

Original Article

PUMA enhances sensitivity of anaplastic thyroid carcinoma cells to cisplatin

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Abstract: Background and aims: p53 upregulated modulator of apoptosis (PUMA) is a potent proapoptotic molecule that is rapidly induced in cells following DNA damage. We evaluated whether adenovirus mediated exogenous PUMA expression suppresses growth of anaplastic thyroid cancer (ATC) SW1736 cells and sensitizes it to chemotherapeutic agent Cisplatin. Methods: The recombinant adenoviruses expressing wild-type PUMA (Ad-PUMA) and PUMA lacking the BH3 domain (Ad-ΔBH3), alone or in combination with chemotherapeutic agent Cisplatin, was used to treat SW1736 cells in vitro and in vivo. Cell apoptosis, cell viability and proliferation was detected by flow cytometry, TUNEL, MTT and BrdU proliferation assay. Western blot assay was used to detect caspases 3 and 9, as well as cytochrome c expression. PUMA immunohistochemistry was used to detect PUMA expression in frozen sections of the tumors. Results: Infection with Ad-PUMA resulted in more powerful cytotoxicity in SW1736 cells compared with Ad-ΔBH3. Furthermore, we assessed the efficacy of a combined treatment with Ad-PUMA and anticancer drug cisplatin for SW1736 cells and found that PUMA significantly increased the chemosensitivity of SW1736 cells, which may result from more abundant apoptosis induction. Similar results were observed in vivo. Xenograft tumors were inhibited by Ad-PUMA. Combined treatment with Ad-PUMA and cisplatin resulted in more lower viability. Conclusion: Ad-PUMA is a potent cytotoxic agent and could be a promising alternative in the ATC gene therapy in combination with chemotherapeutic agents.

Keywords: Anaplastic thyroid cancer, p53 upregulated modulator of apoptosis, apoptosis, chemotherapeutics

Introduction

ATCs are aggressive undifferentiated tumors, with a disease-specific mortality approaching 100% [1]. No curative therapy exists for ATC, it is almost uniformly fatal [2]. If the patient appears to have resectable disease, an attempt at total thyroidectomy with complete gross tumor resection should be made. However, total thyroidectomy with attempted complete tumor resection has not been shown to prolong survival except for the few patients whose tumors are small and confined entirely to the thyroid or readily excised structures [3, 4]. External-beam radiation therapy (EBRT)/intensity-modulated radiation therapy (IMRT) is used for patients of total thyroidectomy, but can only increase short-term survival, not long-term survival in some patients [5].

Chemotherapy alone can be considered for patients with unresectable or metastatic disease. Single-agent doxorubicin is the only agent that is approved by the FDA for ATC [6]. Single-agent paclitaxel may benefit some newly diagnosed patients; increased survival has been reported in patients with stage IVB disease [7]. Cisplatin is one of the most widely-used drugs to treat cancers. However, its nephrotoxic and ototoxic side-effects remain major clinical limitations. Single-agent cisplatin was deleted, because it is not recommended for patients with advanced/metastatic ATC or those with impaired renal function because of its chemoresistance, nephrotoxicity and ototoxicity [6]. The molecular mechanisms underlying this resistance, nephrotoxicity and ototoxicity have not been clearly elucidated. Improved understanding of the molecular mechanisms involved

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in cisplatin toxicity and overcoming the cisplatin toxicity will provide more insight to open new clinical strategies to treat ATC.

p53 upregulated modulator of apoptosis (PUMA) as a BH3-only Bcl-2 family protein plays an essential role in p53-dependent and -independent apoptosis [8, 9]. PUMA deficiency blocks apoptotic responses to p53 activation, DNA-damaging agents, and hypoxia in various tissue and cell types, including human cancer cells, mouse thymocytes, embryonic fibroblasts (MEFs), hematopoietic cells, and developing neurons [10-12]. Upon transcriptional induction in response to DNA damage, PUMA functions through other Bcl-2 family members, including Bax, Bcl-2, and Bcl-xL, to induce mitochondrial dysfunction and caspase activation [10]. Previous studies demonstrated that PUMA plays an important role in promoting the apoptosis of pancreatic cancer [13] and cholangiocarcinoma cell [14, 15]. In lung cancer cells, PUMA overexpression sensitizes chemotherapeutic agents induced apoptosis [16]. In colon cancer cells, chemotherapeutic agent oxaliplatin-induced PUMA expression was increased in a time- and dose-dependent manner and suppression of PUMA expression decreased oxaliplatin-induced apoptosis [17]. In addition, PUMA knockout mice recapitulated several major apoptotic deficiencies observed in p53-knockout mice [10, 12]. However, the role of PUMA in therapeutic responses in ATC cells remains unclear.

In this study, we examined the regulation of PUMA by commonly used anticancer agent cisplatin in ATC cells. We also showed that PUMA is a potent inducer of apoptosis in ATC cells and that expression of PUMA enhances the therapeutic responses of ATC cells to chemotherapeutic agents. Our data suggest that PUMA may be therapeutically useful to sensitize ATC cells to cisplatin.

Materials and methods

Cell line and culture

The human anaplastic thyroid cancer cell lines SW1736 cell lines were purchased from DSMZ (Beijing, China). The cells were cultured as the DSMZ's instruction. Briefly, the cells were grown in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS), penicillin, sodium

pyruvate, and non-essential amino acids. Adherent monolayer cultures were maintained on plastic and incubated at 37°C in 5% carbon dioxide and 95% air. The cultures were free of *Mycoplasma* species. In all of the assays, a monolayer of cells that was 50-70% confluent was used. All the methods used were according to the manufacturer's instruction.

Adenoviruses and treatment

The recombinant adenoviruses expressing wild-type PUMA (Ad-PUMA) and PUMA lacking the BH3 domain (Ad-ΔBH3) was kindly gifted by Dr. Pie (State Key Laboratory of Molecular Oncology, Cancer Institute/Hospital, Peking Union Medical College and Chinese Academy of Medical Sciences). High-titer viruses were produced in HEK-293 cells and purified by CsCl gradient ultracentrifugation. SW1736 cells were treated with 10 and 100 MOI Ad-PUMA or Ad-ΔBH3 for 48 h.

Drug treatment

The anticancer drug Cisplatin was purchased from Sigma (St. Louis, Qingdao, Jinan). 1.5 μg/ml of cisplatin was dissolved in DMSO and diluted to appropriate concentrations with cell culture media. SW1736 cell lines were treated with Cisplatin (1.5 μg/ml) for 48 hrs. For combination treatments, cells were treated together with (10 MOI) Ad-PUMA or Ad-ΔBH3 and Cisplatin (1.5 μg/ml) for 48 hours. To detect the role of caspase in PUMA-induced apoptosis, cells were pretreated with the pan-caspase inhibitor Z-VAD-fmk (20 μmol/L) for 4 hours and then subjected to the cisplatin (1.5 μg/ml) and 10 MOI ad-PUMA for 48 hours.

Cell viability assay

Cell viability was measured using the MTT assay following the manufacturer's instructions. Briefly, cells were seeded on a 96-well cell culture cluster (Corning Inc., Hangzhou, China) at a concentration of 10⁴ cells/well in a volume of 100 μl, and grown overnight. Cell viability was measured at 48 h. At the end of 48 h, 20 μl of 5 mg/ml MTT (Sigma, St. Louis, MO) was added to each well. Four hours later, 150 μl of dimethylsulfoxide was added to the MTT-treated wells for 10-15 min and the absorption was determined at 570 nm using an automated plate reader. Each experimental condition was performed in triplicate.

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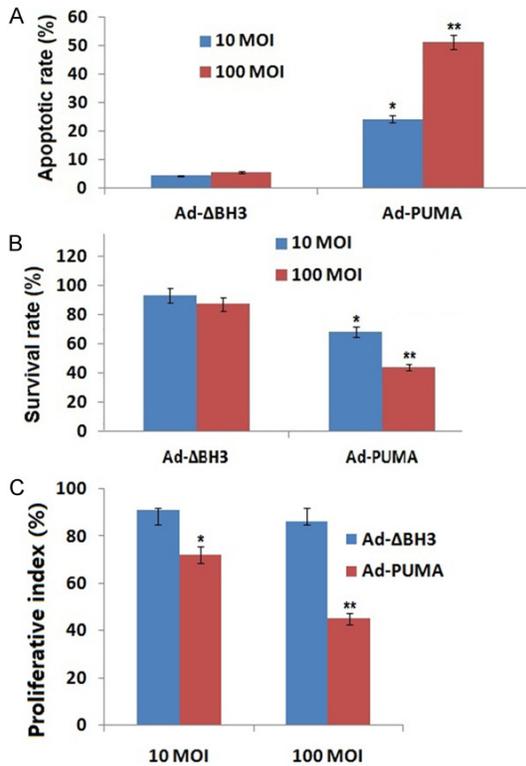


Figure 1. Ad-PUMA induces apoptosis and inhibits viability of SW1736 cells *in vitro*. SW1736 cells were treated with 10 and 100 MOI Ad-PUMA or Ad-ΔBH3 for 48 h. A. Cell apoptosis was analyzed by flow cytometry; B. Cell viability was measured by MTT assay. C. Cell proliferation by BrdUrd cell proliferation assay; * $P < 0.05$; ** $P < 0.01$.

Cell proliferation assays

Cell growth was evaluated by BrdU proliferation assay. Briefly, cells (5.0×10^3) at every experimental end point were plated onto a 24-well plate and allowed to attach for 24 h. The culture medium was then replaced with fresh medium. The cells were then permitted to incorporate with BrdU (50 μ M) for 1.5 hours. Cells were then washed with PBS, trypsinized, and washed again. BrdU staining was performed according to an adaptation of the manufacturer's protocol. BrdU-incorporation was detected according to the manufacturer's protocol for flow cytometry analysis on a FACScan instrument.

Apoptotic assay

Cells were collected at the indicated times PI, washed once with PBS, and suspended in 0.5 ml of PBS containing 0.1% (v/v) Triton X-100 for nuclei preparation. The suspension was filtered through a nylon mesh and then adjusted to a

final concentration of 0.1% (w/v) RNase and 50 μ g/ml propidium iodide. Apoptotic cells were quantified by FACScan cytometer. The DNA fragmentation assay was carried out as the manufacturer's instruction.

Western blot assay

Cells were lysed using Cellytic™ M cell lysis reagent (Sigma) that contained 1 mM PMSF, 1 mM NaF and protease inhibitors cocktail (Roche). Equal amounts of protein were subjected to SDS-PAGE on either a 10 or 12% polyacrylamide gel. The resolved proteins were transferred onto a PVDF membrane (Millipore), which was then exposed to 5% non-fat dried milk in TBS-Tween buffer for an hour at room temperature before incubation overnight at 4°C with primary antibodies. Mouse polyclonal antibodies against PUMA and β -actin were obtained from Santa Cruz Biotechnology (Shanghai, China). The PVDF membrane was then washed three times with TBS containing 0.05% Tween-20 before incubation for an hour at room temperature with HRP-conjugated goat antibodies to rabbit IgG. Immune complexes were finally detected with chemiluminescence reagents (Millipore). Experiments were repeated at least three times.

Xenograft tumors

All animal experiments were approved by the Institutional Animal Care and Use Committee at the affiliated hospital of Qingdao University. Xenograft tumors (50-100 mm³) (4 weeks) were established by s.c. injection of 5×10^6 SW1736 cells into both flanks of 5- to 6-week-old female athymic nude mice (Harlan). Tumor treatment was initiated by injecting each tumor (~50-100 mm³) with Ad-PUMA or Ad-ΔBH3 at 5×10^8 plaque-forming units in 100 μ L of PBS (on days 1, 3 and 5). In the combination model, cisplatin (3 mg/kg/d) was injected i.p. into the mice (on days 6-10). Each treatment was repeated twice. Tumor growth was monitored thrice a week for 6 weeks by calipers to calculate tumor volumes according to the formula (length \times width²)/2.

TUNEL assay

Terminal deoxyribonucleotidyl transferase-mediated dUTP nick end labeling staining on frozen sections was done using recombinant terminal transferase (Roche, Hangzhou, China)

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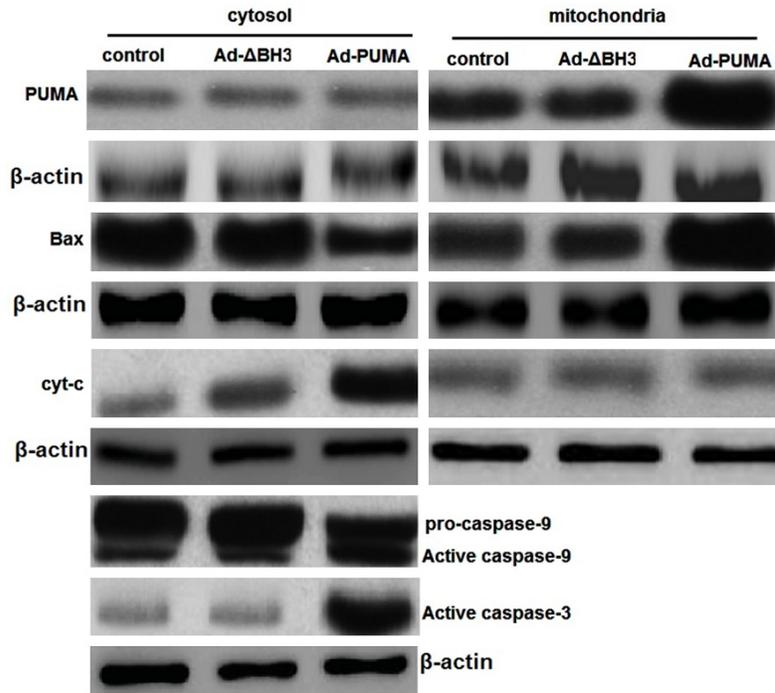


Figure 2. Ad-PUMA induced mitochondria-mediated apoptosis. Release of cytochrome c, Bax and caspase-3, 9 was examined in the cytosolic fractions of the SW1736 cells infected with ad-PUMA for indicated times by Western blotting.

and dUTP-Alexa 594 (Molecular Probes) according to the instructions of the manufacturers and counter stained by 4',6-diamidino-2-phenylindole. Apoptotic cells were counted under a fluorescence microscope in randomly chosen fields, and the apoptosis index was calculated as a percentage of TUNEL-positive cells in at least 1,000 scored cells.

Statistical analyses

Data were analyzed by unpaired *t*-test or ANOVA in which multiple comparisons were performed using the least-significant difference method. *P*-values < 0.05 were considered to be statistically significant. The means \pm one standard deviation (S.D.) are displayed in the figures.

Results

Ad-PUMA induces apoptosis and inhibits viability of SW1736 cells in vitro

The ability of PUMA to induce cell death has been well documented in various human cancer cell lines, including colorectal, lung, head and neck, osteosarcoma, and ATC cells. Other study has previously shown that adenoviral-

mediated expression of PUMA causes massive death of melanoma cells within 72 hours of infection. Here, we show that infection of ad-PUMA (10 and 100 MOI) for 48 h produced a large proportion (23% and 51%) of apoptotic cells (**Figure 1A**), whereas Ad-ΔBH3 had virtually no effect on cell apoptosis compared with the untreated cells, suggesting that ad-PUMA induced apoptosis of SW1736 cells in dose-dependent way.

Cell viability by MTT assay shown that ad-PUMA was found to cause profound growth suppression in SW1736 cells in a dose-dependent way, compared with the Ad-ΔBH3 groups (**Figure 1B**). To confirm cell growth inhibition, we have also

conducted the cell proliferation assay using the BrdU labeling and Detection Kit. We found similar results as MTT assay using this method (**Figure 1C**).

Ad-PUMA kills SW1736 cells via rapid induction of mitochondrial-mediated apoptosis

PUMA reportedly induces apoptosis through the intrinsic, mitochondrial-mediated pathway [18]. Activation of this pathway is characterized by translocation of cytosolic Bax to the mitochondrial membrane, release of cytochrome c from mitochondria, subsequent activation of caspase-3 and caspase-9, inevitably, cell death. To confirm that our ad-PUMA construct activates this pathway in SW1736 cells, we examined the cytosolic and mitochondrial protein expression. Cells infected with ad-PUMA (100 MOI) for 48 hours were subjected to subcellular fractionation to separate cytosolic and mitochondrial proteins. Western blot analysis of the protein extracts showed that PUMA was strongly expressed in mitochondria after infection (**Figure 2**), showing the ability of ad-PUMA to rapidly induce gene expression, but not in those infected with Ad-ΔBH3. PUMA expression was accompanied by translocation of cytosolic

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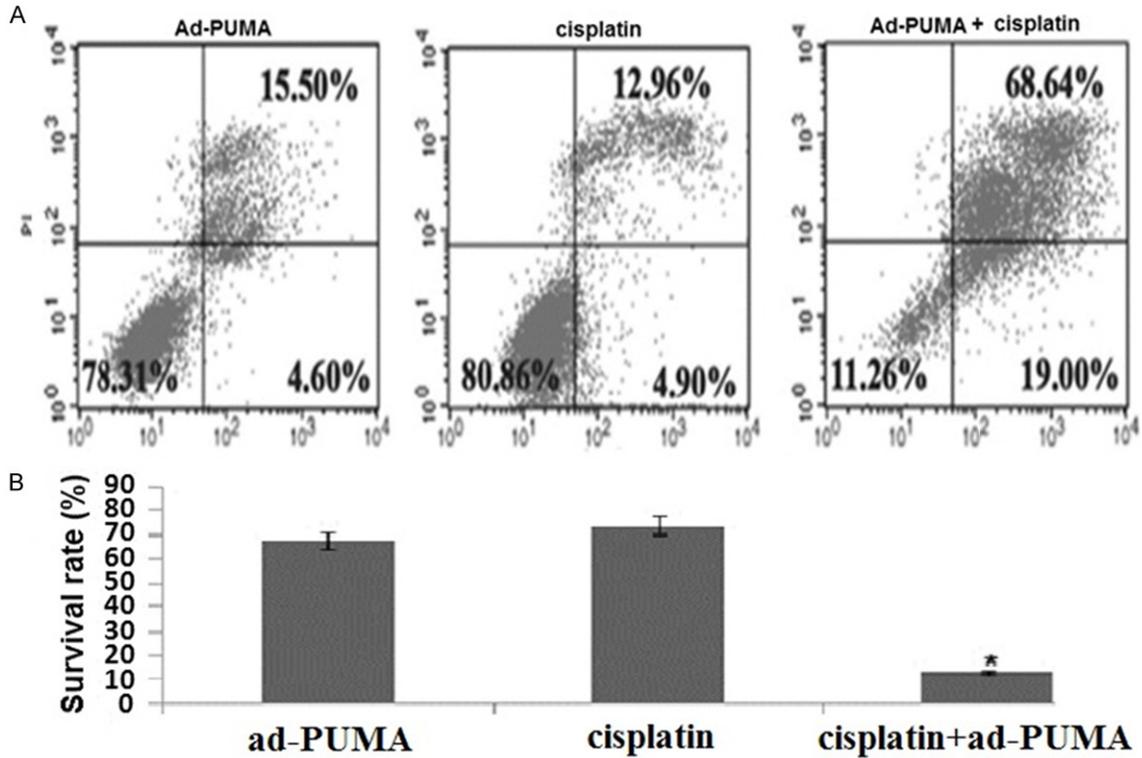


Figure 3. The sensitizing effects of PUMA are mediated by enhanced apoptosis induction. SW1736 cells were subjected to cisplatin (1.5 $\mu\text{g/ml}$) and analyzed for apoptosis. Adenoviruses were used at 10 MOI. A. Apoptosis was analyzed by Flow cytometry assays. B. Cell viability was detected by MTT assay. * $P < 0.01$.

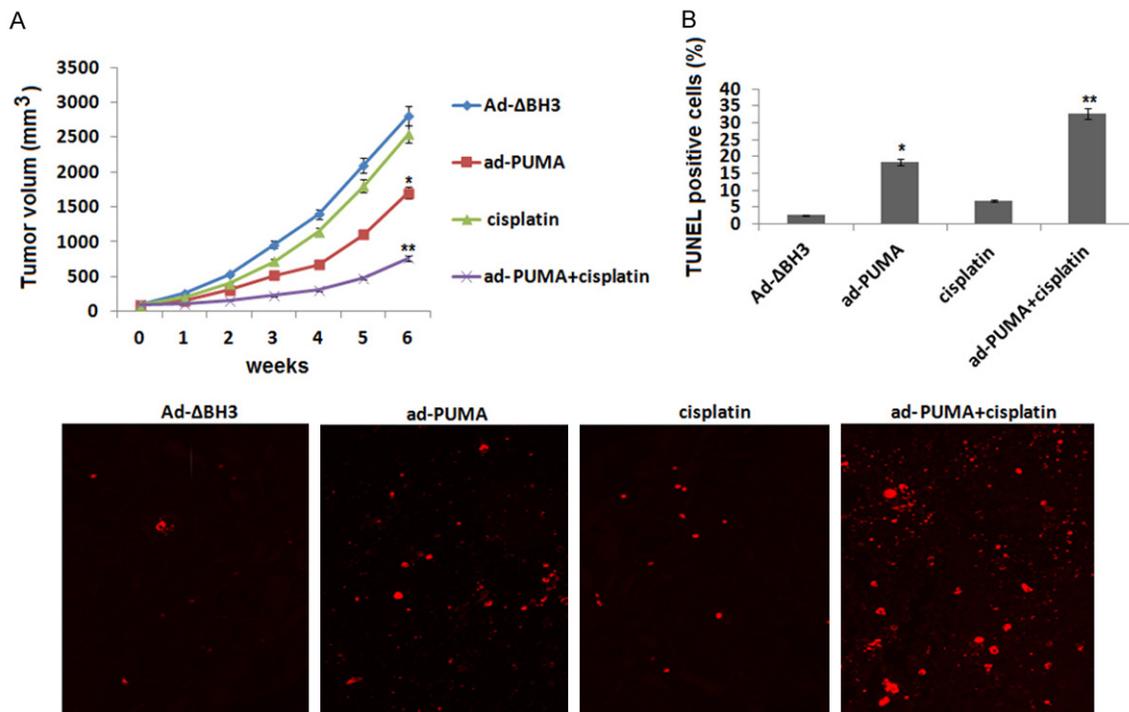


Figure 4. Ad-PUMA enhances cisplatin-mediated growth inhibition both in vivo. A. The growth curve of SW1736 tumors ($n = 6$ per group) subjected to Ad-PUMA or/and cisplatin treatments as described in Materials and Methods. B. Apoptosis was analyzed by TUNEL staining. * $P < 0.05$; ** $P < 0.01$.

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Bax to the mitochondria, release of cytochrome c (cyt-c) from mitochondria into the cytosol, and cleavage of procaspase-3 and procaspase-9 (**Figure 2**). These data confirm that ad-PUMA induces SW1736 cell death via mitochondrial-mediated apoptosis.

PUMA sensitizes SW1736 cells to cisplatin

Abnormalities of apoptosis regulation have been shown to contribute to the development of resistance to chemotherapy in cancer cells. The important role of PUMA in DNA damage-induced apoptosis suggests that elevated PUMA expression may restore sensitivity of cancer cells to anticancer agents. To test this hypothesis, SW1736 cells were treated with low dose of Ad-PUMA [10 multiplicity of infection (MOI)], alone or in combination with cisplatin (1.5 µg/ml). PUMA was found to significantly enhance the growth inhibitory effects of cisplatin, with the synergy most pronounced when combined with DNA-damaging agent cisplatin (**Figure 3A**).

We then determined whether PUMA expression sensitizes SW1736 cells to cisplatin through induction of apoptosis. Ad-PUMA (10 MOI) and cisplatin (1.5 µg/ml) alone did not induce significant apoptosis in SW1736 cells (**Figure 3B**). However, almost 87% of cells underwent apoptosis after the combination treatment for 48 hours (**Figure 3B**). In contrast, the control Ad-ΔBH3 had no effect when combined with cisplatin (**Figure 3B**).

Ad-PUMA inhibits growth and sensitizes of ATC tumor xenografts to cisplatin

In light of the potent cell killing ability of ad-PUMA *in vitro*, we hypothesized that ad-PUMA would be highly effective at inhibiting ATC tumor growth and sensitizes of ATC tumor xenografts to Cisplatin *in vivo*. Animals bearing SW1736 tumors ($N = 24$) were randomized into 4 groups and treated with either (a) ad-PUMA (b) Ad-ΔBH3 (c) cisplatin (d) ad-PUMA and cisplatin. As expected, ad-PUMA significantly inhibited SW1736 tumor growth (**Figure 4A**). After 3 times of ad-PUMA treatment, tumors treated with ad-PUMA were 38% smaller than those of the untreated SW1736 tumor ($P < 0.05$, *t* test). Treatment of cisplatin showed slowed SW1736 tumor growth rates, but only 10% smaller than those of the untreated SW1736 tumor ($P >$

0.05, *t* test). When cisplatin and ad-PUMA treatments were combined, however, a marked enhancement of growth inhibition was observed; the combination treatment (ad-PUMA and cisplatin) yielded tumors that were 78% smaller than those of the untreated SW1736 tumor ($P < 0.01$, *t* test). Ad-ΔBH3 did not show any effect on tumor growth (data not shown).

Analyzing tumor histology by apoptosis in situ by terminal deoxyribonucleotidyl transferase-mediated dUTP nick end labeling staining revealed significant apoptotic cells in the tumors treated with Ad-PUMA or Ad-PUMA+ cisplatin groups. Furthermore, apoptotic cells significantly increased in the Ad-PUMA+cisplatin groups than that of in the Ad-PUMA or cisplatin groups (Figure 4B). Our data indicate that ad-PUMA inhibits SW1736 tumor growth and clearly augments the inhibitory effect of cisplatin on SW1736 tumor growth *in vivo*.

Discussion

Anaplastic thyroid cancer (ATC) represents 1%-2% of all thyroid tumors and is characterized by aggressive, local invasion and common distant metastases. Due to the extremely aggressive behavior of ATC, the American Joint Committee on Cancer (AJCC) defines all of its stages as stage IV [18]. The mean survival time is around 6 months from diagnosis an outcome that is frequently not altered by treatment. The majority of patients with ATC die from aggressive local regional disease, primarily from upper airway respiratory failure. For this reason, aggressive local therapy is indicated in all patients who can tolerate it [19].

In the event that patients present with surgically resectable disease, without distant metastasis, treatment plans are multi-modal and include surgery, radiation, with or without the addition of chemotherapy [20]. Aggressive surgery for ATC is especially worthwhile when the disease is unilateral in location. Chemotherapy appears to increase survival; however, toxic regimens can be poorly tolerated [21]. Current clinical trials have investigated the use of a combination of doxorubicin and cisplatin, in addition to docetaxel or paclitaxel in this setting. However, it has demonstrated a response in only 20% of patients [22-24], suggesting that ATC is drug-resistance. Research into novel molecular targeting agents is urgently required.

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PUMA was initially identified as a transcriptional target of p53 and a potent apoptosis inducer in various cancer cells [8-10]. PUMA is localized in the mitochondria and induces apoptosis through the Bcl-2 family proteins Bax/Bak and the mitochondrial pathway [25]. Previous studies have shown that elevated PUMA expression, either alone or in combination with chemotherapy or irradiation, induced profound toxicity to a variety of cancer cells such as lung, head and neck, esophagus and breast cancer cells [16-19]. However, the role of PUMA in the therapeutic responses of ATC cells to Cis-based anticancer drugs remains unclear.

In the present study, SW1736 cells were infected with an adenovirus expressing PUMA (Ad-PUMA) or a control adenovirus (Ad- Δ BH3) expressing PUMA lacking the BH3 domain. After infection for 48 hours, cells were analyzed by MTT assay and BrdU proliferation assay. Ad-PUMA was found to cause profound growth suppression in SW1736 cells whereas Ad- Δ BH3 had virtually no effect on cell growth compared with the untreated cells. The cells infected by Ad-PUMA, but not those infected by Ad- Δ BH3, underwent massive apoptosis revealed by FCM analysis. Activation of caspases 3 and 9 was detected in SW1736 cell lines after Ad-PUMA infection and correlated with growth suppression. To examine whether PUMA-induced apoptosis in SW1736 cells is mediated through mitochondrial pathway, cytosolic fractions were isolated from cells infected with Ad-PUMA and Ad- Δ BH3 for 48 hours, and then analyzed by Western blotting. Cytochrome c and Bax was found to be released into the cytosol in the cells infected with Ad-PUMA but not in those infected with Ad- Δ BH3. These data show that PUMA is a potent inducer of growth suppression and apoptosis in SW1736 cells and that PUMA promotes the release of mitochondrial apoptogenic proteins and caspase activation to induce apoptosis in SW1736 cells.

SW1736 tumors injected with ad-PUMA grew more slowly than those injected with Ad- Δ BH3-negative control virus. After 42 days of treatment, Ad- Δ BH3 tumors treated with ad-PUMA were 48% smaller than controls. More apoptosis cells were shown in the ad-PUMA groups. Our results indicate that ad-PUMA indeed inhibits SW1736 tumor growth by inducing apoptosis *in vivo*.

Treatment with single-drug chemotherapy is not very effective in ATC patients, although some patients may respond or have stable disease [29]. The addition of larger doses of other chemotherapeutic drugs has not been associated with improved control of distant disease or with improved survival. In the present study, SW1736 cells or SW1736 tumors treated with cisplatin alone resulted in fewer cell apoptosis and growth inhibition. SW1736 cells were treated with low dose of Ad-PUMA [10 MOI], alone or in combination with cisplatin. PUMA was found to significantly enhance the growth inhibitory effects of cisplatin, with the synergy most pronounced when combined with cisplatin. We then determined whether PUMA expression sensitizes SW1736 cells to cisplatin through induction of apoptosis. Ad-PUMA (10 MOI) alone did not induce significant apoptosis in SW1736 cells. However, almost 75% of cells underwent apoptosis after the combination treatment for 48 hours. Further study has indicated PUMA can synergize with cisplatin to trigger the release of cytochrome c to initiate apoptosis in ATC cells. *In vivo*, PUMA and cisplatin has the same results as that of *in vivo*. It was shown that PUMA seems to be the most effective in enhancing growth suppression and apoptosis when combined with DNA-damaging agents, such as cisplatin. This observation is consistent with the notion that PUMA plays a critical role in DNA damage-induced apoptosis.

In summary, we have presented the first evidence that PUMA play an important role in inhibiting growth and inducing apoptosis in ATC cells *in vitro* and *in vivo*. Our novel finding that combined PUMA and cisplatin results in a strong therapeutic effect for ATC cells. These results, taken together, form the basis for a novel and potentially more effective therapeutic strategy in the treatment of ATC.

Disclosure of conflict of interest

None.

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