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Original Article

ING4 enhances paclitaxel's effect on colorectal cancer growth in vitro and in vivo

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Abstract: Inhibitor of growth 4 (ING4) is a tumor suppressor that can inhibit cell growth and induce apoptosis. ING4 expression levels show negative correlation with the clinical stage, histological grade, and lymph node metastasis of colorectal cancer. Further insights are needed to analyze the effect of adenovirus-mediated ING4 on colorectal cancer cell growth and the response to paclitaxel treatment. In this study, we found adenovirus-mediated ING4 expression reduced proliferation and enhanced apoptosis in the SW1116 cells. p-Stat3 and Ki-67 expression significantly decreased in the SW1116 cells treated with Ad-ING4, PTX, or Ad-ING4 + PTX compared with those treated with PBS or Ad-GFP both in vitro and in vivo ($P < 0.05$). In animal experiments, the mice treated with Ad-ING4, PTX, or Ad-ING4 + PTX exhibited significantly inhibited growth of SW1116 xenografts compared with those treated with PBS or Ad-GFP ($P < 0.05$) and the combination (Ad-ING4 + PTX) treatment exhibited the highest inhibition. Our results highlight that Ad-ING4 significantly inhibits growth and induces apoptosis in SW1116 colorectal cancer cells and suppresses tumor growth in SW1116 xenografts by downregulating p-Stat3 and Ki-67 expression. A combination of Ad-ING4 and PTX exhibits the highest inhibition, indicating that ING4 enhances sensitivity to chemotherapy.

Keywords: Colorectal cancer, adenovirus, inhibitor of growth family, paclitaxel, signal transducers and activators of transcription

Introduction

Colorectal cancer is one of the most common malignancies of the digestive system and the second most lethal malignancy in the United States and Europe [1]. Environmental, hereditary, and dietary factors, ethnicity, as well as diseases such as colorectal adenoma and chronic inflammation play a role in the incidence of colorectal cancer. Surgery and chemotherapy are currently the primary treatment strategies for colorectal cancer [2].

One of the approaches used for cancer treatment is gene therapy. Recombinant adenoviral human p53 is a gene therapy agent that has been approved for clinical trials for liver cancer and has displayed obvious curative effects [3]. The inhibitor of growth (ING) family includes five members (ING1 to ING5), which play important roles in tumor inhibition. As a new candidate tumor suppressor, the effect and mechanism of ING4 activity has attracted increasing attention. ING4 protein is localized in the nucleus. Mutations leading to functional inactivation of the ING4 protein is associated with the development and progression various tumors. Loss of ING4 expression increases microvessel density in tumor tissue [4]. The tumor inhibitory effect of ING4 is mediated through negative regulation of transcription factors and promoters of tumor-induced genes [5]. Several reports indicate that the ING4 expression level correlates negatively with tumor grade in many types of tumors including glioma [6], melanoma [7], breast cancer [8], and lung cancer [9]. In addition, ING4 increases sensitivity to chemotherapeutic drugs in cancer patients [10]. A combination of adenovirus-mediated ING4 gene transfer and cisplatin displays enhanced antitumor effects in hepatocarcinoma cells [11].

To date, ING4 has been experimentally used for the treatment of glioma [12], pancreatic cancer [13], and breast cancer [14]. Adenoviral vectors were employed in most of the clinical trials involving gene therapy because of their wide host range, ability to infect tumor cells in interphase and anaphase stages of the cell cycle, high transfection efficiency generation of high-titer virus, and because this virus cannot integrate into the genome of host cells, and hence cannot cause any mutation or cancer [15, 16]. In this study, the SW1116 colorectal cancer cell line was infected with ING4-expressing adenovirus (Ad-ING4), and the effects of Ad-ING4 on the growth of tumor xenografts in nude mice as well as the sensitivity to paclitaxel treatment were explored.

Materials and methods

Virus production and cell culture

Adenoviral empty vector (Ad-GFP), ING4-expressing recombinant adenovirus (Ad-ING4), and QBI-293A cells were gifts from the Department of Cell and Molecular Biology, Suzhou University. QBI-293A cells were transfected with Ad-ING4 or Ad-GFP to obtain high-titer virus. The SW1116 colorectal cancer cell line (a gift from the Nanjin Medical University) was maintained in NOE medium consisting of RPMI 1640 (Sigma-Aldrich Co.) supplemented with 10% fetal bovine serum (Intergen), 10 ng/mL epidermal growth factor (Sigma-Aldrich Co.), and 100 U/mL penicillin or streptomycin (Sigma-Aldrich Co.).

Cell line generation and treatment

SW1116 cells in the exponential growth phase were treated/infected with 100 MOI of Ad-GFP, 100 MOI of Ad-ING4, paclitaxel (PTX) to a final concentration of 0.5 μg/mL, 100 MOI of Ad-ING4 along with 0.25 μg/mL PTX, or 0.1 mol/L PBS. The corresponding treated cells are hereafter referred to as Ad-GFP, Ad-ING4, PTX, Ad-ING4 + PTX, or PBS groups, respectively. The cells were incubated under conditions of 5% CO_2 at 37°C.

ING4 expression analysis in SW1116 cells by RT-PCR

Total RNA was isolated from SW1116 cells subjected to various treatments (as detailed above) using Trizol (Invitrogen), according to the manufacturer's instructions. One microgram of RNA from each treatment was used for cDNA synthesis involving reverse transcription at 42°C for 60 min, followed by incubation at 70°C for 5 min. The following primers were used for ING4 amplification: ING4 sense or forward primer (5'-GCGTCGACATGGATGATGGGATGTATTTGGAAC-3') and reverse primer (5'-GCAAGCTTCTATTTCTTCTTCCGTTCTTGGGAG-3'). Glyceraldehyde-3-phosphate dehydrogenase was used as an internal control, with the forward or sense primer (5'-CAAGGTCATCCATGACAACTTTG-3') and reverse primer (5'-GTCCACCACCCTGTTGCTGTAG-3'). The PCR conditions included initial denaturation at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 65°C for 30 s, and elongation at 72°C for 1 min, followed by a final elongation for 15 min. The amplified products were visualized by agarose gel electrophoresis.

Immunohistochemical staining

Immunohistochemical staining was performed by the avidin-biotin-peroxidase method. In brief, sections from the formalin-fixed and paraffin-embedded tissue were deparaffinized in xylene, rehydrated by washing with 1 × PBS, and subjected to antigen retrieval. After blocking endogenous peroxidase activity and nonspecific protein binding, the sections were incubated overnight with ING4 (Sanying Inc., Wuhan, 1:100), Stat3 (Santa Cruz, USA, 1:100), p-Stat3 (Cell Signaling, USA, 1:100), and Ki-67 (Boaoseng Inc., Beijing, 1:100) antibodies at 4°C in a humid chamber. After a reaction with MaxVision (Maixing, Fujian) for 20 min, the signal was detected using the chromogen 3,3'-diaminobenzidine (Maixing, Fujian). The nuclei were counterstained with hematoxylin and eosin (H&E), followed by dehydration and mounting on the coverslip.

Analysis of ING4-, Stat3-, p-Stat3-, and Ki-67-stained tissue sections

In tissue sections stained for ING4, Stat3, p-Stat3, and Ki-67, the presence of tumor cells with a brown cytoplasm and nucleus was considered positive. We reviewed 5 fields per section at 400 × magnification. Positively stained cells per 100 tumor cells were counted in each field, and the mean percentage of positively stained cells was used to determine the expression of the target protein in a given section. All these counts were performed in an unbiased

manner in at least 3 randomly selected sections from each mouse.

Western blot analysis

Western blot analysis was performed as described previously [17]. The cells and tumor tissues were lysed in ice-cold radioimmunoprecipitation assay buffer. Protein concentrations were determined, and equal amounts of protein were separated on a 10% sodium dodecyl sulfate-polyacrylamide gel. The proteins were then transferred to a polyvinylidene fluoride membrane (Amersham Hybond-P PVDF Membrane; GE Healthcare) and probed with primary antibodies of interest (described in the section on IHC staining) or β-actin antibody (Zhongshan Inc., Beijing). Horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG secondary antibodies were also purchased from Zhongshan Inc., Beijing.

MTT assay

Following various treatments, SW1116 cells in the logarithmic growth phase were diluted to 1 × 10^5 cells/mL in culture medium, and 100 μL of the diluted cell suspensions were added to the wells of 96-well plates in replicates of six. The cells were incubated for 24 h, 48 h, and 72 h, following which 20 μL of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma) reagent was added to each well, including three control wells (containing only medium) that serve as blanks for absorbance measurement. After 4 h, when the purple precipitate was clearly visible, 100 μL of detergent reagent was added to all of the wells, including controls, for precipitate solubilization, following which the absorbance in each well was measured at 570 nm using a microplate reader. The average values for triplicate samples were calculated, from which the average value for the blanks were subtracted. The inhibition rate (IR) of the various treatment groups was calculated using the following formula: IR (%) = (1 - $OD_{570\ nm}$ of the treated group/$OD_{570\ nm}$ of the control group) × 100%.

Apoptosis assay

To analyze apoptosis, cells subjected to various treatments were trypsinized, and 5 μL of 7-amino-actinomycin D (7-AAD) was added to 1 × 10^5 cells in 50 μL of binding buffer. The cells were then stained with annexin V-Phycoerythrin (PE) using the Annexin V kit (Apoptosis kit, Kaiji Biotechnology Inc., Nanjing, China), according to the manufacturer's instructions. Apoptosis was analyzed by flow cytometry using FACStation (Beckman Inc., USA). The experiment was repeated three times. The apoptotic rate (AR) of different treated groups of cells was calculated using the following formula: AR (%) = (1-apoptotic cell numbers in the treated group/apoptotic cell numbers in the control group) × 100%.

Animal experiments

SW1116 cells subjected to various treatments were amplified for obtaining sufficient cells for animal injection. Twenty-five nude mice aged 6-8 weeks were obtained from the animal center of the Nanjin Medical University. Each mouse was subcutaneously injected with 0.1 mL of PBS containing 3.0 × 10^6 untreated SW1116 cells. Twenty days after injection, when the tumors had grown to an average volume of 0.5 mm^3, the mice were randomly divided into five groups of five mice each and administered one of the following by peritumoral injections at multiple sites: PBS (50 μL/mouse), Ad-GFP (50 μL media containing 1 × 10^7 pfu Ad-GFP/mouse), Ad-ING4 (50 μL media containing 1 × 10^7 pfu Ad-ING4/mouse), PTX (10 mg/kg body weight of mouse), and Ad-ING4 + PTX (50 μL media containing 1 × 10^7 pfu Ad-ING4 + PTX 5 mg/kg body weight). These treatments were repeated every 2 days, and the tumor volume measured every week. Thirty-one days after the initial injection, the mice were sacrificed by cervical decapitation, and the tumor masses were removed and weighed. The tumor tissue was fixed in 10% formalin and embedded in paraffin for H&E and immunohistochemical staining. This study was approved by the Animal Welfare Committee of Tianjin Union Medicine Center.

Tumor growth analysis

The length and width of each tumor were measured using a vernier caliper every day from Day 20 to Day 41 after inoculation. Tumor size was determined using the following formula: Tumor volume (mm^3) = (length × $width^2$)/2. Tumor growth curves were established on the basis of tumor size. The IR of the engrafted tumor was determined using the following formula: IR (%) = (1-tumor weight in the treated group/tumor weight in the control group) × 100%.

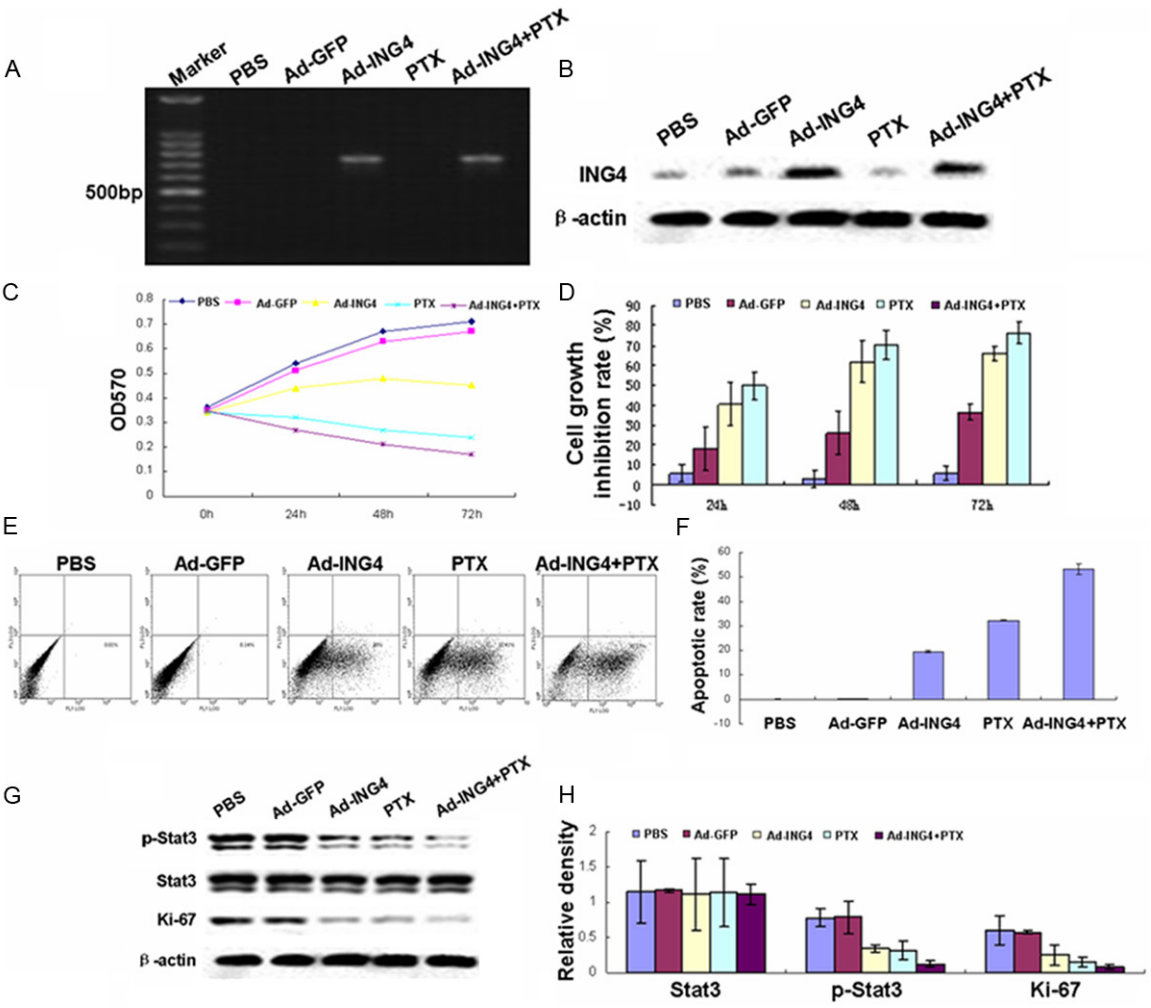

Figure 1. ING4 inhibits growth, proliferation, and expression of relevant proteins in SW1116 cells subjected to various treatments. A: Analysis of ING4 transcription by RT-PCR. B: Analysis of ING4 expression using western blot analysis. C: MTT proliferation assay using SW1116 cells subjected to various treatments. D: IR of proliferation of SW1116 cells subjected to various treatments, with varying treatment duration. E: Apoptosis analysis in different treatment groups of SW1116 cells using flow cytometry. F: Determination of AR for SW1116 cells using Annexin V-PE/7-AAD double staining. G: Expression analysis of different proteins in SW1116 cells using western blot analysis. H: Gray value comparison for determining the relative expression levels of the proteins of interest among the different treatment groups.

Statistical analysis

Statistics software package SPSS 10.0 (Chicago, Illinois) was used for the analysis. $P < 0.05$ was considered significant. Differences among groups were assessed using ANOVA.

Results

Expression analysis of ING4 in SW1116 cells

SW1116 cells subjected to various treatments were harvested, and total mRNA was extracted. RT-PCR analysis followed by agarose gel electrophoresis revealed a band of 747 bp, corresponding to an ING4-specfic product, in the Ad-ING4- and Ad-ING4 + PTX groups, confirming successful infection and adenovirus-mediated ING4 expression in the SW1116 cells (**Figure 1A**).

ING4 expression was further confirmed by western blot analysis, performed in triplicate, using cell lysates generated from the different treatment groups of SW1116 cells. β-actin was used as a loading control. A band corresponding to 29 kDa indicated ING4 expression in all the five groups (representing endogenous

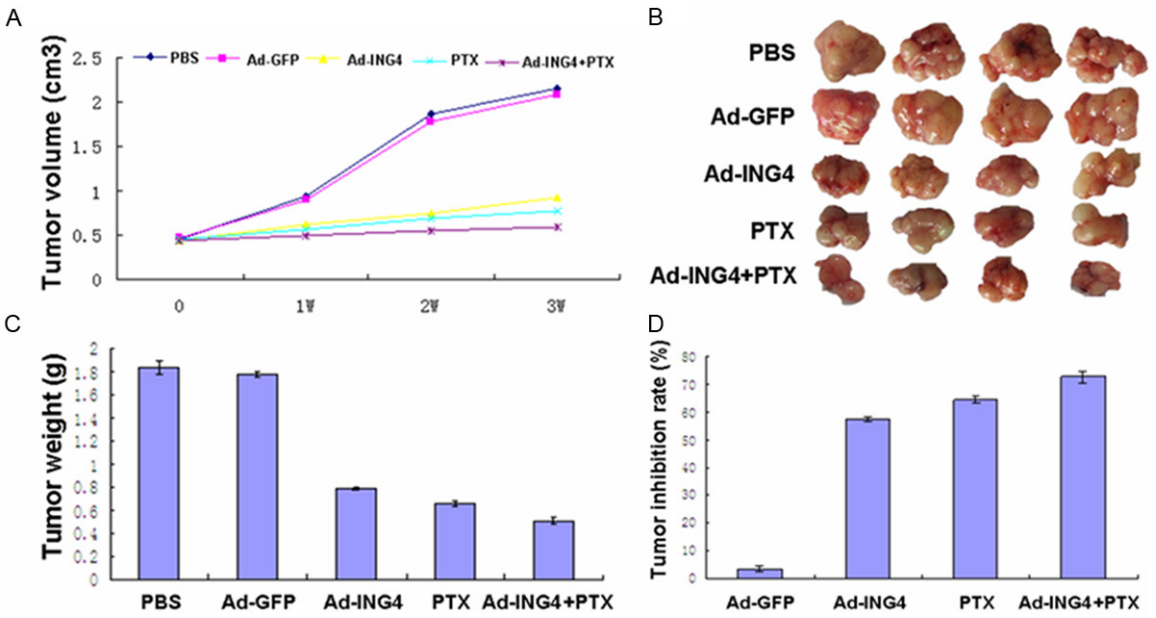

Figure 2. Analysis of tumor growth in mice subjected to various treatments. A: Tumor growth curve in different treatment groups. B: Gross images of tumors from mice subjected to various treatments. C: Comparison of tumor weight in the different treatment groups. D: Histogram representing tumor IRs after various treatments.

ING4), with increased ING4 expression in the Ad-ING4 and Ad-ING4 + PTX groups (**Figure 1B**).

MTT cell proliferation assay using different treatment groups of SW1116 cells

The proliferative potential of the five groups of SW1116 cells treated for 0 h, 24 h, 48 h, and 72 h was compared using the MTT assay, which revealed a decrease in the growth of Ad-ING4, PTX, and Ad-ING4 + PTX groups, with the corresponding IR values being (36.60 ± 4.01)%, (66.11 ± 3.85)%, and (76.11 ± 5.50)% after 72 h treatment, respectively. The inhibition was time dependent, with the highest IR observed at 72 h for these treatment groups (**Figure 1C**). Moreover, the treatment groups showed significant ($P < 0.05$) IR values, with the combination (Ad-ING4 + PTX) group exhibiting the highest IR, significantly higher than the Ad-ING4 and PTX groups ($P < 0.05$; **Figure 1D**).

Analysis of apoptosis in the five treatment groups

SW1116 cells subjected to different treatments for 72 h were harvested for apoptosis analysis using flow cytometry (**Figure 1E**). Ad-ING4, PTX, and Ad-ING4 + PTX treatments were found to induce apoptosis, with AR values of (19.46 ± 0.47)%, (32.28 ± 0.12)%, and (53.15 ± 2.25)%. The treatment groups showed significant ($P < 0.05$) AR values, with the combination group (Ad-ING4 + PTX) exhibiting the highest AR, significantly higher than the Ad-ING4 and PTX groups ($P < 0.05$; **Figure 1F**).

Western blot analysis of ING4, Stat3, p-Stat3, and Ki-67 expression in SW1116 cells and mouse tumors

Protein extracts from cells and mouse tumor tissue from the five treatment groups were used for the determination of the expression levels of Stat3, p-Stat3, and Ki-67 using western blot analysis. The Ad-ING4, PTX, and Ad-ING4 + PTX treatments decreased p-Stat3 and Ki-67 expression levels both in vitro and in vivo, with the lowest expression found after Ad-ING4 + PTX treatment (**Figures 1G** and **3B**). These differences in expression levels were significant ($P < 0.05$; **Figures 1H** and **3C**). On the other hand, Stat3 expression levels did not exhibit significant differences between the five treatment groups in vitro or in vivo.

Animal experiments

Twenty days after injection with SW1116 cells, all the nude mice had palpable tumors, 10 mm in diameter; these mice were then divided into

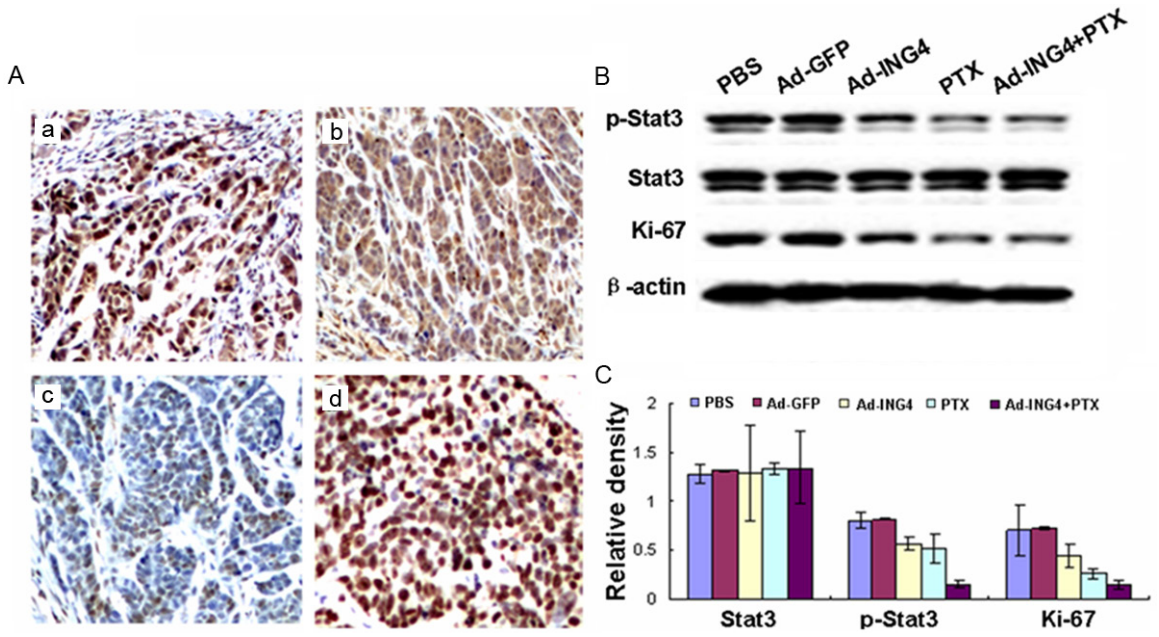

Figure 3. Expression analysis of different proteins in SW1116-derived tumors in mice subjected to various treatments. A: Immunohistochemical staining. a. IHC staining for ING4. Tumor cells stained positive for ING4 in the Ad-ING4 group (IHC, 100 ×). b. Tumor cells in the PBS group expressed Stat3 (SP × 100). c. IHC staining for p-Stat3. Tumor cells in the PBS group stained positive for p-Stat3 (SP × 100). d. IHC staining for Ki-67. The percentage of cells positive for Ki-67 staining was high in the tumors in the PBS group (SP × 100). B: Detection of various proteins in tumor tissues by Western blot. C: Comparison of expression of various proteins in different treatment groups.

five groups, and each group was administered a particular treatment (refer to Materials and methods) every 2 days. The tumor volumes were measured every week to obtain tumor growth curves. The average tumor volume was significantly reduced in the Ad-ING4 + PTX group compared with the other groups (**Figure 2A**), with significant differences observed between these groups ($P < 0.05$). Ad-ING4 and PTX treatments also inhibited tumor growth. Forty-one days after injections with SW1116 cells, the mice were killed and the tumor mass removed and measured. The average tumor weight in the PBS, Ad-GFP, Ad-ING4, PTX, and Ad-ING4 + PTX groups was (1.836 ± 0.056) g, (1.775 ± 0.022) g, (0.786 ± 0.012) g, (0.655 ± 0.025) g, and (0.505 ± 0.035) g, respectively (**Figure 2B**). Significant differences in the tumor weight were observed between these groups ($P < 0.05$; **Figure 2C**), with the PTX and Ad-ING4 + PTX groups having the least average tumor weight. The tumor IR was calculated from the tumor weight. **Figure 2D** shows a histogram of IR among the five groups. IR values in the Ad-ING4, PTX, and Ad-ING4 + PTX groups were (57.18 ± 0.67)%, (64.34 ± 1.35)%, and (72.48 ± 1.89)%, respectively, and exhibited significant differences ($P < 0.05$).

Assessment of ING4, Stat3, p-Stat3, and Ki-67 expression in tumors

All the treatment groups contained tumor cells that stained positive for ING4 (**Figure 3A-a**), Stat3 (**Figure 3A-b**), p-Stat3 (**Figure 3A-c**), and Ki-67 (**Figure 3A-d**). ING4 and p-Stat3 proteins were detected in both the cytoplasm and nucleus of tumor cells, while Stat3 protein was detected only in the cytoplasm. Ki-67 expression was observed in the nuclei of cells during the DNA synthesis (S) phase of the cell cycle and provides an important marker for tumor proliferation. Immunohistochemical analysis revealed significant differences in the mean percentage of cells staining positive for ING4 ($F = 78.37$, $P = 0.0013$), p-Stat3 ($F = 28.15$, $P = 0.048$), and Ki-67 ($F = 53.5$, $P = 0.0387$) among the five treatment groups. The Ad-ING4 + PTX group had the lowest p-Stat3 and Ki-67 expression, while the Ad-ING4 group showed the highest ING4 expression. Immunohistochemistry results obtained for Ki-67 indicated that the combination treatment (Ad-ING4 + PTX) inhibits tumor growth by decreasing tumor cell replication, as suggested by the lower mean percentage of positively stained cells (**Table 1**). Taken together, these results reveal that Ad-ING4 +

Table 1. Comparison of the mean percentage of cells staining positive for ING4, Stat3, p-Stat3 and Ki-67 among the five groups ($\bar{X}$ ± SD)

Group	n	ING4	Stat3	p-Stat3	Ki-67
PBS	5	0.393 ± 0.167	0.855 ± 0.272	0.392 ± 0.098	0.952 ± 0.327
Ad-GFP	5	0.280 ± 0.137	0.875 ± 0.187	0.355 ± 0.187	0.928 ± 0.355
Ad-ING4	5	0.847 ± 0.193	0.869 ± 0.193	0.196 ± 0.134	0.731 ± 0.433
PTX	5	0.296 ± 0.124	0.898 ± 0.297	0.180 ± 0.087	0.686 ± 0.377
Ad-ING4 + PTX	5	0.792 ± 0.242	0.916 ± 0.243	0.078 ± 0.078	0.579 ± 0.287

PTX treatment results in tumor inhibition by downregulation of p-Stat3 and Ki-67 expression, as well as ING4 overexpression.

Discussion

Inhibitor of growth 4 is a protein that, in humans, is encoded by ING4. It is a tumor suppressor that can interact with TP53, inhibit cell growth, and induce apoptosis [18]. The C terminus of ING4 protein contains a plant homeodomain [19], which can interact with phosphatidylinositol phosphate kinases, which in turn regulate cell survival, growth, and proliferation. As mentioned previously, ING4 interacts with TP53 and with EP300/p300, a component of the histone acetyltransferase complex. ING4, aided by its nuclear localization signal (NLS), controls p53 transcriptional activity by regulating p53 gene acetylation, which results in cell growth arrest and apoptosis, suppressing tumor formation [20]. Abnormal ING4 protein, as a consequence of mutation in its NLS, is not able to combine with TP53, resulting in inhibition of TP53 activity, which results in tumor formation [21-23].

ING4 expression levels show negative correlation with the clinical stage, histological grade, and lymph node metastasis of colorectal cancer. Moreover, high microvessel density is observed in colorectal cancer with low ING4 expression, which prompts the hypothesis that ING4 suppresses tumor growth through regulation of tumor angiogenesis [24]. In the present study, we infected SW1116 colorectal cancer cell line with adenovirus expressing ING4, to observe the inhibitory effect of ING4 on tumor growth. Our results show that Ad-ING4, PTX, and Ad-ING4 + PTX treatments inhibit the proliferation of SW1116 cancer cells, with IR increasing with the time elapsed, both in vitro and in vivo. Moreover, AR was higher in Ad-ING4 and Ad-ING4 + PTX groups than in the PBS control group.

The family of signal transducers and activators of transcription (STATs), which mediate the transcription of several cytokines and growth factors, includes STAT1, STAT2, Stat3, STAT4, STAT5a, STAT5b, STAT6, and Stat3, which are associated with tumor development and progression [25-27]. Among the members of the STAT family, Stat3 plays an important role in tumor cell proliferation, differentiation, and apoptosis [25, 28]. Stat3 induces tumor formation through the JAK/Stat3 signaling pathway, which results in Stat3 phosphorylation (p-Stat3). p-Stat3 is activated upon tyrosine phosphorylation [29], resulting in dimerization of p-Stat3 in the cytoplasm and its translocation into the nucleus. Nuclear p-Stat3 interacts with the promoter of target genes and induces the expression of anti-apoptotic genes and genes related to tumor angiogenesis [26, 30]. Cyclin D1 is an important cell cycle regulatory protein. p-Stat3 interacts with the cyclin D1 promoter and induces its overexpression, which results in cell proliferation [31]. High p-Stat3 expression levels in colorectal cancer are important for tumor growth and metastasis [32-34] and are associated with tumor stage and histological grade [19]. Fan et al. reported that the silencing of Stat3 expression by RNA interference downregulated BcL-xL and survivin, inducing tumor apoptosis [35]. The present study shows that Ad-ING4 and PTX downregulate p-Stat3 and Ki-67 expression but do not affect Stat3 expression. Ad-ING4 + PTX treatment had the highest inhibitory effect in the present study, indicating that ING4 regulates SW1116 cell proliferation and apoptosis; it also enhances the sensitivity of cells to chemotherapy by regulating p-Stat3 expression. Besides PTX, recent reports show that ING4 increases the sensitivity of the HepG2 hepatocellular cancer cell line to doxorubicin and etoposide by promoting G1/M arrest and reducing the S phase [36]. Such enhancement in the sensitivity to chemotherapy suggests that combining ING4 complementation with chemotherapy in colorectal cancer treatment would increase the curative effect, decrease dosage, and relieve the side effects of chemotherapy.

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Disclosure of conflict of interest

None.

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