Study on the Antiproliferation Effect of Targeting of Survivin siRNA Transfection Combined with 5-FU on Liver Cell Line HepG2

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ABSTRACT Objective: To investigate the anti-proliferation effect of (small interference RNA) siRNA against survivin combined with(fluorouracil) 5-FU on human liver cancer cell line HepG2. Methods: HepG2 cells cultures were divided into five groups: blank control group, negative control group, 5-FUgroup, siRNA-survivin group, siRNA-survivin+5-FU group. Lipofectamine TM 2000 was used to transfect HepG2 cell. The expression of survivin mRNA was detected by RT-PCR. Inhibition rate of each group on HepG2 was assayed by MTT method, and apoptosis was analyzed by flow cytometry respectively. Results: The experiment of RT-PCR showed that the expression of survivin mRNA in siRNA-survivin group and siRNA-survivin+5-FU group were lower than that in other groups($P<0.05$), but its levels in blank control group, negative control group and 5-FU group had no significant change ($P>0.05$). The MTT assay indicated that the antiproliferation rate of siRNA-survivin+5-FU group was 51.38%± 1.35. The antiproliferation rate of this group was higher than that in any other groups($P<0.05$). Flow cytometry results indicated that the apoptosis rate of siRNA-survivin+5-FU group was also higher than any other groups ($P<0.05$). Conclusion: siRNA against survivin combined with 5-FU can specifically suppress its expression, inhibit the proliferation of HepG2 and induce apoptosis in common.

Key words: siRNA; Survivin; Liver neoplasms; 5-FU

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Introduction

Hepatocelluar carcinoma is commonly higher prevalence of human malignancies, with the characteristics of early recurrence and metastasis, so much has advanced clinical findings. Currently the main treatment is the combined therapy of surgery and chemotherapy, but long-term efficacy is not obvious[1,2]. Tumorigenesis is connected with the imbalance of cell proliferation and apoptosis. As one of the inhibitor of apoptosis protein (IAP) members, survivin was found in 1997. It had a unique gene and protein structure, and was expressed in most tumor tissues. Survivin is an ideal target in a targeted cancer therapy [3,4], and showed high expression state in the hepatocarcinoma. Several studies have shown, survivin has dual functions of inhibition of apoptosis and cell division regulation and helps a variety of tumor cells resistance to various apoptotic stimuli [5,6]. RNA interference is a widely used gene knockdown technology, with an equal effect to gene knockout,and often used in gene therapy research. This experiment will combine the targeting survivin's siRNA and traditional chemotherapy drugs 5-FU, and then investigate its effect on inhibition liver cancer cells proliferation, to provide new therapy and experimental evidence for improving the efficacy of liver cancer treatments.

1 Materials and methods

1.1 Materials

1.1.1 Main reagents and apparatus Fetal bovine serum, RPMI 1640 culture medium (the Hang Zhou Evergreen Biological Engineering Materials Company's products); Lipofectamine 2000 transfection kit (Invitrogen products); MTT(Sigma company); RT-PCR (Dalian PoSang company); small molecular weight proteins (Galen's Marker).

1.1.2 Cell line Human hepatoma cell line HepG2 is derived from Affiliated Hospital of Qingdao University Medical Cancer laboratory, cultured with 10 % fetal bovine serum in RPMI 1640 culture medium.

1.2 Methods

1.2.1 Design of survivin recombinant plasmid According to RNA design principles and survivin (NM-001168) gene coding sequence and referring to BLAST, based on the preliminary experimental results [7], synthetized one fragment of siRNA coded by sequences of survivin, build specifically the recombinant carrier of survivin-pshRNA survivin 387, the target gene is in the 387 points of survivin gene sequence. The target genes is survivin 5' GAAAGTGCGCCGTGCCATC 3'. Both ends of the recombinant plasmid have Bam HI or Bbs I restriction sites, and are easy to link with pGPH1/GFP/Neo carrier. According to the experimental requirements negative control carrier (pshRNA survivin NC)and positive control carrier (pshRNA survivin GAPDH) respectively, all the carriers were synthesized by Shanghai Gene Pharma company.

1.2.2 Cell grouping and transfection The HepG2 cells in the logarithmic phase are passaged growth strongly, and then inoculated in 6-well plates, so that each hole is approximately the number of cells 1×10^5, cultured them at 37℃, 5 % CO_2 for 24 hours until

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the cells are adherent. The experiments were divided into control group(without liquid), the negative control group(plus pshRNA survivin NC 3.2μg/well)[8], 5-FU treatment group (without liquid), siRNA transfection group(plus pshRNA survivin 387 3.2 μg/well) 5-FU+siRNA transfection group (plus pshRNA survivin 387 3.2 μg/well). 4 h after transfection, medium changed, the control group, negative control and siRNA transfection group changed for RPMI1640 culture medium with 10 % fetal bovine serum. 5-FU treatment group and 5-FU+siRNA transfection group changed for RPMI1640 culture medium with 10 % fetal bovine serum which contained 5-FU 20 μg/ml [9]. Each group had five repetitive holes, the process was in accordance with instructions of Lipofectamine 2000 transfection kits.

1.2.3 Cell proliferation measured by the MTT The concentration of cells was 20 μL in logarithmic growth phase, the cell was seeded in 96-well, each well with 100 μL, after cultured for 48 h MTT, added 100 μg, then continue to incubate 4 h. liquid supernatant was abandoned and DMSO was added 200 μL into each hole, then the cell was shocked 10min, and purple crystals completely was dissolved and the absorbance value was measured at the 570 nm wavelength by microplate reader. The control group was as a reference group. The cell survival rate was 100 %, and the inhibition rate of tumor cell proliferation was calculated according to the following formula: inhibition rate(%)=(1-OD of experimental hole/OD of control well) × 100 %.

1.2.4 Detection of apoptosis rate by flow cytometry Transfection cells were collected into test tubes after 48 h; prepared the cell suspension for 1×10^6, fixed them by 70 % ethanol; 4 ℃ for overnight, centrifuged 1200r/min ,10min. Discarded the supernatant, rinsed twice with PBS; cells were resuspended in 0.4 mL PBS, and then added 5 g/L Rnase A 10 μL, 37 ℃ water bathed for 1 h, and plus propidium bromide staining at 4 ℃ for overninght. Cell apoptosis were measured at excitation wavelength 488 nm of flow cytometry. Rate of apoptosis(AI)by the following formula:AI (%)= twice the peak of sube-cells/ total cells × 100%.

1.2.5 The level of survivin mRNA detected by RT－PCR Transfection cells were collected after cultured 48 h, the concentration of cell was adjusted for 1×10^7 /mL, the total RNA was extracted by Trizol. The cDNA was synthesized in the 20 μL reaction system; PCR was reacted in the 50 μL reaction system, containing the template 2 μL, Taq enzyme 5U, and each of purpose primer is 0.5 μmol / L, and was made up to 50 μL. The reaction conditions of PCR were 94 ℃ 1 min, 55 ℃ 1 min, 72 ℃ 2 min, and all were 35 cycles of reaction. The obtained PCR products were separated in 1.5 % agarose gel electrophoresis with EB, the results were analyzed under UV light.(PCR primers were synthesized by Bio-Technology Co., Ltd. Chongqing Bo, survivin primers: upstream 5'-GGGCATGGGTGCCCCGACGTT-3', downstream 5'-AGAGGCCTCAATCCATGGCA-3', with GAPDH as an internal reference). Analysis software was applied for the relative quantitative of survivin mRNA, the level of survivin mRNA was showed by the absorbance (A) value of survivin mRNA expression / the A values of GAPDH mRNA expression.

1.2.6 Statistical analysis The data was analyzed by SPSS 13.0 statistical software, results expressed in $\bar{x}\pm s$, compare groups by single-factor analysis of variance, pairwise comparison between groups used the Q test.

2 Results

2.1 Comparison of the proliferation rates between each group of HepG2 cell

The HepG2 cell proliferation rates of 5-FU treatment group, siRNA transfection group, 5-FU + SiRNA transfected group were higher than those in the negative control group and blank control group (F = 280.326, * q = 4.72 ~ 7.34, P <0.05), while the rate of 5-FU + SiRNA transfection group was significantly higher than SiRNA transfection group and 5-FU treatment group (F = 280.326, # q = 5.27 ~ 9.84, P <0.05).

Table 1 Inhibition rate of cell proliferation by MTT assay (n=5, $\bar{x}\pm s$)

Groups	OD value	Inhibition rate(%)
Blank control group	0.265± 0.151	--
Negative transfected group	0.250± 0.147	7.46± 1.20
5-FU treatment group	0.217± 0.132*#	15.67± 1.07*#
siRNA transfection group	0.156± 0.006*#	29.35± 1.16*#
5-FU + SiRNA transfection group	0.102± 0.004*	51.58± 1.35*

Note: compared with the blank control group and the negative transfected group, F = 280.326, * q = 4.72 ~ 7.34, P <0.05; and compared with siRNA transfection +5- FU treatment group #q = 5.27 ~ 9.84, P <0.05.

2.2 Comparison the rate of cell apoptosis between groups

Through the technology of RNAi pshRNA survivin 387 which transfected HepG2 cells can induce cell apoptosis, cells of 5-FU + siRNA transfection group could significantly induce apoptosis, its apoptosis rate was 43.36 ± 2.03%. Compared with that in siRNA transfection group, 5-FU treatment group, negative control group, the apoptosis rate was significantly higher (F = 13568.68, q = 110.47 ~ 327.16, P <0.01). The apoptosis rate all in-

creased in the siRNA transfection group and 5-FU treatment group respectively compared with that in the negative control group, and their differences were statistically significant (F = 46.523, q = 3.36 ~ 5.21, P<0.05).

2.3 Detect the level of survivin mRNA by RT-PCR

The levels of survivin mRNA were significantly lower in the siRNA transfection group (the ratio of survivin / GAPDH was 0.162 ± 0.044) , 5-FU + SiRNA transfection group (the ratio of survivin / GAPDH was 0.298 ± 0.075) compared with those in the blank control group (the ratio of survivin / GAPDH was 0.318 ± 0.080), negative control group (the ratio of survivin / GAPDH was 0.307 ± 0.078) and 5-FU treatment group (the ratio of survivin / GAPDH was 0.302 ± 0.071), and the difference were statistically significant (F=326.782, q=5.78~7.12, P<0.05), while the former two groups compared with each other, and the latter three pairwise compared with each other, there was respectively no statistically significant difference (P>0.05).

3 Discussion

Primary liver cancer is very common in clinical practice. China is a country with a high incidence of liver cancer. Compared with low-incidence areas, the pathogenetic age was younger and was rapid progression. Recent epidemiological studies have showed that even in western countries its incidence and mortality have increased. The current treatment to liver cancer is mainly surgery, but its efficacy is not perfect [10,11]. Studies have shown, the survivin gene is relevance on the development and progression of hepatoma, and on the drug resistance of chemotherapy, then it can increase their sensitivity to chemotherapeutic drugs[12].

Survivin is a member of the IAP genes which recently discovered more popular therapy targets, and survivin gene could be detected to express different levels in a variety of malignant tumors, that survivin has the characteristics of tumor-selective. A number of studies have shown targeting closure of survivin can induce apoptosis of hepatoma cells, and inhibit of cell proliferation, thereby inhibit tumor growth [13]. While other studies showed that: closed survivin had no effect on some tumor cells apoptosis and proliferation, but the combination of chemotherapy drugs can promote tumor cell apoptosis[14,15].

RNAi is a gene silencing mechanism of transcription, reverse transcription and translation, its effective molecules siRNA can specifically close the expression of target gene. Compared with the conventional genetic closed techniques, it has the advantages of simple operation, low-cost, high transfection, stronger inhibition, and as a means of gene therapy it has broad application prospects [16,17]. In the experimental treatment to tumors, RNAi can inhibit the expression of oncogenes, knockout oncogene which were activated by mutant, inhibit gene amplification or confertus gene expression and inhibit other tumor-related gene expression [18,19]. Cheng used RNA interference (RNAi) technology to transfect human hepatoma cell line which was recombinant plasmid, the survivin expression decreased, as a result, the number of cells was significantly reduced in G2 / M phase, while apoptosis index increased and cell growth slow down[19,20]. Due to the high expression of survivin gene in hepatocellular carcinoma, it can become an ideal target to gene therapy.

This study, showed that siRNA of targeting survivin and 5-FU in combination can significantly inhibit survivin gene expression in liver cancer cells, and inhibit HepG2 cell proliferation, so play a common role in induction of apoptosis. Therefore, RNAi of targeting survivin in combination with chemotherapy drugs may be an effective way of treating liver cancer. But in the process of transfection, liposome had stronger toxicity, and the issue that how to reduce their toxicity remains to be further studied.

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靶向 survivin 的 siRNA 联合 5-FU 转染抑制肝细胞株 HepG2 增殖的研究

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摘要 **目的**：研究靶向 survivin 的（小分子干扰 RNA）siRNA 和（氟尿嘧啶）5-FU 联用对肝癌细胞 HepG2 的增殖抑制及凋亡的影响。**方法**：将 HepG2 细胞分为空白对照组、阴性对照组、5-FU 处理组、siRNA 转染组、5-FU+siRNA 转染组。转染采用脂质体法。RT-PCR 法检测 HepG2 细胞 survivin mRNA 转录水平；MTT 法检测靶向 survivin 的 siRNA 和 5-FU 对 HepG2 细胞增殖的抑制作用；流式细胞术检测 HepG2 细胞凋亡情况。**结果**：空白对照组、阴性对照组、5-FU 处理组 survivin mRNA 表达无明显变化(P>0.05)，siRNA 转染组、5-FU+siRNA 转染组 survivin mRNA 表达明显下降(F=280.326，q=4.72~7.34，P<0.05)。5-FU+siRNA 转染组增殖抑制率为 51.58 %± 1.35 %，与其它各组相比抑制率明显增高 (F=280.326，q=5.27~9.84，P<0.05)。5-FU+siRNA 组与其它各组相比细胞凋亡率明显增高 (F=13568.68，q=110.47～327.16，P＜0.01)。**结论**：将靶向 survivin 的 siRNA 和 5-FU 联合应用可以显著抑制肝癌细胞 survivin 基因表达，并协同抑制 HepG2 细胞增殖，共同发挥诱导细胞凋亡作用。

关键词：SiRNA；survivin；肝癌；5-FU

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