

Association between endogenous gene expression and growth regulation induced by TGF- β 1 in human gastric cancer cells

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Abstract

AIM: To investigate the association between endogenous gene expression and growth regulation including proliferation and apoptosis induced by transforming growth factor- β 1 (TGF- β 1) in human gastric cancer (GC) cells.

METHODS: Reverse transcription polymerase chain reaction (RT-PCR) was performed to detect the main components of the TGF- β 1/Smads signal pathway in human poorly differentiated GC cell line BGC-823. Localization of Smad proteins was also determined using immunofluorescence. Then, the BGC-823 cells were cultured in the presence or absence of TGF- β 1 (10 ng/mL) for 24 and 48 h, and the effects of TGF- β 1 on proliferation and apoptosis were measured by cell growth curve and flow cytometry (FCM) analysis. The ultrastructural features of BGC-823 cells with or without TGF- β 1 treatment were observed under transmission electron microscope. The apoptotic cells were visualized by means of the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP *in situ* nick end-labeling (TUNEL) method. Meanwhile, the expression levels of endogenous *p15*, *p21* and *Smad7* mRNA and the corresponding proteins in the cells were detected at 1, 2 and 3 h after culture in the presence or absence of TGF- β 1 (10 ng/mL) by semi-quantitative RT-PCR and Western blot, respectively.

RESULTS: The TGF- β 1/Smad signaling was found to be intact and functional in BGC-823 cells. The growth curve revealed the most evident inhibition of cell proliferation by TGF- β 1 at 48 h, and FCM assay showed G1 arrest accompanied with apoptosis induced by TGF- β 1. The typical morphological changes of apoptosis were observed in cells exposed to TGF- β 1. The apoptosis index (AI) in TGF- β 1-treated cells was significantly higher than that in the untreated controls ($10.7 \pm 1.3\%$ vs $0.32 \pm 0.06\%$, $P < 0.01$). The levels of *p15*, *p21* and *Smad7* mRNA and corresponding proteins in cells were significantly up-regulated at 1 h, but gradually returned to basal levels at 3 h following TGF- β 1 (10 ng/mL) treatment.

CONCLUSION: TGF- β 1 affects both proliferation and apoptosis of GC cells through the regulation of p15 and p21, and induces transient expression of Smad 7 as a negative feedback modulation of TGF- β 1 signal. Our results suggest a novel functional role of p21 as an accelerant of TGF- β 1-mediated apoptosis in GC cells.

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Key words: Gastric cancer; Transforming growth factor- β 1; Apoptosis; Gene expression

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INTRODUCTION

Gastric cancer is one of the most common malignant tumors in the world^[1]. Gastric carcinogenesis refers to accumulation of genetic alterations of multiple genes such as oncogenes, tumor suppressor genes and mismatch repair genes. However, the exact molecular mechanism remains to be fully elucidated.

Numerous data have shown that TGF- β 1, as a prototypic member of TGF- β superfamily of signaling molecules, has a diverse range of functions, including cell growth control (both negative and positive), differentiation, apoptosis (inducement or inhibition) and synthesis of extracellular matrix proteins^[2]. The TGF- β 1 signal is transduced across the membrane by the type I and II receptors (T β RI and II), which contain intrinsic serine/threonine kinase activities. Identification and isolation of the Smads as TGF- β 1's intracellular signaling mediators have enabled the TGF- β 1 pathway to be traced from the cell surface to the nuclei^[3]. Smad proteins can be classified into three categories^[4]: receptor-specific Smads (R-Smads: Smad2 and Smad3) that interact with and are directly phosphorylated by a particular T β RI; common partner Smad (Co-Smad: Smad4) that acts as a common mediator of TGF- β 1 signaling by interacting with all R-Smads; inhibitory Smad (I-Smad: Smad7) that competitively inhibits R-Smads' association with activated T β RI and thereby prevents ongoing signaling^[5]. TGF- β 1 signal transduction relies upon the rapid T β RI-mediated phosphorylation of R-Smads followed by R-Smad/Co-Smad dimerization and translocation to the nuclei, where the dimer can directly or indirectly regulate specific gene transcription, through interactions with other transcription factors. I-Smad functions to antagonize this signal by a negative feedback loop in response to the same stimulus of TGF- β 1. The TGF- β 1/Smad signal pathway can widely inhibit proliferation of various cell types of epithelial origin, and modulate cell apoptosis and differentiation^[3]. It has been noted that TGF- β 1 shows biphasic effects on contributing to tumorigenesis^[6]. In the initial stage of tumorigenesis, TGF- β 1 may play a negative or tumor suppression role in tumor development by inhibiting cell growth. However,

a requirement for TGF- β 1 in progression of late stage tumor, which allows the tumor to invade and metastasize through its effects on angiogenesis, immunosuppression and synthesis of extracellular matrix proteins, suggests a second role of TGF- β 1 in cancer^[7-9].

Previous studies have suggested that TGF- β 1 not only inhibits cell proliferation^[10], but also induces cell apoptosis^[11] by regulating the activities of cyclin, cyclin-dependent kinases (CDKs) and CDK inhibitors (CDKIs), which lead to the arrest of the cell cycle at G1 phase. Nevertheless, the possible role of TGF- β 1 signal in the simultaneous modulation of GC cells' proliferation and apoptosis remains unclear. Therefore, elucidation of the mechanism by which TGF- β 1 inhibits cell growth in GC cells not only provides insights into the molecular events involved in tumor progression, but also allows us to better understand how cells regulate those events contributing to tumor progression through the cell cycle. In this study, we investigated the effects of TGF- β 1 on cell kinetics and the expression of *p15*, *p21* and *Smad7* genes and the potential role of these genes in regulation of cell kinetics in human GC cells, in order to reveal the role of this signaling in gastric carcinogenesis.

MATERIALS AND METHODS

Cell culture

Human poorly-differentiated gastric adenocarcinoma cell line, BGC-823, was obtained from Shanghai Institute of Cell Biology, Chinese Academy of Sciences. Cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), L-glutamine and penicillin/streptomycin (100 U/mL), and maintained at 37 °C in an atmosphere containing 50 mL/L CO₂ and 95% air.

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from BGC-823 cells by TRIzol RNA isolation (GIBCO-BRL, USA) and purified with DNase I (Invitrogen, USA). The integrity of RNA was determined by electrophoresis on 1% agarose gel containing ethidium bromide (EB). The yield of RNA was quantified using DNA/RNA calculator (Pharmacia, England). RNA (1 μ g) was reverse-transcribed to the first strand of cDNA using superscript reverse transcriptase system (Invitrogen, USA) according to the

manufacturer's instructions. cDNA amplification was used to detect expression of the main components of TGF- β 1/Smad signaling, including TGF- β 1, T β RI, T β RII, Smads 2, 3, 4 and 7. PCR was performed under the following conditions: first at 95 °C for 3 min, 30 cycles at 95 °C for 30 s, at different annealing temperatures for 30 s, at 72 °C for 30 s, and finally at 72 °C for 10 min (Table 1). PCR products were detected by electrophoresis on 1.5% agarose gel containing EB.

Immunofluorescence assays

BGC-823 cells were seeded coverslips, and allowed to grow at 20-30% confluence. After washing with cold 1 \times PBS, cells were fixed in 4% paraformaldehyde for 15 min, and then treated with 0.1% Triton X-100 for 15 min. Subsequently, cells were incubated with Smads 2, -3, -4 or -7 primary antibodies (Santa Cruz, USA) overnight at 4 °C. Primary antibodies were diluted at 1:25 with PBS containing 1% BSA. After washing with cold PBS, cells were incubated with Fluorescein (FITC)-conjugated affinity-pure anti-goat IgG secondary antibodies (1:50 with 1% BSA in PBS, Zhongshan, Beijing) for 2 h at room temperature. After washed with cold PBS, slides were immersed for 30 s in 10 ng/mL Hoechst dye 33258 to stain cellular nuclei, and then subjected to an additional wash in cold PBS. All slides were observed under a microscope equipped with epifluorescence optics (Zeiss Axioskop, Germany).

Cell growth curve

Cells were seeded in 24-well plates at a concentration of 5 \times 10⁴/well, and allowed to attach overnight, followed by a 24-h serum starvation to synchronize and subsequently treated with 1640 culture containing 1% FBS in the presence or absence of TGF- β 1 (10 ng/mL, R&D systems, USA). Duplicate wells were trypsinized, stained with trypan blue and counted manually per 12 h for 3 d. The cell growth curve was produced from the mean data of three independent experiments using Microsoft Excel 2000.

Flow cytometry (FCM) analysis

Cells were plated in 25 cm² culture flasks at a density of 5 \times 10⁵ cells, followed by synchronization, and then incubated in 1640 medium containing 1% FBS in the presence or absence of TGF- β 1 (10 ng/mL) for 24 and 48 h. All floated and adherent cells were harvested, centrifuged at 1 000 r/min for 10 min. Cell pellet was washed with 1 \times PBS, centrifuged at 1 000 r/min for

Table 1 Oligonucleotide primers for RT-PCR (Sangon, Shanghai, China)

Gene	cDNA primer sequence	Annealing temperature (°C)	Product size (bp)
<i>TGF-β1</i>	Forward: 5'-GCGACTCGCCAGAGTGTAT-3' Reverse: 5'-ATAGTTGGTGTCCAGGGCTCG-3'	61	284
<i>TβRI</i>	Forward: 5'-ACGGCGTTACAGTGTCTG-3' Reverse: 5'-CCACACCGTCTATATCTGG-3'	57	358
<i>TβRII</i>	Forward: 5'-AGCAACTGCAGCATCACCTC-3' Reverse: 5'-ACTACAGACTCTTCTACAGG-3'	57	688
<i>Smad2</i>	Forward: 5'-ATCCTAACAGAACTCCGCC-3' Reverse: 5'-GAGTCGTTTTTGAAGGGGTG-3'	57	489
<i>Smad3</i>	Forward: 5'-GGAGGGCAGGCTTGGGGAAAATG-3' Reverse: 5'-CCCCTCCCCCGCCACCACATTAT-3'	57	284
<i>Smad4</i>	Forward: 5'-AAGGTGAAGGTGATGTTTG-3' Reverse: 5'-CTCGATAAGGTGGATGACTA-3'	56	264
<i>Smad7</i>	Forward: 5'-GTGGGGAGGCTCTACTGTGTC-3' Reverse: 5'-CAGCTTTCGGAACCTCTTTGG-3'	57	294
<i>p15</i>	Forward: 5'-TTCTTTAAATGGCTCCACCTGCCT-3' Reverse: 5'-AACCGTCGGAAGTAGCTTAATCCA-3'	60	436
<i>p21</i>	Forward: 5'-AGTGGACAGCGAGCAGCTGA-3' Reverse: 5'-ATCTTAGACAGTACGACCAGAC-3'	58	380

10 min, fixed with 70% ethanol, made into a single cell suspension and stored at 4 °C overnight. Then the cells were stained with propidium iodid (PI) solution (with the final concentration of 100 μ g/mL) on ice for 30 min and assayed using a FCM (Bio-Rad, USA).

Transmission electron microscopy (TEM)

Cells were plated in 25 cm² culture flasks at a density of 5×10^5 cells, and then incubated in 1640 medium containing 1% FBS in the presence or absence of TGF- β 1 (10 ng/mL) for 48 h. All floated and adherent cells were collected, centrifuged at 1 000 r/min for 15 min, fixed in 2.5% cold glutaraldehyde and stored at 4 °C overnight. Then the cells were dehydrated, embedded, cut into ultrathin sections and stained routinely. The ultrastructural features of cells were observed under JEM-1220 transmission electron microscope (JEOL, Japan).

Apoptosis assay

BGC-823 cells were seeded on coverslips, and then incubated in 1640 medium containing 1% FBS in the presence or absence of TGF- β 1 (10 ng/mL) for 48 h. Terminal deoxynucleotidyl transferase (TdT)-mediated dTUP *in situ* nick end-labeling (TUNEL) assay was performed using an *in situ* Apoptosis Detection kit (Boehringer Mannheim, Germany) according to the manufacturer's instructions. After washing with cold $1 \times$ PBS, the cells were fixed in 4% paraformaldehyde for 30 min, then immersed for 30 min in 0.3% H₂O₂ to block endogenous peroxidase at room temperature. Subsequently, cells were treated with 0.1% TritonX-100 (containing 0.1% sodium citrate) on ice for 2 min to increase the penetration of cell membrane. After washing with cold PBS, cells were incubated with 50 μ L TUNEL reaction solution (5 μ L TdT +45 μ L dUTP solution) in a humidified box for 1 h at 37 °C. After washed with cold PBS, cells were combined with POD-horseradish peroxidase in a humidified box for 30 min at 37 °C, and then subjected to an additional wash in cold PBS. Cells were colorized with diaminobenzidine tetrahydrochloride (DAB, Zhongshan, Beijing), and the reaction was terminated with a wash in cold PBS after the appearance of positive cells with brown nuclei under a light microscope. Slides were restained with hematoxylin slightly and sealed up with neutral resin. Five high power microscopic views ($\times 200$) were selected on each slide, and 1 000 cells were counted. The apoptotic index (AI) was calculated as follows: AI = (number of apoptotic cells/total number counted) \times 100%. The cells with addition of 50 μ L dUTP solution were used as negative controls, and cells treated with DNase I for 20 min were used as positive controls. The experiments were repeated three times independently. All data were summarized as mean \pm SD and analyzed using χ^2 test. $P < 0.01$ was considered statistically significant.

Semi-quantitative RT-PCR

Cells were plated in 25 cm² culture flasks at a density of 10^6 cells, followed by synchronization and then incubated in 1640 medium containing 1% FBS in the presence or absence of TGF- β 1 (10 ng/mL) for 1, 2 and 3 h. Total RNA was extracted and reverse-transcribed to the first strand of cDNA. *p15*, *p21* and *Smad7* cDNA were amplified by PCR under the conditions in Table 1. β -actin cDNA fragment was used as an internal control to eliminate the systematic and quantitative errors. The experiments were repeated three times independently.

Western blot analysis

Cells were plated in 25 cm² culture flasks at a density of 10^6 cells, followed by synchronization. After incubated in 1640 medium containing 1% FBS in the presence or absence of TGF- β 1 (10 ng/mL) for 1, 2 and 3 h. Total proteins were extracted^[12]

and quantified using DU 640 protein analyzer (BECKMAN, USA). A prestained low-molecular-weight rainbow marker (Invitrogen, USA) and the protein sample (50 μ g) were separated on 12% or 15% SDS gels by SDS-PAGE. Separated proteins were transferred onto a 0.45 μ mol/L polyvinylidene difluoride membrane (Millipore, USA) that was blocked at room temperature for 2 h in Tris-buffered saline with 0.1% Tween-20 (TBS-T) containing 5% skim milk and probed with primary antibodies. The primary antibodies against P15, P21 (Zhongshan, Beijing) and Smad7 (Santa Cruz, USA) were diluted at 1:200 in TBS-T, and the β -actin primary antibody (Santa Cruz, USA) was diluted at 1:500 in TBS-T. Secondary antibodies included horseradish peroxidase (HRP)-labeled anti-rabbit IgG against P15, anti-mouse IgG against P21 and anti-goat IgG against Smad 7 and β -actin. All secondary antibodies were diluted at 1:1 000 with TBS-T. Protein bands were visualized by ECL (Amersham, UK) according to the manufacturer's instructions and developed on a film (Kodak, USA). Western blot of β -actin was performed for an internal control.

RESULTS

Localization and expression of main TGF- β 1/Smad signal pathway components

The results of RT-PCR demonstrated that the BGC-823 cell line expressed all main TGF- β 1/Smad signaling pathway components, including TGF- β 1, T β RI, T β RII, Smads 2, -3, -4, and -7 (Figure 1). In addition, all Smad proteins were located in the cytoplasm as demonstrated by immunofluorescence (Figure 2).

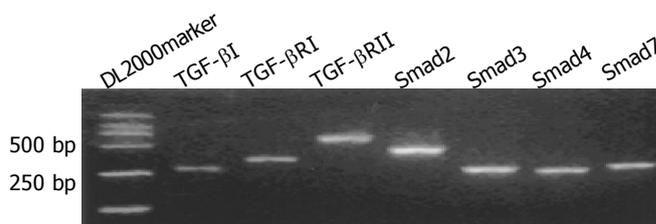


Figure 1 RT-PCR data from BGC-823 cell line demonstrate complete expression of all main components of TGF- β 1/Smad signal pathway, including TGF- β 1 (284 bp), T β RI (358 bp), T β RII (688 bp), Smad2 (489 bp), Smad3 (284 bp), Smad4 (264 bp) and Smad7 (294 bp). Products were electrophoresed on 1.5% agarose gel containing ethidium bromide.

Table 2 Cell cycle alterations of BGC-823 cells treated with TGF- β 1

Time of TGF- β 1 treatment (h)	Cell cycle (%)			
	G0-G1	S	G2-M	Apoptosis
0	52.72	32.39	14.89	0.34
24	57.07	33.82	9.11	6.61
48	60.20	29.54	10.26	9.86

Effect of TGF- β 1 on proliferation and apoptosis of BGC-823 cells

BGC-823 cell growth was inhibited by TGF- β 1 treatment, and the phase of significant inhibition was at 48 h. Thenceforth, this inhibitory effect attenuated gradually, and cells resumed normal growth speed (Figure 3). FCM analysis showed that cells at G1 phase increased while cells at S phase decreased, and the hypodiploid peak, called apoptotic peak indicating reduced DNA content in apoptotic cells, appeared before the G1 phase after exposure to TGF- β 1 (Figure 4). Table 2 shows that TGF- β 1 treatment induced G1 arrest and apoptosis of tumor cells in a time-dependent manner and apoptotic rate reached 6.61% and 9.86%, respectively, at 24 h and 48 h after TGF- β 1 stimulation.

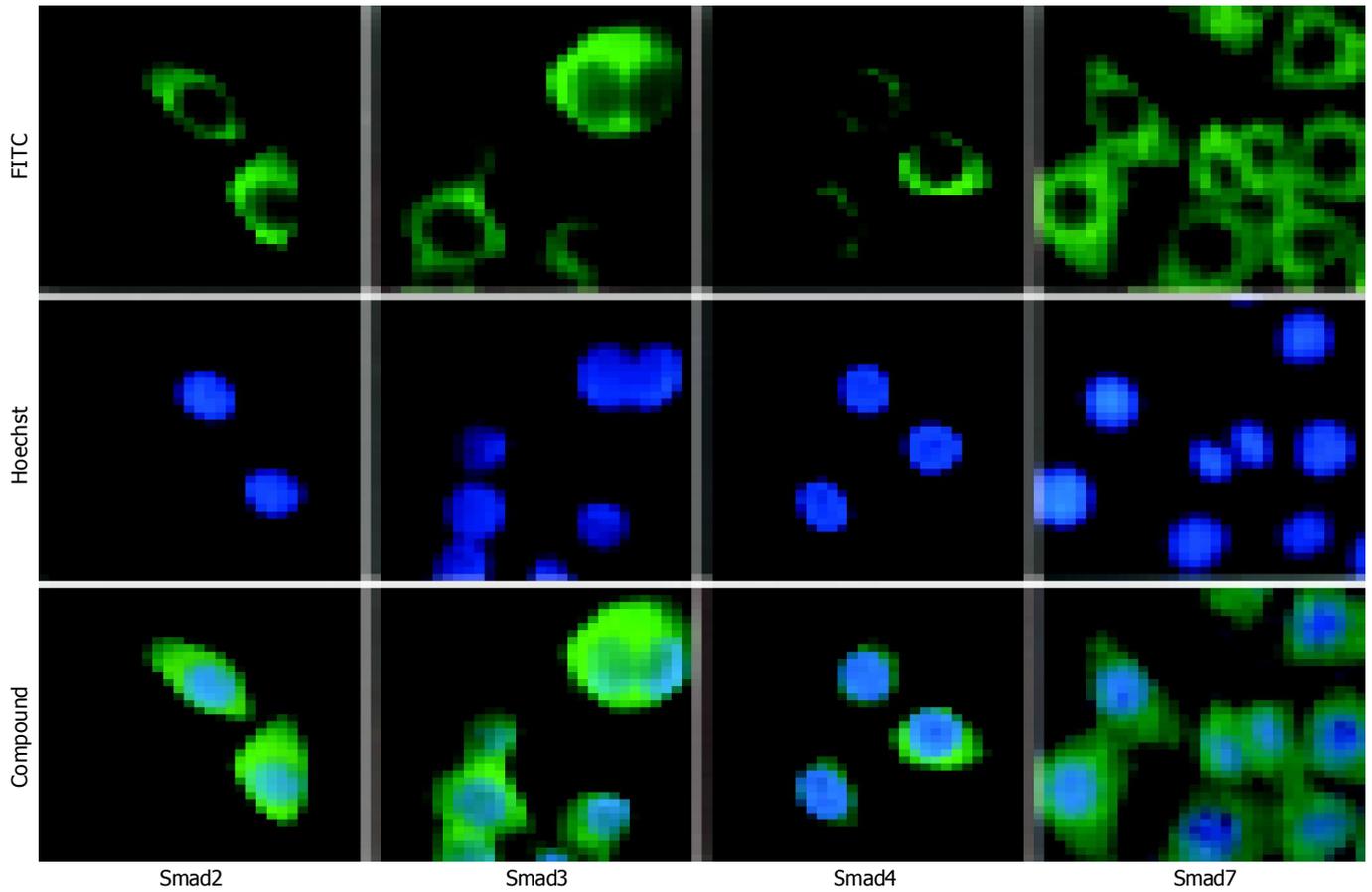


Figure 2 Localization and expression of Smads in BGC-823 cells by immunofluorescence.

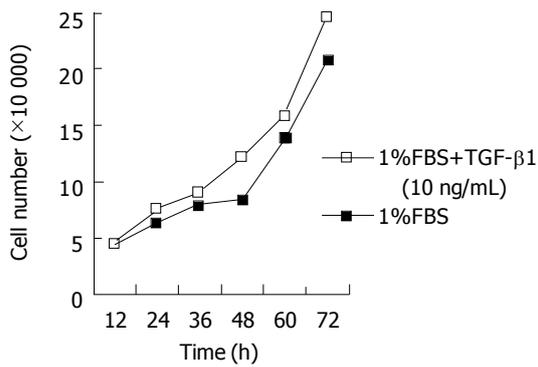


Figure 3 Effect of TGF-β1 on proliferation of BGC-823 cells.

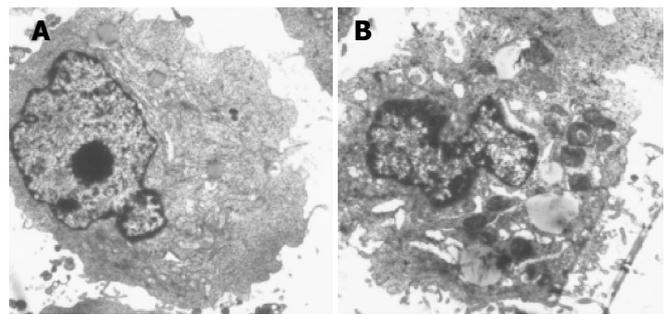


Figure 5 TGF-β1-induced ultrastructural changes in BGC-823 cells under TEM. A: Controls, ×5 000; B: Treated with TGF-β1 for 48 h, ×8 000.

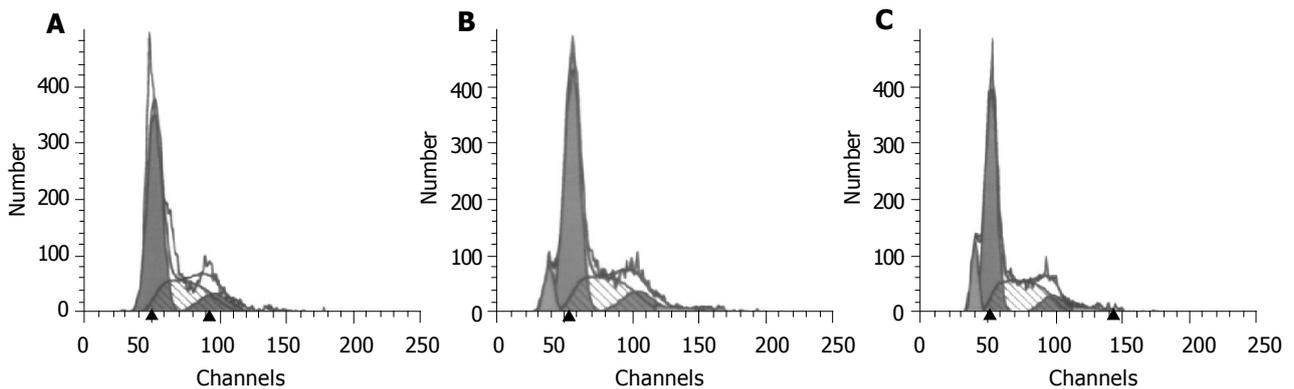


Figure 4 FCM analysis of BGC-823 cells treated with TGF-β1. A: Controls; B: Treated with TGF-β1 for 24 h; C: Treated with TGF-β1 for 48 h.

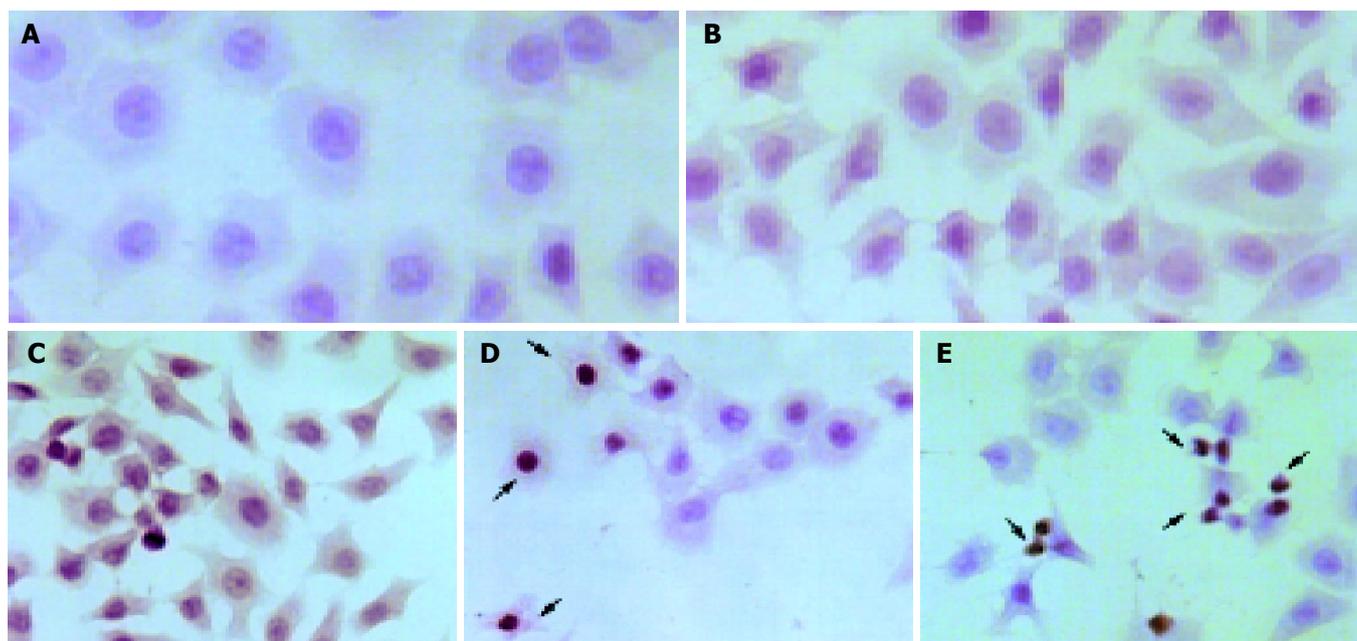


Figure 6 Apoptotic cells induced by TGF- β 1 in BGC-823 cells with TUNEL assay ($\times 200$). A: Normal BGC-823 cells; B: Negative controls; C: Positive controls; D and E: Treated with TGF- β 1 for 48 h. The arrows in picture D and E are pointed to positive cells.

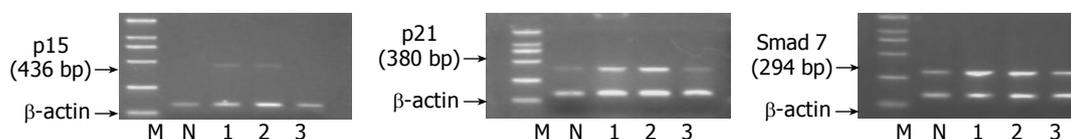


Figure 7 Up-regulation of endogenous genes in BGC-823 cells treated with TGF- β 1. RT-PCR products were electrophoresed on 1.5% agarose gel containing ethidium bromide. The level of β -actin was used as an internal control. M: DL2000 marker; N: the basal level; lanes 1, 2 and 3: 1-, 2- and 3-h exposure to TGF- β 1, respectively.

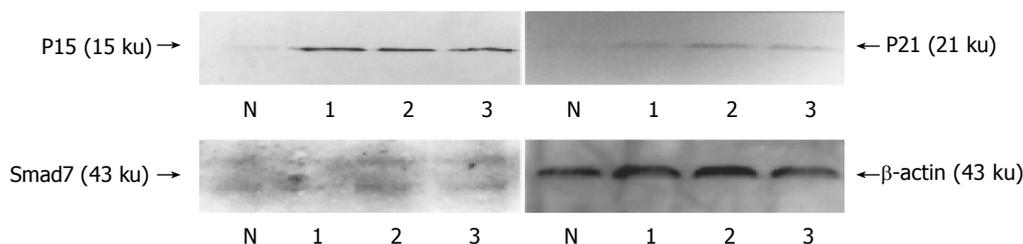


Figure 8 Increase of p15, p21 and Smad7 proteins confirmed by Western blot analysis. N: basal level; lanes 1, 2 and 3: 1-, 2- and 3-exposure to TGF- β 1, respectively.

Under transmission electron microscope, control cells were big and round, with normal organelles, intact nuclear membrane, obvious nucleoli and low density in nuclear chromatin (Figure 5A). However, the cells treated with TGF- β 1 exhibited typical characteristics of apoptosis including cell membrane shrinkage, vacuole formation and mitochondria swelling in cytoplasm, morphologic alteration of nuclei, condensation and fragmentation of nuclear chromatin adjacent to nuclear membrane, although the cell membrane and a few organelles were relatively intact. Cells in late phase of apoptosis with pyknotic nuclei were occasionally observed (Figure 5B). The TUNEL assay revealed that TGF- β 1 induced evident apoptosis of BGC-823 cells, and AI ($10.7 \pm 1.3\%$) at 48 h after TGF- β 1 treatment was significantly higher than that of control group ($0.32 \pm 0.06\%$, $P < 0.01$) (Figure 6).

Up-regulation of endogenous genes, p15, p21 and Smad 7 by TGF- β 1 treatment

Transcript levels of *p15*, *p21* and *Smad7* in BGC-823 cells were

significantly elevated, and gradually recovered after 1 to 3 h following TGF- β 1 (10 ng/mL) treatment using semi-quantitative RT-PCR (Figure 7). Subsequently, the accordant changes of p15, p21 and Smad7 proteins were confirmed by Western blot analysis. Although the basal levels of those proteins were relatively low or almost undetectable, they were rapidly increased in response to TGF- β 1 treatment (Figure 8). Up-regulation of p15 protein peaked at 1 h after TGF- β 1 stimulation, whereas the induction of p21 and Smad7 proteins was most significant at 2 h. The alteration of p15 was ahead of p21 appreciably. Thereafter those protein levels decreased but were still paranormal 3 h after TGF- β 1 administration. These observations suggested that *p15*, *p21* and *Smad7* gene were rapidly but transiently induced by TGF- β 1 treatment.

DISCUSSION

The dynamic balance between cell proliferation and apoptosis is very important to maintain the homeostasis in human body,

and gastric carcinogenesis is related to this imbalance^[13]. Many cancers develop resistance to the growth-inhibitory effects of TGF- β 1, and mutations in signaling pathway components may underlie tumor progression. Several researches on human GC cell lines have revealed that transcriptional repression of the *T β RI* gene by 5' CpG island hypermethylation and decreased or truncated expression of *T β RII* gene result in the development of resistance to antiproliferation of TGF- β 1^[14,15]. Apoptosis of GC cells induced by TGF- β 1 is also mediated by functional T β R^[16]. Both the *T β RI* expression and AI show negative correlations with the degree of gastric mucosal lesions such as chronic superficial gastritis, intestinal metaplasias, atypical hyperplasia to gastric adenocarcinoma. The loss of T β RI expression may play an important role in the inhibition of apoptosis and is involved in the development of GC^[17]. No mutation of *Smad2* gene is detected in human sporadic gastric carcinomas^[18]. In digestive tumors, mutations of *Smad4* gene are mainly found in terminal cancer and correlate with tumorigenesis and metastasis^[19,20]. In this study, BGC-823 cells expressed all main components of TGF- β 1/Smad signal pathway, including TGF- β 1, T β RI, T β RII, Smads 2, -3, -4 and -7, and Smads were located in the cytoplasm. However, it has been shown that the subcellular localization of Smad7 has specificity in various cell types^[12].

TGF- β 1 either simultaneously regulates cell proliferation and apoptosis or just inhibits proliferation without induction of apoptosis^[21,22]. In this study, growth of BGC-823 cells was inhibited in response to TGF- β 1 treatment, and the phase with significant inhibition was at 48 h after exposure to TGF- β 1. Thenceforth, this effect attenuated gradually, and cells resumed normal growth speed. FCM analysis revealed the existence of G1 arrest and apoptosis in tumor cells treated with TGF- β 1, and the decreased proportion of cells at S phase. TEM and TUNEL also indicated that TGF- β 1 induced apoptosis in BGC-823 cells. Our results suggest an intact and functional TGF- β 1/Smad signal pathway in this cell line, which supports the responsiveness of this cell line to TGF- β 1. The negative growth regulation includes both proliferation inhibition and apoptosis enhancement.

In diverse cell types, the mechanism of TGF- β 1 in modulating the activity of CDKs and consequent antiproliferation involve the direct induction of *p15* and *p21* gene transcription^[23-25] and the regulation of p27 binding shift from CDK4 to cyclinE-CDK2^[26]. *p15* and *p21* are known to regulate the activity of cyclin-CDK complexes which is essential to the evolution from G₁ to S phase, the key process of cell cycle^[27,28]. Thus, the transcriptional induction of *p15* and *p21* genes has been postulated at least as partially responsible for the anti-proliferative action of TGF- β 1^[29]. TGF- β 1 has been shown to regulate *p15* gene expression at two levels, i.e., mRNA accumulation and protein stability^[26]. Furthermore, Smads 2, -3 and -4 cooperate with Sp1 to induce *p15* transcription in response to TGF- β 1^[30]. The increased transcripts of *p15* gene result in combination of p15 protein with cyclinD-CDK6/CDK4 in G₁ phase and subsequent repression of kinase activity, suggesting that the role of p15 is to prevent the phosphorylation of intracellular targets including retinoblastoma protein (Rb) by cyclin-CDK complexes during TGF- β 1-mediated arrest. On the other hand, accumulation and overexpression of *p21* mRNA inhibits the activities of multiple cyclins-CDKs such as cyclinE-CDK2 and cyclinD-CDK4, and prevents cells from entering into S phase.

The mechanism of cell apoptosis stimulated by TGF- β 1 has not been fully elucidated. TGF- β 1 may induce apoptosis of multiple tumor cells and consequent inhibition of carcinogenesis^[31,32] by down-regulating the expression of *Bcl-2* and *Bcl-xl* and

promoting the transcription of the death-associated protein kinase and activating caspases^[33-38]. It has been found that TGF- β 1 induces apoptosis in some GC cell lines *in vitro*, which is related to the activation of caspase-3^[16,36,39].

Recently, the effect of CDKs, especially p21, on cell apoptosis has attracted more and more attentions^[40]. Patients with multiple myeloma have a delayed cell apoptosis if *p15* gene is methylated^[41]. A number of studies have shown that endogenous or exogenous p21 may assume both pro-^[42-45] or anti-apoptotic^[46-48] functions in response to different anti-tumor agents depending on cell type and cellular context^[49]. The uncertain function of p21 in the apoptosis of GC cells is bewildering^[47,50]. The precise mechanism of p21 in regulating apoptosis is still poorly understood, but some researchers believe that p21 activates some pro- or anti-apoptotic signal pathways. There is a possible relation between the enhancement of apoptosis by p21 and activation of caspase-3 and caspase-9^[44,51]. p21 promotes apoptosis by increasing the expression of *Bax*, thus modulating the molecular ratio of *Bcl-2*: *Bax* in human hepatocarcinoma cells^[45]. On the other hand, p21 inhibits apoptosis by down-regulating the *Bax/Bcl-2* ratio in human colon cancer cells^[52], suggesting that the role of p21 in apoptosis remains uncertain^[45] and the association between p21 and cell apoptosis induced by TGF- β 1 is still unclear. TGF- β 1 induces apoptosis of cultured bronchiolar epithelial cells and GC cell line SNU-16 via caspase-3 activation and p21 repression^[36,53]. However, TGF- β 1-induced apoptosis of head and neck squamous cell carcinoma cells and hepatoma cells is associated with increased expression of p21 and p15 and reduced ratio of phosphorylated Rb (pRb), implicating that TGF- β 1-induced apoptosis occurs downstream of the pRb/E2F pathway^[54,55].

In our experiments, the expression levels of *p15*, *p21* and *Smad7* mRNA were rapidly and significantly up-regulated after exposure to TGF- β 1, which is in agreement with previous findings^[56,57]. The accordant changes in p15, p21 and Smad7 proteins were confirmed by Western blot analysis. Cells showed modest reduction at S phase, and the obvious cell apoptosis may be associated with the up-regulation of p21 by TGF- β 1 and consequent activation of some pro-apoptotic pathways. Based on our data, we believe that p15 and p21 synergistically inhibit the cellular transition from G₁ to S phase^[10] in response to TGF- β 1 by acting on different cyclin-CDK complexes in BGC-823 cells. The net effects of this responsiveness are to repress the proliferation and enhance the apoptosis, in which p21 may serve as a critical regulator. Therefore, our results suggest a novel functional role of p21 as an accelerant of TGF- β 1-mediated apoptosis in GC cells.

The idea that Smad7 antagonizes TGF- β 1-mediated antiproliferation has been widely accepted. The rapid and transient induction of *Smad7* gene in this study suggests that *Smad7*, as an antagonist of TGF- β 1 antiproliferation, is also a TGF- β 1-inducible early target gene, and functions as a negative feedback loop in GC cells^[5]. Recent studies have shown that Smad7 may modulate the apoptosis induced by TGF- β 1 as either an accelerant^[58-64] or a depressor^[59,65] depending on various cell type and cellular context. The molecular mechanism of apoptosis regulated by Smad7 is largely undefined, and may be related to inhibition of the activity of NF κ B and activation of p38 and caspases^[59,60,62,65]. It is postulated that Smad7 modulates TGF- β 1-mediated apoptosis independent of its role in antagonizing the antiproliferative function of TGF- β 1. Further studies should focus on the exact mechanisms of how endogenous p21 and Smad7 regulate apoptosis induced by TGF- β 1 and their interactions with known apoptosis-associated proteins.

It is noticed that a rapidly growing list of physiological and

pathological factors such as other members of the TGF- β superfamily, tumor suppressor genes, other growth factors, cytokines, and hormones may enhance or antagonize the function of *p15*, *p21* and *Smad7* genes, and regulate their expression by known or unknown mechanisms. The full scope of the interplay of those ligands and TGF- β 1 is unclear, and requires further studies^[66-68].

In conclusion, all observations in this study provide strong evidence that the TGF- β 1/Smad signal pathway is intact and capable of regulating expression of endogenous genes, consequently inhibiting cell growth in BGC-823 cells. Thus manipulation of *p15*, *p21* and *Smad7* may provide a novel strategy to cancer therapy.

REFERENCES

- 1 **Parkin DM.** Global cancer statistics in the year 2000. *Lancet Oncol* 2001; **2**: 533-543
- 2 **Massague J.** The transforming growth factor-beta family. *Annu Rev Cell Biol* 1990; **6**: 597-641
- 3 **Rooke HM, Crosier KE.** The smad proteins and TGFbeta signalling: uncovering a pathway critical in cancer. *Pathology* 2001; **33**: 73-84
- 4 **Maduzia LL, Padgett RW.** Drosophila MAD, a member of the Smad family, translocates to the nucleus upon stimulation of the dpp pathway. *J Biochem Biophys Res Commun* 1997; **238**: 595-598
- 5 **Nakao A, Afrakhte M, Moren A, Nakayama T, Christian JL, Heuchel R, Itoh S, Kawabata M, Heldin NE, Heldin CH, ten Dijke P.** Identification of Smad7, a TGFbeta-inducible antagonist of TGF-beta signalling. *Nature* 1997; **389**: 631-635
- 6 **Blobe GC, Schiemann WP, Lodish HF.** Role of transforming growth factor beta in human disease. *N Engl J Med* 2000; **342**: 1350-1358
- 7 **Xiong B, Gong LL, Zhang F, Hu MB, Yuan HY.** TGF beta1 expression and angiogenesis in colorectal cancer tissue. *World J Gastroenterol* 2002; **8**: 496-498
- 8 **Xiong B, Yuan HY, Hu MB, Zhang F, Wei ZZ, Gong LL, Yang GL.** Transforming growth factor-beta1 in invasion and metastasis in colorectal cancer. *World J Gastroenterol* 2002; **8**: 674-678
- 9 **Hasegawa Y, Takanashi S, Kanehira Y, Tsushima T, Imai T, Okumura K.** Transforming growth factor-beta1 level correlates with angiogenesis, tumor progression, and prognosis in patients with nonsmall cell lung carcinoma. *Cancer* 2001; **91**: 964-971
- 10 **Reynisdottir I, Polyak K, Iavarone A, Massague J.** Kip/Cip and Ink4 Cdk inhibitors cooperate to induce cell cycle arrest in response to TGF-beta. *Genes Dev* 1995; **9**: 1831-1845
- 11 **Tavassoli M, Soltaninia J, Rudnicka J, Mashanyare D, Johnson N, Gaken J.** Tamoxifen inhibits the growth of head and neck cancer cells and sensitizes these cells to cisplatin induced-apoptosis: role of TGF-beta1. *Carcinogenesis* 2002; **23**: 1569-1575
- 12 **Wang B, Hao J, Jones SC, Yee MS, Roth JC, Dixon IM.** Decreased Smad 7 expression contributes to cardiac fibrosis in the infarcted rat heart. *Am J Physiol Heart Circ Physiol* 2002; **282**: H1685-H1696
- 13 **Ishida M, Gomyo Y, Tatebe S, Ohfuji S, Ito H.** Apoptosis in human gastric mucosa, chronic gastritis, dysplasia and carcinoma: analysis by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labelling. *Virchows Arch* 1996; **428**: 229-235
- 14 **Kang SH, Bang YJ, Im YH, Yang HK, Lee DA, Lee HY, Lee HS, Kim NK, Kim SJ.** Transcriptional repression of the transforming growth factor-beta type I receptor gene by DNA methylation results in the development of TGF-beta resistance in human gastric cancer. *Oncogene* 1999; **18**: 7280-7286
- 15 **Yang HK, Kang SH, Kim YS, Won K, Bang YJ, Kim SJ.** Truncation of the TGF-beta type II receptor gene results in insensitivity to TGF-beta in human gastric cancer cells. *Oncogene* 1999; **18**: 2213-2219
- 16 **Ohta S, Yanagihara K, Nagata K.** Mechanism of apoptotic cell death of human gastric carcinoma cells mediated by transforming growth factor beta. *Biochem J* 1997; **324(Pt 3)**: 777-782
- 17 **Zhuang ZH, Chen YL, Wand CD, Chen YG.** The expression of TGF β receptor I and apoptosis in gastric carcinoma and precancerous lesions. *Zhongguo Shiyong Neike Zazhi* 2001; **21**: 342-344
- 18 **Shitara Y, Yokozaki H, Yasui W, Takenoshita S, Kuwano H, Nagamachi Y, Tahara E.** No mutations of the Smad2 gene in human sporadic gastric carcinomas. *Jpn J Clin Oncol* 1999; **29**: 3-7
- 19 **Maitra A, Molberg K, Albores-Saavedra J, Lindberg G.** Loss of Dpc4 expression in colonic adenocarcinomas correlates with the presence of metastatic disease. *Am J Pathol* 2000; **157**: 1105-1111
- 20 **Miyaki M, Kuroki T.** Role of Smad4 (DPC4) inactivation in human cancer. *Biochem Biophys Res Commun* 2003; **306**: 799-804
- 21 **Wimmel A, Wiedenmann B, Rosewicz S.** Autocrine growth inhibition by transforming growth factor beta-1 (TGFbeta-1) in human neuroendocrine tumour cells. *Gut* 2003; **52**: 1308-1316
- 22 **Hague A, Bracey TS, Hicks DJ, Reed JC, Paraskeva C.** Decreased levels of p26-Bcl-2, but not p30 phosphorylated Bcl-2, precede TGFbeta1-induced apoptosis in colorectal adenoma cells. *Carcinogenesis* 1998; **19**: 1691-1695
- 23 **Pardali K, Kurisaki A, Moren A, ten Dijke P, Kardassis D, Moustakas A.** Role of Smad proteins and transcription factor Sp1 in p21(Waf1/Cip1) regulation by transforming growth factor-beta. *J Biol Chem* 2000; **275**: 29244-29256
- 24 **Hannon GJ, Beach D.** p15INK4B is a potential effector of TGF-beta-induced cell cycle arrest. *Nature* 1994; **371**: 257-261
- 25 **Datto MB, Li Y, Panus JF, Howe DJ, Xiong Y, Wang XF.** Transforming growth factor beta induces the cyclin-dependent kinase inhibitor p21 through a p53-independent mechanism. *Proc Natl Acad Sci USA* 1995; **92**: 5545-5549
- 26 **Sandhu C, Garbe J, Bhattacharya N, Dakis J, Pan CH, Yaswen P, Koh J, Slingerland JM, Stampfer MR.** Transforming growth factor beta stabilizes p15INK4B protein, increases p15INK4B-cdk4 complexes, and inhibits cyclin D1-cdk4 association in human mammary epithelial cells. *Mol Cell Biol* 1997; **17**: 2458-2467
- 27 **Sherr CJ.** Mammalian G1 cyclins. *Cell* 1993; **73**: 1059-1065
- 28 **Sherr CJ, Roberts JM.** CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes Dev* 1999; **13**: 1501-1512
- 29 **Hu PP, Datto MB, Wang XF.** Molecular mechanisms of transforming growth factor-beta signaling. *Endocr Rev* 1998; **19**: 349-363
- 30 **Feng XH, Lin X, Derynck R.** Smad2, Smad3 and Smad4 cooperate with Sp1 to induce p15(Ink4B) transcription in response to TGF-beta. *EMBO J* 2000; **19**: 5178-5193
- 31 **Totth A, Sebestyen A, Barna G, Nagy K, Gondor A, Bocsi J, Mihalik R, Petak I, Houghton J, Kopper L.** TGF beta 1 induces caspase-dependent but death-receptor independent apoptosis in lymphoid cells. *Anticancer Res* 2001; **21**: 1207-1212
- 32 **Guo Y, Kyprianou N.** Restoration of transforming growth factor beta signaling pathway in human prostate cancer cells suppresses tumorigenicity via induction of caspase-1-mediated apoptosis. *Cancer Res* 1999; **59**: 1366-1371
- 33 **Francis JM, Heyworth CM, Spooncer E, Pierce A, Dexter TM, Whetton AD.** Transforming growth factor-beta 1 induces apoptosis independently of p53 and selectively reduces expression of Bcl-2 in multipotent hematopoietic cells. *J Biol Chem* 2000; **275**: 39137-39145
- 34 **Chipuk JE, Bhat M, Hsing AY, Ma J, Danielpour D.** Bcl-xL blocks transforming growth factor-beta 1-induced apoptosis by inhibiting cytochrome c release and not by directly antagonizing Apaf-1-dependent caspase activation in prostate epithelial cells. *J Biol Chem* 2001; **276**: 26614-26621
- 35 **Jang CW, Chen CH, Chen CC, Chen JY, Su YH, Chen RH.** TGF-beta induces apoptosis through Smad-mediated expression of DAP-kinase. *Nat Cell Biol* 2002; **4**: 51-58
- 36 **Kim SG, Kim SN, Jong HS, Kim NK, Hong SH, Kim SJ, Bang YJ.** Caspase-mediated Cdk2 activation is a critical step to execute transforming growth factor-beta1-induced apoptosis in human gastric cancer cells. *Oncogene* 2001; **20**: 1254-1265
- 37 **Schiffer M, Bitzer M, Roberts IS, Kopp JB, ten Dijke P, Mundel**

- P, Bottinger EP. Apoptosis in podocytes induced by TGF-beta and Smad7. *J Clin Invest* 2001; **108**: 807-816
- 38 **Shima Y**, Nakao K, Nakashima T, Kawakami A, Nakata K, Hamasaki K, Kato Y, Eguchi K, Ishii N. Activation of caspase-8 in transforming growth factor-beta-induced apoptosis of human hepatoma cells. *Hepatology* 1999; **30**: 1215-1222
- 39 **Yanagihara K**, Tsumuraya M. Transforming growth factor beta 1 induces apoptotic cell death in cultured human gastric carcinoma cells. *Cancer Res* 1992; **52**: 4042-4045
- 40 **Gartel AL**, Tyner AL. The role of the cyclin-dependent kinase inhibitor p21 in apoptosis. *Mol Cancer Ther* 2002; **1**: 639-649
- 41 **Chen W**, Wu Y, Zhu J, Liu J, Tan S, Xia C. Methylation of p16 and p15 genes in multiple myeloma. *Chin Med Sci J* 2002; **17**: 101-105
- 42 **Chopin V**, Toillon RA, Jouy N, Le Bourhis X. P21(WAF1/CIP1) is dispensable for G1 arrest, but indispensable for apoptosis induced by sodium butyrate in MCF-7 breast cancer cells. *Oncogene* 2004; **23**: 21-29
- 43 **Zhang L**, Sharma S, Zhu LX, Kogai T, Hershman JM, Brent GA, Dubinett SM, Huang M. Nonradioactive iodide effectively induces apoptosis in genetically modified lung cancer cells. *Cancer Res* 2003; **63**: 5065-5072
- 44 **Yang HL**, Pan JX, Sun L, Yeung SC. p21 Waf-1 (Cip-1) enhances apoptosis induced by manumycin and paclitaxel in anaplastic thyroid cancer cells. *J Clin Endocrinol Metab* 2003; **88**: 763-772
- 45 **Kang KH**, Kim WH, Choi KH. p21 promotes ceramide-induced apoptosis and antagonizes the anti-death effect of Bcl-2 in human hepatocarcinoma cells. *Exp Cell Res* 1999; **253**: 403-412
- 46 **Martinez LA**, Yang J, Vazquez ES, Rodriguez-Vargas Mdel C, Olive M, Hsieh JT, Logothetis CJ, Navone NM. p21 modulates threshold of apoptosis induced by DNA-damage and growth factor withdrawal in prostate cancer cells. *Carcinogenesis* 2002; **23**: 1289-1296
- 47 **Liu ZM**, Chen GG, Ng EK, Leung WK, Sung JJ, Chung SC. Upregulation of heme oxygenase-1 and p21 confers resistance to apoptosis in human gastric cancer cells. *Oncogene* 2004; **23**: 503-513
- 48 **Niibe Y**, Nakano T, Ohno T, Tsujii H, Oka K. Relationship between p21/WAF-1/CIP-1 and apoptosis in cervical cancer during radiation therapy. *Int J Radiat Oncol Biol Phys* 1999; **44**: 297-303
- 49 **Liu S**, Bishop WR, Liu M. Differential effects of cell cycle regulatory protein p21(WAF1/Cip1) on apoptosis and sensitivity to cancer chemotherapy. *Drug Resist Updat* 2003; **6**: 183-195
- 50 **Zheng SY**, Ge JF, Shen ZY, Zhao J, Qin Y. Apoptosis of human gastric cancer cell line SGC-7901 by p21 gene transduction. *Zhongguo Aizheng Zazhi* 2002; **12**: 9-12
- 51 **Agrawal S**, Agarwal ML, Chatterjee-Kishore M, Stark GR, Chisolm GM. Stat1-dependent, p53-independent expression of p21 (waf1) modulates oxysterol-induced apoptosis. *Mol Cell Biol* 2002; **22**: 1981-1992
- 52 **Javelaud D**, Besancon F. Inactivation of p21WAF1 sensitizes cells to apoptosis via an increase of both p14ARF and p53 levels and an alteration of the Bax/Bcl-2 ratio. *J Biol Chem* 2002; **277**: 37949-37954
- 53 **Hagimoto N**, Kuwano K, Inoshima I, Yoshimi M, Nakamura N, Fujita M, Maeyama T, Hara N. TGF-beta 1 as an enhancer of Fas-mediated apoptosis of lung epithelial cells. *J Immunol* 2002; **168**: 6470-6478
- 54 **Tavassoli M**, Soltaninia J, Rudnicka J, Mashanyare D, Johnson N, Gaken J. Tamoxifen inhibits the growth of head and neck cancer cells and sensitizes these cells to cisplatin induced-apoptosis: role of TGF-beta1. *Carcinogenesis* 2002; **23**: 1569-1575
- 55 **Fan G**, Ma X, Wong PY, Rodrigues CM, Steer CJ. p53 dephosphorylation and p21(Cip1/Waf1) translocation correlate with caspase-3 activation in TGF-beta1-induced apoptosis of HuH-7 cells. *Apoptosis* 2004; **9**: 211-221
- 56 **Dunfield LD**, Dwyer EJ, Nachtigal MW. TGF beta-induced Smad signaling remains intact in primary human ovarian cancer cells. *Endocrinology* 2002; **143**: 1174-1181
- 57 **Akagi M**, Yasui W, Akama Y, Yokozaki H, Tahara H, Haruma K, Kajiyama G, Tahara E. Inhibition of cell growth by transforming growth factor beta 1 is associated with p53-independent induction of p21 in gastric carcinoma cells. *Jpn J Cancer Res* 1996; **87**: 377-384
- 58 **Schiffer M**, Bitzer M, Roberts IS, Kopp JB, ten Dijke P, Mundel P, Bottinger EP. Apoptosis in podocytes induced by TGF-beta and Smad7. *J Clin Invest* 2001; **108**: 807-816
- 59 **Edlund S**, Bu S, Schuster N, Aspenstrom P, Heuchel R, Heldin NE, ten Dijke P, Heldin CH, Landstrom M. Transforming growth factor-beta1 (TGF-beta)-induced apoptosis of prostate cancer cells involves Smad7-dependent activation of p38 by TGF-beta-activated kinase 1 and mitogen-activated protein kinase kinase 3. *Mol Biol Cell* 2003; **14**: 529-544
- 60 **Lallemant F**, Mazars A, Prunier C, Bertrand F, Kornprost M, Gallea S, Roman-Roman S, Cherqui G, Atfi A. Smad7 inhibits the survival nuclear factor kappaB and potentiates apoptosis in epithelial cells. *Oncogene* 2001; **20**: 879-884
- 61 **Landstrom M**, Heldin NE, Bu S, Hermansson A, Itoh S, ten Dijke P, Heldin CH. Smad7 mediates apoptosis induced by transforming growth factor beta in prostatic carcinoma cells. *Curr Biol* 2000; **10**: 535-538
- 62 **Okado T**, Terada Y, Tanaka H, Inoshita S, Nakao A, Sasaki S. Smad7 mediates transforming growth factor-beta-induced apoptosis in mesangial cells. *Kidney Int* 2002; **62**: 1178-1186
- 63 **Kanamaru C**, Yasuda H, Fujita T. Involvement of Smad proteins in TGF-beta and activin A-induced apoptosis and growth inhibition of liver cells. *Hepatol Res* 2002; **23**: 211-219
- 64 **Patil S**, Wildey GM, Brown TL, Choy L, Derynck R, Howe PH. Smad7 is induced by CD40 and protects WEHI 231 B-lymphocytes from transforming growth factor-beta -induced growth inhibition and apoptosis. *J Biol Chem* 2000; **275**: 38363-38370
- 65 **Kim SG**, Jong HS, Kim TY, Lee JW, Kim NK, Hong SH, Bang YJ. Transforming growth factor-beta 1 induces apoptosis through Fas ligand-independent activation of the Fas death pathway in human gastric SNU-620 carcinoma cells. *Mol Biol Cell* 2004; **15**: 420-434
- 66 **Afrakhte M**, Moren A, Jossan S, Itoh S, Sampath K, Westermarck B, Heldin CH, Heldin NE, ten Dijke P. Induction of inhibitory Smad6 and Smad7 mRNA by TGF-beta family members. *Biochem Biophys Res Commun* 1998; **249**: 505-511
- 67 **Ulloa L**, Doody J, Massague J. Inhibition of transforming growth factor-beta/SMAD signalling by the interferon-gamma/STAT pathway. *Nature* 1999; **397**: 710-713
- 68 **Kardassis D**, Papakosta P, Pardali K, Moustakas A. c-Jun transactivates the promoter of the human p21(WAF1/Cip1) gene by acting as a superactivator of the ubiquitous transcription factor Sp1. *J Biol Chem* 1999; **274**: 29572-29581