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ARL6IP1 mediates cisplatin-induced apoptosis in CaSki cervical cancer cells

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Received October 30, 2009; Accepted January 28, 2010

DOI: 10.3892/or_00000783

Abstract. Cisplatin has been shown to induce apoptosis in various types of cancer cells. Despite the great efficacy at treating certain kinds of cancers, cisplatin introduced into clinical use shows side effects and the acquisition or presence of resistance to the drug. Thus, it is important that we further understand the anti-cancer mechanism of cisplatin with the goal of enhancing its efficacy. ADP-ribosylation factor-like 6 interacting protein 1 (ARL6IP1) is an apoptotic regulator. We studied cisplatin-induced apoptosis with suppression of ARL6IP1 expression in CaSki cervical cancer cells. Exogenous expression of ARL6IP1 suppressed cisplatin-induced apoptosis in CaSki cells, and siRNA-induced silencing of ARL6IP1 triggered apoptosis in CaSki cells even in the absence of other apoptotic stimuli. Cisplatin treatment induced caspase-3, -9, p53, Bax, NF-κB and MAPK expression, and suppressed Bcl-2 and Bcl-xl expression, whereas cells transfected with pcDNA3.1-ARL6IP1 showed lower levels of cisplatin-induced caspase-3, -9, p53, Bax, NF-κB and MAPK up-regulation and higher levels of cisplatin-suppressed Bcl-2 and Bcl-xl down-regulation. These novel findings collectively suggest that ARL6IP1 may play a key role in cisplatin-induced apoptosis in CaSki cervical cancer cells by regulating the expression of apoptosis-associated proteins such as caspase-3, -9, p53, NF-κB, MAPK, Bcl-2, Bcl-xl, and Bax.

Introduction

Cisplatin, a critical component of therapeutic regimens in a broad range of malignancies, has been shown to induce apoptosis in various types of cancer cells (1-3). Despite the great efficacy at treating certain kinds of cancers, cisplatin, carboplatin, and other cisplatin analogs introduced into clinical use have side effects, and the acquisition or presence of resistance to these drugs undermine their curative potential (4). Thus, it is important that we understand the anticancer mechanism of cisplatin with the goal of enhancing its efficacy as valuable adjunct or single agent in anticancer therapy.

The caspase cascade is activated in response to cisplatin insult; this activation leads to an irreversible commitment to apoptotic cell death (5). It has been observed that cisplatin-induced apoptosis in both sensitive and resistant ovarian cancer cells is associated with an increased level of Bax and Bak proteins (6). In addition, a decrease in Bcl-2 expression has been reported in cisplatin resistant ovarian cancer cells after cisplatin treatment (7). Tumor suppressor genes p53 and p73 also influence cisplatin-induced apoptosis (8,9). Signals from the extracellular environment, such as certain growth factors and cytokines, may also modulate cisplatin-induced apoptosis because they can regulate the apoptotic response to chemotherapeutic drugs (10). It has been also reported that activation of c-jun NH2-terminal kinase/p38 is involved in the cisplatin-induced apoptosis in some cancer cells, such as A431 epidermoid carcinoma cells, HT29 colon cancer cells, and a human ovarian carcinoma cell line (11,12). Multiple mechanisms have been implicated in the development of cisplatin resistance, including increased expression levels of Bcl-2-related antiapoptotic genes, and alterations in signal transduction pathways involved in apoptosis (13,14). It is generally accepted that cytotoxicity of cisplatin is mediated through induction of apoptosis which activates multiple signaling pathways including p53, p73, Bcl-2 family, caspases, cyclins and cyclin-dependent kinases (15,16).

ADP-ribosylation factor-like 6 interacting protein 1 (ARL6IP1) is an apoptotic regulator. ARL6IP1 was first identified as an ADP-ribosylation factor-like 6 (ARL-6)-association factor by yeast two-hybrid screening (17). Previous studies have shown that ARL6IP1 protects HT1080 fibrosarcoma cells by inhibiting caspase-9 activity and reveal a possible role for ARL6IP1 in cell survival (18). ARL6IP1 was also isolated as a down-regulatory factor during myeloid differentiation by differential display and ARL6IP1 gene is suggested to be involved in protein transport, membrane trafficking, or cell signaling during hematopoietic maturation (19).

We evaluated the role of ARL6IP1 expression and regulation in cisplatin-induced apoptosis system in CaSki cells, providing important new insight into the anticancer effects of cisplatin.

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Key words: ARL6IP1, cisplatin, cervical cancer, apoptosis

Table I. Primer pairs used in semi-quantitative RT-PCR.

Genes	Forward primer (5'-3')	Reverse primer (5'-3')
Caspase-3	CAAAGATCATACATGGAAGCG	TGAAAAGTTTGGGTTTTCCAG
Caspase-9	TGGTGGAAGAGCTGCAGGT	TGGGCAAACTAGATATGGCGT
p53	CTGTGCAGCTGTGGGTTGATT	TCTTGCGGAGATTCTCTTCCT
Bcl-2	CATCCATTATAAGCTGTCGCA	TGCCGGTTCAGGTACTCAGT
Bcl-xL	AACTCTTCCGGGATGGGGTAA	AATTCTGAGGCCAAGGGAACT
Bax	ATGAAGACAGGGGCCCTTT	ATGGTGAGTGAGGCGGTGA
GAPDH	AATCCCATCACCATCTTCCA	CCTGCTTCACCACCTTCTTG

Materials and methods

Cell lines. CaSki cells were maintained in our laboratory and cultured in Dulbecco's-modified Eagle's medium (DMEM; Gibco) supplemented with 10% bovine calf serum (BCS) (Gibco). Cells were maintained at 37˚C in an atmosphere of humidified air with 5% CO_2.

MTT assay. Cells at 10^3/well were cultured in 96-well plates with 10% FBS DMEM at 37˚C 5% CO_2 for varying periods and exposed to fresh media every other day. During the last 4 h of culture each day, the cells were treated with methyl thiazolyl tetrazolium (MTT, 50 μg per well, Sigma, USA). The generated formazan was dissolved in DMSO and measured at OD (490 nm) to determine the concentration demonstrating 50% cytotoxicity on the tumor cells (IC50 value).

Construction and transfection of the ARL6IP1 expression vector. For transfection of the plasmid expression vector encoding human ARL6IP1 the DNA sequencing containing the ARL6IP1 open reading frame flanked by *Hin*dIII-*Bam*HI restriction sites was PCR amplified from CaSki cells. Primer sequences used were sense 5'-ATTAAGCTTATGGCGGAG GGAGAT-3' and antisense 5'-GAACTCGAGTCATTCGT TTTTCTTT-3'. The resulting fragment was inserted into *Hin*dIII/*Bam*HI-precut pcDNA3.1 (+) (Invitrogen) to generate pcDNA3.1-ARL6IP1. The desired sequence was confirmed by direct DNA sequencing.

For transfection, cervical cancer cells were grown to 70% confluence and transfected in serum-free medium for 6 h with Lipofectamine 2000 (Invitrogen) and pcDNA3.1-ARL6IP1 or empty vector (control). After 48 h, cells were harvested, followed by limited dilution in 96-well plates for the generation of individual cell clones. Three weeks later, the levels of ARL6IP1 expression in cell clones that had been infected with pcDNA3.1-ARL6IP1, were characterized by Western blot analysis for the ARL6IP1 protein.

Total RNA isolation and RT-PCR analysis. RNA isolated from cells was reverse-transcribed and amplified using the One-Step RT-PCR System (Fermentas, Vilnius, Lithuania). Primer sequences used are shown in Table I. After heating at 95˚C for 1 min, samples were exposed to 30 cycles (GAPDH, 25 cycles) of 95˚C for 30 sec, 60˚C for 30 sec and 68˚C for 1 min 30 sec with a final extension at 68˚C for 10 min.

Western blot analysis. The cells were washed with cold phosphate-buffered saline (PBS) and lysed in Laemmli buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 50 mM dithiothreitol, and 0.01% bromophenol blue) for 5 min at 95˚C. Cell lysates were analyzed by SDS-PAGE and transferred electrophoretically to polyvinylidene difluoride membrane. The blots were probed with specific antibodies for ARL6IP1, phosphorylated NF-κB, phosphorylated p44/p42 MAPK, and ß-actin (Cell Signaling Technology) by a secondary detection step with goat anti-rabbit IgG-HRP (horseradish peroxidase) (Santa Cruz Biotechnology, Santa Cruz, CA). The immunoreactive proteins were revealed by an ECL kit.

Construction of silencing RNA (siRNA). To knock down ARL6IP1 expression, we used pGCsi-U6/Neo/GFP vector encoding a small hairpin RNA directed against the target gene in CaSki cells. The target sequence for ARL6IP1 was 5'-CTCCTTGGAAAGCTTCTTA-3' (siARL6IP1). As a negative control, we used shRNA vector without hairpin oligonucleotides (sictrl). The individual cell clones that had been infected with siRNA-ARL6IP1 were obtained as mentioned above.

Flow cytometry analysis of cell cycle. Cells were harvested at 70-80% confluence and resuspended in fixation fluid at a density of 106/ml, 1500 μl propidium iodide (PI) solution was added, and the cell cycle was detected by FACS Caliber (Becton-Dickinson).

Statistical analysis. Data are expressed as mean ± SEM. The difference among groups was determined by ANOVA analysis and comparison between two groups was analyzed by the Student's t-test using the GraphPad Prism software version 4.0 (GraphPad Software, Inc., San Diego, CA). A value of $P<0.05$ was considered as statistical significance.

Results

Cisplatin induces apoptosis in CaSki cervical cancer cells. To investigate the effect of cisplatin on CaSki cervical cancer cell, MTT assays were used. CaSki cells were cultured with or without cisplatin (0, 1, 2 and 3 mg/l) for 96 h, removing aliquots every 24 h to evaluate cell viability. Fig. 1 shows that CaSki cells cultured in the absence of cisplatin maintained an

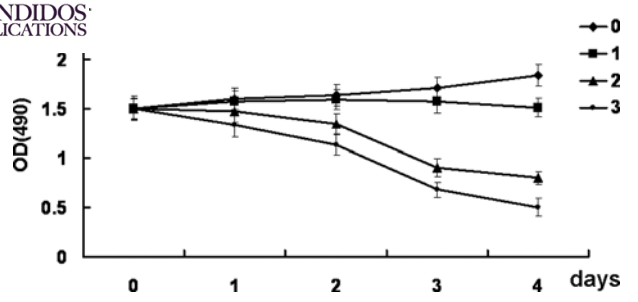

Figure 1. Effect of cisplatin on CaSki cervical cancer cell viability. After treatment with various concentrations of cisplatin (0, 1, 2 and 3 mg/l) and times (0, 24, 48, 72 and 96 h), the changes of cell viability of CaSki cervical cancer cell were observed by MTT assay. Data are presented as mean ± SD (n=10); p<0.05.

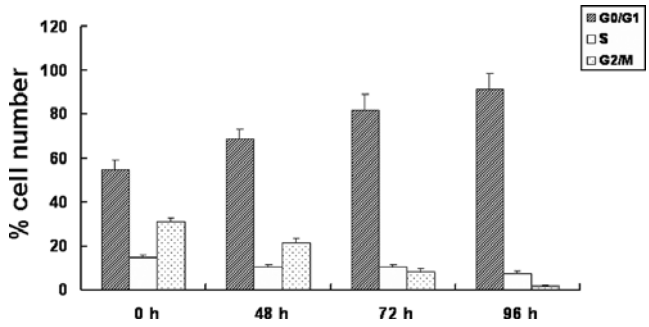

Figure 2. Flow cytometry analysis of the CaSki cells affected by the cisplatin. Analysis of the cell cycle of the CaSki cells incubated with cisplatin (2 mg/l) and 0, 48, 72 and 96 h. The percentage of sub-G1 phase cells was determined based on the DNA content histograms and represented as the mean ± SD from three independent experiments; p<0.05.

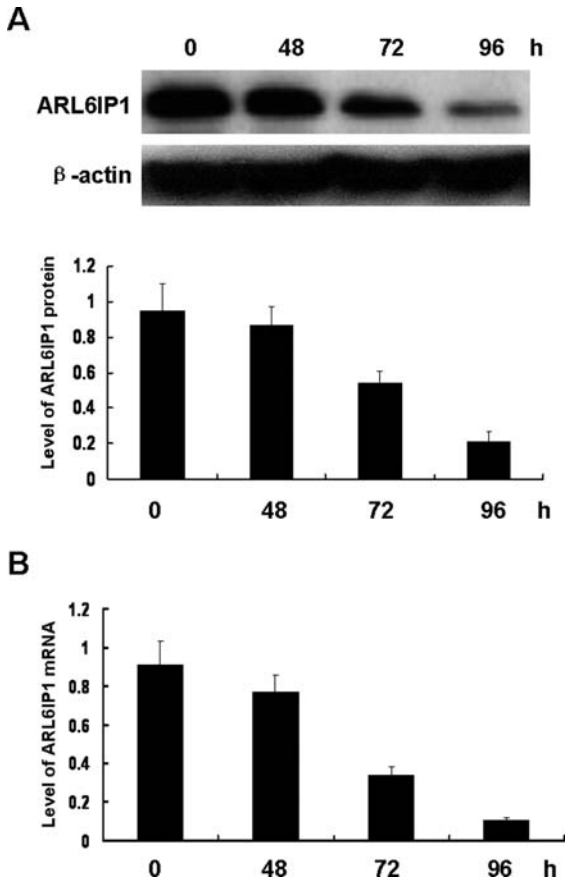

Figure 3. Inhibition of ARL6IP1 expression in CaSki cells. CaSki cells were treated with cisplatin (2 mg/l) for the indicated times. (A) Western blotting was used to assess cellular ARL6IP1 protein levels and the relative protein levels were normalized to ß-actin and ratio of ARL6IP1 to ß-actin was calculated. (B) The relative mRNA levels were normalized to that of GAPDH and ratio of ARL6IP1 to GAPDH was calculated. The graph represents the mean ± SD from three independent experiments; p<0.05.

increased number of viable cells, while the numbers of viable CaSki cells cultured in the presence of cisplatin with time were reduced, especially with 2 and 3 mg/l cisplatin. The presence of cisplatin (2, 3 mg/l) in CaSki cultures resulted in absence of expansion, especially in days two, three, and four. Cisplatin reduced CaSki cell numbers with an IC50 of 2 mg/l after 72 h of treatment. Overall, a marked reduction in cell viability was observed in cultures containing cisplatin. Cell cycle analysis was then performed by flow cytometry on CaSki cervical cancer cells cultured with (2 mg/l) cisplatin at 0, 48, 72 and 96 h to assess the effect of cisplatin upon cell cycle and ascertain whether cell cycle status was related to cisplatin-induced cell apoptosis. This treatment also resulted in accumulation of cells in the G1 phase along with blockage of cell proliferation (Fig. 2). The number of G0/G1 phases of apoptotic CaSki cells increased from 54.5±4.3% to 68.7±4.6% 81.7±7.4% and 91.2±7.3% after 48, 72 and 96 h of exposure to the cisplatin. The G1 phase arrest eventually led to cell apoptosis with cisplatin.

Cisplatin suppresses ARL6IP1 expression in CaSki cells. Previous studies have shown that ARL6IP1 protects HT1080 fibrosarcoma cells and reveal a possible role for ARL6IP1 in cell survival. To examine ARL6IP1 expression in cervical cancer cells exposed to apoptotic stimuli, CaSki cells were treated with cisplatin (2 mg/l). Western blot analysis was used to examine cisplatin-induced changes in ARL6IP1 protein levels over time in CaSki cells. ARL6IP1 protein and mRNA levels decreased in a time-dependent manner in cisplatin-treated CaSki cervical cancer cells (Fig. 3).

Inhibition of cisplatin-induced apoptosis by overexpression of ARL6IP1. To determine the importance of the ARL6IP1 in cisplatin-induced apoptosis, an expression plasmid containing the full-length cDNA of ARL6IP1 was transfected into CaSki cells, which were then subjected to cisplatin treatment with (2 mg/l) cisplatin for 72 h. Western blotting revealed that transfection of cervical cancer cells with ARL6IP1 effectively suppressed cisplatin-induced decreases in ARL6IP1 expression (Fig. 4A). Interestingly, overexpression of ARL6IP1 decreased the apoptotic sub-G1 fraction even in the presence of cisplatin (Fig. 4B).

Effect of downregulation of ARL6IP1 expression with siRNA on apoptosis in CaSki cervical cancer cells. To further determine the effect of ARL6IP1 on apoptosis, we used siRNA methodology to silence the ARL6IP1 gene. siRNA oligonucleotide specific for ARL6IP1 (siARL6IP1) or control

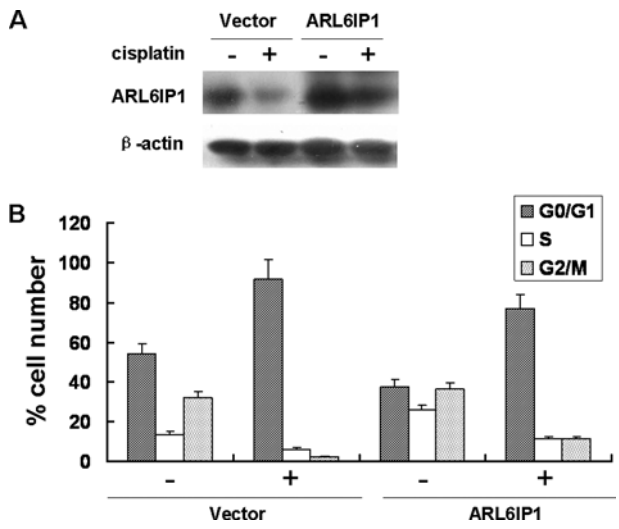

Figure 4. Inhibition of cisplatin-induced apoptosis by overexpression of ARL6IP1. CaSki cells were transfected with vector control (pcDNA3.1) or the ARL6IP1 expression plasmid (pcDNA3.1-ARL6IP1). After transfection, cells were treated with DMSO or cisplatin (2 mg/l) for 72 h. (A) Whole cell lysates were prepared for Western blot analysis of ARL6IP1 protein and ß-actin. (B) Each of these groups was analyzed by flow cytometry after propidium iodide staining. The percentage of sub-G1 phase cells is presented as the mean ± SD from three independent experiments; p<0.05.

oligonucleotide (sictrl) were transfected into CaSki cells. As shown in Fig. 5, siARL6IP1 increased the apoptotic sub-G1 fraction of the cells up to 83.4%, whereas transfection with the empty vector was associated with 55.8% sub-G1 fraction. Furthermore, MTT assay was used to investigate the dynamics of siARL6IP1 CaSki cells treated with cisplatin (2 mg/l) (Fig. 5C). Following a 4-day period, the growth of siARL6IP1 cells was much slower, as compared with control groups (sictrl). A significant reduction of cell viability was observed in siARL6IP1 cells exposed to cisplatin compared with the control group. These results indicate that the down-regulation of ARL6IP1 expression is capable of inducing apoptosis in CaSki cells even in the absence of other apoptotic signals.

Cisplatin-induced downregulation of ARL6IP1 is dependent of caspase-3, -9, p53, Bcl-2, Bcl-xl, and Bax. The molecular basis for cisplatin-induced downregulation of ARL6IP1 in CaSki cells was also investigated, and the expression of various apoptosis-regulatory genes was examined. To validate whether cisplatin-induced downregulation of ARL6IP1 was mediated via molecular pathways related to caspase, we analyzed the gene expression levels of caspase-3 and -9 in CaSki cells (Fig. 6A). The caspase-3 and -9 mRNA expression level was remarkably reduced in cells transfected with pcDNA3.1-ARL6IP1. Many apoptosis-related genes are transcriptionally regulated by p53. Next, we examined the expression of p53 (Fig. 6A). The p53 mRNA expression in CaSki cells trans-fected pcDNA3.1-ARL6IP1 was down-regulated. Bcl-2 family are central regulators of programmed cell death, and members that inhibit apoptosis, such as Bcl-2 and Bcl-xL, are overexpressed in many cancers and contribute to tumour initiation, and progression (20,21), but, Bax may lead to the caspase activation and apoptosis (22,23). Here, we examined the gene expression levels of Bcl-2 and Bcl-xL. RT-PCR analysis showed that the expression levels of Bcl-2 and Bcl-xL in CaSki cells

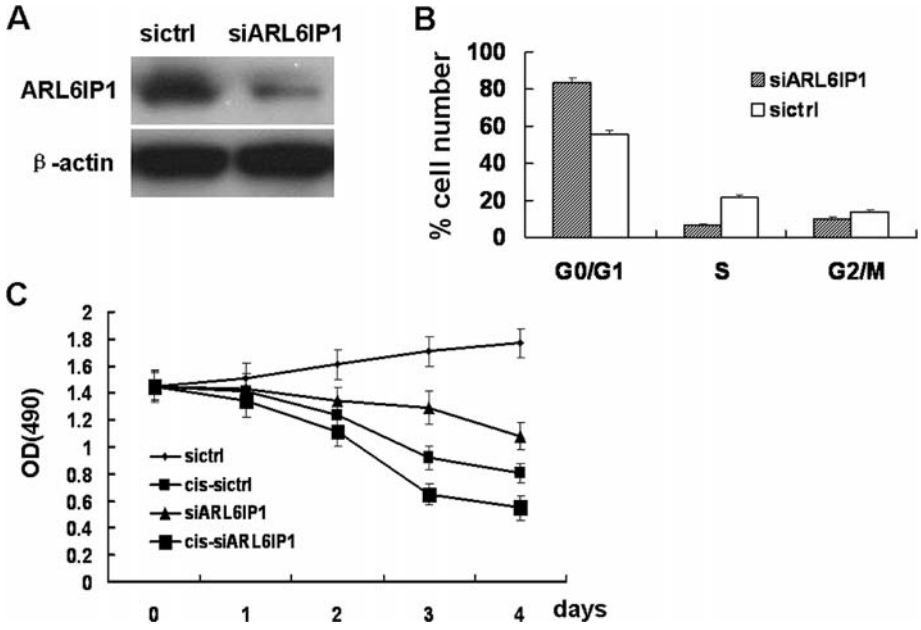

Figure 5. Effect of downregulation of ARL6IP1 expression with small interfering RNA (siRNA) on apoptosis in CaSki cells. (A) Western blot analysis of ARL6IP1 in CaSki cells transfected with control (sictrl) or small interfering RNA (siARL6IP1). (B) The cells were then harvested for flow cytometric analysis for the percentages of cells with sub-G1 DNA content. The percentage of sub-G1, S, G2 phase cells is presented as the mean ± SD from three independent experiments. (C) MTT assay was used to investigate the dynamics of siARL6IP1 CaSki cells treated with cisplatin (2 mg/l). Data are presented as mean ±SD (n=10); p<0.05.

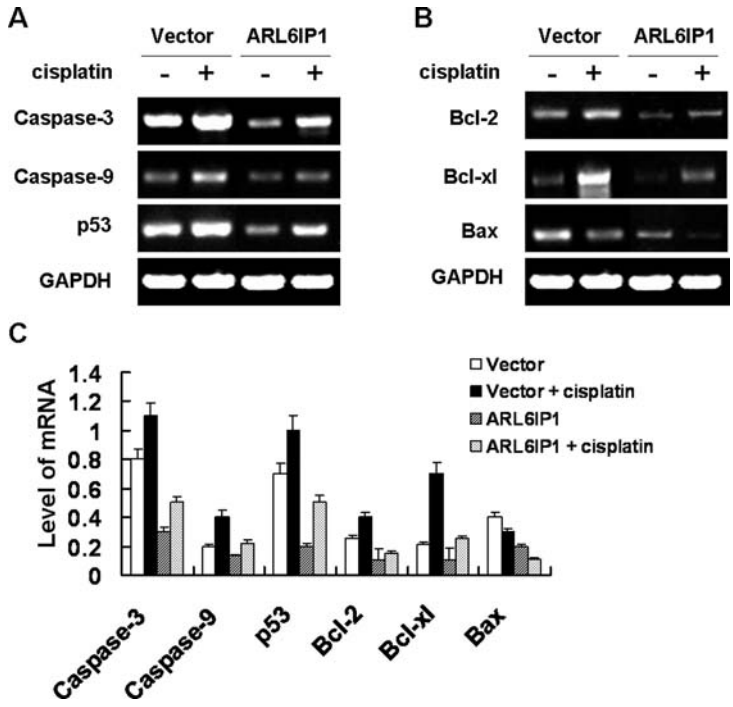

Figure 6. Cisplatin-induced downregulation of ARL6IP1 is dependent of caspase-3, -9, p53, Bcl-2, Bcl-xl, and Bax. (A) Caspase-3, -9, and p53 expression levels were examined in CaSki cells treated with cisplatin (2 mg/l) for 72 h. (B) Expression levels of Bcl-2, Bcl-xl, and Bax were examined in CaSki cells treated with cisplatin (2 mg/l) for 72 h. (C) The relative mRNA levels were normalized to that of GAPDH and ratio of caspase-3, -9, p53, Bcl-2, Bcl-xl, and Bax to GAPDH was calculated. The present results are representative of three independent experiments showing similar trends; $p<0.05$.

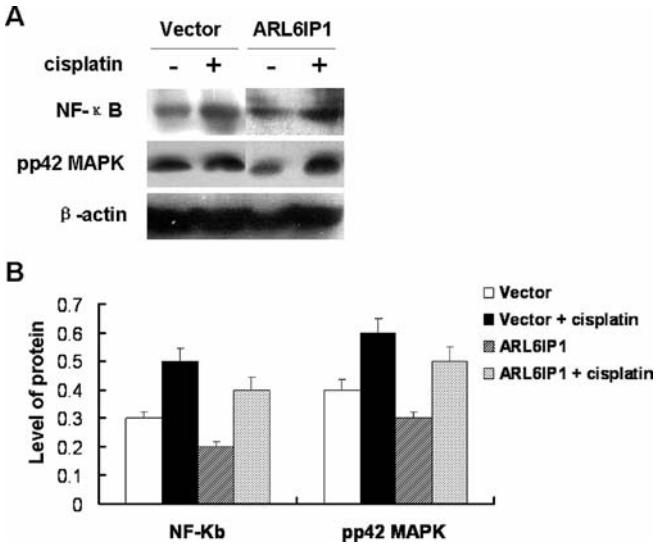

Figure 7. Cisplatin-induced downregulation of ARL6IP1 involves the activity of NF-κB and p42 MAPK. (A) The activity of NF-κB and p42 MAPK was analyzed by Western blot in CaSki cells treated with cisplatin (2 mg/l) for 72 h. (B) The relative protein levels were normalized to ß-actin and ratio of NF-κB and p42 MAPK to ß-actin was calculated. The present results are representative of three independent experiments showing similar trends; $p<0.05$.

transfected with pcDNA3.1-ARL6IP1 was upregulated, however, Bax expression was decreased (Fig. 6B).

Cisplatin-induced downregulation of ARL6IP1 involves the activity of NF-κB and MAPK. NF-κB and MAPK are known to promote apoptosis in various cancer cells, and we further probed NF-κB and MAPK activity (24,25). The protein expression level of phospho-NF-κB and -p42 MAPK in CaSki cells transfected with pcDNA3.1-ARL6IP1 decreased compared with control cells (Fig. 7).

Discussion

We herein showed that ARL6IP1 is critical for cisplatin-induced apoptosis of CaSki cells. Two lines of evidence support this conclusion: i) overexpression of the ARL6IP1 gene blocked cisplatin-induced apoptosis in CaSki cells; and ii) silencing of ARL6IP1 by siRNA was sufficient to induce apoptosis in CaSki cells even in the absence of additional apoptotic stimuli.

ARL6IP1 has been reported to protect HT1080 fibrosarcoma cells by inhibiting caspase-9 activity and that it may act as a novel inhibitor of apoptosis (18). Thus suggesting that ARL6IP1 might play an important role in tumor cell apoptosis.

In the present study, we showed that ARL6IP1 is downregulated during the apoptotic pathway. Interestingly, overexpression of the ARL6IP1 blocked apoptosis in cisplatin- treated CaSki cells, indicating that ARL6IP1 downregulation was not merely a consequence of apoptosis and ARL6IP1 may play a role in cisplatin resistance. Furthermore, cisplatin combination with downregulation of ARL6IP1 expression with siRNA effectively suppressed cells growth, suggesting ARL6IP1 may be applied in new chemotherapeutic strategies using cisplatin.

Previous studies have reported association between caspase family members and cisplatin-induced apoptosis. Cisplatin may cause mitochondrial release of cytochrome c and caspase-9 and caspase-3 activation (26,27). To examine

the role of caspase in cisplatin-mediated apoptosis, we investigated the expression levels of caspase-9 and caspase-3. Our results revealed that caspase-9 and caspase-3 expression was induced in cisplatin-treated CaSki cells, whereas transfection with pcDNA3.1-ARL6IP1 reduced cisplatin-induced caspase expression. Cisplatin DNA damage may lead to expression of p53 protein that subsequently induces expression of downstream protein and G1 phase cell cycle arrest (28,29). Our finding that p53 expression increased in cisplatin-treated cells and was blocked by overexpression of ARL6IP seems to suggest that ARL6IP may at least partly mediate cisplatin-induced p53 upregulation in CaSki cells.

Bcl-2 family members are also involved in cisplatin-induced apoptosis (30,31). It has been observed that cisplatin-induced apoptosis in both sensitive and resistant ovarian cancer cells is associated with an increased level of Bax and Bak proteins (32). In addition, a decrease in Bcl-2 expression has been reported in cisplatin-resistant ovarian cancer cells after cisplatin treatment (33). The present study showed that Bcl-2 and Bcl-xl was downregulated and Bax was up-regulated in cisplatin-treated CaSki cells, however, the expression was changed by overexpression of ARL6IP1. These findings suggest a relationship between Bcl-2 family and ARL6IP, and provide evidence that Bcl-2 family participates in ARL6IP1-mediated apoptosis induced by cisplatin.

In the apoptosis pathway, NF-κB and MAPK are the critical transcription factors that regulate the transcription of many genes associated with cisplatin-induced apoptosis (34,35). We examined the importance of NF-κB and MAPK in ARL6IP1-mediated apoptosis. Our results revealed that NF-κB and MAPK expression was induced in cisplatin-treated cells and blocked by overexpression of ARL6IP1.

In summary, we showed that ARL6IP1 is critical for cisplatin-induced apoptosis in CaSki cervical cancer cells. Cisplatin-suppressed ARL6IP1 expression is mediated in CaSki cervical cancer cells by regulating the expression of apoptosis-associated proteins such as caspase-3, -9, p53, NF-κB, MAPK, Bcl-2, Bcl-xl, and Bax. As ARL6IP1 is suppressed by cisplatin in CaSki cervical cancer cells and may play a key role in drug-induced apoptosis, these findings provide important new insight into signaling involved in cisplatin-induced apoptosis by ARL6IP1 mediation and may facilitate the development of chemotherapeutic or chemopreventive strategies using cisplatin.

Acknowledgements

This study was supported by a grant from the National Natural Science Foundation of China (No. 30672352).

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