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FBXL5 attenuates RhoGDI2-induced cisplatin resistance in gastric cancer cells

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Abstract. – OBJECTIVE: The hemerythrin-like domain of F-box and leucine-rich repeat protein 5 (FBXL5), an E3 ubiquitin ligase subunit, has critical roles in the regulation of cancer cells metastasis and chemoresistance by targeting diverse substrates for ubiquitin-mediated destruction.

MATERIALS AND METHODS: Here, we report that FBXL5 interacts with Rho GDP dissociation inhibitor 2 (RhoGDI2) and attenuates RhoGDI2-induced cisplatin resistance in gastric cancer cells. By utilizing immunoprecipitation (IP) coupled with mass spectrometry assay, we found that FBXL5 regulated gastric cancers migration via cortactin destruction.

RESULTS: Depletion of FBXL5 enhances cisplatin resistance of gastric cancer cells through Erk and p38 activation. However, FBXL5 did not affect the abundance and stability of RhoGDI2. Instead, FBXL5 was rapidly degraded in response to cisplatin treatment in RhoGDI2-overexpressing gastric cancer cells.

CONCLUSIONS: Collectively, our data suggested the existence of a FBXL5-RhoGDI2 negative feedback loop in RhoGDI2-induced cisplatin resistance in gastric cancer cells, implicating FBXL5 as a novel and promising therapeutic target for RhoGDI2-induced cisplatin resistance gastric cancers.

Key Words:
FBXL5, RhoGDI2, Cisplatin resistance, Gastric cancers.

Introduction

Gastric cancer is the fourth most common cancer and the second leading cause of cancer mortality worldwide, with nearly one million new cases diagnosed per year[1]. In China, it has become the second most frequently diagnosed cancer and the third leading cause of cancer death and Chinese gastric cancer patients have a worse outcome than US gastric cancer patients[2]. Although multiple oncogenes and tumor suppressors have been identified to participate in the development and progression of gastric cancer and chemotherapy is commonly used for metastatic cancer, it's still poor for the prognosis of advanced gastric cancer, and usually unsuccessful for the treatment[3,4]. Therefore, identification of critical players in chemo-resistance will help to discover new therapeutic targets for gastric cancer.

RhoGDI2 belongs to a family of Rho GTPase dissociate inhibitors (RhoGDIs), which play significant roles in regulating the actin cytoskeleton, cell polarity, microtubule dynamics, membrane transport pathways, and transcription factor activities[5]. It has been reported that the RhoGDI2 expression was positively correlated with tumor progression and metastatic potential in gastric cancer[6]. RhoGDI2 is associated with the acquisition of resistance to chemotherapeutic agent cisplatin by repressing cisplatin-induced apoptosis in gastric cancer cells[7,8]. A recent work suggests that repressed p38/JNK kinase activities contributed to RhoGDI2-induced cisplatin resistance in gastric cancer cells[9].

The Skp-Cullin-F box (SCF) ubiquitin E3 ligase machinery consists of Skp1, Cullin 1, and a F-box protein that interacts with and targets many substrates for proteasome-dependent destruction[10]. The accumulated evidences have shown that this complex plays an important role in tumorigenesis, by controlling cell proliferation, apoptosis, metastasis and angiogenesis[11]. For instance, recent researches have identified FBXW7 as a haploinsufficient tumor suppressor that targets several proto-oncoproteins for degradation, including cyclin E, c-Myc, c-jun and Mcl1[12-15].

Previously, we have shown that another F-box protein family member FBXL5 interacted with Cortactin and mediated the ubiquitination and degradation of Cortactin to regulate gastric cancers migration[16]. We have also demonstrated that

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FBXL5 inhibited metastasis of gastric cancer through suppressing Snail1[17]. In the present study, we report that FBXL5 interacts with Rho GDP dissociation inhibitor 2 (RhoGDI2) and attenuates RhoGDI2-induced cisplatin resistance in gastric cancer cells in a proteasome independent manner.

Materials and Methods

Cell Culture and Reagents

Gastric cancer cell lines (MKN-28, MKN-45 and AGS) were obtained from American Type Culture Collection (Rockville, MD, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum. Cultures were maintained at 37°C in a humidified atmosphere with 5% CO_2. All chemical reagents were purchased from Sigma-Aldrich.

RNAi and Cell Transfection

Small interfering (si) RNA against FBXL5 was purchased from Dharmacon (catalog number D-012424-04) and a non-targeting siRNA (Dharmacon, catalog number D-001210-01). For cell transfection experiments, MKN-28 and/or MKN-45 cells were grown to 70% to 80% confluence in 6-well plates or 10-cm dishes. Cells were transiently transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

Immunoprecipitation

The immunoprecipitation (IP) procedure was described previously[18]. Briefly, cells after transfection were lysed in 2 mL of lysis buffer (50 mM Tris-HCl pH 7.5, 200 mM NaCl, 0.5% Nonidet P40, protease inhibitor cocktail) for 30 min at 4°C. Lysates were cleared using centrifugation at 15,000 rpm for 10 min; the supernatant was then subjected to immunoprecipitation with anti-FLAG M2 affinity resin (Sigma) overnight. The resin containing immune complexes was washed with cold lysis buffer 5 times. Proteins were eluted with 2X SDS buffer for 10 min at 95 . Immunoprecipitation samples were directly subjected to SDS-PAGE.

Western Blotting

MKN-28 and MKN-45 cells were harvested and lysed with ice-cold lysis buffer (50 mM Tris-2-M HCl, pH 6.8, 100 mM β-Mercaptoethanol, 2% w/v SDS, 10% glycerol). After centrifugation at 20000 × g for 10 min at 4°C, proteins in the supernatants were quantified and separated by 10% SDS-PAGE, transferred to NC membrane (Amersham Bioscience, Uppsala, Sweden). After blocking with 10% nonfat milk in PBS, membranes were immunoblotted with antibodies as indicated, followed by HRP-linked secondary antibodies purchased from Cell Signaling (Danvers, MA, USA). The signals were detected by Super-Signal West Pico Chemiluminescent Substrate kit (Pierce, Rockford, IL, USA) according to manufacturer's instructions.

Antibodies

Rabbit anti-FBXL5, RhoGDI2, p44/42 Map Kinase, anti-Phospho-p44/42 Map Kinase (Thr202/Tyr204), anti-p38 MAP Kinase, and anti-Phospho-p38 MAP Kinase (Thr180/Tyr182) antibodies were purchased from Cell Signaling Technology. Antibodies against Cullin1 and SKP1 were purchased from Santa Cruz (Santa Cruz, CA, USA). Antibodies against Flag and Ubiquitin were purchased from Sigma. Protein levels were normalized to total GAPDH, using a rabbit polyclonal anti-GAPDH antibody (Santa Cruz, CA, USA).

Apoptosis Assays

Annexin-V assay was carried out by Annexin V-FITC Apoptosis Detection Kit according to manufacturer's instruction (BD, San Diego, CA, USA). Briefly, 4×10^5 MKN-28 and/or MKN-45 cells were collected, washed twice with cold PBS, resuspended in binding buffer and incubated with Annexin V-FITC at room temperature, followed by addition of propidium iodide for 5 min. Fluorescent intensities were determined by flow cytometry (Becton Dickinson, San Jose, CA, USA).

Statistical Analysis

Data are expressed as the mean ± SEM from at least three separate experiments. Differences between groups were analyzed using Student's *t*-test and Chi-square test. A value of $p < 0.05$ was considered statistically significant.

Results

FBXL5 Interacted with RhoGDI2 in Gastric Cancer Cells

In our previous studies, we have uncovered a critical role of FBXL5 in the regulation of migration and metastasis of gastric cancer cells via in-

teracting with different substrates[16,17]. We purified FBXL5 interacting protein complexes and found that Rho GDP dissociation inhibitor 2 (RhoGDI2) could be a potential interacting protein of FBXL5. To this end, we performed an immunoprecipitation assay using FBXL5 and control IgG antibodies to capture endogenous FBXL5 protein complexes in AGS cells. As expected, we could detect the present of RhoGDI2, as well as SKP1 and Cullin1, in FBXL5 immunoprecipitate (Figure 1A). We then overexpressed Flag- RhoGDI2 in MKN-45 cells and exogenous Flag-RhoGDI2 was captured by Flag-M2 beads. As depicted in Figure 1B, we could clearly observe the present of FBXL5 in RhoGDI2 immunoprecipitate, but not SKP1 and Cullin1. Taken together, these data suggested that RhoGDI2 was interacted with FBXL5 but not with SCF-FBXL5 complex.

FBXL5 Did Not Affect the Abundance and Stability of RhoGDI2

Because FBXL5 could form an E3 ligase with skp1 and cullin1 and recognize substrates, it is possible that FBXL5 may destabilize RhoGDI2. However, the protein level of RhoGDI2 was barely affected by over-expression of Flag-FBXL5 in MKN-28 cells (Figure 2A). Silencing the expression of FBXL5 by siRNAs in MKN-28 cells did not affect both the abundance and stability of RhoGDI2 in MKN-28 cells (Figure 2B-C), suggesting that FBXL5 interacted with RhoGDI2 but did not target RhoGDI2 to destruction.

FBXL5 Sensitized RhoGDI2-Overexpressing Gastric Cancer Cells to Cisplatin-Induced Apoptosis

Because RhoGDI2 is associated with the acquisition of resistance to chemotherapeutic agent cisplatin by repressing cisplatin-induced apoptosis in gastric cancer cells[7,8], we sort to ask whether FBXL5 affect the cisplatin resistance function of RhoGDI2. To test this possibility, Flag-con or Flag-FBXL5 was transfected with into MKN-28 cells for 48h, in which contained a higher endogenous RhoGDI2 protein level. The expression of exogenous FBXL5 was detected by Western blot with Flag antibody (Figure 3A). We then treated those cells with 10 μg/ml cisplatin for 36h, and the apoptosis ratios of those cells were determined by FACS assay using Annexin-V and PI staining. We found that MKN-28 cells with FBXL5 overexpression have higher apoptosis ratio than control cells (58.3 ± 2.7 *vs.* 24.4 ± 3.9) (Figure 3B), suggesting that FBXL5 could sensitize RhoGDI2-overexpressing gastric cancer cells to cisplatin-induced apoptosis.

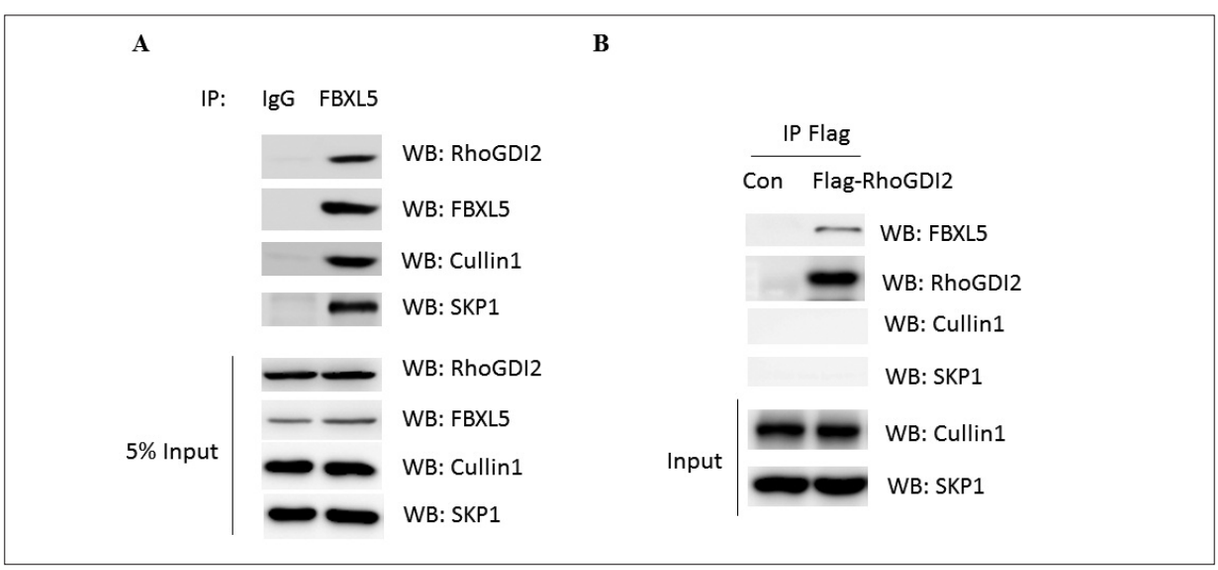

Figure 1. FBXL5 interacted with RhoGDI2 in gastric cancer cells. ***A,*** Lysate from human gastric carcinoma AGS cells was incubated with either anti- FBXL5 antibody or normal rabbit IgG, and the immunocomplexes were probed with the indicated endogenous antibodies. 5% percent lysate was used as input. ***B,*** Human gastric cancer MKN-45 cells were transfected with Flag-con or Flag- RhoGDI2 for 48h. Cells were harvested and incubated with Flag-M2 beads overnight. The immunocomplexes were probed with the indicated antibodies.

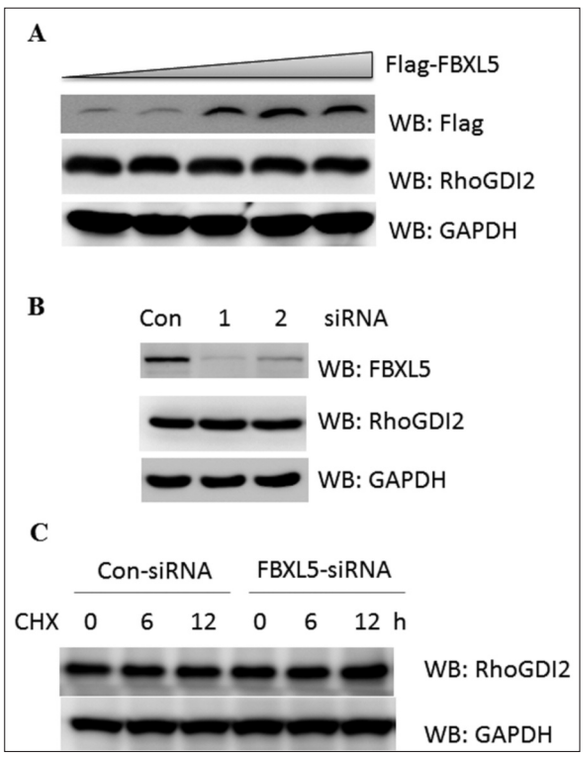

Figure 2. FBXL5 did not affect the abundance and stability of RhoGDI2. ***A,*** Human gastric cancer MKN-28 cells were transfected with indicated increase amount of Flag-FBXL5 plasmid for 36h. Cells were harvested and the cell lysate was subjected to Western blot with indicated antibodies. ***B,*** MKN-28 cells were transfected with indicated siRNAs for 36h. Cells were harvested and the cell lysate was subjected to Western blot with indicated antibodies. ***C,*** MKN-28 cells were transfected with indicated siRNAs for 24h. Cells were then treated with cycloheximide (CHX) for the indicated time. Cells were harvested and the cell lysate was subjected to Western blot with indicated antibodies.

Depletion of FBXL5 Enhances Cisplatin Resistance of Gastric Cancer Cells Through Erk and p38 Activation

Since overexpression of FBXL5 could sensitize RhoGDI2-overexpressing gastric cancer cells to cisplatin-induced apoptosis, we asked whether silencing of FBXL5 in MKN-28 cells affects DNA damage sensitivity. As shown in Figure 4A, we found that knockdown of FBXL5 in MKN-28 cells caused decreased apoptosis induced by DNA damage reagent cisplatin. Since mitogen-activated protein kinase (MAPK) pathways are implicated in the execution of apoptosis by cisplatin and suppression of Erk and p38 activation attenuates cisplatin-induced apoptosis in gastric cancer cells[9], we then asked whether MAPK pathway was altered in FBXL5-depleted cells. MKN-45 cells transfected with siRNAs targeting FBXL5 or non-specific siRNAs were treated with 10 μg/ml cisplatin for 4h to activate MAPK pathway. We could clearly observe that the phosphorylation levels of Erk and p38 were significantly decreased in FBXL5-depleted MKN-45 cells compared with control siRNA transfected cells (Figure 4B). Taken together, these data suggested that depletion of FBXL5 enhanced cisplatin resistance of gastric cancer cells through Erk and p38 activation

FBXL5 was Degraded in Response to Cisplatin Treatment in RhoGDI2-Overexpressing Gastric Cancer Cells

The above findings suggested that although FBXL5 did not target RhoGDI2 for destruction, FBXL5 indeed played a role in cisplatin resistance of gastric cancer cells. Because RhoGDI2 could induce cisplatin resistance in gastric cancer cells and RhoGDI2 interacted with FBXL5, we then asked whether RhoGDI2 could affect the expression of FBXL5 in return. To this end, we detected the expression of FBXL5 in two kinds of gastric cancer cells MKN-28 (with endogenous RhoGDI2 overexpression) and MKN-45 (without endogenous RhoGDI2 overexpression) in response to cisplatin treatment. The endogenous FBXL5 protein levels were declined after cisplatin administration in MKN-28 cells but not in MKN-45 cells, indicating that in the present of RhoGDI2, the protein expression of FBXL5 was decreased upon cisplatin treatment (Figure 5A). However, without RhoGDI2, cisplatin alone could not reduce the expression of FBXL5 (Figure 5A). Given the interaction between RhoGDI2 and FBXL5, we assumed that RhoGDI2 might regulate FBXL5 in the posttranscriptional level. To test this hypothesis, we expressed Flag-FBXL5 in MKN-28 cells. The exogenous expressed Flag-FBXL5 protein was purified by Flag M2 beads, and detected by Western blot with anti-ubiquitin antibody. We found that after cisplatin treatment, the ubiquitinated FBXL5 was significantly increased (Figure 5B). Taken together, these data suggested that FBXL5 was degraded in response to cisplatin treatment in RhoGDI2-overexpressing gastric cancer cells.

Discussion

Fbxl5 knockout mice showed an embryonic lethality phenotype, with growth defects readily apparent prior to day E9 despite normal placenta-

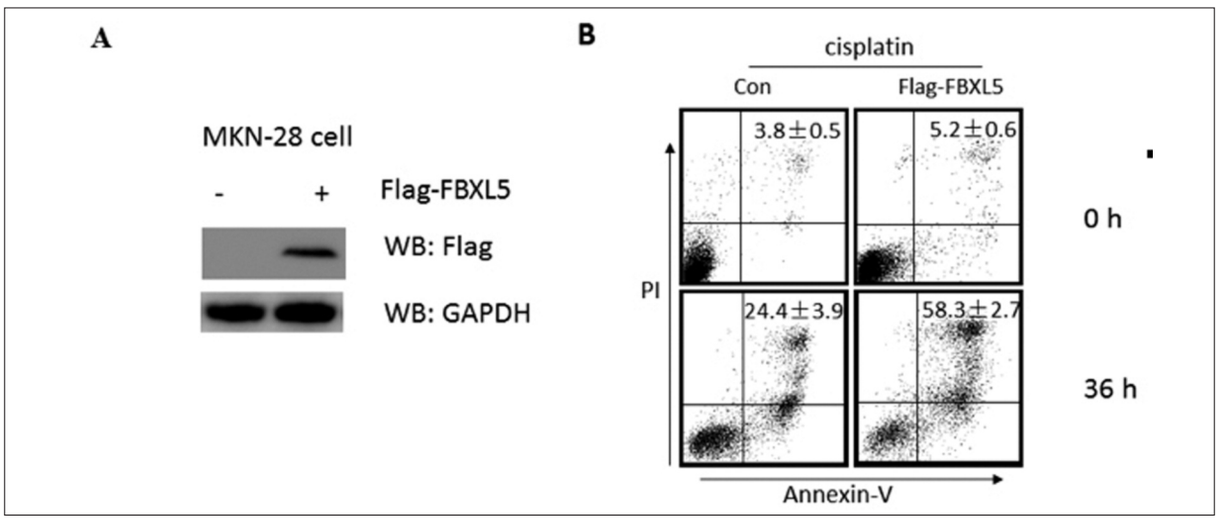

Figure 3. FBXL5 sensitized RhoGDI2-overexpressing gastric cancer cells to cisplatin-induced apoptosis. ***A,*** MKN-28 cells were transfected with Flag-con or Flag-FBXL5 for 48h. Cells were harvested and the cell lysate was subjected to Western blot with indicated antibodies. ***B,*** MKN-28 cells were transfected with Flag-con or Flag-FBXL5 for 24h. Cells were then treated with or without 10 ug/ml cisplatin for 36h. Cells were then harvested. The apoptotic cells were detected by Annexin-V and PI staining.

tion, gastrulation and cardiovascular development[19]. Biochemical studies suggest that FBXL5 has a critical role in the maintenance of cellular iron homeostasis by targeting IRP1 and IRP2 for ubiquitination[20]. Cells from Fbxl5 knockout mice were trended to apoptosis due to unrestrained IRP activity which was critical for embryonic development[19]. In spite of the essential role in embryonic development, recent studies uncovered a critical role of FBXL5 in tumorigenesis. It has been reported[21] that lung cancer cells with FBXL5 overexpression showed a waked cellular

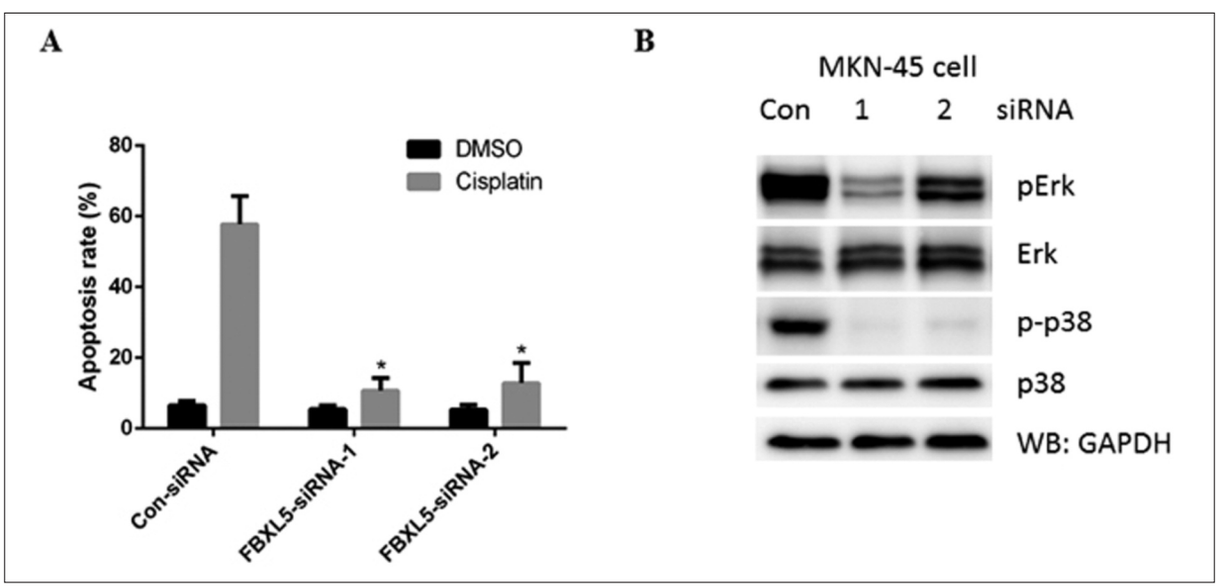

Figure 4. Depletion of FBXL5 enhances cisplatin resistance of gastric cancer cells through Erk and p38 activation. ***A,*** MKN-45 cells were transfected with indicated siRNAs for 24h. Cells were then treated with or without 10 μg/ml cisplatin for 36h. Cells were then harvested. The apoptotic cells were detected by Annexin-V and PI staining. The data presented the percentages of apoptotic cells. ***B,*** MKN-45 cells were transfected with indicated siRNAs for 36h. Cells were treated with 10 μg/ml cisplatin for 4h. Cells were harvested and the cell lysate was subjected to Western blot with indicated antibodies.

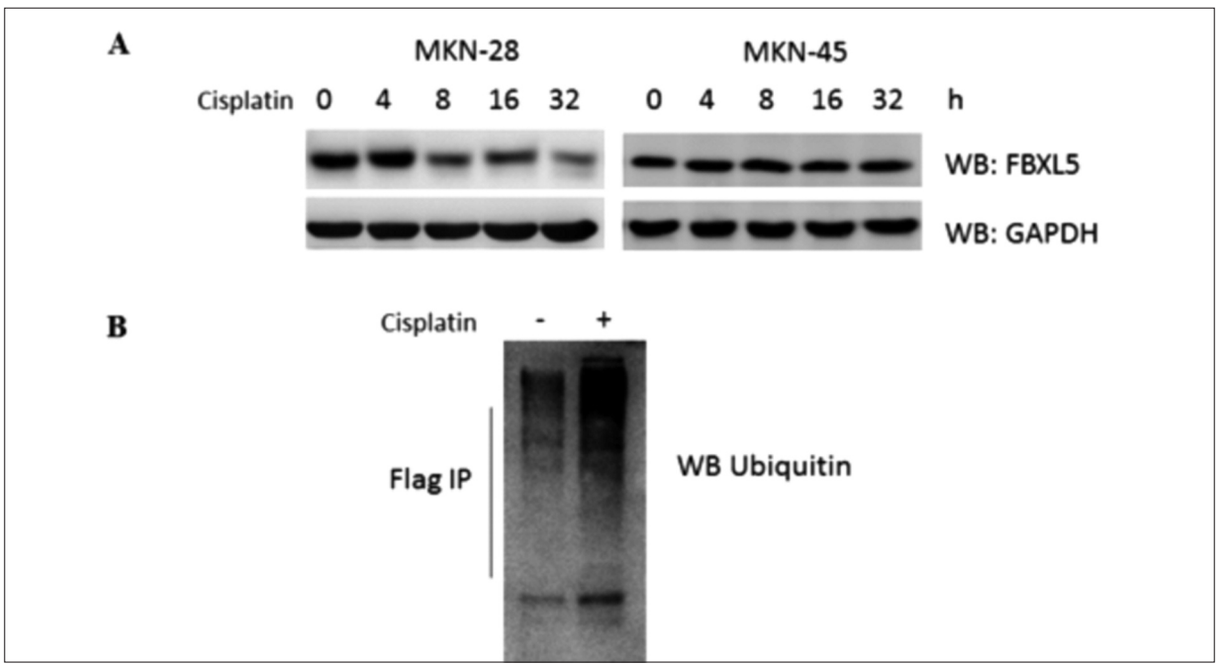

Figure 5. FBXL5 was degraded in response to cisplatin treatment in RhoGDI2-overexpressing gastric cancer cells. ***A,*** MKN-28 and MKN-45 cells were treated with10 ug/ml cisplatin for the indicated time. Cells were harvested and the cell lysate was subjected to Western blot with indicated antibodies. ***B,*** Lysate from MKN-28 cells was incubated with anti-FBXL5 antibody and the immunocomplexes were probed with anti-ubiquitin antibodies.

response to DNA double-strand breaks by targeting single-stranded DNA-binding protein hSSB1 for destruction. We showed that FBXL5 interacted with Cortactin and mediated the ubiquitination and degradation of Cortactin to regulate gastric cancers migration[16]. We have also demonstrated that FBXL5 inhibited metastasis of gastric cancer through suppressing Snail1[17]. However, whether Fbxl5 also participates in cisplatin resistance in gastric cancer cells remains largely unexplored.

In the present work, our results reveal a novel role of FBXL5 in cisplatin resistance in gastric cancer cells. The initial purpose of this study is to further identify the substrates of FBXL5. By searching our FBXL5-interacting protein database, RhoGDI2, a critical player in cisplatin resistance of gastric cancer cells, was selected for further research. Firstly, we have confirmed the interaction between FBXL5 and RhoGDI2. However, we found that RhoGDI2 only interacted with FBXL5, but not with Cullin1 and SKP1, suggesting that RhoGDI2 did not form a complex with these proteins. Then we found that FBXL5 could not target RhoGDI2 for ubiquitination and destruction. Thus, we turned to study whether FBXL5 could affect the biological function of RhoGDI2. Next, we found that FBXL5 could sensitize RhoGDI2-overexpressing gastric cancer cells to cisplatin-induced apoptosis via MAPK pathway. These findings drove us to test whether RhoGDI2 might affect the expression of FBXL5. Finally, we found that FBXL5 was rapidly degraded after cisplatin treatment in the present of RhoGDI2, indicating that RhoGDI2 might interact with FBXL5 and promote the ubiquitination and degradation of FBXL5 by proteasome system in response to cisplatin treatment.

Conclusions

Collectively, we suggested the existence of a FBXL5-RhoGDI2 negative feedback loop in RhoGDI2-induced cisplatin resistance in gastric cancer cells, implicating FBXL5 as a novel and promising therapeutic target for RhoGDI2-induced cisplatin resistance gastric cancers.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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