Functions of the AP-2α gene in activating apoptosis and inhibiting proliferation of gastric cancer cells both \textit{in vitro} and \textit{in vivo}

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Abstract

Introduction: This study was designed to investigate the potential function of the activating protein 2α (AP-2α) gene in controlling the proliferation and apoptosis of gastric cancer.

Material and methods: Gastric cancer cell line MCG-803 cells and normal cell line GE5-1 cells were selected to transfec trans pcDNA3.1(+)-AP-2α and pcDNA3.1(+) plasmids, respectively. Both mRNA and protein levels of AP-2α in each group transfected with the pcDNA3.1(+)-AP-2α plasmids were up-regulated after 48 h by real-time PCR and Western blotting analysis, leading to marked proliferation inhibition and significant cell cycle arrest.

Results: pcDNA3.1(+) reduced tumor tissue growth in a subcutaneous tumor gastric carcinoma nude mouse model. Protein over-expression of AP-2α in the nude mouse model was accompanied by down-regulation of Bcl-2 and ErbB2, resulting in the up-regulation of caspase-3, -8, and -9, ERα and p21 WAF1/CIP1.

Conclusions: The reintroduction of the AP-2α gene by pcDNA3.1 could inhibit gastric tumor growth \textit{in vitro} and \textit{in vivo}, which may be an alternative future therapeutic molecular target for human gastric cancer.

Key words: activating protein 2α, gastric cancer, gene therapy.

Introduction

Gastric cancer, a high-risk malignancy, is a genetic disease developing from the cooperation of multiple gene mutations and a multistep process [1–4]. Gene therapy as a novel treatment method has achieved improvements over past decades, particularly for treating malignant cancer [5–7]. However, there are still some unsolved problems, limiting the further development of gene therapy for malignant cancer. Thus, it is cru-
cial to choose an appropriate target therapy gene such that the effects of gene therapy are limited to cancer cells. Recently, several preliminary studies of targeted genes in gastric cancer have been performed [8, 9].

The activating protein 2 family is a sequence-specific DNA-binding transcription factor [10]. These factors are required for normal growth and morphogenesis during mammalian development and are expressed in neural crest lineages and regulated by retinoic acid [11, 12]. Recent studies have shown that activating protein 2α (AP-2α) can regulate cell function by targeting related cancer genes such as the ErbB 2 proto-oncogene, the cell-cycle control gene CDKN1A, and the cell adhesion and invasion genes MMP-2, NMP-9, and PAR-1 [13]. Jean et al. found that the AP-2 protein is less expressed in melanoma cells than in normal epithelium cells [14]. The expression of the AP-2 protein shows a lower regulatory or even a deficiency state in other malignant cancer cells, such as in cervical intraepithelial neoplasia, ovarian cancer, breast cancer, colorectal cancer, and prostate cancer [15–17]. Thus far, the role of the AP-2α gene in primary gastric cancer tissue has received little study.

Over-expression of the AP-2α protein can inhibit the expression of Bcl-2 protein, stimulate the bax/cytochrome/Apaf1/caspase-9 signaling pathways, and finally induce apoptosis in tumor cells. In addition, the AP-2α protein can enhance the efficacy of chemotherapy drugs against tumors [18, 19]. Previous studies not only observed clear inhibition of tumor growth in a mouse pancreatic cancer tumor model, but also revealed that the efficacy of gemcitabine is enhanced by up-regulating the expression of AP-2α protein [20]. As mentioned above, the AP-2α protein is closely associated with tumor cells and shows great potential as a targeted biomarker in clinical therapy.

Based on our robust previous studies, 41 pairs of primary gastric cancer tissues including tumor tissues and tumor-adjacent tissues were selected to investigate the differences in expression of AP-2α protein by real time-polymerase chain reaction and Western blotting. The results showed that the expression of AP-2α in tumor tissue was dramatically lower than that in tumor-adjacent tissue [21]. Prognosis analysis indicates that the 5-year survival rate of patients who have low AP-2α expression is lower than that of patients who have high AP-2α expression. Taken together, these data strongly support an inverse correlation between the AP-2α gene and the development of primary gastric cancer, and based on these studies we can investigate the feasibility of suppressing cancer growth and inducing cancer apoptosis by regulating the expression of the AP-2α protein [22, 23].

Thus, the present study was aimed at identifying novel clinical therapeutic approaches to gastric cancer with the aid of further in-depth studies of the AP-2α protein.

Material and methods

Cell culture and stable transfection

The gastric cancer cell line MCG-803 was purchased from the Shanghai Cell Bank (Shanghai, China). Gastric cancer cells were cultured in Roswell Park Memorial Institute medium (RPMI) 1640 supplemented ( Gibco, Shanghai, China) with 10% fetal bovine serum (FBS) (Sijixin, Beijing, China). The cells were incubated at 37°C under 5% carbon dioxide. The cells were seeded into 6-well plates overnight at a total of 2 x 10^5 cells per well until the cultures reached approximately 90% confluence. The cells were then transfected with a mixture of either 0.4 μg of pcDNA3.1(+)-AP-2α, 0.4 μg of pcDNA3.1(+)-AP-2α, or 0.4 μl phosphate-buffered saline plus 8 μl of Effectene transfection reagent (Qiagen, Shanghai, China) in 1000 μl of fresh serum-free RPMI 1640 culture medium. The transfected cells were selected using G418 (400 μg/ml). After 4 weeks of exposure to selection pressure, monoclonal cells were selected and further cultured for subsequent experimentation.

Real-time polymerase chain reaction

Total RNA from cells and tissues samples was extracted using Trizol reagent (Invitrogen) according to the manufacturer’s instruction. The extracted RNA was pretreated with RNase-free DNase, and about 1 mg of total RNA was used for reverse transcription by using a one-step real-time quantitative reverse transcription polymerase chain reaction (RT-PCR) kit (Invitrogen) in a thermocycler (Bio-rad iQ5, Bio-rad) under the following reaction conditions: cDNA synthesis, 45°C for 30 min; inactivation, 94°C for 2 min; PCR amplification of 40 cycles, denature at 94°C for 30 s, annealing at 55°C for 30 s, chain extension at 72°C for 45 s, and a final chain extension at 72°C for 10 min. Expression data were normalized to the geometric mean of the housekeeping β-actin gene to control the variability in expression levels. Real time-PCR was designed using the Primer Express Software version 3.0 (Applied Biosystems). The same method was used to detect Bic-2, ErbB2, Ero1, caspase-9, p21^{WAF1/CIP1}, and caspase-3, -8, and -9 protein expression in a primary-tumor nude mouse model.

Western blotting analysis

Transfected cells were solubilized in lysis buffer (150 mM sodium chloride, 0.1 M Tris (pH 8), 1% Tween 20, 50 mM diethylthiocarbamic
acid, and 1 mM ethylenediaminetetra acetic acid (pH 8) containing protease inhibitors, before being subjected to sonication and centrifugation at 4°C for 3 min. Loading buffer (2×) was added to each of the protein solutions, which were then boiled for 5 min and separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis before being transferred to nitrocellulose membranes (Amer sham Biosciences, Beijing, China). The membranes were blocked for 1 h with 5% bovine serum albumin in phosphate-buffered saline, incubated with polyclonal antibodies against Ap-2α (1 : 250; Santa Cruz Biotechnology, Beijing, China), and incubated with horseradish peroxidase-conjugated secondary antibodies (1 : 2,000; Amersham Pharmacia Biotech). Protein bands were visualized using an enhanced chemiluminescence kit, according to the manufacturer’s specifications (Boster, Wuhan, China).

**MTT assay for cell proliferation**

After transfection, MTT assay was applied to evaluate the proliferation of both introduced MGC-803, AGS, and SGC-7901 cells and normal GES-1 cells. A total of 2 × 10^4 cells per well were plated in 96-well plates that were cultured for 24, 48, 72, 96, and 120 h. Before removal from incubation, the cells were incubated with 200 μl of 0.5 mg/ml MTT (Sigma, Guangzhou, China) for an additional 4 h. The medium was then replaced with 200 μl of dimethyl sulfoxide (Sigma, Guangzhou, China) to resolve the crystals. The absorbance at 490 nm was then measured for each well.

**Flow cytometry-based cell cycle assay**

Transfected cells (1 × 10^6) were trypsinized and fixed with prechilled 75% ethanol at 4°C for 24 h. The cells were then treated with 1% RNase at 37°C for 30 min and stained with 50 μg/ml propidium iodide (Sigma, Aldrich, China) for 1 h at 4°C. Cell cycle analysis was then performed using flow cytometry (Beckman-Coulter, Brea, CA), and the data were analyzed using multicycle DNA content and cell analysis software.

**Establishment of animal models**

All animal studies complied with current ethical considerations with the approval of the Institutional Animal Care and Use Committee of Shanghai. Mice (male, 20–22 g, 5 weeks old) were obtained from the Experimental Animal center of Chinese Academy of Sciences, Shanghai, China. Female BALB/c mice 4–6-weeks old were subcutaneously injected in the right side of the back with 1 × 10^6 normal MGC803 cells/mouse. Both AP-2α protein recombinant plasmids and blank gene vectors were injected into mice. Tumor volumes were measured every 2 days for post-injection tumor growth and recorded at each measured point. Mice were killed 30 days after injection of cells and tumor weights were recorded. Tumor growth inhibition rate was calculated as follows: percentage of tumor growth inhibition = (1 – M_T / M_C) * 100%, where M_T and M_C represent the mean tumor masses in treatment and control groups, respectively.

**Statistical analysis**

All statistical analyses were carried out using the SPSS 17.0 statistical software package. All statistical analysis was performed using the one-way ANOVA with a value of p less than 0.05 or 0.01 considered to represent a significant difference (p < 0.05 or p < 0.01). Each experiment was performed independently at least twice with similar results.

**Results**

**Reintroduction of AP-2 protein by pcDNA3.1(+)–AP-2α**

Protein levels of transfected cells were detected by RT-PCR analysis (Figure 1). Compared with the controls in both GES-1 and MGC-803 cell groups, the expression of AP-2α protein was increased in pcDNA3.1(+)–AP-2α transfected cells (p < 0.05; Figure 1 B). The results demonstrated that the ex-

![Figure 1. Western blotting analysis of AP-2α expression in both GES-1 (A) cells and MGC-803 (B) cells before and after transfecting pcDNA3.1(+)–AP-2α](image-url)
pression of AP-2α protein was successfully up-regulated by the pcDNA3.1(+)-AP-2α gene in MGC-803 cells.

**AP-2α insertion resulted in cell cycle arrest of MGC 803 cells**

After transfection, both flow cytometry and MTT assay were used to estimate the cell cycle and viability status, respectively (Figure 2). The results showed a significant reduction of pcDNA3.1(+)-AP-2α transfected cells in the G2 and S phases compared with the pcDNA3.1 and blank group (Figures 2 A–C). However, AP-2α overexpressing cells had a prolonged G1 phase with numerous cells arrested in G1. These results indicated that the expression of pcDNA3.1(+)-AP-2α resulted in G1 phase arrest in MGC-803 cells. Proliferation status of the transfected cells was assessed by MTT assay at 12, 24, 36, 72, and 96 h (Figure 2 D). The results revealed that gastric cancer cells transfected with pcDNA3.1(+)-AP-2α showed robust growth inhibition \( (p < 0.05) \) relative to the control group. The differences were significant at 36, 72, and 96 h. These results showed that the proliferation of MGC-803 cells was significantly inhibited by up-regulated AP-2α.

**AP-2α expression attenuated gastric tumor growth in nude mice**

A primary gastric tumor animal model was established. As shown in Figure 3 A, the growth curve of the primary gastric cancer model present-
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Wajapeyee et al. [15] showed that AP-2α is a potent growth inhibitor gene. Our results also indicated that AP-2α protein induces cytotoxicity by inducing cell cycle arrest and apoptosis [18].

As shown in Figure 4, protein and mRNA levels of Bcl-2, ErbB2, p21\(^{WAF1/CIP1}\) and caspase-3, -8, and -9 proteins in the primary gastric cancer model were determined by western blotting and real-time PCR (Figure 4 A). The results showed that pcDNA3.1(+)−AP-2α inhibited the levels of proteins Bcl-2 and ErbB2, but up-regulated the level of ERα, p21\(^{WAF1/CIP1}\), and caspase-3, -8, and -9. These results revealed that activation of pcDNA3.1(+)−AP-2α could down-regulate Bcl-2 and ErbB2 and up-regulate caspase-3, -8, and -9, p21, and ERα expression in vivo.

Discussion

Previous studies suggested that the AP-2α gene is involved in the etiology of human breast cancer, bladder cancer, and colon cancer [17]. The AP-2α protein induces cytotoxicity by inducing cell cycle arrest and apoptosis [18]. Our results also show that AP-2α is a potent growth inhibitor gene of MGC-803 gastric cancer cells.

Earlier studies showed that Bcl-2 is up-regulated in gastric cancer cells [15]. Meanwhile, Wajapeyee et al. found that AP-2α represses Bcl-2 to induce apoptosis by stimulating the bax/cytochrome/Apaf1/caspase-9 signaling pathways [18]. Kuida et al. found that the proapoptotic molecules Bim and caspase-9 control the apoptosis of gastric epithelial cells not only in vitro but also in vivo [22]. To further clarify the mechanism of suppression of gastric cancer proliferation regulated by AP-2α, expression of another two proteins, Bcl-2 and caspase-9, associated with carcinogenesis was investigated. In the present study comparing the expression of AP-2α, Bcl-2, caspase-3, -8, and -9, an inverse relationship was found between AP-2α and both of these proteins. The expression of caspase-3, -8, and -9 was down-regulated along with the activation of the AP-2α gene. In contrast, Bcl-2 shows an inverse relationship with the upregulation of AP-2α. These results suggest that Bcl-2 and caspase-3, -8, and -9 may play a role in gastric carcinogenesis, consistent with previous studies by Wajapeyee et al. [23]. In cell culture models, AP-2α has been reported to activate p21\(^{WAF1/CIP1}\) expression, resulting in an inhibition of both cell division and stable colony formation [26]. We also investigated the correlation between AP-2α and p21\(^{WAF1/CIP1}\).

A positive correlation of AP-2α and p21\(^{WAF1/CIP1}\) results was obtained. With the upregulation of AP-2α, the expression level of p21\(^{WAF1/CIP1}\) increased significantly [24]. Müller et al. reported that the cancer cell suppression of AP-2α is associated with its interaction with p53 and activation of the p21\(^{WAF1/CIP1}\) expression [15, 23]. Another study found that expression of p21\(^{WAF1/CIP1}\) was induced by AP-2α and suggested that AP-2α directly targeted the p21 promoter [25]. Identification of the exact mechanism still needs further studies. Functional promoter studies indicate that AP-2α can activate the ERα promoter and may be involved in repression of ErbB2 expression [26, 27]. In this study, the expression level of ErbB2 clearly decreased after the activation of AP-2α protein in gastric tumor cells, and a positive up-regulation level of ERα was found, supporting promoter studies that suggested that AP-2α induces activation of the ERα promoter and inhibits ErbB2 gene expression.

Based on our findings, although the apoptosis mechanism still lacks sufficient evidence, it is possible that AP-2α stimulates gastric cancer cell

![Figure 3](image-url)
Figure 4. Western blotting (A) and real-time PCR (B–H) analysis of the changes of and other gastric cancer-associated proteins including: Bcl-2 (B), caspase-3 (C), caspase-8 (D), caspase-9 (E), p21WAF1/CIP1 (F), ErbB2 (G) and ERα (H) after pcDNA3.1(+)-AP-2α transfection, respectively.
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apoptosis through inhibition of Bcl-2 and ErbB2 and up-regulation of caspase-3, -8, and -9, ERα, and p21WAF1/CIP1. Our results clearly demonstrated that AP-2α plays a significant role in regulating the proliferation and apoptosis of gastric cancer in vivo and in vitro with great potential to act as a target gene of genetic therapy for gastric cancer.

In conclusion, in this study, we provided the first evidence that the AP-2α-expressing plasmid represents a promising approach to activating expression of AP-2α, and the AP-2α gene has great potential to be a novel target gene for gastric cancer gene therapy.

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Conflict of Interest

The authors declare no conflict of interest.

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