14 inhibits chemotherapeutic drugs-induced apoptosis in leukemia cells via Aurora B. (A) U937 cells were transfected with vector or Flag-USP14 for 24 hours and then treated with 1μM VP16 for additional 12 hours. Cells were harvested and the percentage of apoptotic cells was determined by Annexin-V/PI staining assay. (B) NB4 cells were transfected with vector or Flag-USP14 for 24 hours and then treated with 5nM PTX, for additional 12 hours. Cells were harvested and the percentage of apoptotic cells was determined by Annexin-V/PI staining assay. (C) U937 cells were transfected with vector or Flag-USP14 and shRNA against Aurora B or non-specific target for 24 hours and then treated with 0.5μM VP16 for additional 12 hours. Cells were harvested and the percentage of apoptotic cells was determined by Annexin-V/PI staining assay. (D) Jurkat cells were transfected with vector or Flag-USP14 and shRNA against Aurora B or non-specific target for 24 hours and then treated with 5nM PTX for additional 12 hours. Cells were harvested and the percentage of apoptotic cells was determined by Annexin-V/PI staining assay. (E)U937 cells were treated with increased dose of b-AP15, a specific inhibitor of USP14 for 24 hours. Cells were harvested and the percentage of apoptotic cells was determined by Annexin-V/PI staining assay.

treated with b-AP15, a specific inhibitor of USP14 for the indicated time course [14]. We found that b-AP15 significantly increased U937 cells apoptosis in a dose-dependent manner (Fig. 6E). Taken together, these data indicated that USP14-medated Aurora B stabilization played a role in chemotherapeutic drugs-induced apoptosis of leukemia cells.

Discussion

Aurora B has been found to be overexpressed in 88 % of acute promyelocytic leukemia (APL) patients which made it an important drug target for APL treatment [2]. However, how to regulate Aurora B in leukemia cells remain largely unknown. Here we demonstrated that in response to apoptosis-inducing drugs stimuli, Aurora B protein was rapidly degraded by the proteasome system. We first demonstrated that apoptosis-inducing drugs could rapidly decrease the protein of Aurora B via the proteasome system, without affecting its mRNA expression. It has been reported that the ubiquitin E3 ligase FBXW7 and FBXL2 could mediated Aurora B ubiquitination and degradation. We further found that FBXW7 was required for Aurora B degradation in response to leukemic chemotherapy drugs-induced cell apoptosis. However, VP16 treatment did not alter the expression of endogenous FBXW7 drove us to ask whether other mechanisms might be involved. As deubiquitination enzymes can cleave ubiquitin from proteins to prevent proteasome-mediated destruction, we then reasoned that impaired deubiquitination toward Aurora B might also cause it degradation.

We found that one of the deubiquitination enzymes USP14 was a critical regulator of Aurora B in leukemic chemotherapy drugs-induced cell apoptosis. This was supported by

several lines of evidence. First, our biochemical assay proved that USP14 interacted with Aurora B. Second, USP14 overexpression largely decreased the ubiquitination of Aurora B during leukemic chemotherapy drugs-induced cell apoptosis. Moreover, our CHX assay found that in the present of USP14, the half-live of Aurora B was increased, suggesting that USP14 protected Aurora B from ubiquitin-mediated degradation. Third, apoptosis-inducing reagents treatment prevents the interaction between USP14 and Aurora B. Finally, USP14 inhibits DNA damage reagents-induced apoptosis in leukemic cells via Aurora B.

Several studies found that Aurora B was amplified and overexpressed in various tumors, including several types of leukemia [15, 16]. These discoveries provided a rationale for the development of small-molecule inhibitors of Aurora B as leukemia therapies. Because of the high toxicity and poor efficacy, the first generation of Aurora B inhibitors did not fare well in clinical trials [17, 18]. However, the second-generation, highly selective Aurora B inhibitors have increased the enthusiasm for targeting Aurora B in leukemia [19]. The amplification and overexpression USP14 of have been observed in many different cancers [20]. The highly selectively USP14 inhibitor b-AP15 has anti-tumor activity against several kind of cancers [21, 22]. We also found that b-AP15 induced leukemic cells apoptosis in a dose-dependent manner. Thus, further studied will be warranted to test the anti-leukemia effects of the combination of b-AP15 with Aurora B inhibitors.

In the present study, our results uncovered a dynamic mode of controlling Aurora B stability upon apoptosis in leukemia cells involving both ubiquitination and deubiquitination activities. Our data demonstrated USP14 played a novel critical role of in leukemic chemotherapy drugs-induced cell apoptosis and by suppressing ubiquitination of Aurora B and suggested that USP14 could be a potential therapeutic target for leukemia patients.

Disclosure Statement

None.

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