Original Article
p53β expression in the MKN45 gastric cancer cell line responds to 5-fluorouracil growth inhibition treatment

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Received January 3, 2016; Accepted March 17, 2016; Epub April 1, 2016; Published April 15, 2016

Abstract: This study aims to explore the role of the p53β isoform in fluorouracil-induced growth inhibition of MKN45 gastric cancer cells. A Cell Counting Kit 8 (CCK8) assay was used to measure inhibition rates in gastric cancer cell cultures (lines MKN45 and SGC7901) treated with different concentrations of 5-fluorouracil (5-FU). The abundance of p53β and p53 mRNA was measured using the nested reverse transcription polymerase chain reaction (nRT-PCR) method; protein abundance of p53β and p53 was quantified using western blots. In MKN45 cells, the growth inhibition differed significantly between groups treated with different 5-FU doses (F = 31.682, P < 0.01); this phenomenon was not observed for SGC7901 cells (F = 0.014, P > 0.05). In addition, growth inhibition differed significantly between groups measured at different time-points for MKN45 cells treated with 25 µg/mL 5-FU (F = 225.304, P < 0.01), but not for SGC7901 cells (F = 0.043, P > 0.05). The mRNA expression for p53 and p53β differed significantly between groups treated with different 5-FU doses (Fp53 = 187.992, Fp53β = 23.020, P < 0.01) in MKN but not in SGC7901 cells (Fp53 = 1.912, P = 0.206; Fp53β = 1.626, P = 0.259). Protein expression for p53 and p53β differed significantly between groups treated with different 5-FU doses (Fp53 = 80.646, Fp53β = 213.419, P < 0.01) in MKN but not in SGC7901 cells (Fp53 = 0.055, P = 0.982; Fp53β = 1.613, P = 0.261). p53β is an important oncotarget of 5-FU in the growth inhibition of MKN45 gastric cancer cells.

Keywords: p53β isoform, gastric cancer, oncotarget, fluoracil

Introduction

Although mutations frequently deactivate p53 in malignant diseases such as gastric carcinoma [1-3], these changes rarely show clinical significance [4-10]. However, studying p53 isoforms may yield alternative pharmacological strategies. To date, more than 12 isoforms of p53 have been reported, including p53α, p53β, p53γ, Δ40p53α, Δ40p53β, Δ40p53D, Δ133p53α, Δ133p53β, Δ133p53D, Δ160p53α, Δ160p53β, and Δ160p53γ. Isoforms of p53 express atypically in cancers of the breast [11], kidney [12, 13], colon [14], ovary [15-18], bone marrow [19], and nervous system [20, 21]. Some of these isoforms are p53 co-activators (e.g., p53β), whereas others are p53 antagonists (e.g., Δ40p53α and Δ133p53α) [22-24]. Preliminary measurements in our lab indicated that in gastric cancer cells, the expression rates of p53β and Δ133p53α are 20% and 75%, respectively; meanwhile, in tissues of superficial atrophic gastritis or para-cancerous lesions, p53β expression is significantly higher than baseline but Δ133p53α expression is significantly lower than baseline. Recent results indicate a link between Helicobacter pylori, Δ133p53α, and gastric carcinogenesis [25, 26].

In the present work, the role of p53β in gastric carcinogenesis was studied, and this molecule’s potential as a chemotherapy target was evaluated. After culturing two different gastric cancer cell lines under different doses of the growth inhibitor 5-fluorouracil (5-FU), the cells’ p53 and p53β expression levels were measured. The two cell lines under study have different p53 background: MKN45 cells have wild-type p53, whereas SGC7901 cells have mutant p53 (204 codon GAG→GCG mutation in the sixth exon, Glu→Ala) [27-29]. This study aimed to elucidate processes governing apoptosis by perturbing growth inhibition and gene expression in cells with different p53 backgrounds that were exposed to different levels of 5-FU.
**Materials and methods**

**CCK-8 analysis**

MKN45 and SGC7901 cells in the exponential growth phase were digested, seeded in a 96-well plate (5 × 10⁴ cells/well), and cultured for 24 hrs. 20 µL of 5-FU (No. H31020593, Xudong Hai-pu Pharmaceutical Co., Ltd., Shanghai, China) in concentration of 25, 50, 100 μg/mL, or an equal volume of phosphate-buffered saline (PBS), was added to the cells, which were cultured for another 48 h. Next, the culture medium was discarded. A 20-μl mixture of CCK-8 (No.CK-04, Xusheng Biotechnology Co., Shanghai, China) and 10X-diluted growth medium was added to each well; cells were cultured for another 1 h. The optical density (OD) of the cells was measured at a wavelength of 450 nm by a Multiskan FC microplate reader (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The growth inhibition rate (IC) was calculated using Equation 1.

\[
(1)IC = \frac{(A_{\text{Exp}} - A_{\text{C}})}{A_{\text{C}}} \times 100%
\]

As Equation 1 shows, the growth inhibition rate IC is 100% multiplied by the ratio of two differences: the difference between \( A_{\text{Exp}} \), absorbance in experimental group, and \( A_{\text{C}} \), absorbance in control group; and the difference between \( A_{\text{C}} \) and \( A_{\text{Emp}} \), absorbance in empty group.

**RT-PCR**

MKN45 and SGC-7901 cells in the exponential growth phase were digested, seeded in a 96 well plate (5 × 10⁴ cells/well), and then cultured for 24 h. 20 µL of 5-FU (No. H31020593, Xudong Hai-pu Pharmaceutical Co., Ltd., Shanghai, China) in concentration of 25, 50, 100 μg/mL, or an equal volume of phosphate-buffered saline (PBS), was added to the cells, which were cultured for another 48 h. Next, the culture medium was discarded. A 20-μl mixture of CCK-8 (No.CK-04, Xusheng Biotechnology Co., Shanghai, China) and 10X-diluted growth medium was added to each well; cells were cultured for another 1 h. The optical density (OD) of the cells was measured at a wavelength of 450 nm by a Multiskan FC microplate reader (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The growth inhibition rate (IC) was calculated using Equation 1.

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**Table 1. Primer sequences and length of fragments**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer sequence(5’–3’)</th>
<th>Length (base pairs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P53</td>
<td>Sense: GGTCTCCCTCAGCCCTTCTGTC</td>
<td>690</td>
</tr>
<tr>
<td></td>
<td>Antisense: GGCCTCATGAGCTTGTC</td>
<td></td>
</tr>
<tr>
<td>p53β</td>
<td>Outer Sense: GTCAGTCGCTAGGAGGAGGCAA</td>
<td>1050</td>
</tr>
<tr>
<td></td>
<td>Antisense: GAGGACGACCTATTGCAAGGAGGTTC</td>
<td></td>
</tr>
<tr>
<td>Inner</td>
<td>Sense: GTGAGGAGGAGGACAGGCTAGAT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Antisense: TGGAAAGCTGCTGTGCTCTGA</td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td>Sense: GTGCGCGCCGCAGGCAACCA</td>
<td>539</td>
</tr>
<tr>
<td></td>
<td>Antisense: CTCCTTAATGTCACGCAGATTTC</td>
<td></td>
</tr>
</tbody>
</table>

**Table 2. Effect of 5-FU with different concentrations on inhibition rate of gastric cancer cells ( \( \bar{x} \pm s, n = 5 \))**

<table>
<thead>
<tr>
<th></th>
<th>MKN45</th>
<th>SGC-7901</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-FU (25 µg/mL)</td>
<td>18.204±3.950a</td>
<td>26.305±8.677a</td>
</tr>
<tr>
<td>5-FU (50 µg/mL)</td>
<td>31.354±5.576a</td>
<td>26.363±3.597a</td>
</tr>
<tr>
<td>5-FU (100 µg/mL)</td>
<td>52.969±9.960b</td>
<td>25.780±4.849b</td>
</tr>
<tr>
<td>F</td>
<td>31.682</td>
<td>0.014</td>
</tr>
<tr>
<td>P</td>
<td>0.001</td>
<td>0.954</td>
</tr>
</tbody>
</table>

24 h cocultured with 5-FU. \( P_{de} = 0.011 \) (P < 0.05), \( P_{se} = 0.000 \) (P < 0.01). In SGC-7901 cells, there are no significant differences between the different groups cocultured with different 5-FU.

**Table 3. Effect of 5-FU with different time on cell inhibition rate of gastric cancer cells ( \( \bar{x} \pm s, n = 5 \))**

<table>
<thead>
<tr>
<th></th>
<th>MKN45</th>
<th>SGC-7901</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 h</td>
<td>18.204±3.950d</td>
<td>26.305±8.677d</td>
</tr>
<tr>
<td>48 h</td>
<td>37.219±5.350e</td>
<td>27.428±8.136e</td>
</tr>
<tr>
<td>72 h</td>
<td>79.099±4.516f</td>
<td>26.209±4.171f</td>
</tr>
<tr>
<td>F</td>
<td>225.304</td>
<td>0.043</td>
</tr>
<tr>
<td>P</td>
<td>0.000</td>
<td>0.985</td>
</tr>
</tbody>
</table>

Concentration of 5-FU: 25 µg/mL. \( P_{de} < 0.001, P_{se} < 0.001, \) \( P < 0.001 \).

Greater understanding of p53-related regulatory networks may accelerate discovery of therapeutics for gastric cancer.

**Materials and methods**

**CCK-8 analysis**

MKN45 and SGC7901 cells in the exponential growth phase were digested, seeded in a 96-well plate (5 × 10⁴ cells/well), and cultured for 24 hrs. 20 µL of 5-FU (No. H31020593, Xudong Hai-pu Pharmaceutical Co., Ltd., Shanghai, China) in concentration of 25, 50, 100 μg/mL, or an equal volume of phosphate-buffered saline (PBS), was added to the cells, which were cultured for another 48 h. Next, the culture medium was discarded. A 20-μl mixture of CCK-8 (No.CK-04, Xusheng Biotechnology Co., Shanghai, China) and 10X-diluted growth medium was added to each well; cells were cultured for another 1 h. The optical density (OD) of the cells was measured at a wavelength of 450 nm by a Multiskan FC microplate reader (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The growth inhibition rate (IC) was calculated using Equation 1.

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As Equation 1 shows, the growth inhibition rate IC is 100% multiplied by the ratio of two differences: the difference between \( A_{\text{Exp}} \), absorbance in experimental group, and \( A_{\text{C}} \), absorbance in control group; and the difference between \( A_{\text{C}} \) and \( A_{\text{Emp}} \), absorbance in empty group.

**RT-PCR**

MKN45 and SGC-7901 cells in the exponential growth phase were digested, seeded in a 96 well plate (5 × 10⁴ cells/well), and then cultured for 24 h. 20 µL of 5-FU (No. H31020593, Xudong Hai-pu Pharmaceutical Co., Ltd., Shanghai, China) in concentration of 25, 50, 100 μg/mL, or an equal volume of phosphate-buffered saline (PBS), was added to the cells, which were cultured for another 48 h. mRNA extraction and cDNA synthesis were performed using the TRIzol extraction kit (No.14033089C, Sangon Biotech Co., Ltd., Shanghai, China) and reverse transcription PCR kit (No.901KA241, Sangon Biotech Co., Ltd., Shanghai, China), according to manufacturer’s instructions. PCR was performed to amplify p53 and p53β. **PCR primers** (Table 1): p53 primer forward, 5’-GCT CTC CTC CAC CGC TTC TGT C-3’ and reverse, 5’-GAC GCA CAC CTA TTC TGG CAA GCA AGG GTT C-3’; p53β outer primer forward, 5’-GTC ACT GCC ATG GAG GAG CCG CA-3’ and reverse, 5’-GAC GCA CAC CTA TTC TGG CAA GCA AGG GTT C-3’; p53β internal primer forward, 5’-ATG GAG GAG CCG CAG TCA GAT-3’ and reverse, 5’-TTT GAA AGC TGG TCT GAG CCG TAC-3’. 4674 Int J Clin Exp Pathol 2016;9(4):4673-4679
GGT CCT GA-3'; β-actin primer forward, 5'-GTG GGG CGC CCC AGG CAC CA-3' and reverse, 5'-CTC CTT AAT GTC ACG CAC GAT TTC-3'. The lengths of the amplified products were 690 bp for p53, 1050 bp for p53β, and 539 bp for β-actin.

PCR conditions: 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 with a Biospectrum AC Gel Imaging system (Alpha Innotech Corp., San Leandro, CA, USA).

Western blot

MKN45 cells treated by 20 µL of 5-FU in concentration of 25, 50, 100 µg/mL, or equal volume of phosphatebuffered saline, were scraped into a lysis buffer (50 mM Tris, pH 7.6, 150 mM NaCl, 1% Nonidet P-40, 10 mM sodium phosphate, 10 mM NaF, 1 mM sodium orthovanadate, 2 mM phenylmethylsulfonyl fluoride, 10 µg/mL aprotinin, 10 µg/mL leupeptin, and 10 µg/mL pepstatin) and incubated on ice for 30 min. After centrifuging the lysates at 12000 rpm for 10 min at 4°C, aliquots of the supernatant were electrophoresed on 10% SDS-polyacrylamide gels and then transferred to PVDF membranes. The membranes were incubated with primary antibody DO-1 (No. ab80645, Abcam Inc., MA, USA) or mouse anti-β-actin at 4°C overnight, and washed with Tris-Buffere Saline and Tween 20. After washing, the membranes were incubated with goat-anti-mouse IgG coupled to horseradish peroxidase, for 1 h. Protein abundances were measured using chemiluminescence.

Statistical analysis

The software package SPSS version 17.0 (International Business Machines, Armonk, NY USA) was used for statistical analyses. Significant differences between two experimental groups were determined using Fisher’s Least

Figure 1. Effects of 5-FU on p53 and p53β mRNA expression in MKN45 and SGC7901 cells. A and C. Result from agarose gel electrophoresis. a: blank control; b: 25 µg/mL 5-FU; c: 50 µg/mL 5-FU; d: 100 µg/mL 5-FU. B. Compari-son of relative mRNA expression (*P < 0.05, **P < 0.01). Pa = 0.035; Pb = 0.000; Pc = 0.000; Pd = 0.133; Pe = 0.012; Pf = 0.027. D. Comparison of relative mRNA expression. No significant difference of relative expression of target genes between each group.
Significant Difference (LSD-t) test, and one-way analyses of variance (ANOVA) was employed to test for significant differences between more than two groups. Pearson linear relevancy analysis was used to test for correlation between variances.

**Results**

**Effect of 5-FU on growth inhibition**

Table 2 shows the growth inhibition rate of gastric cancer cells. For MKN45 cells, growth inhibition differs significantly between groups with different 5-FU doses (F = 31.682, P < 0.01); such differences were not observed for SGC7901 cells (F = 0.014, P > 0.05). In addition, growth inhibition of MKN cells treated with 25 µg/mL 5-FU differs significantly between groups of measurements made at different time points (Table 3, F = 225.304, P < 0.01); such differences were not observed over time for SGC7901 cells (F = 0.043, P > 0.05).

**Effect of 5-FU on expression of p53 and p53β mRNA**

p53 and p53β mRNA expression differed significantly between groups exposed to different concentrations of 5-FU for MKN cells (F\_p53 = 187.992, F\_p53β = 23.020, P < 0.01; Figure 1A and 1B) but not for SGC7901 cells (F\_p53 = 1.912, P = 0.206; F\_p53β = 1.626, P = 0.259; Figure 1C and 1D).

**Effect of 5-FU on expression of p53 and p53β proteins**

Western blot data (Figure 2) indicate that p53 and p53β protein expression differ significantly between MKN45 groups exposed to different concentrations of 5-FU (F\_p53 = 80.646, F\_p53β = 213.419, P < 0.01).

**Discussion**

TP53 is among the most scrutinized of the tumor-suppressive genes, and it is relevant to malignancy in multiple tissues. Though mutations are common at many sites, mutations with pharmacological significance were not commonly reported for this gene [30-32]. As indicated by recent research, atypical expression levels of p53 isoforms is common for some malignant diseases, and this observation may indicate a productive avenue of pharmacological research [33-39]. Moreover, the preliminary data indicated that the expression profile of p53 isoforms, including p53β and Δ133p53α, differs between tissues of gastric carcinoma, gastritis, or precancerous lesions [29]. Particularly for patients with wild-type expression, p53 isoforms such as p53β and Δ133p53α are promising pharmacological targets.

5-FU induced growth inhibition of two gastric cancer cell lines was observed. These two cell lines had distinct p53 backgrounds. 5-FU inhibited growth of (wild-type p53) MKN45 cells in a dose- and time-dependent manner; such inhibition for (mutant p53) SGC7901 cells was not observed. For MKN45 but not for SGC7901 cells, p53 and p53β mRNA and protein expression levels differed significantly between groups exposed to different concentrations of 5-FU. This result indicates that the p53β isoform plays an important role in 5-FU-induced growth inhibition of wild-type p53 MKN45 cells but not mutant-p53 SGC7901 cells.
p53β in fluoracil-induced growth inhibition

In the last ten years, the profile of p53 isoforms has been shown to have meaningful changes during the process of carcinogenesis; some of these changes might be promising for pharmacological research. Our group has devoted much effort to uncovering the role of p53 isoforms in the growth inhibition of clinically applied gastric carcinoma therapeutics. Early work indicated that Δ133p53 is correlated with tumor-necrosis-factor-induced growth inhibition [40], but p53β is relevant to cisplatin-induced growth inhibition of MKN45 gastric cancer cells [28]. The present study indicates that p53β is also related to 5-FU-induced growth inhibition of MKN45 gastric cancer cells. In summary, p53β is a promising oncotarget for 5-FU-related therapies on gastric cancer cells with wild-type p53.

Acknowledgements

This study was supported by Shandong Provincial Award Foundation for Youth and Middle-aged Scientist (BS2010SW034).

Disclosure of conflict of interest

None.

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p53β in fluoracil-induced growth inhibition

