

Ectopic expression of p33^{ING1b} suppresses proliferation and induces apoptosis in colonic adenocarcinoma cells

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Abstract. Inhibitor of growth 1b (ING1b) is considered to be a class II tumor suppressor gene. Although decreased expression of p33^{ING1b} has previously been reported in colorectal cancer (CRC), its role in CRC has remained to be elucidated. The present study was designed to assess the function of p33^{ING1b} in CRC and to further evaluate its underlying mechanisms of action. Western blot analysis confirmed that ING1b gene expression was significantly decreased in CRC tissues compared with that of adjacent non-tumorous colorectal tissues. Furthermore, recombinant adenovirus-mediated ectopic expression of p33^{ING1b} resulted in growth inhibition, G1-phase cell cycle arrest and apoptosis in the SW480, HT29 and LoVo colorectal adenocarcinoma cell lines. The results suggested that the downregulation of ING1b contributes to colorectal carcinogenesis and that ectopic expression of ING1b may be a potentially useful therapeutic approach for CRC.

Introduction

The inhibitor of growth 1 (ING1) gene was previously identified and characterized as a type-II tumor suppressor gene. The ING1 gene is located on chromosome 13q34 and encodes a minimum of 4 protein isoforms (p47^{ING1a}, p33^{ING1b}, p24^{ING1c}, and p27^{ING1d}), as a result of the effects of various promoters, exons and alternative splicing (1). The ING1b gene exists in numerous species, including humans, mice, rats, frogs and yeast (2). The ING1b proteins have significant roles in the regulation of chromatin structure, inhibition of cell proliferation (3), cell cycle regulation (4), apoptosis (5), damaged DNA repair (6), regulation of gene transcription

and other biological functions (7). Of these four variants, p33^{ING1b} is the isoform which is most extensively expressed in human tissues, and has therefore been the most intensively analyzed. Studies have indicated that p33^{ING1b} is expressed in the majority of normal cells and tissues, suggesting that it may have fundamental functions (8-10). The expression of p33^{ING1b} is significantly downregulated in multiple human malignancies, including breast, esophageal, gastric and brain cancer, as well as leukemia (11-15). Blocking ING1 expression has been shown to enhance cell proliferation *in vitro* and tumor formation *in vivo* (16). Additionally, adenovirus-mediated ING1b gene transfer has been demonstrated to significantly suppress growth and increase apoptosis in glioma (17) and gastric adenocarcinoma cells (18). These results suggest that ING1b may be a universal tumor suppressor gene.

Colorectal cancer (CRC) is the third most common human malignancy amongst males and the second most common amongst females, with >1.2 million novel cases diagnosed and 608,700 associated mortalities worldwide, in 2008 (19). It has been generally accepted that CRC develops through step-wise genetic alterations (20). Analysis of the molecular mechanism of carcinogenesis facilitates the development of novel approaches for the prevention and treatment of a particular cancer (21). However, the carcinogenic mechanisms of CRC remain far from being fully elucidated. Although previous reports have demonstrated that the expression of p33^{ING1b} is downregulated at the mRNA level in CRC (22), the role of p33^{ING1b} in colorectal carcinogenesis has yet to be investigated.

In the present study, the expression of p33^{ING1b} in CRC tissues was evaluated, and the effects of adenovirus-mediated p33^{ING1b} ectopic expression in colorectal adenocarcinoma cells *in vitro* were investigated.

Materials and methods

Patients and tissue preparation. CRC samples and adjacent non-malignant tissues, which were ≥ 2 cm from the tumor, were collected endoscopically from 10 patients with histologically verified colorectal adenocarcinomas at the First Affiliated Hospital, School of Medicine, Xi'an Jiaotong University (Xi'an, China). The samples were immediately snap-frozen in liquid nitrogen and stored at -80°C. This study was approved by the ethics committee of The First Affiliated Hospital, School of

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Medicine, Xi'an Jiaotong University (Xi'an, China). Written informed consent was obtained from all patients.

Cell culture and adenoviral infection. The human colorectal adenocarcinoma cell lines SW480, HT29 and LoVo were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Grand Island, NY, USA) supplemented with 10% fetal calf serum (FCS; Gibco-BRL), 100 U/ml penicillin [Runze Pharmaceutical (Suzhou) Co., Ltd., Suzhou, China] and 100 µg/ml streptomycin [Runze Pharmaceutical (Suzhou) Co., Ltd.] at 37°C in a humidified 5% CO₂ atmosphere.

Recombinant adenoviruses. Recombinant adenoviruses (type 5) encoding ING1b (Ad-ING1b) or green fluorescent protein (Ad-GFP), were constructed as previously reported (18). Briefly, p33ING1b was cloned into the adenoviral shuttle plasmid, pShuttle-CMV (Stratagene, La Jolla, CA, USA). The recombinant pShuttle-CMV-p33ING1b plasmid was digested by PmeI (New England Biolabs, Ipswich, MA, USA) to linearize, followed by homologous recombination with adenoviral bone plasmid pAdEasy-1 (Stratagene) in *E.coli* BJ5183. The positive recombinant adenoviral plasmid, pAd-p33ING1b, was identified by PacI digestion (New England Biolabs) and the recombinant adenovirus, Ad-p33ING1b, was obtained following transfection in HEK293 cells (Xi'an Huaguang Biological Engineering Co., Ltd., Xi'an, China). The cytopathogenic effect was observed under a fluorescent microscope (IX-50; Olympus Corporation, Tokyo, Japan). The titers of adenoviral stocks were determined by polymerase chain reaction (Qiagen, Hilden, Germany) performed under the following conditions: Initial denaturation at 95°C for 10 sec followed by 40 cycles of denaturation at 95°C for 5 sec, annealing at 50°C for 15 sec and elongation at 72°C for 23 sec. The viral stocks were aliquoted and stored in 10% glycerol at -80°C prior to use.

Adenovirus-mediated gene transfer. The extent of the adenovirus-mediated gene transfer in the three colorectal adenocarcinoma cell lines was determined by measuring the expression of GFP 72 h post-infection with Ad-GFP. Briefly, when the cells reached ~70-90% confluence, the medium was aspirated and the cell monolayer was washed with pre-warmed sterile phosphate-buffered saline (PBS; Gibco-BRL). The cells were incubated with Ad-GFP at various multiplicity of infections (MOIs: 0, 10, 20, 40, 80 and 160) at 37°C. Two hours later, 2 ml of fresh growth medium was added and the cells were incubated for an additional 72 h at 37°C. Subsequently, the transduction efficiency was evaluated under a fluorescence microscope (BX61; Olympus Corporation). When the cells were infected with Ad-GFP at an MOI of 40, ~70% of the cells were identified to be GFP-positive, without exhibiting marked toxic effects. Higher MOIs did not result in higher transduction efficiencies but did result in toxicity. Therefore, the subsequent experiments were performed using Ad-ING1b and Ad-GFP at an MOI of 40.

Western blot analysis. Colonic tissues and cultured cells were homogenized in radioimmunoprecipitation lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% SDS, 0.02% sodium azide,

1% Nonidet P-40, 0.5% sodium deoxycholate and protease inhibitor cocktail; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) in an ice-bath for 30 min. The extracts were clarified by centrifugation at 17,465 x for 10 min at 4°C. Following measurement of the protein concentrations using the Bradford assay (23), 100 µg total protein was subjected to 10% SDS-PAGE (Santa Cruz Biotechnology, Inc.). Following electrophoresis, the proteins were electrophoretically transferred onto a nitrocellulose membrane (EMD Millipore, Bedford, MA, USA), and blocked with 5% nonfat dry milk in PBS with 0.2% Tween-20 for 1 h. Subsequently, the membrane was incubated overnight at 4°C with primary mouse anti-human antibodies against p33^{ING1b} (polyclonal antibody prepared in the Reproductive Medical Laboratory of Xi'an Jiaotong University; 1:400 dilution) or β-actin (1:1,000 dilution; Sigma-Aldrich, St. Louis, MO, USA). Following three 15-min washes with Tris-buffered saline with 0.1% Tween-20 (Sigma-Aldrich), the membrane was incubated with horseradish peroxidase-conjugated goat anti-mouse Immunoglobulin G as a secondary antibody (1:2,000 dilution; Sigma-Aldrich) for 1 h at room temperature. Visualization of the blots was accomplished using enhanced chemiluminescence solution (Pierce, Rockford, IL, USA).

MTT assay. An MTT assay was conducted to assess the number of viable cells pre- and post-infection at 24 h intervals. The cells were seeded in 96-well plates at a density of 1x10³ cells/well and cultured for 24 h at 37°C with 5% CO₂. Subsequently, the cells were infected with Ad-GFP or Ad-ING1b, or treated with PBS, as described above. At the indicated time-points (day 1, 2, 3, 4, 5, 6, 7 and 8), 5 mg/ml MTT (Sigma-Aldrich) was added to each well, and the cells were incubated at 37°C for 4 h prior to removal of the supernatants. The crystals were dissolved in 150 µl dimethyl sulfoxide (Sigma-Aldrich). The absorbance was examined using an automated microplate reader (BioTek Instruments, Inc., Winooski, VT, USA) at an absorption wavelength of 490 nm. Each sample was evaluated in triplicate, and three independent experiments were conducted.

Cell cycle analysis. The cells were cultured in 6-well plates (Corning Inc., Corning, NY, USA) with growth medium for 24 h prior to adenoviral infection. Twenty-four hours post-infection, the cells were cultured with DMEM without bovine serum (serum starvation) for 24 h to achieve cell cycle synchronization. The cells were then cultured with complete growth medium (Gibco-BRL) for 48 h. The harvested cells were washed twice with ice-cold PBS and fixed with 70% ethanol at 4°C overnight. Following RNase A (20 mg/ml; Sigma-Aldrich) digestion at 37°C for 30 min, the cells were stained with 50 mg/ml propidium iodide (PI; Sigma-Aldrich) at 4°C for 30 min in the dark. The cell cycle distribution was analyzed by flow cytometry (FCM; FACSCalibur; BD Biosciences, San Jose, CA, USA).

Apoptosis detection. Apoptosis was determined by staining cells with Annexin V (ApoScreen Annexin V; Southern-Biotech, Birmingham, AL, USA) and PI (Sigma-Aldrich) according to the manufacturer's instructions. Briefly, the cells were cultured in 6-well plates (Corning Inc.) and infected with adenoviruses as described. Seventy-two hours post-infection, the cells were harvested and resuspended in binding buffer (10 mM HEPES,

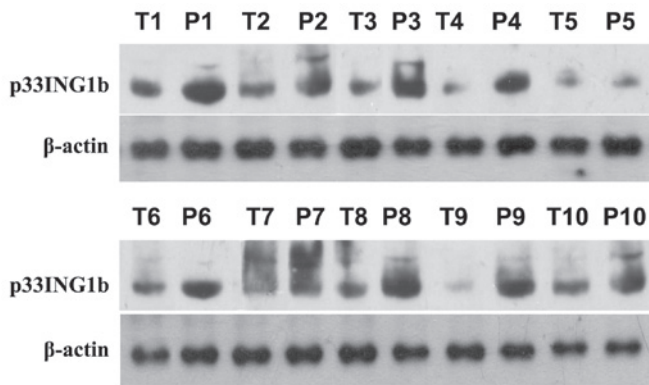


Figure 1. Expression of p33^{ING1b} is downregulated in colorectal cancer. Western blot analysis of 10 samples of colorectal cancer and adjacent non-malignant tissues. T, tumor tissue; P, peritumoral non-malignant tissue.

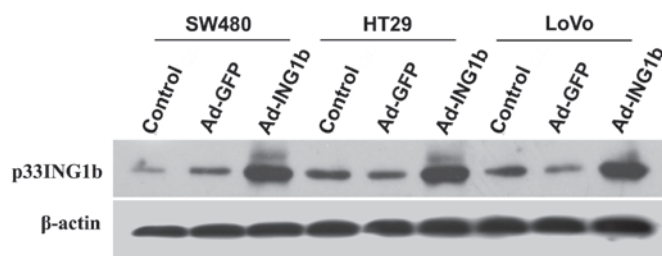


Figure 2. Adenoviral transfection with Ad-ING1b enhances p33^{ING1b} expression in colorectal carcinoma cells. Western blot analysis of adenovirus-mediated ectopic expression of p33^{ING1b} in three colorectal adenocarcinoma cell lines. Ad, adenovirus; GFP, green fluorescent protein; ING1b, inhibitor of growth 1b.

pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂ and 0.5% bovine serum albumin; Southern-Biotech) at a concentration of 1x10⁶ cells/ml. Following incubation with Annexin V-fluorescein isothiocyanate (Southern-Biotech) in an ice bath in the dark for 15 min, binding buffer and PI were added to the cells. The cells were then analyzed immediately using a FACScan flow cytometer (BD Biosciences).

Statistical analysis. All statistical analyses were performed using SPSS software version 19.0 (SPSS, Inc., Chicago, IL, USA). All values are presented as the mean ± standard deviation. Statistical analyses of the data were performed using one-way analysis of variance followed by the Student-Newman-Keuls method. P<0.05 was considered to indicate a statistically significant difference.

Results

p33^{ING1b} expression is downregulated in CRC tissues. Western blot analysis of p33^{ING1b} expression in CRC tissues demonstrated that p33^{ING1b} was steadily expressed in the non-tumorous colonic tissues. However, in the colonic adenocarcinoma tissues, the expression of p33^{ING1b} was markedly lower than that in the adjacent non-tumorous colonic tissues. The results indicated that the expression of p33^{ING1b} was downregulated in CRC tissues (Fig. 1; P<0.05).

Recombinant adenovirus-mediated p33^{ING1b} expression in colorectal adenocarcinoma cells. To verify the ectopic

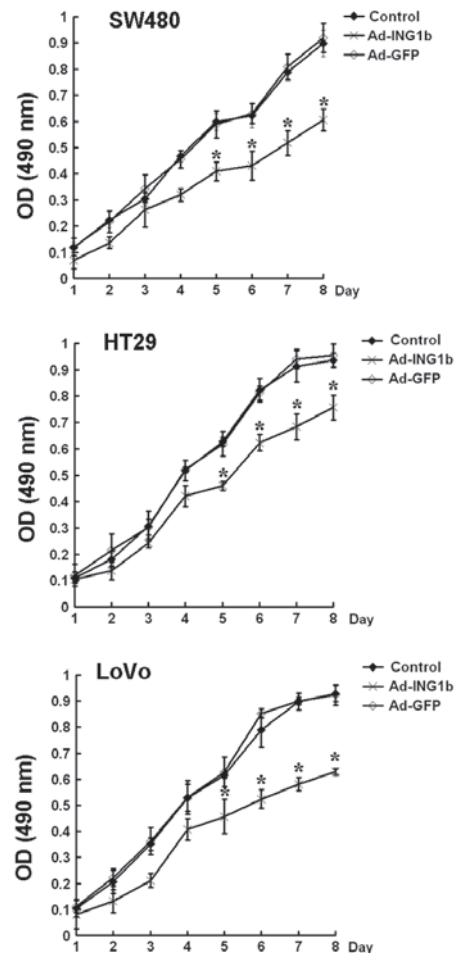


Figure 3. Ectopic p33^{ING1b} expression inhibits the proliferation of colorectal adenocarcinoma cells. Expression was analyzed by MTT assay. The results are presented as the mean ± standard deviation from three independent experiments. *P<0.05 vs. Ad-GFP and PBS control groups at analogous time-points. Ad, adenovirus; GFP, green fluorescent protein; ING1b, inhibitor of growth 1b; OD, optical density.

expression of p33^{ING1b} mediated by adenoviral infection, western blot analysis with a specific antibody against p33^{ING1b} was performed. As shown in Fig. 2, low levels of p33^{ING1b} expression were detected in all three CRC cell lines following infection with Ad-GFP, which had no notable effect on the expression of p33^{ING1b} protein. Ad-ING1b-infected cells demonstrated markedly higher p33^{ING1b} expression levels than those of the Ad-GFP-infected or blank control cells (P<0.05).

p33^{ING1b} suppresses proliferation of colorectal adenocarcinoma cells. Subsequently, the effect of p33^{ING1b} on the proliferation of colorectal cancer cells *in vitro* was examined using an MTT assay. The results demonstrated that the three colorectal cancer cell lines exhibited slower growth rates following Ad-ING1b infection than those of cells infected with Ad-GFP at the same MOI or the blank control (P<0.05). No significant difference in growth rate was observed between the Ad-GFP and blank control groups (Fig. 3).

Ectopic expression of p33^{ING1b} induces apoptosis in colorectal adenocarcinoma cells. Apoptosis, or programmed cell death, has a significant role in the development of CRC. To examine

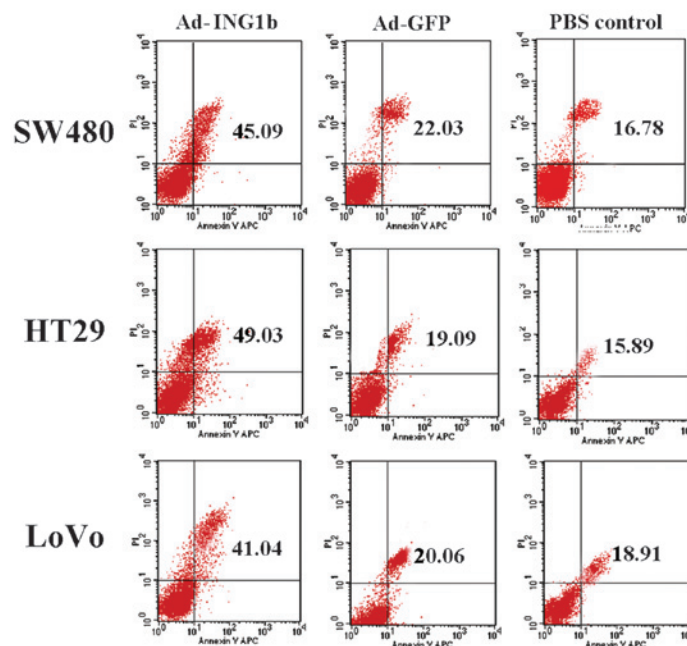


Figure 4. Ectopic expression of p33^{ING1b} induces apoptosis in colorectal adenocarcinoma cells. The cells were infected with Ad-GFP or Ad-ING1b at a multiplicity of infection of 40. Apoptosis was determined by Annexin V and PI staining followed by flow cytometric analysis 48 h following infection. PI, propidium iodide; Ad, adenovirus; GFP, green fluorescent protein; ING1b, inhibitor of growth 1b.

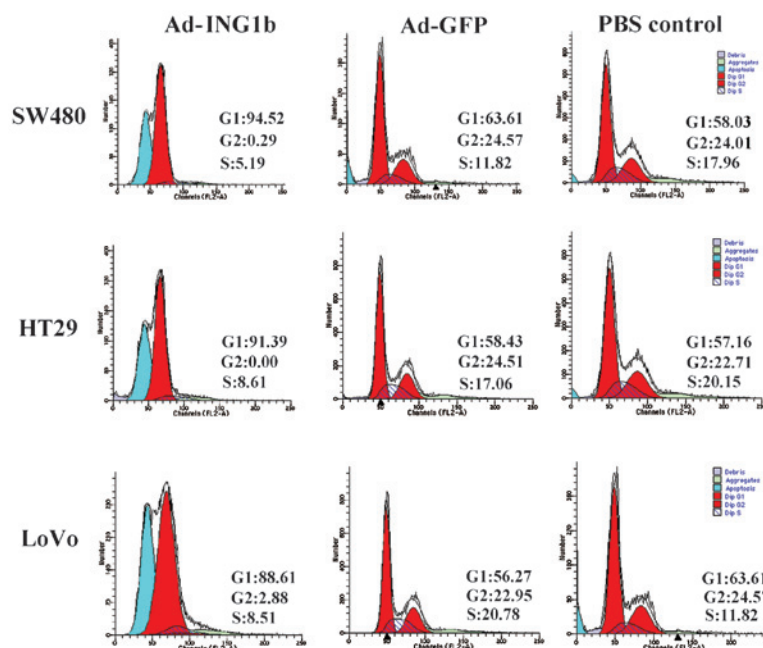


Figure 5. Ectopic expression of p33^{ING1b} induces G1 phase cell cycle arrest. Colorectal adenocarcinoma cells were transduced with Ad-GFP or Ad-ING1b, at a multiplicity of infection of 40. Forty-eight hours later, cell cycle distribution was analyzed using flow cytometry. Ad, adenovirus; GFP, green fluorescent protein; ING1b, inhibitor of growth 1b.

the role of p33^{ING1b} in the apoptosis of CRC cells, apoptotic cells were evaluated following Ad-ING1b infection by Annexin V-PI staining followed by FCM analysis. For all three cell lines, Ad-ING1b transduction resulted in an increase in apoptosis, compared with that of cells infected with Ad-GFP or the blank control. The Ad-GFP-infected cells demonstrated a slight increase in apoptotic ratio compared with that of the blank control group (Fig. 4). These results demonstrated that ectopic expression of p33^{ING1b} induces apoptosis in colorectal adenocarcinoma cells.

p33^{ING1b} induces G1 phase cell cycle arrest in colorectal adenocarcinoma cells. To further study the potential mechanisms underlying the growth-inhibitory and pro-apoptotic effects of p33^{ING1b} in colorectal adenocarcinoma cells, alterations in the cell cycle distribution following adenoviral infection were analyzed. As shown in Fig. 5, the Ad-GFP and blank control groups exhibited similar cell cycle profiles, indicating that the expression of Ad-GFP exerted little effect on the cell cycle. However, Ad-ING1b infection resulted in an

enlarged proportion of cells in G1 phase in all three cell lines. These results suggested that ectopic p33^{ING1b} may inhibit proliferation and induce apoptosis in colorectal adenocarcinoma cells by arresting the cells in G1 phase.

In accordance with the results of Annexin V-PI staining and FCM analysis of apoptotic cells (Fig. 4), analysis of the cell cycle distribution by FCM also indicated that Ad-ING1b infection induced an increase in the number of apoptotic cells (Fig. 5).

Discussion

It has previously been demonstrated that the expression of ING1b is downregulated in malignancies derived from various organs and tissues, and that downregulation of p33^{ING1b} has a significant role in carcinogenesis (24-28). The function of p33^{ING1b} in colorectal carcinogenesis has not been extensively studied; however, a previous study has demonstrated that the mRNA expression of ING1b was downregulated in human sporadic colorectal cancer (29). In the present study, the expression of p33^{ING1b} in CRC specimens was detected using western blot analysis. The results indicated that the expression of p33^{ING1b} in CRC tissues was markedly reduced compared with that of their paired peritumoral mucosa tissues. This result, along with the data obtained by Chen *et al* (24), indicated that the expression of p33^{ING1b} was downregulated in CRC, as previously described for malignancies derived from other tissues (11,13-15,24). This evidence suggests that the downregulation of p33^{ING1b} may be involved in the development and progression of CRC.

Subsequently, the effects of adenovirus-mediated ectopic expression of p33^{ING1b} on the proliferation of three colorectal adenocarcinoma cell lines were evaluated. A recombinant adenovirus was used to deliver ING1b into the *in vitro* cultured colorectal adenocarcinoma cells, as this approach facilitated guaranteed transduction efficiency, whilst avoiding the potential adoptive alterations that may occur during long-term selection following transfection with plasmids. The results revealed that infection with the reporter adenovirus, Ad-GFP, at an MOI of 40, resulted in GFP expression in ~70% of the cultured colorectal adenocarcinoma cells and exerted no notable effects on p33^{ING1b} expression, proliferation, cell cycle distribution or apoptosis. However, the cells infected with Ad-ING1b exhibited significantly decreased growth rates due to the ectopic expression of p33^{ING1b}. This result was in agreement with previous reports using adenoviral transduction in gastric adenocarcinoma cells (18) and using plasmid transfection in human fibroblast cells and breast cancer cell lines (22,30).

Early studies revealed that one of the major biological functions of p33^{ING1b} is to promote apoptosis, via a p53-dependent pathway (31-35). In the present study, three colorectal adenocarcinoma cell lines with different p53 statuses were used, in order to observe the effects of p33^{ING1b} on apoptosis *in vitro*. The three cell lines comprised the LoVo cell line, which has wild-type p53, the HT29 cell line, which has a heterozygous mutation of p53 and the SW480 cell line, which has two copies of mutated p53 (36,37). The results revealed that, despite the varying p53 statuses, the ectopic expression of p33^{ING1b} exerted similar pro-apoptotic effects in all three adenocarcinoma cell lines, suggesting that the pro-apoptosis effect of p33^{ING1b} may

not be fully dependent on the presence of wild-type, functional p53.

Cell cycle progression is correlated with proliferation and apoptosis. G1 phase of the cell cycle involves a critical DNA-damage checkpoint, which functions as a safeguard against genomic instability (38). Cells that arrest in G1 phase may undergo apoptosis, or may recover from the G1 phase arrest and enter into S phase. In a previous study, the overexpression of p33^{ING1b} in human diploid fibroblasts was shown to induce a 50% increase in the number of cells in the G₀/G₁ phase of the cell cycle, whereas knockdown of p33^{ING1b} in these cells resulted in abrogation of this arrest and the entry of the cells into S phase (39). These results indicate that p33^{ING1b} may have a role in mediating the G₁-S phase transition (2). Accordingly, in the present study, cell cycle distribution analysis revealed that cells infected with Ad-ING1b were arrested in G1 phase, whereas the normal control and Ad-GFP-infected cells proliferated normally, suggesting that the inhibitory effect on proliferation and the pro-apoptotic effects of p33^{ING1b} may be, at least partially, attributed to its influence on cell cycle progression.

In conclusion, the results of the present study provide substantial evidence indicating that the downregulation of p33^{ING1b} has a significant role in CRC development. Furthermore, gene therapy based on the ectopic expression of p33^{ING1b} may provide a promising therapeutic approach for the treatment of CRC.

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