Δ133p53表达状态对rmhTNF效应的影响及机制研究

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［摘要］ **背景与目的：**p53异构体在胃癌发生中的作用报道较少。该研究旨在探讨p53异构体Δ133p53在重组改构人肿瘤坏死因子(recombinant mutant human tumor necrosis factor，rmhTNF)干预胃癌细胞系生物学效应中的作用，为胃癌诊断和治疗提供新的依据。**方法：**采用细胞增殖/毒性检测试剂盒(CCK-8)和流式细胞术，检测不同浓度的rmhTNF单独或联合氟尿嘧啶(5-FU)应用于MKN-45(表达Δ133p53)和SGC-7901(不表达Δ133p53)细胞，观察细胞抑制率和细胞凋亡情况。通过巢式逆转录多聚酶链反应(nested reverse transcriptase-polymerase chain reaction，nRT-PCR)和实时荧光定量多聚酶链反应(real-time polymerase chain reaction，RT-PCR)检测Δ133p53、Gadd45α和CyclinB1 mRNA的表达变化。**结果：**rmhTNF单独作用于Δ133p53表达阳性的MKN-45细胞有抑制作用，浓度为50、500 IU/mL的rmhTNF作用24 h后，细胞抑制率分别为24.82%、72.33%(t=-9.558，P<0.01)，并可提高5-FU的抑制率，且具有显著的量效和时效关系，5-FU(25 μg/mL)、rmhTNF(50 IU/mL)+5-FU(25 μg/mL)、rmhTNF(500 IU/mL)+5-FU(25 μg/mL)作用于MKN-45细胞24 h后，抑制率分别为18.20%、48.66%、59.83%(F=82.742，P<0.01)；rmhTNF(50 IU/mL)+5-FU(25 μg/mL)作用于MKN-45细胞24、48、72 h后，抑制率分别为48.66%、68.20%、85.23%(F=128.583，P<0.01)。而对于Δ133p53表达阴性的SGC-7901细胞，浓度为50、500 IU/mL的rmhTNF单独抑制率为2.74%、3.25%，抑制作用不明显(t=-0.121，P>0.05)。流式细胞术显示，rmhTNF不仅单独可引起MKN-45细胞凋亡，而且可显著增强5-FU促细胞凋亡作用，rmhTNF(50 IU/mL)、rmhTNF(50 IU/mL)+5-FU(25 μg/mL)、rmhTNF(500 IU/mL)+5-FU(25 μg/mL)作用于MKN-45细胞24 h后，凋亡率分别为7.21%、10.13%、15.28%(F=123.931，P<0.05)。在MKN-45中，rmhTNF单独或联合5-FU可下调*Δ133p53*和*CyclinB1*基因，上调*Gadd45α*基因表达水平。nRT-PCR检测对照组及实验组*Δ133p53*基因相对表达量分别为0.886、0.499、0.330、0.161(F=240.927，P<0.01)；Real-time PCR检测实验组*Gadd45α*基因相对表达量分别为1.227、1.694、3.394，*Cyclin B1*基因相对表达量分别为1.221、0.722、0.316。*Δ133p53*表达水平与*CyclinB1*呈正相关(r=0.977，P<0.01)，与*Gadd45α*呈负相关(r=-0.950，P<0.01)。**结论：**rmhTNF对表达Δ133p53胃癌细胞展现出显著的抑制效应，并能增加传统化疗药物5-FU的疗效，其中部分效应可能是通过调节*p53*下游分子*CyclinB1*和*Gadd45α*表达实现的，提示*Δ133p53*可能是rmhTNF治疗胃癌生物学效应的关键靶点。

［关键词］ 重组改构人肿瘤坏死因子；5-FU；胃癌细胞系；Δ133p53

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Effect and mechanism of rmhTNF on gastric cancer cell lines with different Δ133p53 status JIANG Qiqi[1], ZHANG Hongmei[2], GUO Ai[1], ZHANG Na[1], GAO Zhixing[2], JI Wansheng[2] (1.Weifang Medical College, Weifang Shandong 261053, China; 2. Department of Gastroenterology, Affiliated Hospital of Weifang Medical College, Weifang Shandong 261031, China)

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［**Abstract**］ **Background and purpose:** Little about the function of p53 isoforms in gastric cancer was reported. This study was designed to explore the role of Δ133p53 in the effect of recombinant mutant human tumor necrosis factor (rmhTNF) on gastric cancer cells, and provide a new basis for the diagnostics and therapeutics of gastric carcinoma. **Methods:** MKN45 (with Δ133p53 expression) or SGC7901 (without Δ133p53 expression) cells

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were treated with rmhTNF of different concentrations only or combined with 5-FU (a traditional gastric cancer cellular killer), and the growth inhibition rate and apoptosis was detected by CCK-8 and flow cytometry. mRNA expressions of Δ133p53, Gadd45α and CyclinB1 were measured by nested reverse transcription-polymerase chain reaction (nRT-PCR) or real-time polymerase chain reaction(RT-PCR). **Results:** On MKN-45 cells with positive Δ133p53 expression, the inhibitory effect of rmhTNF was significant, the inhibition rates of 50 and 500 IU/mL rmhTNF were 24.82%, 72.33% after culturing for 24 h (t=-9.558, P<0.01); also, the inhibitory effect of 5-FU was improved by rmhTNF remarkably in time- and dose-dependence, the inhibition rates of 5-FU (25 μg/mL), rmhTNF (50 IU/mL) combined with 5-FU (25 μg/mL), rmhTNF (500 IU/mL) combined with 5-FU (25 μg/mL) were 18.20%, 48.66%, 59.83%, separately, after culturing for 24 h (F=82.742, P<0.01); the inhibition rates of rmhTNF (50 IU/mL) combined with 5-FU (25 μg/mL) were 48.66%, 68.20%, 85.23%, separately, after culturing for 24 h, 48 h and 72 h (F=128.583, P<0.01). However, on SGC-7901 cells with negative Δ133p53 expression, no growth inhibition was showed by rmhTNF only, the inhibition rates of 50 and 500 IU/mL were 2.74%, 3.25% after culturing for 24 h (t=-0.121, P>0.05). In apoptosis test, the apoptosis-enhancing effect of rmhTNF was significant on MKN45 cells, and the apoptosis-enhancing effect of 5-FU was further promoted significantly by rmhTNF, the apoptosis of rmhTNF (50 IU/mL), rmhTNF (50 IU/mL) combined with 5-FU (25 μg/mL), rmhTNF (500 IU/mL) combined with 5-FU (25 μg/mL) were 18.20%, 48.66%, 59.83%, separately, after culturing for 24 h (F=123.931, P<0.05). In mRNA measurement, down-regulation of Δ133p53 and CyclinB1, up-regulation of Gadd45α were significant in MKN45 cells treated by rmhTNF alone or combined with 5-FU. In nRT-PCR analysis, the mRNA levels of *Δ133p53* were relatively 0.886, 0.499, 0.330, 0.161 (F=240.927, P<0.01); In real-time PCR analysis, the mRNA levels of *Gadd45α* were 1.227, 1.694, 3.394, and the mRNA levels of *CyclinB1* were 1.221, 0.722, 0.316, relatively. The expression of *Δ133p53* was positively related to *CyclinB1* (r=0.977, P<0.01), but negatively related to *Gadd45α* (r=-0.950, P<0.01). **Conclusion:** In Δ133p53 positively expressed MKN45 cells, rmhTNF showed as an effective tumor inhibitor and an enhancer of 5-FU as well, and this effect might be helped by two *p53* down-stream molecules *CyclinB1* and *Gadd45α*. The results suggest that Δ133p53 might be a key target for the biological effect of rmhTNF against gastric cancer.

［**Key words**］ Recombinant mutant human tumor necrosis factor; 5-FU; Gastric cancer cell lines; Δ133p53

作为基因组卫士，*p53*基因失活是多数肿瘤发生的重要环节。不仅基因突变或缺失，而且选择性剪接也可造成*p53*基因失活。目前已发现至少12种p53剪切异构体，并在人类不同类型组织包括正常组织、癌前病变和肿瘤组织中表达。p53异构体与乳腺癌、结肠癌、肾癌、卵巢癌、骨肉瘤及子宫内膜癌等肿瘤的发生密切相关[1-6]。胃癌发生常伴有*TP53*基因突变，但目前尚不清楚这些突变与胃癌发生之间的关系。p53异构体在胃癌发生中的作用鲜有报道。在正常胃黏膜、慢性萎缩性胃炎、胃癌组织中，Δ133p53表达呈上升趋势，而p53β表达呈下降趋势[7]。Wei等[8]的研究显示，Δ133p53所致的*p53*基因失活可能是幽门螺旋杆菌感染相关慢性炎性反应、乃至胃癌发生的重要诱导因素。上述研究提示p53异构体可能在胃癌发生中起着重要作用，Δ133p53可能是胃癌诊断和治疗的新靶点。

本研究利用重组改构人肿瘤坏死因子(recombinant mutant human tumor necrosis factor，rmhTNF)作用于表达Δ133p53的MKN-45和不表达Δ133p53的SGC-7901胃癌细胞系，观察*Δ133p53*及*p53*下游基因*Gadd45α*和*CyclinB1*的表达，以探索*Δ133p53*在rmhTNF治疗效应中的作用。

1 材料和方法

1.1 胃癌细胞培养

将MKN-45和SGC-7901细胞(由第四军医大学提供)置于含100 mL/L胎牛血清、100 U/mL青链霉素混合液的RPMI-640培养基中，置于37 ℃、CO_2体积分数为5%、饱和湿度的培养箱中培养，取对数生长期细胞用于实验。

1.2 CCK-8测定细胞增殖抑制率

将MKN-45和SGC-7901细胞消化成单细胞悬液，以5×10^4/mL的密度接种于96孔板

中，每孔100 μL。在培养箱中温育相同时间后加药。设置空白组、对照组和实验组，实验组分为：①5-FU(25 μg/mL)；②rmhTNF(50 IU/mL)；③rmhTNF(500 IU/mL)；④5-FU(25 μg/mL)+ rmhTNF(50 IU/mL)；⑤5-FU(25 μg/mL)+ rmhTNF(500 IU/mL)。对照组为不含药物的培养基。空白组为不含细胞和药物的培养基。培育24、48及72 h，弃去各孔培养基，加入CCK-8试剂和培养基混合液(1∶10比例)，继续温育60 min，用酶标仪(波长450 nm)测各孔吸光度(*D*)，计算抑制率。

1.3 流式细胞术检测细胞凋亡率

取对数生长期MKN-45细胞，设置空白组、对照组及实验组。实验组分为：①rmhTNF(50 IU/mL)；②rmhTNF(50 IU/mL)+5-FU(25 μg/mL)；③rmhTNF(500 IU/mL)+ 5-FU(25 μg/mL)。培养24 h后收集细胞，调整细胞浓度至1×10^6/mL，取100 μL细胞悬液，加入5 μL Annexin V-FITC和5 μL PI，混匀细胞，同时设置阴性对照组(没有染色的细胞、仅用Annexin V或PI染色的细胞)，室温下避光温育15 min。用流式细胞仪检测细胞凋亡率。

1.4 巢式逆转录多聚酶链反应(nested reverse transcriptase–polymerase chain reaction，nRT–PCR)和实时荧光定量多聚酶链反应(real–time polymerase chain reaction，RT–PCR)检测Δ133p53、Gadd45α和CyclinB1 mRNA的表达

取对数生长期MKN-45和SGC-7901细胞，制备单细胞悬液，均匀接种于6孔板中，对照组不加药，实验组分为：①rmhTNF(50 IU/mL)；rmhTNF(50 IU/mL)+5-FU(25 μg/mL)；rmhTNF(500 IU/mL)+5-FU(25 μg/mL)。培养24 h后收集细胞，用TRIzol试剂提取总RNA。nRT-PCR采用M-MULV第一链cDNA合成试剂盒逆转录、Premix Taq PCR扩增；RT-PCR采用PrimeScript™ RT reagent Kit、SYBR® Premix Ex Taq™。以β-actin作为内参，PCR反应体系为25 μL(引物序列见表1)，反应条件为94 ℃预变性5 min；94 ℃变性30 s，56～58 ℃退火30 s，72 ℃延伸30 s，40个循环；72 ℃终延伸10 min。PCR产物各5 μL在2%琼脂糖凝胶上电泳20 min，溴乙锭(EB)染色。凝胶成像分析系统观察nRT-PCR结果。荧光定量PCR仪(Bio-Rad IQ5)检测Ct值，$2^{-\Delta\Delta Ct}$计算相对表达量。

1.5 统计学处理

采用SPSS 17.0软件进行统计分析，各组实验数据以$\bar{x}\pm s$表示，组间差异采用*t*检验，多组间差异比较采用单因素方差分析，两两比较采用LSD-*t*检验，基因表达量的相关性采用Person直线相关分析，$P<0.05$为差异有统计学意义。

2 结　果

2.1 rmhTNF和5–FU对MKN–45和SGC–7901增殖的影响

不同浓度rmhTNF(50、500 IU/mL)分别作用于MKN-45和SGC-7901 24 h后，结果显示，rmhTNF对MKN-45抑制率随浓度增加而增加(t=-9.558，$P<0.01$)，对SGC-7901抑制作用不明显(t=-0.121，$P>0.05$，图1A)。不同浓度的rmhTNF(0、50、500 IU/mL)联合5-FU(25 μg/mL)作用24 h后(表2)，对 MKN-45细胞抑制率随浓度增加而增加，对SGC-7901细胞抑制率差异无统计学意义($P>0.05$)。rmhTNF(50 IU/mL)联合5-FU(25 μg/mL)作用24、48及72 h后(表3)，对MKN-45和SGC-7901细胞抑制率均随时间增加而增加。可见rmhTNF提高MKN-45细胞对5-FU的敏感性，具有量效和时效关系；而对SGC-7901细胞，随时间延长，rmhTNF可提高5-FU的抑制作用(图1B、C)。

2.2 rmhTNF单独或联合5–FU对MKN–45细胞凋亡的影响

收集各组MKN-45细胞行Annexin V/PI染色，设定FITC(+)/PI(-)为凋亡细胞，流式细胞术检测细胞凋亡，结果显示，24 h后各实验组细胞凋亡率均明显增加，其中rmhTNF(500 IU/mL)联合5-FU组细胞凋亡率为[(14.57±1.37)%]，明显高于rmhTNF(50 IU/mL)组[(7.15±0.94)%]，以及rmhTNF(50 IU/mL)联合

表 1 引物序列及片段长度

Tab. 1 Primer sequences and base pairs

Primer	Primer sequence (5'-3')	Base pairs/bp
Δ133p53		750
First:	Sense: CTGAGGTGTAGACGCCAACTCTCTCTAG	
	Antisense: TGTCAGTCTGAGTCAGGCCCTTCTGTC	
Second:	Sense: GCTAGTGGGTTGCAGGAGGTGCTTACGC	
	Antisense: CTCACGCCCACGGATCTGA	
β-actin		539
	Sense: GTGGGGCGCCCCAGGCACCA	
	Antisense: CTCCTTAATGTCACGCACGATTTC	
Gadd45α		197
	Sense: CGAAAGGATGGATAAGGTG	
	Antisense: GGATCAGGGTGAAGTGGA	
CyclinB1		118
	Sense: CTGAAGGTGATGGAGGTAT	
	Antisense: GGATTCGGTGGTAGACTT	

表 2 rmhTNF联合5–FU作用24 h对胃癌细胞抑制率的影响

Tab. 2 Effects of rmhTNF/5-FU on inhibition rate of gastric cancer cells after 24 h

($\bar{x}\pm s$, n=5, %)

Cell	5-FU (25 μg/mL)	rmhTNF (50 IU/mL)+5-FU (25 μg/mL)	rmhTNF (500 IU/mL)+5-FU (25 μg/mL)	F	P value
MKN-45 (IC)	18.20±3.95	48.66±4.50	59.83±6.95	82.742	P<0.01
SGC-7901 (IC)	26.30±8.68	33.51±3.57	25.78±5.32	2.405	P>0.05

表 3 rmhTNF(50 IU/mL)+5–FU(25 μg/mL)作用24、48及72 h对细胞抑制率的影响

Tab. 3 Effects of rmhTNF (50 IU/mL) combined with 5-FU (25 μg/mL) on cell inhibition rate after different time

($\bar{x}\pm s$, n=5, %)

Cell	24 h	48 h	72 h	F	P value
MKN-45 (IC)	48.66±4.50	68.20±4.11	85.23±1.38	128.583	P<0.01
SGC-7901 (IC)	33.51±3.57	65.01±8.34	88.59±2.82	126.901	P<0.01

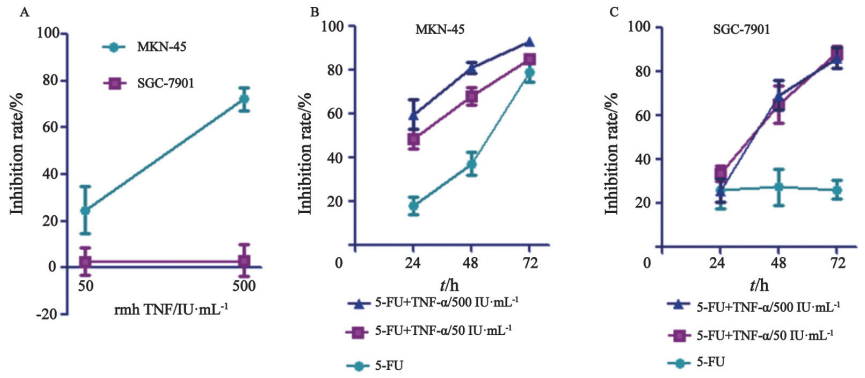

图 1 不同浓度的rmhTNF联合5–FU对MNK–45和SGC–7901细胞增殖的影响

Fig. 1 Inhibitory effects of different concentrations of rmhTNF combined with 5-FU on MNK-45 and SGC-7901 cells

A: Comparisons of cell inhibition rate of MNK-45 and SGC-7901 after 24 h of rmhTNF treatment; B: Comparisons of cell inhibition rate of MNK-45 after 24 h of rmhTNF/5-FU treatment; C: Comparisons of cell inhibition rate of SGC-7901 after 24 h of rmhTNF/5-FU treatment.

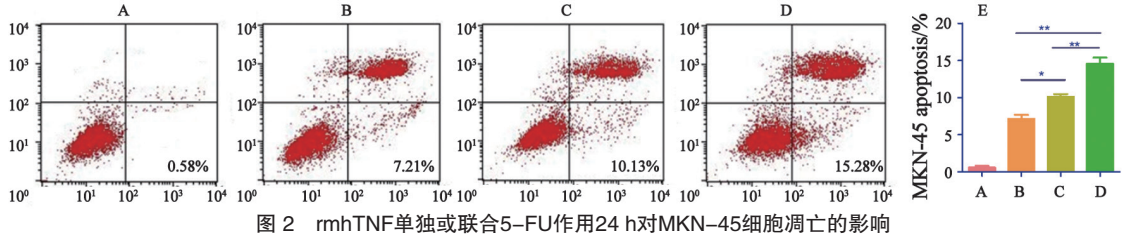

图 2 rmhTNF单独或联合5–FU作用24 h对MKN–45细胞凋亡的影响

Fig. 2 Effects of rmhTNF/5-FU on apoptosis of MNK-45 after 24 h

A: Blank; 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: Cell apoptosis was assessed by FCM assay ($\bar{x}\pm s$, n=3. *: P<0.05; **: P<0.01).

5-FU组［(10.11±0.64)%］(P<0.01，图2)。

2.3 rmhTNF单独或联合5-FU对MNK-45和SGC-7901细胞Δ133p53、Gadd45α和CyclinB1 mRNA的表达影响

rmhTNF单独或联合5-FU作用于MKN-45和SGC-7901细胞24 h后，nRT-PCR结果显示，MKN-45细胞Δ133p53及CyclinB1 mRNA的表达量均随浓度增加而降低(P<0.05)，Gadd45α mRNA的趋势与之相反(P<0.05，图3A)；SGC-7901均不表达*Δ133p53*基因，Gadd45α mRNA的表达量呈上升趋势(P<0.05)，CyclinB1 mRNA的表达量呈下降趋势(P<0.05，图3B)。RT-PCR结果显示，*Gadd45α*和*CyclinB1*基因表达趋势与nRT-PCR结果相同(图3C、D)。在*Gadd45α*基因表达方面，rmhTNF与5-FU联合在2个细胞系中均有促进作用(P<0.01)，而rmhTNF单独使用仅在MKN-45中有促进作用，表达量为对照组的(1.23±0.07)倍；在*CyclinB1*基因表达方面，联合低浓度rmhTNF仅在MKN-45中有抑制作用，为

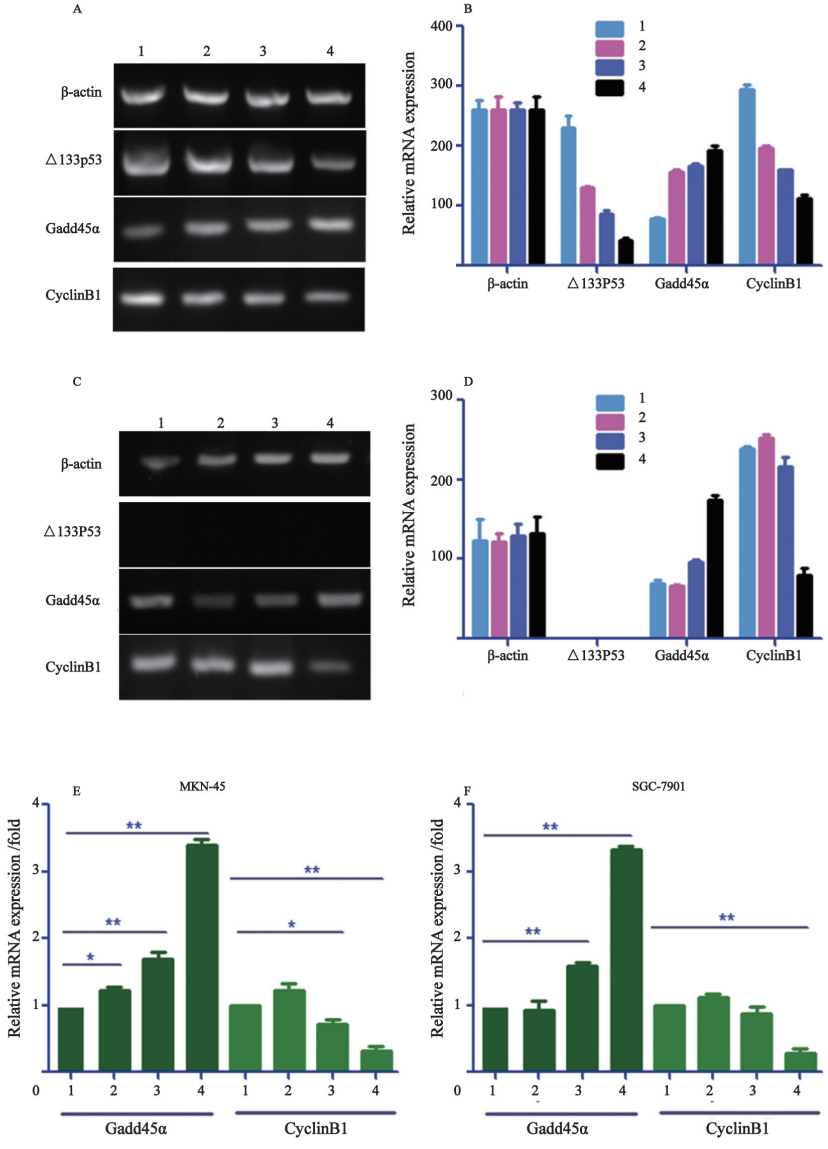

图 3 5-FU、rmhTNF作用24 h对Δ133p53、Gadd45α和CyclinB1 mRNA表达的影响

Fig. 3 Effects of rmhTNF/5-FU on Δ133p53, Gadd45α and CyclinB1 mRNA expressions after 24 h

A: nRT-PCR result of mRNA expression in MNK-45; B: nRT-PCR result of mRNA expression in SGC-7901; C: RT-PCR result of mRNA expression in MNK-45; D: RT-PCR result of mRNA expression in SGC -7901. 1: Blank; 2: rmhTNF (50 IU/mL); 3: rmhTNF (50 IU/mL)+ 5-FU(25 μg/mL); 4: rmhTNF (500 IU/mL)+5-FU (25 μg/mL) ($\bar{x}\pm s$, n=3. *: P<0.05; **: P<0.01).

对照组的(0.72±0.11)倍。

2.4 MKN-45细胞中Δ133p53与Gadd45α和CyclinB1 mRNA表达水平的相关性

在MKN-45细胞中，随rmhTNF浓度增加，Δ133p53 mRNA与CyclinB1 mRNA相对表达呈递减趋势，而Gadd45α相对表达量呈增长趋势。Pearson直线相关分析结果显示，在MKN-45细胞中，Δ133p53 mRNA表达水平与Gadd45α mRNA表达水平呈负相关(r=-0.950，P<0.01)，与CyclinB1 mRNA表达水平呈正相关(r=0.977，P<0.01，图4)。

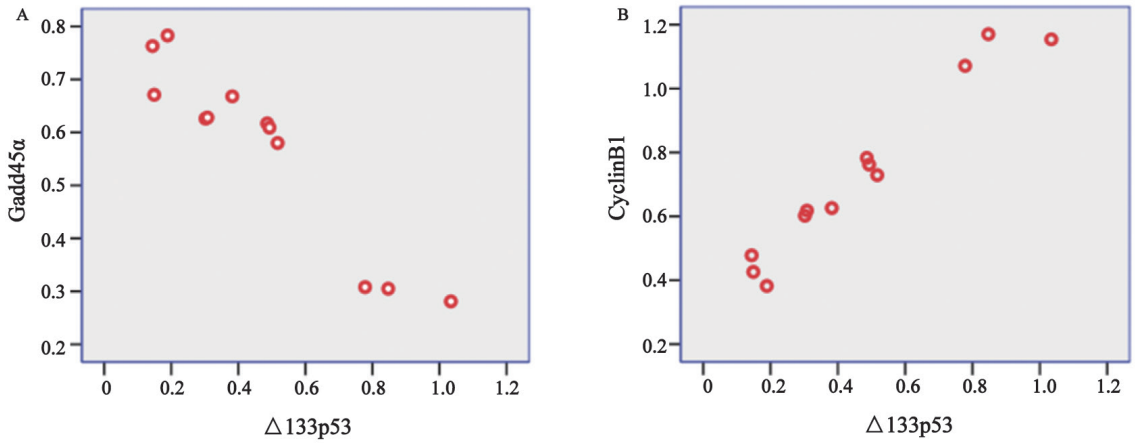

图 4 MKN-45中Δ133p53、Gadd45α和CyclinB1 mRNA表达水平的相关性

Fig. 4 Correlation among the expressions of Δ133p53, Gadd45α and CyclinB1 mRNA in MNK-45

3 讨 论

胃癌的发生是多种遗传变异积累的过程，其中*p53*基因功能丧失起着重要作用[9]。在胃癌组织和细胞系中，*TP53*基因突变和缺失已有详细报道[10-11]，但是鲜有研究聚焦于p53异构体。

Δ133p53作为进展期卵巢癌的一个独立预后指标，也在存在于乳腺癌中，被认为是野生型*p53*功能的负性抑制因子[1,12]。本课题前期研究中发现，Δ133p53 mRNA的阳性率分别为胃腺癌75%(15/20)、慢性胃炎50%(15/30)、浅表性胃炎25%(5/20)和癌旁组织20%(3/15)，差异有统计学意义。Δ133p53的表达状态在两种胃癌细胞系中已证明，MKN-45细胞表达阳性，SGC-7901表达阴性。此外，Wei等[8]研究已证明，Δ133p53在幽门螺杆菌感染相关胃炎发展到胃癌的过程中起着诱导因子的作用。这些发现提示，Δ133p53可能是胃癌发生过程中p53失活的关键分子，是胃癌诊断、治疗及预后研究潜在的靶分子。

本实验结果提示，在Δ133p53阳性MKN-45细胞中，rmhTNF可抑制其生长，并能提高5-FU的抑制作用；而相同条件下，对Δ133p53阴性的SGC-7901胃癌细胞，抑制作用不明显。既往研究发现，Δ133p53具有拮抗野生型*p53*的功能，因此有抗细胞凋亡作用[13-16]。*p53*可激活并上调*Gadd45*基因表达，后者通过与CDC2作用形成复合物，导致CDC2/CyclinB1复合物解离，从而抑制CDC2激酶活性，形成细胞周期G_2/M期阻滞[17]。本实验发现，rmhTNF能下调Δ133p53基因表达，与*CyclinB1*表达水平呈正相关，与*Gadd45α*呈负相关，提示*Δ133p53*下调可选择性释放p53 G_2/M期阻滞功能，抑制细胞增殖，诱导凋亡。

本实验中，rmhTNF对表达*Δ133p53*胃癌细胞展现出显著的抑制效应，并能增加传统化疗药物5-FU的疗效，其中部分效应可能是通过调节*p53*下游分子*CyclinB1*和*Gadd45α*表达实现

的，提示Δ133p53可能是rmhTNF治疗胃癌生物学效应的关键靶点。然而，本研究只是初步研究，在实验设计和实验方法上均有瑕疵。下一步基础研究应引入基因组和蛋白质组学的研究方法，以期得到更加系统的资料。临床研究的重点应集中于整理胃癌组织p53异构体表达的模式，并在此基础上探讨生物学治疗的可行性。

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