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Role of Δ133p53 in Tumor Necrosis Factor-induced survival of p53 functions in MKN45 gastric cancer cell line

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Abstract. – OBJECTIVE: To explore the role of Δ133p53 in the effect of recombinant mutant human Tumor Necrosis Factor (rmhTNF) on two gastric cancer cell lines.

MATERIALS AND METHODS: MKN45 (with Δ133p53 expression) or SGC7901 (without Δ133p53 expression) cells were treated with rmhTNF of different concentrations only or combined with fluorouracil (5-FU), and the growth inhibition rate was detected by a cell counting kit, and apoptosis by flow cytometry. The mRNA of Δ133p53, p53, Gadd45α, MDM2, PTEN and Bax was measured by reverse transcription PCR (RT-PCR) or Nested PCR (nPCR).

RESULTS: On Δ133p53-positive MKN-45 cells, the effect of rmhTNF was significant in growth inhibition test ($t = -9.558$, $p < 0.01$); also, the effect of 5-FU was improved by rmhTNF with remarkable time- and dose-effect ($F = 82.742$, $p < 0.01$; $F = 128.583$, $p < 0.01$). However, on Δ133p53-negative SGC-7901 cells, no growth inhibition was showed by rmhTNF only ($t = -0.121$, $p > 0.05$). In apoptosis test, the effect of rmhTNF was significant on MKN45 cells, and the effect of 5-FU was improved significantly by rmhTNF ($F = 123.931$, $p < 0.05$). In mRNA measurement, rmhTNF-induced up-regulation of p53 accompanied with down-regulation of Δ133p53, which correlated significantly to the change of p53 downstream molecules, including MDM2, PTEN, Gadd45α, and Bax.

CONCLUSIONS: The results in these experiments suggested that Δ133p53 play a pivotal role in rmhTNF-induced survival of p53 functions in Δ133p53-positive MKN-45 cells.

Key Words:
rmhTNF, 5-flurouracil, Gastric cancer cell lines, Δ133p53.

Abbreviations

rmh TNF = Recombinant mutant human Tumor Necrosis Factor; TP53 = tumor protein53; MDM2 = mouse double minute 2 homolog; PTEN = phosphatase and tensin homolog; Bax = bcl-2-like protein 4; gadd45α = growth arrest and DNA damage-45 alpha; RPMI 1640 = Roswell Park Memorial Institute 1640 medium; FITC = fluorescein isothiocyanate; murine leukemia virus, MuLV.

Introduction

p53 has been regarded as the guardian of the genome, and its inactivation contributes to tumors of various tissues[1]. The inactivation of p53 may be caused not only by mutations or deletions of tumor protein53 (TP53) gene, but also by the alternative splicing[2,3]. At least ten p53 isoforms were discovered, and some were reported to appear in different tissues, including various types of normal, precancerous, and malignant tissues[4-16]. These isoforms working together with wild-type p53 or other pathways involve in the process of tumorigenesis[17-25]. Though mutations of p53 were popular in gastric cancer, it was yet unclear about the relationship between these mutations and gastric cancerogenesis[26]. Recently, expression pattern of Δ133p53 and p53β was testified in various gastric tissues in our department: up-regulation of Δ133p53 and down-regulation of p53β accompanied with the process of chronic inflammation to tumorigenesis. The link among p53 isoforms, *Helicobacter pylori* (*Hp*)-related inflammation and gastric carcinoma was firstly reported by Wei et al[27], which suggested that Δ133p53 involve in the process of *Hp*-related chronic inflammation to gastric carcinoma. Therefore, Δ133p53, as one of the hopeful p53 isoform, might be a good target for diagnosis, therapy and prognosis of gastric carcinoma.

To testify the role of Δ133p53 in gastric carcinoma, two gastric cancer cell lines (MKN45 with

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Δ133p53 expression and SGC7901 without Δ133p53 expression) were interfered by rmhTNF – an inhibitor of inflammation and tumors – alone or combining with 5-FU. The expression of Δ133p53, p53 and four p53 downstream molecules (MDM2, PTEN, Bax and gadd45α) was analyzed to explore the possible mechanism.

Materials and Methods

Gastric Cancer Cell Lines

SGC-7901 gastric cancer cell line was stored in our laboratory. MKN45 gastric cancer cell line was offered by Dr. Wang Xin (State Key laboratory of Oncology, Xijing Hospital, Fourth Military Medical University, Xi'an, China). These cell lines had been passaged four times at the time of harvesting for RNA isolation. All human cell lines were cultured in remodified HyClone RPMI-1640 medium (NZD1133, Beijing Corporation, Thermo Fisher Scientific Inc., Waltham, MA, USA) with 5% HyClone fetal bovine serum (FBS, NVD0250, Beijing Corporation, Thermo Fisher Scientific Inc., USA) and maintained in exponential growth at 37°C and 5% CO_2.

Cell Growth Inhibition Measurement

MKN-45 and SGC-7901 cells were digested, harvested and planted in 96-well plate with the density of 5×10^4 cells/mL. Two doses of rmhTNF (50 or 500 IU/mL) alone or combined with 5-FU (25 μg/mL) were added to different wells, incubated for 24, 48, and 72 hours respectively, then the supernatant was replaced by fresh culture medium with cell counting kit (CCK-8, Lot number 105215, Yesen Biotechnology Scientific Inc., Shanghai, China) agent at the ratio of 10:1 and cells were incubated for another hour. The OD value at the wavelength of 450 nm was measured by ELISA microplate reader (Bio Tek Power Wave XS, Bio-Rad Laboratories Inc., Hercules, CA, USA). The growth inhibition rate was calculated and recorded.

Apoptosis Flow Cytometry

MKN-45 cells of each group were digested in exponential phase, collected and regulated to the concentration of 1×10^6 cells/mL, then 5 μL Annexin V-FITC and 5 μL PI were added to 100 μL cell suspension. The contrast groups were designed to add Annexin V-FITC or PI only. The apoptosis was measured by Flow Cytometry (BD, Franklin Lakes, NJ, USA).

RT-PCR and nPCR

A series of keys was used in total RNA isolation, cDNA synthesis, and PCR amplification. RNA was isolated from snap frozen tumor tissue. The concentration of total RNA was determined by spectrophotometer (EV0300, Thermo Electron Corporation, Waltham, MA, USA). M-MuLV first chain synthesis key (Sangon Biotech Shanghai Co., Ltd., Shanghai, China) was used in reverse transcription (RT) of RNA. A 20 μL reaction contained the following: 4 μL of 25 mM $MgCl_2$ solution; 2 μL of 10×PCR Buffer II; 1 μL of H_2O; premixed deoxyribonucleoside triphosphates: 2 μL of dGTP, 2 μL of dATP, 2 μL of dTTP, 2 μL of dCTP (10 mM each), 1 μL of RNA inhibitor (20 U/μL); 1 μL of random hexamers; and 1 μL of MuLV reverse transcriptase as a master mix. Approximately 2 μL of total RNA was added prior to the start of reaction. On the basis of a previous photometric measurement, the total RNA template concentration was below the reaction capacity of ≤1 μg of RNA per reaction. The following adapted time and temperature profiles for the RT were used: incubation for 10 min at 25°C, 30 min at 42°C for RT of RNA, 5 min at 95°C for denaturation, and 5 min at 5°C to cool down the reaction. Negative controls were added to ensure contamination-free consumables for the RT reaction in each series of cDNA reactions. Subsequently, the RT reaction samples underwent 1:4 dilution to obtain a final concentration of 10 ng/μL of cDNA. RT-PCR reaction was under the guidelines of the PCR Amplification Key (Biotechnology Engineering Inc., Dalian, China) in a final volume of 25 μl under the following conditions: 35 cycles at 94°C for 1 min, 58°C for 50 s, and 72°C for 1 min. For Δ133p53, nPCR was performed as follows: the outer primers were used in the first PCR reaction, 2 μl product by first amplification and inner primers was used in the second reaction, and the conditions of both reactions were just the same as mentioned above. Primers for the mRNA of Δ133p53, p53, Gadd45α, Bax, PTEN, MDM2 and β-actin were shown in Table I. The PCR product was run in 1% agarose electrophoresis under the level electrophoresis apparatus (Gulf Gene Group Company, USA). The PCR result was scanned and analyzed by the Biospectrum AC Gel Imaging System (Alpha Innotech Corporation, San Leandro, CA, USA).

Statistical Analysis

The experimental data was analyzed by SPSS16.0 Statistical Software Package (SPSS

Table I. Primers in this experiment.

Primers		Primer sequence (5′-3′)	Length (bps)
Δ133p531	Outer primer	F: CTGAGGTGTAGACGCCAACTCTCTCTAG R: TGTCAGTCTGAGTCAGGCCCTTCTGTC	750
	Inner primer	F: GCTAGTGGGTTGCAGGAGGTGCTTACGC R: CTCACGCCCACGGATCTGA	
p53		F: GGTCTCCTCCACCGCTTCTTGTC R: GGCCTCATCTTGGGCCTGTGT	690
Gadd45α		F: CGAAAGGATGGATAAGGTG R: GGATCAGGGTGAAGTGGA	197
PTEN		F: AGTTCCCTCAGCCGTTACCT R: GGATCAGAGTCAGTGGTGTCAG	436
Mdm2		F: CGCGGGAGTTCAGGGTAAAG R: AGCTGGAGACAAGTCAGGACTTAAC	237
Bax		F: ACCAAGAAGCTGAGCGAGTGTC R: ACAAAGATGGTCACGGTCTGCC R: GGATTCGGTGGTAGACTT	365
β-actin		F: GTGGGGCGCCCCAGGCACCA R: CTCCTTAATGTCACGCACGATTTC	539

Note: 1 amplified by nested PCR. F: forward primer; R: reverse primer.

Inc., Chicago, IL, USA). The difference among groups was testified by one-way analysis of variance, while *LSD-t* test was used to analyze the group-to-group difference. Pearson linear correlation analysis was performed to testify the relevance between Δ133p53 and p53 downstream genes. $p < 0.05$ was considered as the significance difference.

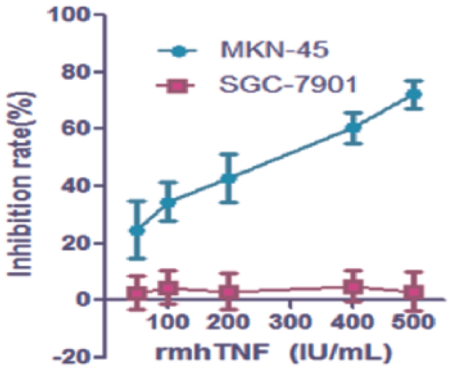

Figure 1. Inhibition effect of rmhTNF on MKN-45 and SGC-7901 cells. Data measured on 24 hours after rmhTNF treatment.

Results

Growth Inhibition of rmhTNF on Gastric Cell Lines

In Figure 1, the growth inhibition of rmhTNF at 24 hours after treatment was remarkable in Δ133p53-positive MKN cells with dose-dependent manner ($t = –9.558$, $p < 0.01$), but insignificant in Δ133p53-negative SGC-7901 cells ($t = –0.121$, $p > 0.05$). The effect of 5-FU was improved by rmhTNF with remarkable time- and dose-effect manner in MKN cells ($F= 82.742$, $p < 0.01$; $F= 128.583$, $p < 0.01$; Figure 2A); though no growth inhibition was showed by rmhTNF only in SGC-7901 cells, rmhTNF was able to enhance 5-FU-induced growth inhibition in both concentration with time- but not dose-dependent manner (Figure 2B).

Pro-apoptotic Effect of rmhTNF on MKN-45 cells

In twenty-four-hour treatment, the result of Flow Cytometry was showed that the apoptotic percentages were 7.15±0.94%, 10.11±0.64%, and 14.57±1.37%, respectively, in 50 IU/mL rmhTNF, 50 IU/mL rmhTNF + 25 μg/mL 5-FU, and 50 IU/mL rmhTNF + 25 μg/mL 5-FU group, and the difference was significance (Figure 3).

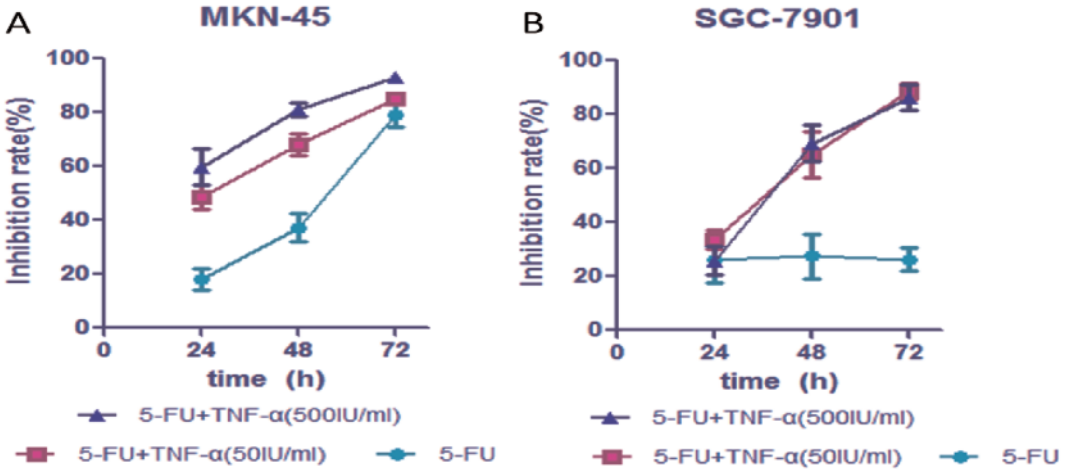

Figure 2. Combining inhibition effect of rmhTNF and 5-Fu on MKN-45 and SGC-7901 cells. Note: concentration of 5-FU: 25 μg/mL.

Effects of rmhTNF on Δ133p53, p53 and p53 Downstream Molecules in MKN Cells

In twenty-four-hour rmhTNF intervention, the result of PCR indicated that Δ133p53 mRNA was lowered, but p53 mRNA increased remarkably. Accompanying with that, the mRNA of p53 downstream molecules changed; in detail, mRNA of MDM2 decreased, while that of PTEN, BAX and Gadd45α declined. All these changes were significant with dose-dependent manner (Figure 4). The result in figure 5 showed that the expression of Δ133p53 was correlated negatively to that of Gadd45α, PTEN and BAX ($r_B = -0.894$, $p_B < 0.01$; $r_C = -0.872$, $p_C < 0.01$; $r_D = -0.971$, $p_D < 0.01$), but did positively to that of MDM2 ($r = 0.924$, $p < 0.01$).

Discussion

Gastric carcinogenesis was accepted as a multiple-step process with accumulating genetic alterations, in which the loss of p53 function plays a pivotal role. The mutation and deletion of TP53 gene in gastric carcinoma and cell lines were reported in details[1,20]; however, rare research focused on p53 isoforms in gastric diseases. In recent years, meaningful discoveries had been coming out and indicated that p53 isoforms involve in the process of gastric tumorigenesis.

Δ133p53, one of the isolated prognostic indicators in invasive ovarian carcinoma[8-10], was also detected and worked as a negative inhibitor against wild-type p53 in breast cancer[5-7]. Previous unpublished study in our group showed that the positive rates of Δ133p53 mRNA were 75% (15/20) in gastric adenocarcinoma, 50% (15/30) in atrophic gastritis, 25% (5/20) in superficial gastritis and 20% (3/15) in para-cancerous tissue; the difference was significant. Expression status of Δ133p53 was also testified in two gastric cancer cell lines, which was positive in MKN45 cells, but negative in SGC7901. Moreover, it was proved by

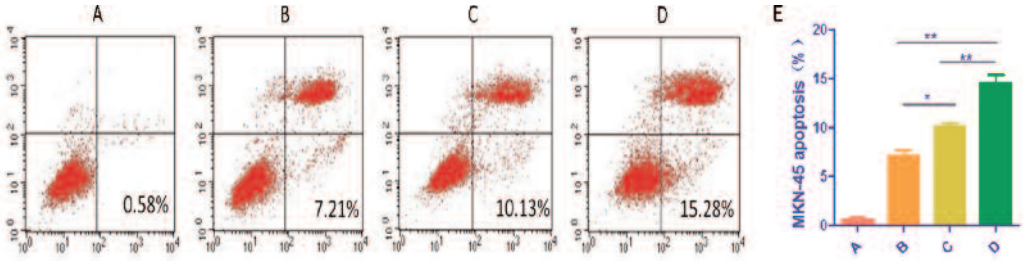

Figure 3. Combining pro-apoptotic effect of rmhTNF and 5-FU on MKN-45 cells. ***A,*** Blank control; ***B,*** rmhTNF (50 IU/mL); ***C,*** rmhTNF (50 IU/mL) + 5-FU (25 μg/mL); ***D,*** rmhTNF (500 IU/mL) +5-FU (25 μg/mL); ***E,*** histogram of apoptosis measured by Flow Cytometry. mean α ± average, n = 3. *$p < 0.05$, **$p < 0.01$.

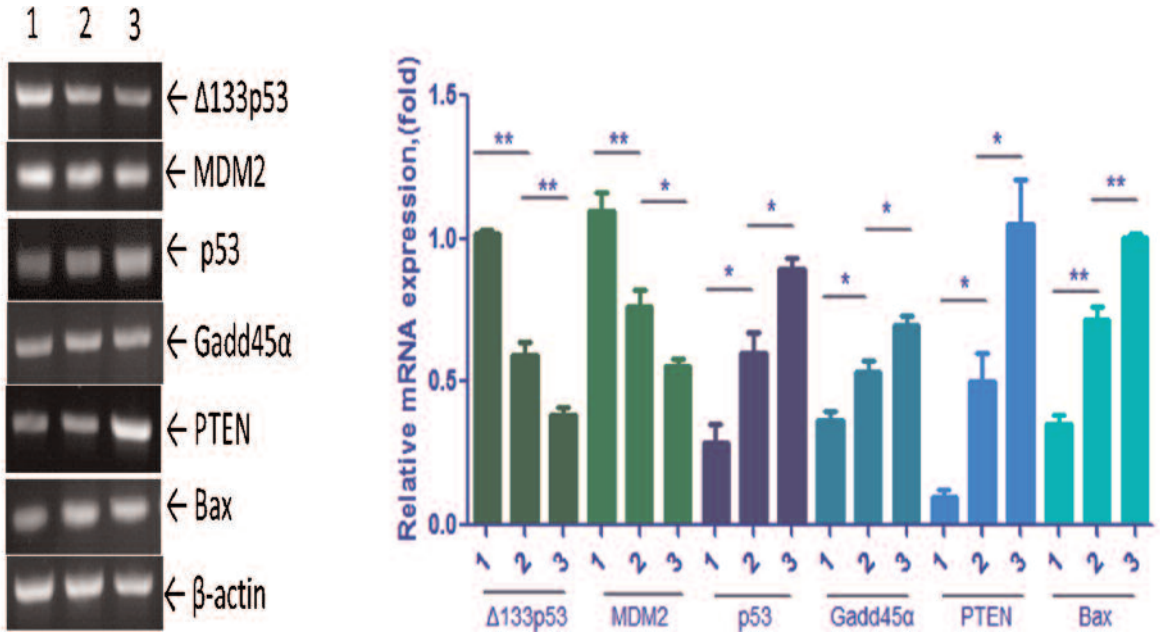

Figure 4. Effect of rmhTNF on Δ133p53, p53 and p53 downstream molecules in MKN cells. Samples collected 24 hours after rmhTNF treatment. (1) Blank control; (2) rmhTNF (50 IU/mL), (3) rmhTNF (500 IU/mL). ± s, n = 3. *$p < 0.05$, **$p < 0.01$.

Wei et al[27] that Δ133p53 acted as an anti-apoptotic inducer in the process of *Hp*-related gastric inflammation to carcinoma. However, more work was required to decipher the hiding story before Δ133p53 becoming a target of diagnostics, therapeutics and prognostics in gastric carcinoma.

In these experiments, Δ133p53 positive or negative gastric cancer line cells were interfered with rmhTNF alone or combining with 5-FU. rmhTNF were able to induce the growth inhibition and enhance the inhibition effect of 5-FU in Δ133p53 positive MKN45 cells; while the same effect was not shown in Δ133p53 negative SGC7901 cells: rmhTNF was unable to induce the growth inhibition of SGC7901 cells alone, but enhance the inhibition effect of 5-FU. In apoptotic analysis, it was showed that rmhTNF was able to induce the apoptotic reaction alone, and enhance the apoptotic effect of 5-FU as well.

In rmhTNF-treated MKN45 cells, the expression of p53 and its function of growth inhibition and apoptosis revived. The apoptotic pattern was shown by p53 downstream molecules, including MDM2, PTEN, BAX and Gadd45α, which varied relatively with Δ133p53. Previously, Δ133p53 was proved to work as an anti-apoptotic factor in various tumors[9,28]. The result in this experiment indicated that Δ133p53 might reverse p53 functions of growth inhibition and apoptosis in MKN45 cells. The function of p53 is finely controlled by PTEN-MDM2 loop[29,30], and Δ133p53 isoform clearly involved in this regulatory loop: Δ133p53 expression correlated positively to MDM2, but negatively to PTEN. Interestingly, this result suggested that rmhTNF-induced survival of p53 functions was caused by the blockade of Δ133p53 (Figure 6).

Conclusions

To be summed up, the results in these experiments suggested Δ133p53 a pivotal point in rmhTNF-induced survival of p53 functions in Δ133p53-positive MKN-45 cells. Historically, no ideal target was clinically applied in the diagnostics and therapeutics of gastric carcinoma, but this original work suggested Δ133p53 a good alternative and worth further exploration. Surely, tremendous work was required to build up a convenient diagnostic method, screen patients, and design new drugs and regimens as well.

Acknowledgements

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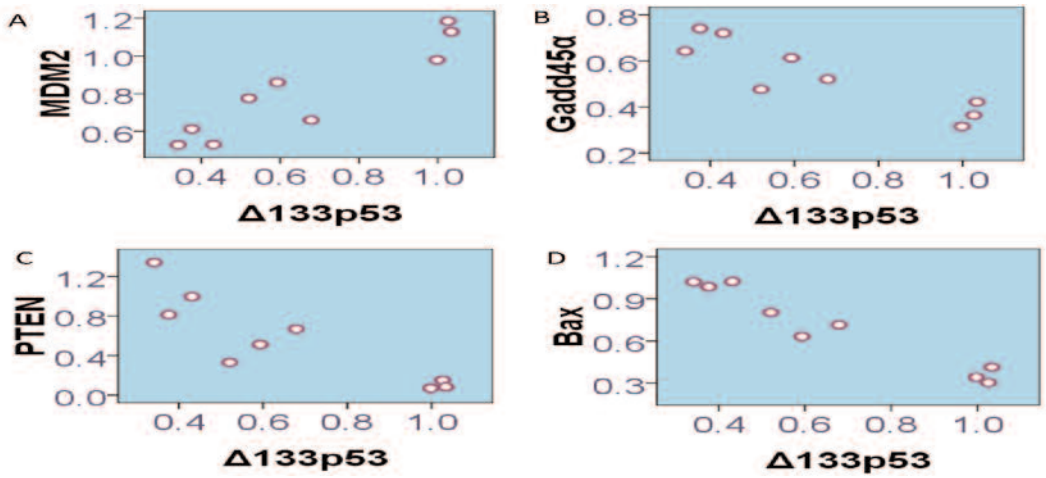

Figure 5. Relevancy of Δ133p53 mRNA with that of p53 and its downstream molecules in MKN cells treated by rmhTNF. Data measured on 24 hours after rmhTNF treatment. Pearson linear relevancy analysis: $r_A = 0.924$, $p_A < 0.01$; $r_B = -0.894$, $p_B < 0.01$; $r_C = -0.872$, $p_C < 0.01$; $r_D = -0.971$, $p_D < 0.01$.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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