Role of p53β in the inhibition of proliferation of gastric cancer cells expressing wild-type or mutated p53

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Abstract. p53 is a tumor suppressor gene whose mutation is highly associated with tumorigenesis. The present study investigated the role of p53β in the inhibition of proliferation of gastric cancer cell lines expressing wild-type or mutated p53. Wild-type p53 is expressed in MKN45 cells, but deleted in KATOIII cells, whereas mutated p53 is expressed in SGC7901 cells. The mRNA expression levels of p53β and Δ133p53 were detected in MKN45, SGC-7901 and KATOIII gastric cancer cell lines using nested polymerase chain reaction (PCR). The mRNA expression levels of p53, p53β and B-cell lymphoma 2-associated X protein (Bax) were detected in the MKN45 and SGC-7901 cells following treatment with cisplatin by reverse transcription-PCR. The inhibition of cellular proliferation following treatment with cisplatin was measured by MTT assay. The results of the present study demonstrated that both p53β and Δ133p53 mRNA were expressed in the MKN45 cells, whereas only p53β mRNA was expressed in the SGC7901 cells. No expression of p53β or Δ133p53 mRNA was detected in the KATOIII cells. Following treatment with cisplatin, the number of both MKN45 and SGC-7901 cells was significantly reduced ($P<0.001$). In the MKN45 cells, p53β, p53 and Bax mRNA expression levels gradually increased with the dose of cisplatin, and the expression of p53β was positively correlated with the expression of p53 ($t_r=6.358$, $P<0.05$) and Bax ($t_r=8.023$, $P<0.05$). In the SGC-7901 cells, the expression levels of p53β, p53 and Bax mRNA did not alter with the dose of cisplatin, and the expression of p53β was positively correlated to the expression of p53 ($t_r=26.41$, $P<0.01$) but not that of Bax. The present study identified the different roles of the p53β isoform in gastric cancer cells with different p53 backgrounds. Enhanced knowledge regarding the p53 status is required for the development of specific biological therapies against gastric cancer.

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Introduction

p53 is a tumor suppressor gene whose mutation status is highly associated with tumorigenesis. Through regulating the transcription of effector molecules, such as B-cell lymphoma 2-associated X protein (Bax), mouse double minute 2 homolog (MDM2), and phosphatase and tensin homolog, p53 is involved in cell cycle arrest, DNA repair and apoptosis (1-3). Besides wild-type p53, other protein isoforms of p53 are expressed under physical and pathological conditions due to promoter selection, alternative splicing and the selection of translation initiators (4-6). The alternative splicing sites and promoters of p53 are presented in the diagram of the human *TP53* gene structure (Fig. 1A), and possible p53 protein isoforms are listed in Fig. 1B. Theoretically, ≥12 isoforms of p53 may be produced by alternative splicing and the selection of different promoters, including p53, p53β, p53γ, Δ40p53, Δ40p53β, Δ40p53γ, Δ133p53, Δ133p53β, Δ133p53γ, Δp53, Δp53β and Δp53γ (7). As compared with the full length p53, the p53β and p53γ isoforms are produced by alternative splicing of intron 9, where 63 carboxyl-terminal amino-acid residues are replaced with 10 or 15 new residues. The Δ133p53 isoform is transcribed by the P2 promoter and lacks 133 amino-terminal residues, including the entire transactivating domain and part of the DNA binding domain. The Δ40p53 isoform is transcribed by the P1 or P1' promoter and lacks 40 amino-terminal residues, including the conservative FxxΨW sequence, due to alternative splicing of intron 2. The Δp53 isoform is produced by non-canonical splicing between exons 7 and 9, and lacks 66 residues in the DNA binding domain, including the highly conserved V region. The selection of different translation initiators may also produce more isoforms; for example, Δ133p53 mRNA may be translated into either the Δ133p53 or Δ160p53 isoform (8).

It was previously reported that p53 isoforms have vital physical functions. Certain isoforms have been shown to be associated with the fine regulation of p53 activities, and are involved in apoptosis, cell cycle arrest and cellular senescence. Furthermore, the abnormal expression of p53 isoforms isolated

from *TP53* gene mutations is associated with the tumorigenesis of various tissues, including renal carcinoma (9), breast carcinoma (10), ovarian carcinoma (11), medulloblastoma (12), neuroblastoma (13), melanoma (14), squamous cell carcinoma (15), and medullar leukemia (16). p53β has previously been shown to coordinate with wild-type p53 to work in a promoter-dependent manner, which helps induce apoptosis and inhibit proliferation, and is therefore associated with the control of carcinogenesis and drug resistance (13,14,17). The p53 status has been shown to differ in various gastric cancer cell lines: Wild-type p53 is expressed in MKN45 cells, but deleted in KATOIII cells (18), whereas mutated p53 is detected in SGC7901 cells (204 codon GAG→GCG mutation in the sixth exon, Glu→Ala) (19). The present study aimed to determine the function of p53β in the inhibition of proliferation of cells expressing wild-type or mutated p53.

Materials and methods

Materials and agents. Cisplatin was purchased from Shandong Platinum Source Industry Co., Ltd. (Jinan, China). MTT, RPMI-1640 medium and fetal bovine serum (FBS) were obtained from Jinan Kiagen Biology Technology Co., Ltd. (Jinan, China). TRIzol® RNA Extraction kit (batch number 14033089C), Reverse Transcription-Polymerase Chain Reaction (RT-PCR) kit (batch number A901KA241), primers, DNA marker (batch number 050714) and agarose (batch number 111860) were all purchased from Sangon Biotech Co., Ltd. (Shanghai, China). MyCycler™ Thermal Cycler, BioSpectrum AC Gel Imaging system and EPS-301 Electrophoresis Meter were purchased from Bio-Rad Laboratories, Inc. (Hercules, CA, USA), UVP, Inc. (Upland, CA, USA) and GE Healthcare Life Sciences (Uppsala, Sweden), respectively.

Cell lines. The MKN45 (batch number CBP60488), SGC7901 (batch number CBP60500) and KATOIII (batch number CBP60483) human gastric cancer cell lines were supplied by the Cell Bank of Chinese Academy of Medical Science (Beijing, China), and had a passage number <10. All cell lines were tested for mycoplasmic infection. The cells were cultured in RPMI-1640 medium supplemented with 10% FBS at 37˚C in an atmosphere containing 5% CO_2. Cells in the exponential growth phase were used for the following experiments.

Measurement of p53β and Δ133p53 mRNA expression levels in gastric cancer cell lines. The MKN45, SGC-7901 and KATOIII cells were isolated in the exponential growth phase. mRNA extraction and cDNA synthesis were performed according to manufacturer's instructions of the TRIzol® and RT-PCR kits. Nested PCR was performed to amplify p53β and Δ133p53, and the primers used were as follows: p53β outer primer forward, 5'-GTCACTGCCATGGAGGAGCCGCA-3' and reverse, 5'-GACGCACACCTATTGCAAGCAAGGGTTC-3'; p53β internal primer forward, 5'-ATGGAGGAGCCGCAGTCAGAT-3' and reverse, 5'-TTTGAAAGCTGGTCTGGTCCTGA-3'; Δ133p53 outer primer forward, 5'-CTGAGGTGTAGACGCCAACTCTCTCTAG-3' and reverse, 5'-AGTCAGTCTGAGTCAGGCCCTTCTGTC-3'; Δ133p53 internal primer forward, 5'-GCTAGTGGGTTGCAGGAGGTGCTTACAC-3' and reverse, 5'-CTCACGCCCACGGATCTGA-3'; β-actin primer forward, 5'-GTGGGGCGCCCCAGGCACCA-3' and reverse, 5'-CTCCTTAATGTCACGCACGATTTC-3'. The lengths of the amplified products were 1050 bp for p53β, 750 bp for Δ133p53, and 539 bp for β-actin. PCR conditions were set as 35 cycles at 94˚C for 1 min for denaturation, 58˚C for 50 sec for annealing and 72˚C for 1 min for extension. The PCR products were subsequently analyzed by 2% agarose gel electrophoresis. The results were scanned and recorded by the Biospectrum AC Gel Imaging system (Alpha Innotech Corp., San Leandro, CA , USA).

MTT analysis of gastric cancer cell proliferation. The MKN45 and SGC-7901 cells were isolated in the exponential growth phase and seeded in a 96-well plate ($5x10^3$ cell/well). The cells were then cultured for 24 h. Cisplatin (1, 2 or 4 μmol/l) or an equal volume of phosphate-buffered saline (PBS) was added to the cells, which were cultured for a further 48 h. Subsequently, the culture medium was discarded and 20 μl MTT solution (5 g/l) and 180 μl fresh RPMI-1640 medium were added to the cells, which were cultured for another 4 h. The supernatant was then discarded, dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO, USA) was added to the cells (150 μl/well) and the plate was oscillated for 10 min. The optical density (OD) of the cells was measured at a wavelength of 490 nm using a Multiskan FC microplate reader (Thermo Fisher Scientific, Inc., Waltham, MA, USA), and the rate of proliferation inhibition was calculated.

Assessment of p53, p53β and Bax mRNA expression levels by RT-PCR analysis. The MKN45 and SGC-7901 cells were isolated in the exponential growth phase and seeded in a 96-well plate ($5x10^3$ cells/well). Cisplatin (1, 2 or 4 μmol/l) or an equal volume of PBS was added to the cells, which were then cultured for 24 h. The cells were then collected and mRNA extraction, cDNA synthesis and PCR amplification were performed according to the manufacturer's instructions of the TRIzol® and RT-PCR kits. The purity and quantity of RNA were analyzed using a 2000c UV-Vis Spectrophotometer (Thermo Fisher Scientific, Inc.). The following primers were used in the RT-PCR in the present study: p53 forward, 5'-GGTCTCCTCCACCGCTTCTTG TC-3' and reverse, 5'-GGCCTCATCTTGGGCCTGTGT-3'; p53β forward, 5'-GTCACTGCCATGGAGGAGCCGCA-3' and reverse, 5'-ATGGAGGAGCCGCAGTCAGAT-3'; Bax forward, 5'-ACCAAGAAGCTGAGCGAGTGTC-3' and reverse, 5'-ACAAAGATGGTCACGGTCTGCC-3'; β-actin forward, 5'-GAGCCACATCGCTCAGACAC-3' and reverse, 5'-TCGAGGAAACAAATTAAGAA-3'. The lengths of the amplified products were 690 bp for p53, 1050 bp for p53β, 365 bp for Bax, and 539 bp for β-actin. PCR conditions were set as 35 cycles at 94˚C for 5 min, 94˚C for 30 sec, 55-58˚C for 30 sec and 72˚C for 30 sec, followed by 72˚C for 10 min. The PCR products were subsequently analyzed by 2% agarose gel electrophoresis. The results were scanned and recorded by the Biospectrum AC Gel Imaging system.

Statistical analysis. SPSS version 17.0 (International Business Machines, Armonk, NY USA) software package was used for all statistical analyses. Comparisons between the measurement data were analyzed by one-way analysis of variance (ANOVA) and correlations between the data were determined by a linear

Table I. Inhibition of MKN45 and SGC7901 gastric cancer cell proliferation by cisplatin.

	Dose of cisplatin (μmol/l)					
Cell line	0	1	2	4	*F*	P-value
MKN45	0.236±0.007	0.200±0.003	0.165±0.004	0.117±0.009	49.768	<0.001
SGC7901	0.269±0.023	0.210±0.006	0.196±0.004	0.156±0.005	52.357	<0.001

Optical density is shown; values are expressed as the mean ± standard deviation (n=5).

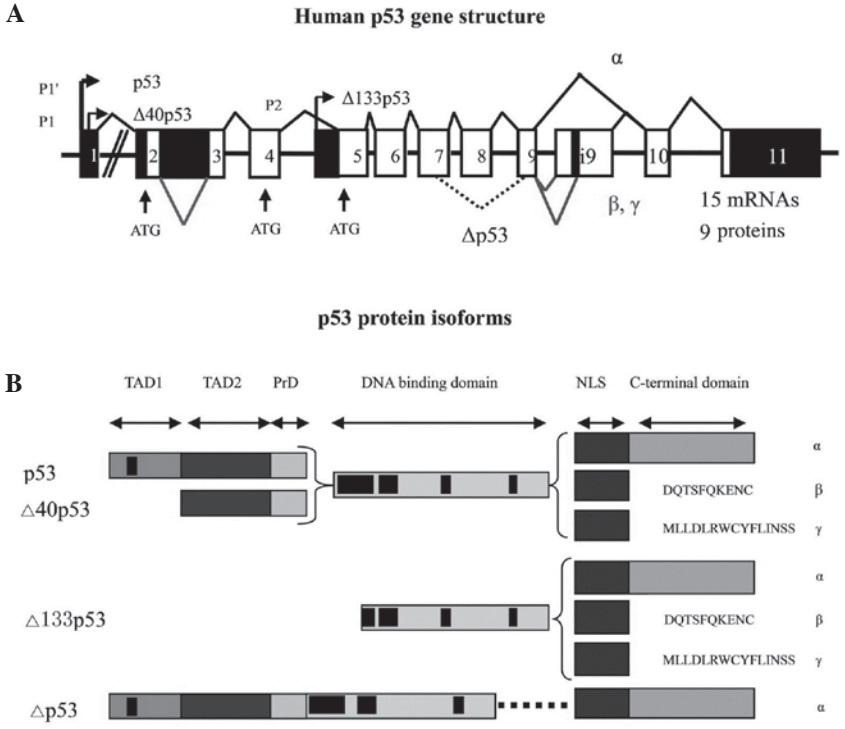

Figure 1. Diagrams of the (A) gene structure of *TP53* and (B) isoforms of p53.

regression ANOVA. P<0.05 was considered to indicate a statistically significant difference.

Results

mRNA expression levels of p53β and Δ133p53 in the three gastric cancer cell lines. p53β and Δ133p53 mRNA was expressed in the MKN45 cell line, whereas only p53β mRNA was expressed in the SGC-7901 cells (Fig. 2). Neither p53β nor Δ133p53 mRNA was expressed in the KATOIII cell line.

Inhibition of MKN45 and SGC7901 cell proliferation by treatment with different doses of cisplatin. Following a 48-h treatment with various doses of cisplatin, the OD value of MKN45 and SGC-7901 cells in the MTT assay was significantly reduced (F_{MKN45}=49.768, *P*<0.001; $F_{SGC7901}$=52.357, P<0.001) (Table I).

mRNA expression levels of p53β, p53 and Bax in the cisplatin-treated cell lines. p53β, p53 and Bax mRNA expression levels were detected in the control and cisplatin-treated groups. In the MKN45 cell line, the expression levels of p53β, p53 and Bax mRNA increased with increasing doses of cisplatin (Fig. 3A). The linear regression ANOVA demonstrated that the expression of p53β was positively correlated with the expression of p53 (n=4, r=0.976, t_r=6.358, P<0.05, y=0.1362+3.217x) (Fig. 4A). The expression of p53β was also positively correlated with the expression of Bax (n=4, r=0.985, t_r=8.023, P<0.05, y=0.423+2.792x). In the SGC-7901 cell line, the mRNA expression levels of p53β, p53 and Bax were not affected by cisplatin at the doses used (Fig. 3B). The linear regression ANOVA demonstrated that the expression of p53β was positively correlated with the expression of p53 (n=4, r=0.999, t_r=26.41, P<0.01, y=0.0004+0.999x) (Fig. 4B); however, the expression of p53β was not correlated with the expression of Bax (n=4, r=–0.067, t_r=0.095, P>0.05, y=0.948-0.135x).

Discussion

Genomic instability, caused by mutations of the *TP53* gene, is widely accepted as a critical event in the carcinogenesis of numerous types of tumor. Furthermore, previous studies have indicated that the p53 isoforms have pivotal roles in various

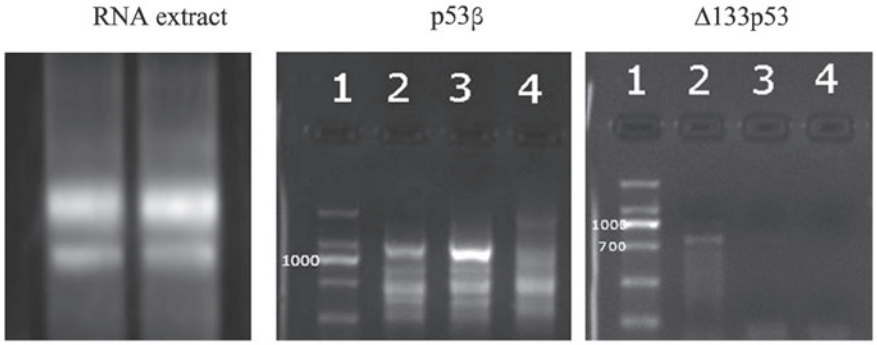

Figure 2. Differential mRNA expression levels of p53 isoforms in MKN45, SGC-7901 and KATOIII human gastric cancer cell lines. Left, RNA extract from the gastric cancer cells; middle, p53β expression in the gastric cancer cells; right, Δ133p53 expression in the gastric cancer cells. Lane 1, DNA marker; lane 2, MKN45 cells; lane 3, SGC7901 cells; lane 4, KATOIII cells.

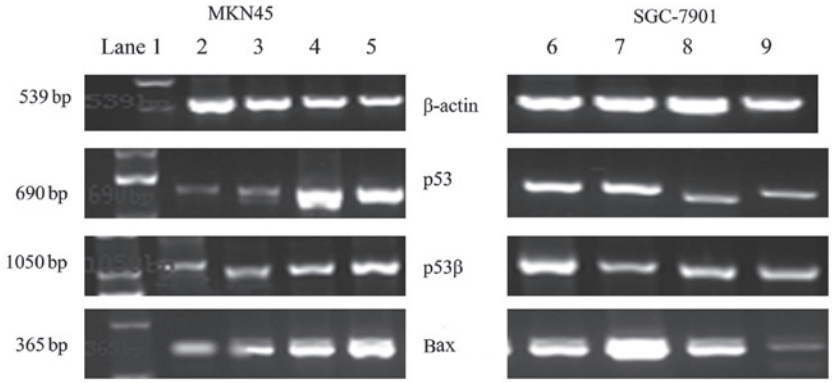

Figure 3. mRNA expression levels of p53, p53β and Bax in MKN45 and SGC7901 human gastric cancer cells treated with cisplantin. Lane 1, DNA marker; lanes 2 and 6, cells prior to treatment with cisplatin; lanes 3 and 7, cells treated with 1 μmol/l cisplatin; lanes 4 and 8, cells treated with 2 μmol/l cisplatin; lanes 5 and 9, cells treated with 4 μmol/l cisplatin. Bax, B-cell lymphoma 2-associated X protein.

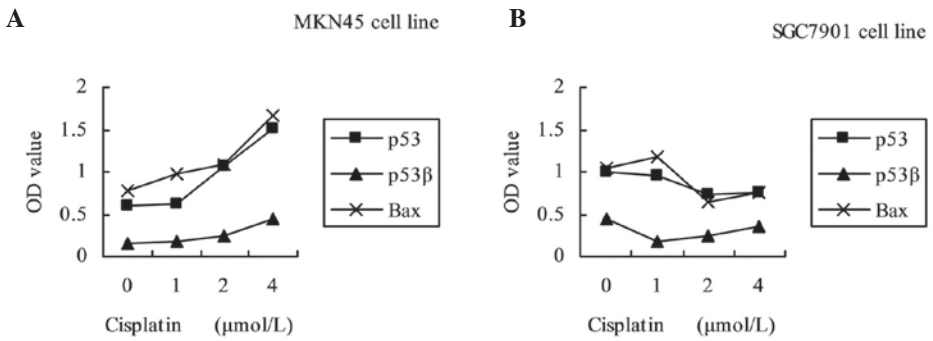

Figure 4. Correlations between the mRNA expression levels of p53β and p53, and p53β and Bax in (A) MKN45 and (B) SGC7901 human gastric cancer cells treated with cisplatin. Bax, B-cell lymphoma 2-associated X protein.

physical and pathological processes. These findings suggested that novel isoform-based gene diagnosis and biological therapy may potentially be developed (10,11,13,15,16,20-23). In previous studies, all p53 isoforms were detected in tissue from superficial gastritis, atrophic gastritis, para-cancerous area, to advanced gastric carcinoma (24,25).

In the present study, mRNA expression levels of p53, p53β and Bax were determined in order to analyze the correlation between p53β and full-length p53, and between p53β and Bax in the cells expressing wild-type or mutated p53. In the MKN45 cells (wild-type p53), p53β and Δ133p53 mRNA was detected, whereas only p53β mRNA was detected in the SGC7901 cells. Neither p53β nor Δ133p53 mRNA was detected in the KATOIII cells. Results of previous studies have indicated that the Δ133p53 isoform has oncogenic effects (22,23,26), whereas the p53β isoform acts as a tumor suppressor gene (13,27). Of note, these results suggested that gastric carcinogenesis may be caused by deletion of the *TP53* gene (KATOIII), muta-

tions of the *TP53* gene (SGC7901) or abnormal expression of p53 isoforms (MKN45). However, the initial results of the present study require further clarification in order to improve knowledge regarding p53-based carcinogenesis, diagnosis and biological therapy.

The growth of MKN45 and SGC7901 gastric cancer cell lines was significantly inhibited by treatment with cisplatin in a dose-dependent manner. Of note, p53β had different roles in the two cell lines. In the MKN45 cell line (expressing wild-type p53), the mRNA expression levels of p53β, p53 and Bax were increased accordingly with the dose of cisplatin, and the linear regression ANOVA indicated that the expression of p53β had a positive linear correlation with the expression of p53 and Bax. However, in the SGC-7901 cell line (expressing mutated p53), the mRNA expression levels of p53β, p53 and Bax were not affected by the dose of cisplatin, and the linear regression ANOVA analysis indicated that the expression of p53β was significantly correlated with the expression of p53; however, p53β was not correlated with the expression of Bax. Previous studies have shown that p53β can work with full-length p53 in a promoter-dependent manner in order to selectively initiate the transcription of Bax, but not MDM2 (17). The results of the present study indicate the existence of a complex regulatory loop between p53 and its downstream target molecules, in which various p53 isoforms may be involved (28,29). Although the expression levels of p53β were associated with the expression of full-length p53 in wild-type and mutated cell lines, only in the cells expressing wild-type p53 could p53β coordinate with p53 and promote the transcription of Bax. In the cells expressing mutated p53, p53β may lose its effects on target gene transcription, apoptosis and proliferative senescence.

In conclusion, based on the p53 status, gastric carcinogenesis may be caused by the deletion, mutation or abnormal expression of p53 isoforms. In cells expressing mutated p53, the p53β isoform lost its effects on the transcription of Bax. Further knowledge regarding the p53 status is required in order to potentially produce p53-based gene diagnosis and individual biological therapy for gastric cancer.

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