Biochemical and Biophysical Research Communications 371 (2008) 531–535

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Downregulation of *BMI-1* enhances 5-fluorouracil-induced apoptosis in nasopharyngeal carcinoma cells

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ARTICLE INFO

Article history:
Received 14 April 2008
Available online 29 April 2008

Keywords:
BMI-1
Chemotherapy
Apoptosis
Nasopharyngeal carcinoma

ABSTRACT

5-Fluorouracil (5-FU) is an important chemotherapeutic agent for nasopharyngeal carcinoma (NPC). However, drug resistance may occur after several cycles of 5-FU-based chemotherapy. The oncogene B-cell-specific Moloney murine leukemia virus insertion site 1 (BMI-1) has been shown to be involved in the protection of cancer cells from apoptosis. In this study, 5-FU treatment could increase the percentage of apoptotic NPC cells among BMI-1/RNAi-transfected cells than that among cells transfected with the empty vector. The 50% inhibitory concentration (IC_{50}) values of 5-FU were significantly decreased to a greater extent in the cells transfected with BMI-1/RNAi. Most importantly, the expression of phospho-AKT and the anti-apoptotic protein BCL-2 were downregulated in the cells in which BMI-1 expression was inhibited, whereas the apoptosis-inducer BAX was observed to be upregulated. Abrogation of AKT pathway by a PI3K inhibitor could not further increase the sensitivity to 5-FU in the cells with reduced BMI-1 expression. Taken together, BMI-1 depletion enhanced the chemosensitivity of NPC cells by inducing apoptosis; which is associated with inhibition of the PI3K/AKT pathway.

Nasopharyngeal carcinoma (NPC) is a common malignancy in southern China with uncertain etiologic factors. The development and progression of NPC are believed to result from the interplay of several factors, including genetic susceptibility, Epstein–Barr virus (EBV) infection, and other environmental factors [1–3].

Currently, the administration of 5-fluorouracil (5-FU)—the most widely used anticancer agent—is one of the standard chemoradiotherapy regimens for NPC [4–6]. However, a sizable proportion of NPC patients show tumor recurrence after 5-FU treatment that is mainly caused by drug-resistant cancer cells. Therefore, during the past 2 decades, new strategies for enhancing the sensitivity of cancer cells to drug-induced apoptosis for cancer therapy have been intensively explored.

The family of polycomb group (PcG) proteins comprises chromatin-modifying proteins that play important roles in embryo development and cancer progression [7]. The B-cell-specific Moloney murine leukemia virus insertion site 1 gene (*Bmi-1*), the first PcG gene to be identified, was originally discovered as an oncogene that cooperated with *c-Myc* in the initiation of lymphoma in murine models [8,9]. Several lines of evidence have indicated that BMI-1 is associated with axial patterning, hematopoiesis, regulation of proliferation, and senescence and that BMI-1 is essential for the self-renewal of normal and malignant stem cells [10–12]. It has also been reported that BMI-1 is extensively upregulated in a variety of malignancies, including non-small cell lung cancer [13], colorectal cancer [14], and oral cancer [15]. Our previous study showed that BMI-1 alone was capable of inducing the immortalization of normal human nasopharyngeal epithelial cells. We also found that the upregulation of BMI-1 in NPC was positively correlated with poor prognosis in NPC patients [16]. Recently, it has been reported that the downregulation of BMI-1 can result in the apoptosis of cancer cells [17,18]. Therefore, we hypothesize that the abrogation of BMI-1 expression may be an effective strategy for sensitizing human cancer cells, including NPC cells, to cancer chemotherapy.

In this study, we report for the first time the anticancer effect of the combination of 5-FU treatment and BMI-1 depletion. After 5-FU treatment, a significantly higher percentage of apoptotic cells was observed among the cells that were transfected with BMI-1/RNAi than among the cells that were transfected with the empty

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doi:10.1016/j.bbrc.2008.04.117

vector. The 50% inhibitory concentration (IC_{50}) values of 5-FU were significantly lower in the cells that were transfected with BMI-1/RNAi than in those that were transfected with the empty vector. We also found that the expression of phospho-AKT was decreased in NPC cells that were transfected with BMI-1/RNAi. These results suggest that the combination of 5-FU treatment and BMI-1 depletion might be a potential clinical strategy for cancer chemotherapy.

Materials and methods

Cell culture and stable infection. The NPC lines CNE2 and HONE1 were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA), and cultured at 37 °C in a 5% CO_2 humidified incubator. BMI-1 small hairpin RNA (shRNA) was designed and cloned into the pSUPER-retro-puro retroviral vector. The shRNA sequence was as follows: 5′-GUUCACAAGACCAGACCAC-3′ [19]. Clone identity was verified by restriction digestion analysis and plasmid DNA sequencing. The BMI-1 shRNA or vector plasmid and the packaging plasmid pIK were cotransfected into 293FT cells using the calcium phosphate precipitation method; this was followed by collection of competent retroviruses 24 h after transfection. The retroviruses harboring BMI-1 shRNA were transfected into NPC cell lines. After selection for 10 d in 0.5 μg/mL puromycin medium, the cells were passaged and harvested.

Measurement of cytotoxicity. After synchronization for 24 h in a serum-free medium, CNE2 and HONE1 cells (5×10^3) were seeded in 96-well plates (Falcon, Lincoln Park, NJ, USA) and treated with various concentrations of 5-FU (Sigma, St. Louis, MO, USA) for 72 h. Cell viability was determined by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. In brief, MTT was dissolved and sterilized in phosphate-buffered saline (PBS) at 5 mg/mL, and 10 μL was added into each well. The plates were incubated at 37 °C and in 5% CO_2 for another 4 h. After removing the medium, 100 μL of dimethyl sulfoxide (DMSO) was added to dissolve the MTT dark blue crystals, and the plate was shaken gently for 5 min. Absorbance (570/650 nm) was measured to determine cell viability. All experiments were carried out in triplicate. The rate of cell growth inhibition (IR) was calculated according to the following equation:

$$\mathrm{IR} = [A_{570}(\mathrm{control}) - A_{570}(\mathrm{drug})]/A_{570}(\mathrm{control}) \times 100\%$$

where A_{570} (control) is the absorbance of the cancer cells without drug treatment and A_{570} (drug) is the absorbance of the cancer cells exposed to the drug.

Propidium iodide staining and flow cytometry. Cultured cancer cells were collected and washed twice with PBS, fixed overnight with precold 70% ethanol at 4 °C, and resuspended in PBS. The cells were incubated with RNase A for 45 min and then stained with 50 mg/mL of propidium iodide in the dark at 4 °C for 60 min. The suspended cells were analyzed using the fluorescence-activated cell sorting (FACS) approach (EPICS-XL; Beckman Coulter, Fullerton, CA, USA). The apoptotic rate was determined on the basis of the "sub-G1" peak.

Western blotting. Western blot analysis was performed as previously described [20]. The following antibodies were used: anti-BMI-1 (Upstate Biotechnology Inc., Norwalk, CT, USA), anti-BCL-2, anti-BAX, anti-AKT, anti-phospho-AKT, and anti-β-actin and horseradish peroxidase-conjugated secondary antibody (Santa Cruz, MA, USA). Enhanced chemiluminescence (ECL) reagents were purchased from Cell Signaling (Beverly, MA, USA).

Hoechst 33258 staining. Cells were harvested and washed with PBS, fixed with 4% paraformaldehyde for 30 min at 25 °C, washed three times with cold PBS, and exposed to 10 mg/L Hoechst 33258 (Beyotime Institute of Biotechnology, Haimen, China) in the dark at room temperature for 10 min. Gray level images were acquired using a laser scanning microscope (Axioskop 2 plus; Carl Zeiss Co. Ltd., Jena, Germany). The cells with condensed chromatin and shrunken nuclei were counted as apoptotic cells.

Statistical analysis. A two-tailed Student's *t*-test was used for statistical comparisons. $P < 0.05$ was considered statistically significant. The results were expressed as means ± SD.

Results

Establishment of BMI-1 shRNA stable cell lines

In order to study the biological role of BMI-1 on cell survival in NPC, 2 BMI-1 shRNA stable cell lines were established. As shown in Fig. 1A and B, BMI-1 expression in the CNE2-BMI-1/RNAi and HONE1-BMI-1/RNAi cells dramatically decreased in comparison to that in the CNE2-vector and HONE1-vector cells.

BMI-1 knockdown made the cells more sensitive to 5-FU

To examine the effect of 5-FU on the survival of BMI-1 knockdown cells, an MTT assay was performed after the CNE2-BMI-1/RNAi, HONE1-BMI-1/RNAi, CNE2-vector, and HONE1-vector cells were treated with 5-FU for 72 h. After treatment with various concentrations of 5-FU, we found that both the BMI-1 shRNA-transfected cells, i.e., the CNE2-BMI-1/RNAi and HONE1-BMI-1/RNAi cells, showed lower cell viabilities than the empty vector-transfected cells. The IC_{50} of 5-FU in the CNE2-vector and CNE2-BMI-1/RNAi cells were 9.1504 ± 0.6997 mg/L and 3.1151 ± 0.8073 mg/L ($P < 0.05$), respectively; the IC_{50} values of 5-FU in the HONE1-vector and HONE1-BMI-1/RNAi cells were 3.9023 ± 0.752 mg/L and 1.5815 ± 0.433 mg/L ($P < 0.05$), respectively (Fig. 1C and D). These results indicated that BMI-1 knockdown made the cells more sensitive to 5-FU.

Depletion of BMI-1 enhanced 5-FU-induced apoptosis

In order to evaluate the effect of BMI-1 knockdown on the induction of apoptosis, the CNE2-BMI-1/RNAi and CNE2-vector cells that were treated with 5 mg/mL of 5-FU for 72 h were subjected to Hoechst 33258 staining. The cells were examined under a fluorescence microscope. As shown in Fig. 2A, typical apoptotic morphological changes, such as condensed chromatin, shrunken nuclei, and loss of cell volume, were frequently observed in the CNE2-BMI-1/RNAi cells. In contrast, only few apoptotic CNE2-vector cells were observed with the same apoptotic morphological changes. In order to further confirm the aforementioned results, we examined the apoptotic rate in the CNE2-BMI-1/RNAi and CNE2-vector cells after treating them with 5 mg/mL of 5-FU for 72 h. The flow cytometry data showed that the apoptotic rate among the CNE2-BMI-1/RNAi cells was 45.4% as compared to 32.1% among the CNE2-vector cells (Fig. 2B and C) ($P < 0.05$).

Knockdown of BMI-1 could inhibit AKT activation and regulate the expression levels of BCL-2 and BAX

To further explore the mechanism underlying the enhancement of 5-FU-induced apoptosis by the silencing of BMI-1, we examined the expression levels of total-AKT, phospho-AKT, BAX, and BCL-2 in the CNE2-BMI-1/RNAi and CNE2-vector cells. Our results showed that the knockdown of endogenous BMI-1 led to substantial reduction in the levels of phospho-AKT, while the total-AKT levels remained uninfluenced. Consistent with this reduction in the phospho-AKT level, Western blot analysis showed significantly decreased expression of BCL-2 in BMI-1-knocked down cells exposed

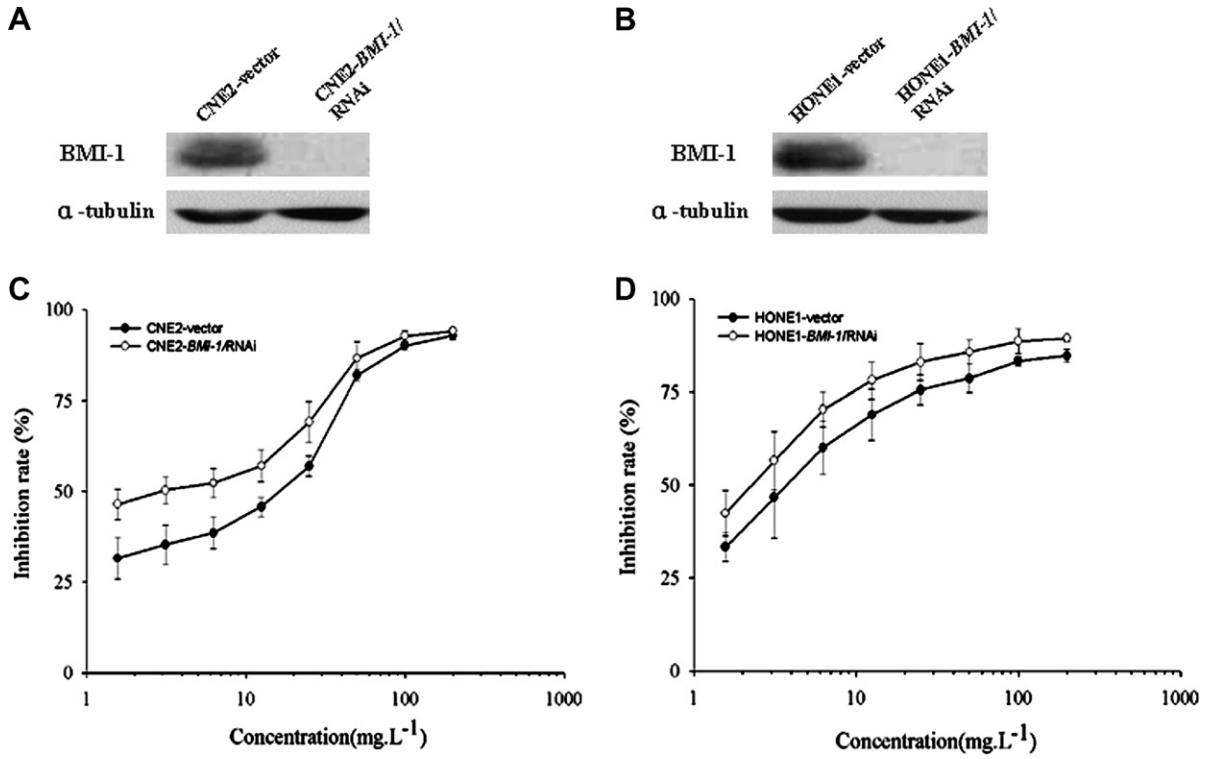

Fig. 1. Effect of *BMI-1* downregulation on the chemosensitivity of CNE2 and HONE1 cells to 5-FU treatment. (A,B) Effect of *BMI-1* shRNA in CNE2 and HONE1 cells. Protein samples were isolated from CNE2-vector, CNE2-*BMI-1*/RNAi, HONE1-vector, and HONE1-BMI-1/RNAi cells. Total protein (10–50 mg per lane) was electrophoresed and separated on a 10% sodium dodecyl sulfate (SDS)–polyacrylamide gel. The protein expression of BMI-1 was detected by Western blot analysis. Triplicate experiments showed consistent results. (C,D) CNE2-vector, CNE2-*BMI-1*/RNAi, HONE1-vector, and HONE1-Bmi-1/RNAi cells were treated with increasing concentrations of 5-FU for 72 h; cell viability was determined by the MTT assay.

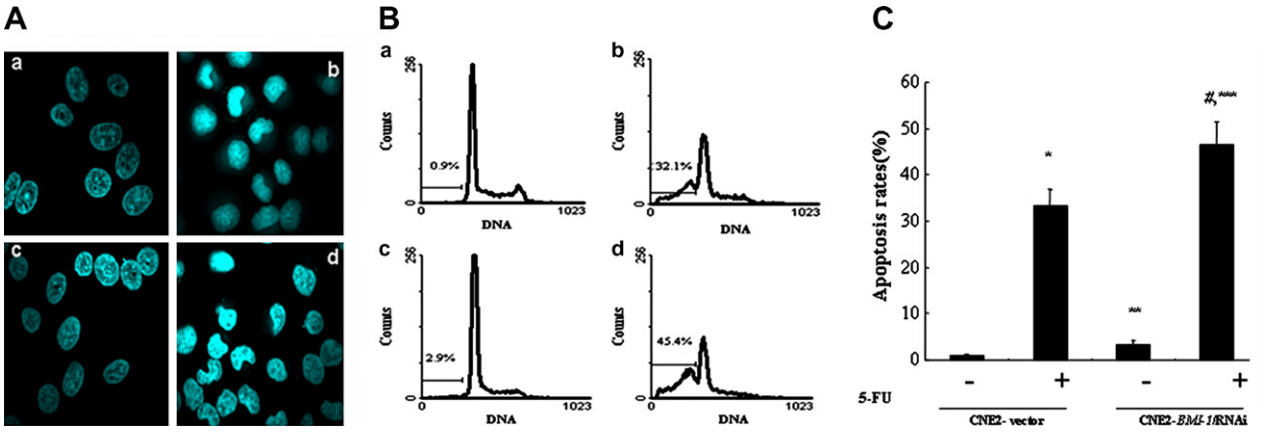

Fig. 2. Effect of *BMI-1* downregulation on 5-FU-induced apoptosis. (A) Fluorescence images (400× magnification) of CNE2 cells stained with Hoechst 33258 show (a) CNE2-vector cells, (b) CNE2-vector cells treated with 5 mg/L 5-FU for 72 h, (c) CNE2-*BMI-1*/RNAi cells, and (d) CNE2-*BMI-1*/RNAi cells treated with 5 mg/L 5-FU for 72 h. (B) Flow cytometry analyses of propidium iodide-stained cells were performed in triplicate, and the percentages of apoptosis cells are shown in (a) untreated CNE2-vector cells, (b) CNE2-vector cells treated with 5 mg/L 5-FU for 72 h, (c) untreated CNE2-*BMI-1*/RNAi cells, and (d) CNE2-*BMI-1*/RNAi cells treated with 5 mg/L 5-FU for 72 h. Columns: means; bars: +SD. Analysis of variance was performed (ANOVA). $^{*}P < 0.01$, $^{**}P < 0.05$ *vs* CNE2-vector; $^{\#}P < 0.01$ *vs* CNE2-*BMI-1*/RNAi; $^{***}P < 0.05$ *vs* CNE2-vector + 5-FU.

to 5-FU. The accumulation of BAX in the BMI-1-knocked down cells was more prominent after the cells were exposed to 5-FU (Fig. 3).

PI3K/AKT pathway was essential for the sensitization effect of BMI-1 to 5-FU treatment

To investigate whether the depletion of BMI-1 enhances 5-FU-induced apoptosis through the PI3K/AKT pathway, the CNE2-BMI-1/RNAi cells were treated with a PI3K inhibitor. The cells were pretreated with 1 μM of the PI3K inhibitor wortmannin for 1 h and then treated with various concentrations of 5-FU; this was followed by the measurement of cell viability determined by the MTT assay. As shown in Fig. 4, the inhibition rates of the CNE2-BMI-1/RNAi and CNE2-vector cells by 5-FU treatment were not significantly different after application of the PI3K inhibitor. However, the rate of inhibition of the CNE2-vector cells by 5-FU increased after pretreatment of the cells with wortmannin; this was in contrast to the low rate of inhibition of the CNE2-vector cells by 5-FU without influencing PI3K activity (Figs. 1C and 4). Taken together, the abrogation of the PI3K/AKT pathway could not further increase

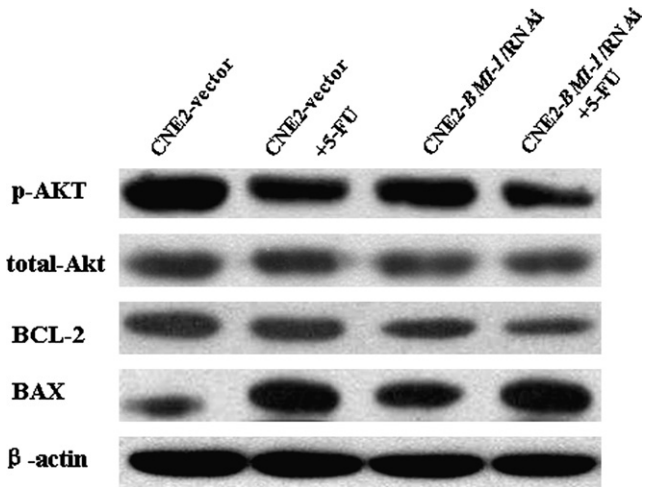

Fig. 3. Effects of knocking down BMI-1 expression on p-AKT, total-AKT, BAX, and BCL-2 levels. CNE2-vector and CNE2-*BMI-1*/RNAi cells were incubated for 72 h in the presence or absence of 5 mg/L 5-FU; total protein was electrophoresed and separated on a 12% sodium dodecyl sulfate (SDS)–polyacrylamide gel. The protein expression levels of p-AKT, total-AKT, BAX, and BCL-2 were determined by Western blot analysis. Triplicate experiments showed consistent results.

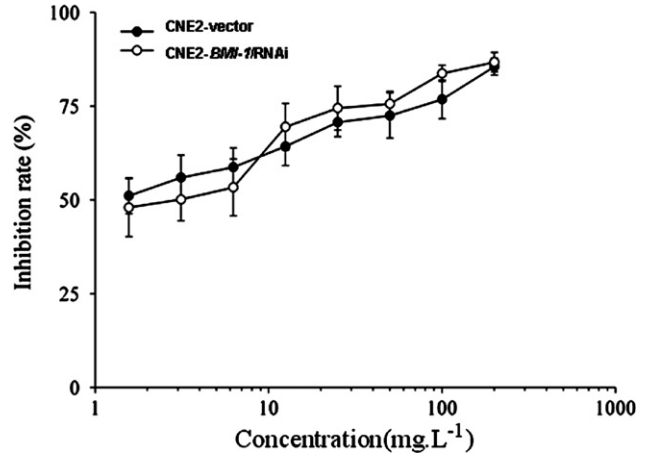

Fig. 4. Effect of the PI3K inhibitor wortmannin on chemosensitivity of CNE2 cells to 5-FU treatment. Pretreatment of CNE2-vector and CNE2-*BMI-1*/RNAi cells with 1 -μM wortmannin for 1 h followed by exposure to increasing concentrations of 5-FU for 72 h. Cell viability was determined by the MTT assay.

the sensitivity of the CNE2-BMI-1/RNAi cells to 5-FU treatment. This suggested that PI3K/AKT pathway was essential for the sensitization effect of BMI-1 to 5-FU treatment.

Discussion

In this study, we demonstrated for the first time that the knockdown of endogenous BMI-1 expression contributed to sensitizing NPC cells to the anticancer drug 5-FU by increasing apoptosis; the mechanism underlying this phenomenon was associated with the PI3K/AKT pathway.

Due to its various therapeutic advantages, such as high efficiency, mild side effects, and easy administration, 5-FU is still one of the most widely used agents in cancer chemotherapy. However, resistance to 5-FU is also commonly observed in cancer chemotherapy [21]. Therefore, enhancing the sensitization of cancer cells to drug-induced apoptosis has become an important strategy for chemotherapy. It has been reported that BMI-1 is extensively upregulated in a variety of malignancies, including non-small cell lung cancer, colorectal cancer, oral cancer, and NPC [13–16]. In our previous study, BMI-1 was found to be upregulated in NPC cells at both the transcriptional and translational levels, and the increased expression of the BMI-1 protein in these tumors correlated with a poor prognosis in NPC patients [16]. The deregulation of *BMI-1* expression has been linked with proliferation and oncogenesis in human cells. Recently it has been reported that BMI-1 is also associated with the protection of tumor cells from apoptosis [17,18,22]. These observations prompted us to investigate the possibility of the combination of 5-FU treatment and BMI-1 depletion as a clinical strategy for cancer chemotherapy.

To examine the role of BMI-1-mediated chemotherapy-induced apoptosis in NPC, 2 NPC cell lines, namely, CNE2 and HONE1, in which BMI-1 is highly expressed, were used in our study. We found that the depletion of BMI-1 in these cells resulted in an increased sensitivity of these cells to 5-FU. Further FACS and morphological evaluation revealed that silencing BMI-1 expression could enhance 5-FU-induced apoptosis. It is popularly known that the wild-type p53 protein induces apoptosis in response to DNA damage by upregulating the expression of BAX and by increasing the formation of BAX/BAX homodimers [23]. The CNE2 cell line was reported to have a heterozygous point mutation at codon 280 in p53 [24]. It was also found that defects in apoptosis due to p53 inactivation could result in treatment-resistant tumors [25]. We assumed that the apoptosis in the CNE2 cells reported herein was p53-independent. The PI3K/AKT pathway is activated in a wide variety of cancers; this results in enhanced resistance to apoptosis through multiple mechanisms. Moreover, the inhibition of PI3K activity has been observed to enhance the effects of chemotherapeutic agents [26,27]. The depletion of BMI-1 expression was observed to reduce PI3K/AKT activity in our previous study [28]. In the present study, the downregulation of the PI3K/AKT pathway by *BMI-1* RNAi was found to play a role in the sensitivity of NPC cells to 5-FU treatment. BMI-1 knockdown resulted in the reduction of the phospho-AKT level in cells treated by 5-FU; this was followed by decreased BCL-2 levels and BAX accumulation. The ratio of BCL-2 to BAX is important in determining cell apoptosis and survival after DNA damage. Our finding revealed that BMI-1 could regulate this ratio significantly in NPC cells.

In conclusion, we report for the first time the anticancer potential of the combination of 5-FU treatment and BMI-1 depletion. We found that the knockdown of BMI-1 makes cancer cells more sensitive to 5-FU treatment and that the depletion of BMI-1 enhances 5-FU-induced apoptosis. Furthermore, we demonstrated that the knockdown of BMI-1 inhibits AKT activation and regulates the expression level of BCL-2 and BAX. Abrogation of the PI3K/AKT pathway was observed to unable to increase the sensitivity of the CNE2-BMI-1/RNAi cells to 5-FU treatment, suggesting an essential role of PI3K/AKT pathway in the sensitization of BMI-1 to 5-FU treatment. Taken together, our study suggested that the combination of 5-FU treatment and BMI-1 depletion might be a potential clinical strategy for cancer chemotherapy.

Acknowledgments

This work was supported by grants from the National Natural Science Foundation of China (30670803, 30770836, 30470666, and 30570701), the Municipal Science and Technology Bureau Foundation of Guangzhou City, China (2060302) and the Major Research Program of Hunan Province, China (05SK1001-2).

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