Effect of p53β on human gastric cancer cells treated with recombinant mutated human TNF and cisplatin

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Abstract. The present study aimed to investigate the role of tumour protein 53 isoform b (p53β) on human gastric cancer (GC) cell lines treated with recombinant mutated human tumour necrosis factor (rmhTNF) and cisplatin. The Cell Counting Kit-8 assay was used to assess growth in the GC cell lines MKN45 and SGC7901, following treatment with rmhTNF in the presence or absence of cisplatin. Levels of p53β and bcl-2 apoptosis regulator (bcl-2) mRNA were assessed using reverse transcription-polymerase chain reaction. The results demonstrated that growth was significantly inhibited by either cisplatin or rmhTNF treatments alone in MKN45 cells, and combination treatment with cisplatin and rmhTNF had a synergistic effect on growth inhibition of MKN45 cells. Notably, these observations were not evident in SGC7901 cells, where a mutant form of p53 is present. Treatment of MKN45 cells with rmhTNF did not affect bcl-2 or p53β mRNA expression levels. However, treatment of MKN45 cells with cisplatin induced upregulation of p53β and downregulation of bcl-2 mRNA expression levels, and these effects were enhanced by combination treatment with rmhTNF. Pearson correlation analysis revealed a negative correlation between the expression of p53β and bcl-2 mRNA, and a negative correlation between bcl-2 mRNA expression and the inhibition of cell growth. In conclusion, the inhibitory effect of cisplatin on the growth of MKN45 GC cells was enhanced by rmhTNF via unknown mechanisms that involved p53β, indicating that p53β may be an appropriate therapeutic target for the treatment of GC.

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Introduction

Gastric cancer (GC) is associated with high mortality worldwide, but its occurrence is particularly high in China and other Asian countries (1,2). Many patients are diagnosed in the late stages of GC, and thus cannot undergo surgery and are subjected to chemotherapy instead. However, the efficacy of 5-fluorouracil- and cisplatin-based regimens during the late stages of GC is limited (3,4). Currently, the prognosis for GC patients is very poor; thus, novel therapeutic targets and alternative treatment strategies are urgently required.

Tumour protein 53 (p53) was first described in 1979 (5), as a protein that bound to the simian virus large T antigen. Inactivation of p53 is evident in more than half of all human cancers (6), and it is caused by mutations or deletions in the *TP53* gene itself (7,8) or by changes in alternative splicing (9-11). Different isoforms of p53 are expressed in different tissues, including various types of normal, precancerous and malignant tissues (12-14). Previous results have linked several factors, including the presence of *Helicobacter pylori* infection, chronic gastritis, and p53 isoforms, with the occurrence of GC (15,16).

Tumour necrosis factor-α (TNF-α) is a potential anticancer agent, effective against various malignant tumours. The therapeutic benefit from TNF-α can be ascribed to its anti-proliferative effects, and its ability to increase the penetration of chemotherapeutic agents into tumour tissues (17). An increasing body of evidence suggests that recombinant mutated human TNF (rmhTNF) acts synergistically with traditional chemotherapeutic drugs to exert enhanced antitumor effects (18). Recently, rmhTNF has been administered to patients with non-small cell lung cancer, non-Hodgkin lymphoma, or malignant pleura and ascites, when other therapies failed (19). In the present study, the effects of combination treatment of the commonly used cytotoxic agent cisplatin and the novel agent rmhTNF were examined on two GC cell lines expressing either wild type or mutated p53 isoform β (p53β).

Materials and methods

Cells. The SGC-7901 (expressing mutant p53) and the MKN45 (expressing wild-type p53) GC cell lines were obtained from the Central Laboratory of Weifang Medical College (Weifang, China). Cell lines were passaged four times prior to harvesting

for RNA isolation. All human cell lines were cultured in RPMI-1640 medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 5% foetal bovine serum (FBS; Thermo Fisher Scientific, Inc.) at 37˚C/5% CO_2.

Inhibition of cell growth. MKN-45 and SGC-7901 cells were seeded in 96-well plates at a density of $5x10^4$ cells/ml and incubated at 37˚C/5% CO_2 for 24 h, in order to achieve the exponential phase of cell growth. The supernatant was then discarded, and fresh culture medium was added to the wells. Treatments were performed by adding to the culture medium the following: i) 4 μg/ml cisplatin (Deyao Pharmaceutical Co., Ltd., Dezhou, China); ii) 50 IU/ml rmhTNF (Shanghai Weike Biopharmaceutical Co., Ltd., Shanghai, China); iii) 100 IU/ml rmhTNF; iv) 200 IU/ml rmhTNF; v) 4 μg/ml cisplatin and 50 IU/ml rmhTNF; and vi) 4 μg/ml cisplatin and 100 IU/ml rmhTNF. Control wells contained medium alone (untreated cells). Following culture for 24 h at 37˚C/5% CO_2, the medium was replaced with 110 μl10% Cell Counting Kit-8 medium (Yesen Biotechnology Scientific Inc., Shanghai, China), and cells were incubated for another 2 h at 37˚C/5% CO_2. The absorbance at 450 nm (A_{450}) was determined using a Bio-Tek PowerWave XS microplate reader (Bio-Rad Laboratories Inc., Hercules, CA, USA). The mean absorbance values were determined from four wells for each treatment group, and the growth inhibition rate was calculated using the following formula:

$$IC = \frac{(A_{Exp}-A_C)}{(A_C-A_{Emp})} \times 100$$

IC denotes the growth inhibition rate (%), A_{Exp} the mean absorbance for the treatment group, A_C the mean absorbance for the control group, and A_{Emp} the mean absorbance for wells without cells or reagents added.

Reverse transcription-polymerase chain reaction (RT-PCR). $4x10^4$ MKN45 and SGC7901 cells in the exponential phase of growth were seeded in per well in 6-well plates and cultured for 24 h in medium supplemented with 4 μg/ml cisplatin, 50 U/ml rmhTNF, 100 U/ml rmhTNF, 200 U/ml rmhTNF, 4 μg/ml cisplatin and 50 U/ml rmhTNF, or 4 μg/ml cisplatin and 100 U/ml rmhTNF. As a control, normal growth medium without supplements was used (untreated cells). TRIzol® RNA isolation, M-MuLV first-chain synthesis and PCR amplification kits were all bought from Shanghai Sangon Biotech Corporation Ltd. (Shanghai, China). The concentration of total RNA in each sample was determined with a 2000c UV-Vis spectrophotometer (Thermo Fisher Scientific, Inc.). Each 20 μl reverse transcription reaction contained 4 μl 25 mM $MgCl_2$, 2 μl 10xPCR Buffer II, 1 μl double-distilled water, 8 μl premixed deoxyribonucleoside triphosphates, 1 μl RNA inhibitor solution (20 U/μl), 1 μl random hexamers, 1 μl MuLV reverse transcriptase, and 2 μl total RNA (≤1 μg), as per the kit's protocols (Shanghai Sangon Biotech Corporation Ltd.). Total RNA was added just prior to the start of the reaction. Reactions were incubated at 25˚C for 10 min, then at 42˚C for 30 min, followed by 95˚C for 5 min and then cooled at 5˚C for 5 min. Samples were then diluted 1:4 to obtain a final concentration of 10 ng/μl cDNA. The PCR step was carried out on a PE-5700 MyCycler (Applied Biosystems; Thermo Fisher Scientific, Inc.) and involved 35 amplification cycles at 94˚C for 1 min, 58˚C for 50 sec and 72˚C for 1 min. Specific oligonucleotide primers were used as listed in Table I. Amplicons were subjected to 1% (w/v) agarose gel electrophoresis, and gels were analysed with a BioSpectrum AC Gel Imaging System (Alpha Innotech Corp., San Leandro, CA, USA). The relative expression levels of target genes were calculated using the following formula:

$$\text{Relative gene expression} = \frac{\text{Absorbance}_{\text{integral area of target gene}}}{\text{Absorbance}_{\text{integral area of β-actin}}}$$

Statistical analysis. SPSS 20.0 (IBM SPSS, Armonk, NY, USA) was used in order to analyse experimental data, with values presented as the mean ± standard deviation. The difference among groups was assessed by one-way analysis of variance. Comparisons between two groups were conducted using the Student-Newman-Keuls method or Student's t-test. The relationship between two variables was analysed using Pearson's correlation coefficient. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effects of cisplatin and rmhTNF on GC cell growth. Following treatment with rmhTNF for 24 h, a significant inhibition of cell growth was observed in MKN45 cells in a dose-dependent manner ($P<0.05$; Fig. 1). However, no significant effect was observed on the cell growth of the mutant p53-expressing SGC7901 cells with rmhTNF treatment (Fig. 1). Combination treatment of cisplatin and rmhTNF acted synergistically to further enhance the inhibitory effect on the growth of MKN45 cells, compared with either cisplatin or rmhTNF treatments alone ($P<0.01$; Fig. 1). A synergistic effect of cisplatin and rmhTNF was not observed in the mutant p53-expressing SGC7901 cells (Fig. 1). The combination treatment of cisplatin was performed with two different doses of rmhTNF (50 and 100 IU/ml) and the growth inhibition observed was dose-dependent in MKN45 cells ($P<0.01$; Fig. 1).

Expression levels of p53β and bcl-2 apoptosis regulator (bcl-2) mRNA in GC cells. The mRNA expression of bcl-2 and p53β in MKN45 cells (Fig. 2A) and bcl-2 in SGC7901 cells (Fig. 2B) were determined. In MKN45 cells, p53β mRNA expression levels were significantly increased by cisplatin alone compared with untreated cells ($P<0.01$; Figs. 2A and 3). Treatment of MKN45 cells with rmhTNF alone had no effect on p53β mRNA expression compared with untreated cells (Figs. 2A and 3). However, when cisplatin was used in combination with rmhTNF, p53β mRNA expression levels were further increased compared with cells treated with cisplatin alone ($P<0.01$; Figs. 2A and 3), suggesting again that rmhTNF and cisplatin act synergistically in MKN45 GC cells.

In MKN45 cells, bcl-2 mRNA expression levels were significantly downregulated by cisplatin alone, or by combined cisplatin and rmhTNF treatment, compared with untreated cells ($P<0.01$; Figs. 2A and 4). However, no significant effect on bcl-2 mRNA expression levels was detected with rmhTNF treatment alone in MKN45 cells, compared with untreated cells ($P>0.05$; Figs. 2A and 4). In the mutant p53-expressing SGC7901 cells, bcl-2 mRNA levels were not significantly

Table I. Primers used for polymerase chain reaction.

Gene	Primer sequence (5'-3')	Product (bp)
β-actin	F: GTGGGGCGCCCCAGGCACCA	539
	R: CTCCTTAATGTCACGCACGATTTC	
bcl-2	F: CGCGACTCCTGATTCATT	316
	R: TGCATTCTTGGACGAGGG	
p53β		1,050
Outer primers	F: GTCACTGCCATGGAGGAGCCGCA	
	R: GACGCACACCTATTGCAAGCAAG GGTTC	
Inner primers	F: ATGGAGGAGCCGCAGTCAGAT	
	R: TTGAAAGCTGGTCTGGTCCTGA	

bcl-2, bcl-2 apoptosis regulator; p53β, tumour protein 53 isoform β.

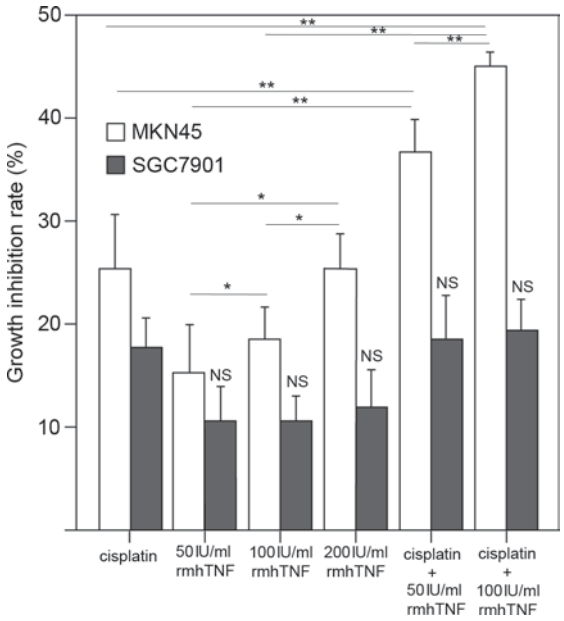

Figure 1. Effect of rmhTNF and/or cisplatin on MKN45 and SGC7901 cell growth. MKN45 and SGC7901 cells were treated with 4 μg/ml cisplatin and or 50, 100 and 200 IU/ml rmhTNF for 24 h. Growth was measured by Cell Counting Kit-8 and growth inhibition rate is reported as a mean ratio relative to untreated cells ± standard deviation (n=3). *P<0.05 and **P<0.01, comparisons indicated by lines. rmhTNF, recombinant mutated human tumour necrosis factor; NS, not significant.

altered in either of the various treatment groups (P>0.05; Figs. 2B and 4).

Correlation between p53β and bcl-2 mRNA expression levels in MKN45 cells. Correlation analysis was performed to assess whether bcl-2 and p53β mRNA expression are associated with each other and with the growth inhibition phenotype. The results indicated that bcl-2 mRNA expression in MKN45 cells was negatively correlated with mRNA expression of p53β (r=-0.897; P<0.01; Fig. 5), and negatively correlated with the cell growth inhibition rate (r=-0.906; P<0.01; Fig. 6).

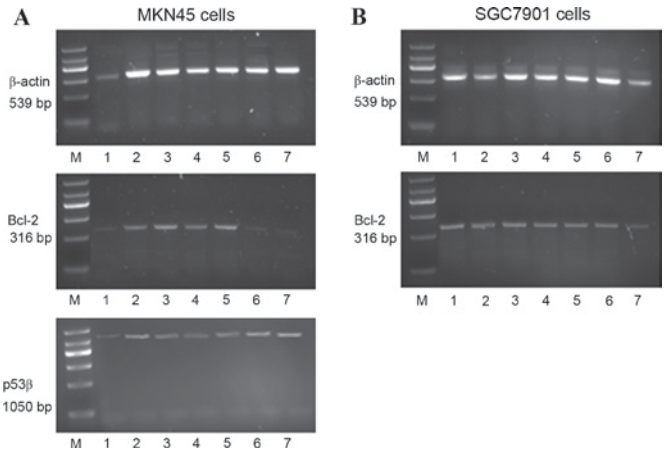

Figure 2. Analysis of β-actin, bcl-2 and p53β mRNA expression in (A) MKN45 and (B) SGC7901 cells. Cells were either untreated (control) or treated with cisplatin and/or rmhTNF for 24 h. Reverse transcription was performed on extracted RNA with resulting cDNA amplified by polymerase chain reaction. The resulting amplicons were visualised by agarose gel electrophoresis. Lane M, DNA marker; lane 1, control; lane 2, cisplatin 4 μg/ml; lane 3, rmhTNF 50 IU/ml; lane 4, rmhTNF 100 IU/ml; lane 5, rmhTNF 200 IU/ml; lane 6, rmhTNF 50 IU/ml plus cisplatin 4 μg/ml; lane 7, rmhTNF 100 IU/ml plus cisplatin 4 μg/ml; Bcl-2, bcl-2 apoptosis regulator; p53β, tumour protein 53 isoform β; rmhTNF, recombinant mutated human tumour necrosis factor.

Discussion

Novel therapeutic targets and alternative therapies are urgently required to improve the prognosis of invasive GC. For more effective therapies, a prerequisite may be that the target of choice is commonly involved in GC. The p53 protein has suppressive effects in tumours, and p53 gene alterations are widespread in cancer (7,8,20-22). Although mutated forms of p53 are common in GC tissues, their pharmacological significance remains unclear. Previous results indicated that p53 isoforms and infection with *H. pylori* have a strong association with the development of GC (13). In a previous small-scale study (23), p53β downregulation and Δ133p53 upregulation was associated with superficial gastritis, atrophic gastritis, paracancerous areas, and eventually invasive GC. These findings indicated that p53 isoforms are involved in gastric carcinogenesis and could be potential targets for GC therapies.

The different p53 isoforms exhibit varying levels of prognostic significance in various cancer types, including breast (24,25) and ovarian (26-28) cancer. It remains unknown whether any of these isoforms could be used as actual targets for therapeutic development. The present study was designed to investigate the role of p53β in two GC cell lines with differing p53 status, that were treated with cisplatin and/or rmhTNF. The MKN45 GC cell line expresses wild-type p53 (29), while the SGC7901 cell line expresses a mutated form of p53 (GAG→GCG in exon six, corresponding to Glu→Ala in codon 204; (30). The growth of MKN45 cells was inhibited by rmhTNF alone, while the growth of SGC7901 cells was unaffected by rmhTNF. The inhibitory effect of cisplatin on the growth of MKN45 cells was enhanced by rmhTNF; however, this was not observed in SGC7901 cells. RT-PCR analysis revealed that cisplatin alone, but not rmhTNF, resulted in significant upregulation of p53β and downregulation of bcl-2 mRNA expression in MKN45 cells. The effect of cisplatin on

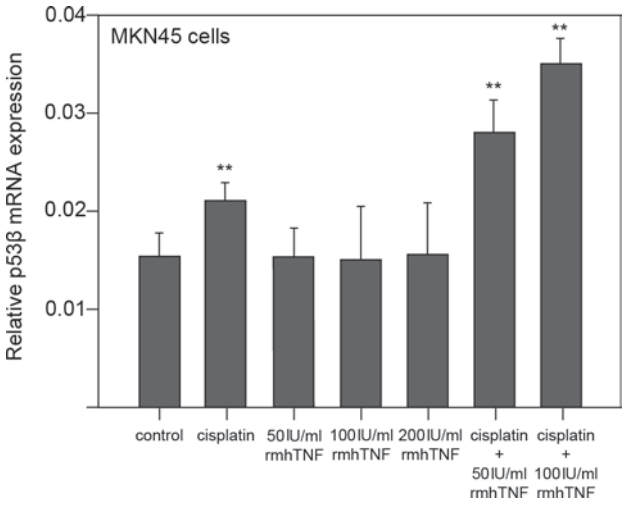

Figure 3. p53β mRNA expression in MKN45 cells. Quantification of the p53β amplicon signals demonstrated in Fig. 2A relative to β-actin. Results are plotted as mean ± standard deviation and each experiment was repeated three times. **P<0.01 vs. control. p53β, tumour protein 53 isoform β; rmhTNF, recombinant mutated human tumour necrosis factor.

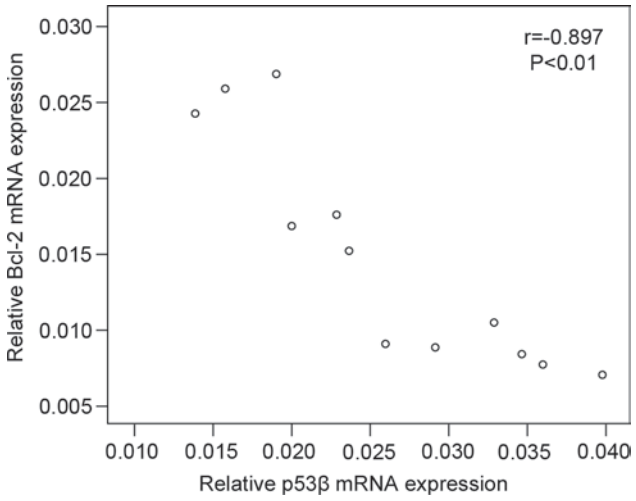

Figure 5. Correlation analysis of p53β and bcl-2 mRNA expression in MKN45 cells. Correlation analysis was performed for the data presented in Figs. 3 and 4 of p53β and bcl-2 mRNA expression. The Pearson's correlation coefficient was r=-0.897, P<0.01. Bcl-2, bcl-2 apoptosis regulator; p53b, tumour protein 53 isoform β.

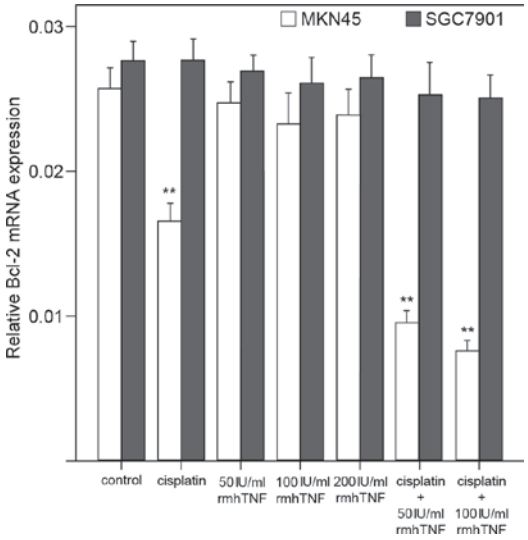

Figure 4. Bcl-2 mRNA expression in MKN45 and SGC7901 cells. Quantification of the bcl-2 amplicon signals demonstrated in Fig. 2A relative to β-actin. Results are plotted as mean ± standard deviation and each experiment was repeated three times. **P<0.01 vs. control. Bcl-2, bcl-2 apoptosis regulator; rmhTNF, recombinant mutated human tumour necrosis factor.

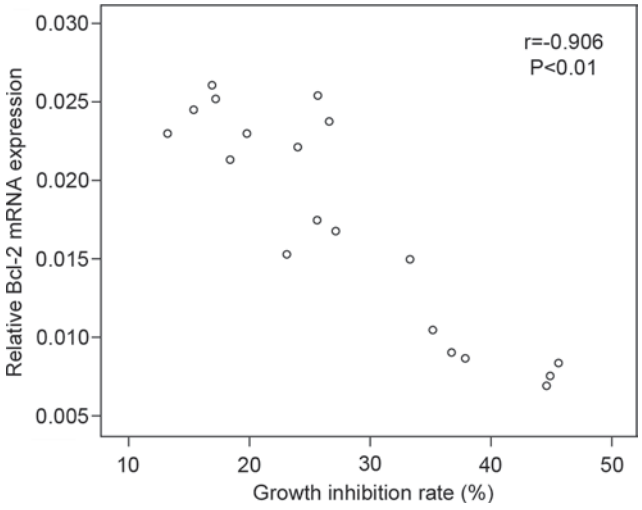

Figure 6. Correlation analysis of inhibition rate and bcl-2 mRNA expression in MKN45 cells. Correlation analysis was performed on the data presented in Figs. 1 and 4 on growth rate inhibition and bcl-2 mRNA expression. The Pearson's correlation coefficient was r=-0.906, P<0.01. Bcl-2, bcl-2 apoptosis regulator.

p53β and bcl-2 mRNA expression was significantly enhanced by rmhTNF.

Taken together, the present study indicated that p53β serves a role in the inhibitory effects exerted by cisplatin on MKN45 GC cells, suggesting that p53β is a key target of cisplatin. Members of the bcl-2 family, which are downstream of p53 signalling, exhibit pro- or anti-apoptotic activity. The final action of bcl-2 family members is determined by the ratio of these mutually antagonistic members (31,32). It is currently unclear how p53β regulates expression of bcl-2 and other downstream targets of p53. One possibility is that tetramers with wild-type p53 are formed (33,34). In wild-type p53-negative H1299 cells, p53β is important in sensitizing these cells to chemotherapy (35). The precise function of p53β requires further clarification; however, the therapeutic significance of this p53 isoform is evident by its negative correlation to breast tumour size, and its positive association with disease-free survival periods (36,37). In the present study, bcl-2 expression was negatively correlated with p53β expression in MKN45 cells, which contain wild-type p53. This was not observed in SGC7901 cells, which contain a mutated form of p53, thereby indicating a p53-dependent mechanism for cisplatin.

The present study indicated that rmhTNF is not able to exert its effects on the p53β-bcl-2 pathway directly, but only when in combination with cisplatin. rmhTNF may function as an enhancer, improving the effects of cisplatin on the inhibition of cellular growth, upregulation of p53β, and downregulation of bcl-2, through mechanisms that are yet

to be elucidated. Previous findings revealed that Δ133p53 is involved in the progression from chronic gastric inflammation to carcinoma (15,38-40). An association between Δ133p53, rmhTNF, and treatment of gastric carcinoma is evident, but further investigations are required to clarify this. In addition, further work is required to screen for targets of rmhTNF and to determine whether Δ133p53 is a target of rmhTNF. Determining the genotype or phenotype that is most closely associated with rmhTNF may improve drug regimens against GC.

In summary, the present study indicated that p53β is involved in the cisplatin-mediated growth inhibition of MKN45 GC cells. The effects of cisplatin on these cells were enhanced when combined with rmhTNF. The mechanism of action for the synergistic effect of cisplatin and rmhTNF remains unknown; however, the present study indicated that p53β may be a critical target in the pharmacology of GC.

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