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siRNA Induced CyclinB1 Knockdown Sensitizes HepG2 Cells to Daunorubicin*

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Abstract The role of CyclinB1 in conferring drug resistance in the treatment of liver cancer was investigated. siRNA delivery was used to knockdown the expression of CyclinB1, and flow cytometry analysis was employed to assess cell apoptosis and cell cycle distribution .Colony formation assay and cytotoxicity assay were used to determine cell growth ability. It was found that siRNA induced CyclinB1 down regulation triggered cell arrest at G2/M by 40%~ 50% and greatly inhibited cell colony growth ability. Daunorubicin in combination with CyclinB1 siRNA induced more apoptosis than that treated with Daunorubicin alone, whereas this combinational effect decreased in HL-7702 cells, a normal human liver cancer cell line. Those data support the notion that targeting CyclinB1 down regulation combined with chemotherapeutical agents would be a promising new strategy in the treatment of liver cancer.

Key words CyclinB1, siRNA, daunorubicin, drug combinational effect, drug resistance
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The change of Cyclin and Cyclin-dependent kinase structure and function is the main reason leading to destruction of cell cycle, and also a focal point of oncogenes and tumor suppressor genes. Cyclin B1 is an important member of Cyclin family and plays a decisive role during the period when cells go through the G2/M checkpoint into M phase. Cyclin B1 combine Cyclin-dependent kinase 1 (CDK1) to form maturation promoting factor (MPF), and MPF activation is necessary for the eukaryotic cell to start mitosis. After the combination, the role of Cyclin B1 is estimated to assist the spindle microtubules to promote cell division [1]. Cyclin B1 can regulate the cell cycle progression through their own biological characteristics, and a variety of factors can also achieve cell cycle regulation by promoting Cyclin B1 synthesis or degradation and thus activating or inhibiting MPF kinase activity.

Cyclin B1 overexpression is a general phenomenon in tumor tissues. As a cell cycle regulator, the level of Cyclin B1 is one of the indicators to determine the malignant degree of tumor cells, and Cyclin B1 is regarded as tumor antigen. The aberrant expression was observed in diverse human tumor tissues including colorectal cancer [2-5], esophageal squamous cell carcinoma [6], gastric carcinoma [7], non-small-cell lung cancer [8], cervical cancer [9], head and neck squamous cell carcinoma[10] and breast cancer[11-13]. The upregulation of Cyclin B1 is closely associated with poor prognosis in various types of cancers[6, 8, 13]. Moreover, overexpression of Cyclin B1 was showed to be involved in the resistance to radiotherapy in head and neck squamous cell carcinoma [10] and breast

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carcinomas with nuclear Cyclin B1-positive are resistant to adjuvant therapy [13]. Therefore, targeting Cyclin B1 would be a promising strategy for cancer treatment.

In the present study, we focused on hepatoma cells and tried to find out the effect of combination Cyclin B1 siRNA with Daunorubicin on tumor cell proliferation. This study provided evidence that the combination of Cyclin B1 siRNA with Daunorubicin substantially enhanced the inhibitory effect on proliferation of liver cancer cells.

1 Materials and methods

1.1 Cell culture

Human hepatoma cells HepG-2 and QGY7703 (American Type Culture Collection, Manassas, VA) were cultured under DMEM medium containing 10% fetal bovine serum (Hyclone, USA), L-glutamine (2 mmol/L), streptomycin (0.1 g/L) and penicillin (100 U/ml). Normal hepatic cells HL7702 (American TypeCulture Collection, Manassas, VA) were cultured under RPMI 1640 containing 10% fetal bovine serum (Hyclone, USA), streptomycin (0.1 g/L) and penicillin (100 U/ml). All the cells were incubated at 37℃ in a humidified incubator supplied with 5% CO_2.

1.2 Cyclin B1 down regulation by siRNA

siRNA targeted to encoding region(776～796) of CyclinB1(5′ CUCGUACAGCCUUGGGAGACAtt 3′) was chemically synthesized (Genepharma, Shanghai) according to Yuan J, *et al*[14]. A scrambled siRNA was used as a negative control. For siRNA delivery, HepG-2, QGY7703 and HL7702 cells (2×10^5 in Opti-MEM Ⅰ medium) were mixed gently with siRNA and OligofectAMINE (Invitrogen, CA) in a volume of 0.5～1.5 ml, following manufacturer′s instructions, and incubated at 37℃, 5% CO_2 for 4 h, followed by the addition of an equal volume of fresh medium containing 20% FBS. Cells were continuously incubated until harvest.

1.3 Western blot analysis

Western blot analysis was performed as previously described [15]. Briefly ,cells were lysed in lysis buffer (10 mmol/L N-2- hydro-xyethylpiperazine-N′-2-ethanesulfonic acid, 10 mmol/L KCl, 1 mmol/L ethylenediaminetetra acetic acid (EDTA, pH 8.0), 0.1% NP-40, 1 mmol/L DTT, 1 mmol/L PMSF, and 0.5 mmol/L Na_3VO_4). Soluble protein (30 μg) was separated on a 12% sodium dodecyl sulfate (SDS)-polyacrylamide electrophoresis gel and blotted onto a pure nitrocellulose membrane (Bio-Rad, Hercules, CA) at 180 mA for 2 h. After blockage with 5% skim milk in phosphate-buffered saline at room temperature for 45 min, membranes were incubated with CyclinB1 antibody (1 ∶ 500, Abcam) in the same buffer for 2 h, followed by incubation with goat anti-rabbit IgG (1 ∶ 2 000) for 1 h. Antibody binding was detected using enhanced chemiluminescence-system (Pierce, Rockford, IL). To correct protein loading amounts, the membranes were reprobed with β-actin monoclonal antibody (1∶40 000) (Sigma, St. Louis, MO).

1.4 Real time PCR

Total RNA was extracted from 293T and HepG 2 cells using Trizol reagent (Invitrogen, CA), and cDNA was synthesized using a reverse transcription kit (TOYOBO, Japan). Cyclin B1 mRNA levels was quantitated relative to GAPDH levels using primer and probes specific for Cyclin B1 and GAPDH. Cyclin B1 primers used for real-time PCR are as follows: forward, 5′ GCCTCTAGAGGTGAAGAGGAAGCCATG 3′; reverse, 5′ GAAGGATCCTTACACCTTTGCCACA-GCC 3′. The primers for GAPDH amplification are as follows: forward, 5′ TTACTCGCACCCCTGGCCAA-GG 3′; reverse, 5′ CTTACTCCTTGGAGGCCATG 3′. Real-time PCR was done using 7 500 Real-time PCR system(Taqman, Applied Biosystems, Foster City, CA) with a buffer kit using SYBR premix Ex TaqTM (TAKARA, Japan). The temperature variables used were 95℃, 10 s, one cycle; followed by T1 (95℃, 5 s), T2 (60℃, 34 s), 40 cycles; and then followed by 95℃ 15 s, 60℃ 1 min, 95℃ 15 s, one cycle, for dissociation stage.

1.5 Cytotoxicity assay

2×10^4 cells were seeded in each well of 24-well plates, and cells were allowed to attach for 24 h. At 24 h after siRNA transfection, cells were incubated with different concentration of Daunorubicin. After an additional 48 h, cells were trypsinized, and cell number was determined by a Coulter counter (Beckman Coulter, Fullerton, CA).

1.6 Colony formation assay

HepG2 or QGY7703 cells treated with Cyclin B1 targeting siRNA or scramble RNA were seeded in 6-well plate at a density of 1 000 cells per well into medium with or without 1 nmol/L Daunorubicin. Cells were cultured at 37℃ with 5% CO_2, and colonies were photographed 2 weeks later.

1.7 Flow cytometry analysis

For flow cytometry analysis, 5×10^5 of cells in 6-well plate were transfected with siRNA and were exposed to different concentration of Daunorubicin for 24 h. Medium was gently removed, and cells were washed with cold PBS and then trypsinized. Cells in PBS and trypsin digestion were pooled, washed with PBS twice at 1 200 *g* for 10 min and then subjected to immediate propidium iodide (PI) and annexin-V- FITC staining for 10 min in the dark. FACScan analysis was performed using a FACScan cytometer (Beckman Coulter, Fullerton, CA). Three repeats were conducted.

1.8 Statistic analysis

Statistic analysis was performed using Student's *t*-test with INSTAT statistical analysis package (Graph Pad Software). Significance was defined as $P < 0.05$.

2 Results

2.1 siRNA induced Cyclin B1 knockdown in HepG2 cells

To determine the effect of Cyclin B1 targeting siRNA, HepG2 and HL-7702 cells were treated with 100 nmol/L of scramble RNA or Cyclin B1 siRNA. At different time points after siRNA treatment, cells were collected for Western blot analysis. As shown in Figure 1, Cyclin B1 targeting siRNA could effectively decrease the expression of Cyclin B1 in both HepG2 and HL-7702 cells. Whereas no obvious alteration of Cyclin B1 expression was observed in cells treated with scramble RNA. Those results suggest that the synthesized Cyclin B1 targeting siRNA could down regulate the expression Cyclin B1 effectively.

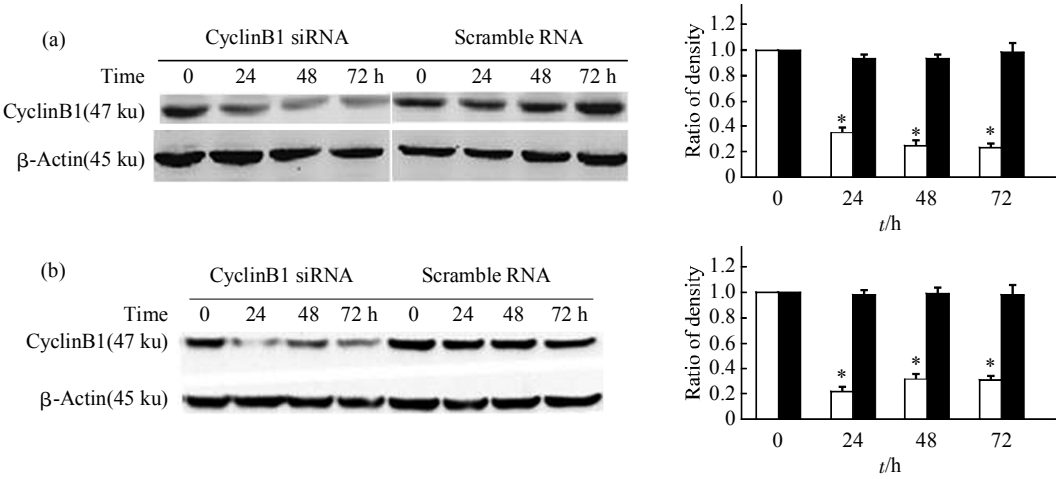

Fig. 1 CyclinB1 siRNA induced CyclinB1 knockdown in HepG2 and HL-7702 cells

HepG2(a) and HL-7702(b) cells incubated with 100 nmol/L CyclinB1 siRNA or scramble RNA for the indicated time were subjected to Western blot analysis with antibodies targeting CyclinB1 and β-actin. Right panels: Quantification from left panels; the data were from three independent measurements ,*$P < 0.01$. □: CyclinB1 siRNA; ■: Scramble RNA.

2.2 Down regulation of Cyclin B1 sensitizes HepG2 cells to treatment of Daunorubicin

To investigate the possible combination of Cyclin B1 knockdown with Daunorubicin in hepatocellular cancer therapy, we treated HepG2 cells with Cyclin B1 siRNA and followed by further incubation of Daunorubicin. The number of apoptotic and survival cells was measured by flow cytometry and as shown in Figure 2a, b, combination of Cyclin B1 siRNA with Daunorubicin triggered more apoptotic response compared to the treatment with Daunorubicin alone in HepG2 cells. To explore the selective effect of Cyclin B1 siRNA combined with Daunorubicin on cancer cells, HL7702 cell, a normal human liver cell line, was used as control. As shown in Figure 2c and Figure 2d, less combinational effects of Cyclin B1 siRNA and Daunorubicin on apoptotic response of HL7702 cell were observed compared to that of HepG2 cell. Those data indicates that Cyclin B1 knockdown might serve as a combination with chemotherapeutical agents in liver cancer treatment.

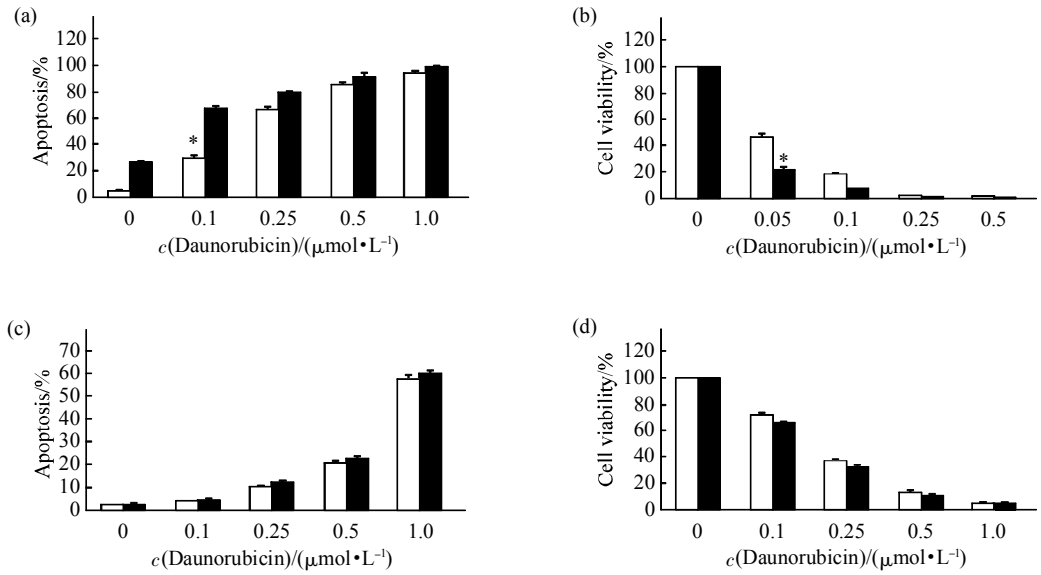

Fig. 2 CyclinB1 down regulation increase cellular sensitivity to Daunorubicin in HepG2 cells

HepG2 cells (a, b) and HL-7702cells(c, d) incubated with 100 nmol/L Cyclin B1 siRNA or scramble RNA for 24 h were treated with Daunorubicin at indicated concentrations for another 48 h, and the cell apoptosis rate and cell viabllity were determined by flow cytometry. The value was expressed as $\bar{x} \pm s$ (n=3), *P < 0.05. □: Scramble RNA; ■: siRNA-Cylin B1.

2.3 Dose kinetics of CyclinB1 siRNA in HepG2 cells

To investigate the dose kinetics of combinational effects, HepG2 cells were first transfected with 0, 1, 50, 100 and 200 nmol/L concentrations of scramble RNA or Cyclin B1 siRNA respectively, followed by the treatment of 0.1 μmol/L Daunorubicin for another 24 h. As shown in Figure 3, the combinational effect of Cyclin B1 siRNA and Daunorubicin on apoptosis and cytotoxicity showed a Cyclin B1 siRNA dose dependent manner in HepG2 cells. HepG2 cells with the treatment of Cyclin B1 siRNA were more sensitive to the incubation of Daunorubicin than that of treated with scramble RNA. Those results further confirm that targeted down regulation of Cyclin B1 would be of a promising strategy in the therapy of hepatocellar carcinoma.

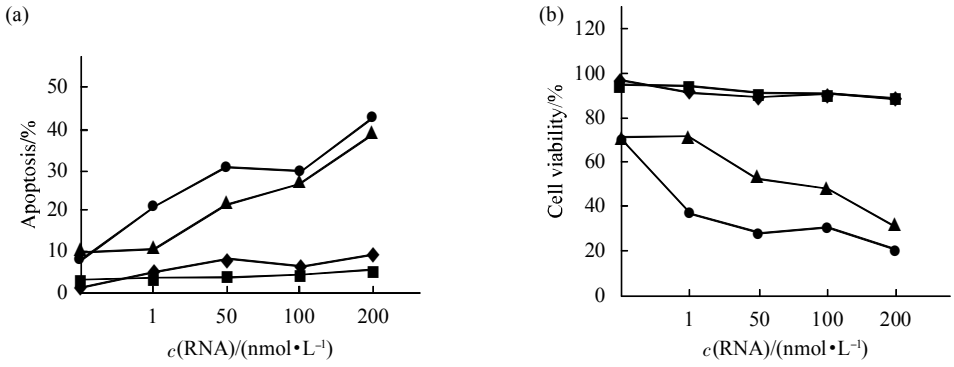

Fig. 3 Dose kinetics of CyclinB1 siRNA on celluar sensitivity to Daunorubicin treatment

HepG2 cells(a, b) incubated with Cyclin B1 siRNA or scramble RNA at the indicated concentrations for 24 h were treated with 0.1 μmol/L Daunorubicin for another 48 h, and the cell apoptosis rate (a) and cell viabllity (b) were determined by flow cytometry.The value was expressed as $\bar{x} \pm s$ (n=3). ◆—◆: Scramble RNA; ▲—▲: Scramble RNA+Dau 0.1 μmol/L; ■—■: siRNA-Cyclin B1; ●—●: siRNA-Cyclin B1+Dau 0.1 μmol/L.

2.4 siRNA induced Cyclin B1 knockdown reduces colony growth ability in HepG2 cells

As Cyclin B1 is an essential modulator of cell cycle progression, we next assessed the effects of Cyclin B1 siRNA on cell cycle distribution using flow cytometry. After incubation with 100 nmol/L of Cyclin B1 siRNA for 48 h, 40% of HepG2 cells were arrested in G2/M phase and no obvious G2/M phase arrest was observed in HepG2 cells incubated with scramble RNA (Figure 4a). In contrast to tumor cell line, only 5% of G2/M phase arrest was observed in HL7702 cell, the human normal liver epithelial cell line (Figure 4b). The difference of CyclinB1 knockdown induced G2/M phase arrest between HepG2 and HL7702 cell might be due to the low proliferation ability of HL7702 cell. It is interesting to find out the effect of Cyclin B1 down regulation on tumor cell colony growth ability. It was found that more and bigger clones appeared in HepG2 cells transfected with scramble RNA compared to that treated with Cyclin B1 siRNA, and colony growth ability of HepG2 cells was shown to be greatly suppressed by application of 0.1 μmol/L of Daunorubicin, and no clone was observed in HepG2 cells incubated with 100 nmol/L of Cyclin B1 siRNA and 0.1 μmol/L of Daunorubicin (Figure 4c).

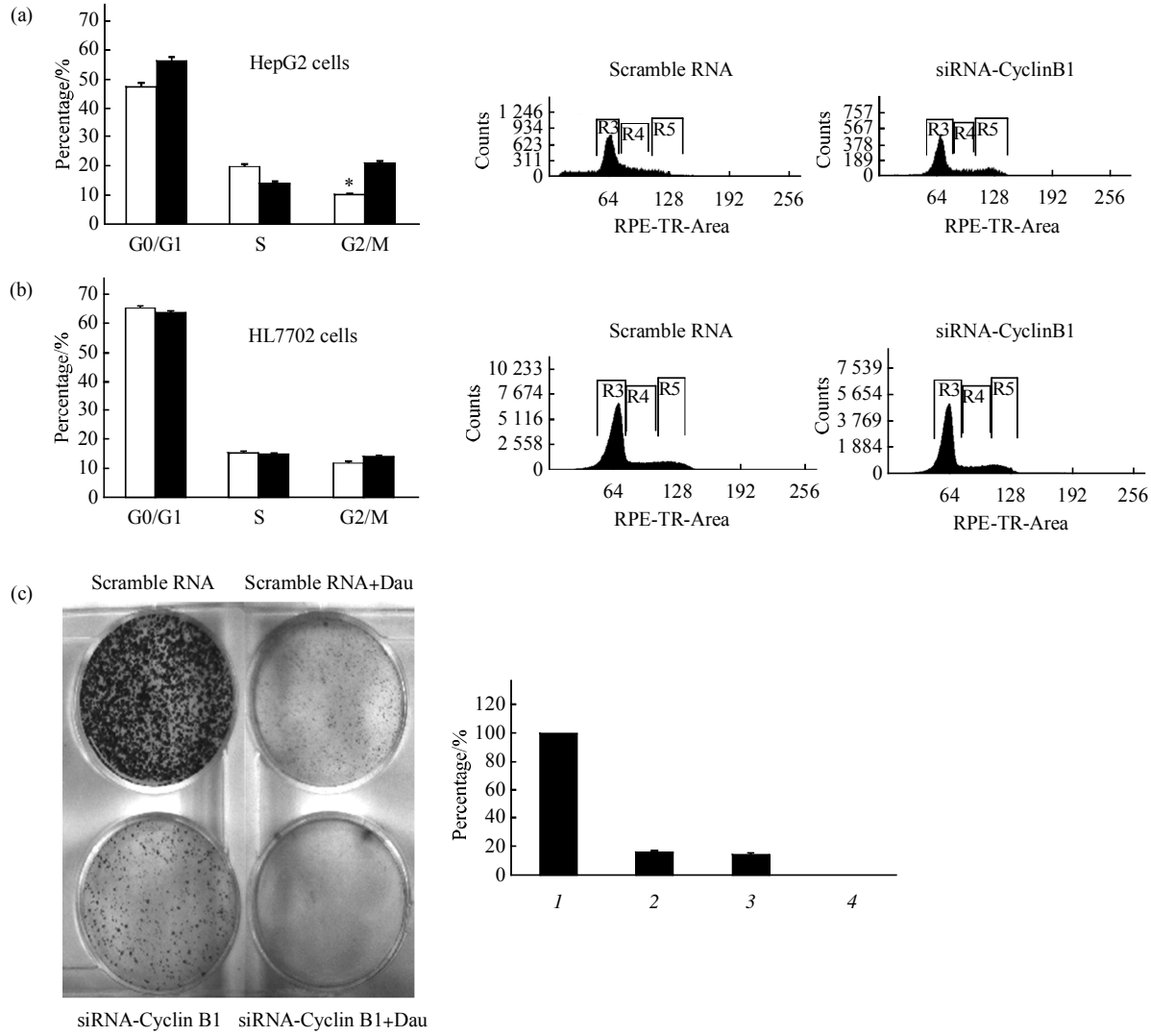

Fig. 4 Cyclin B1 siRNA induced Cyclin B1 knockdown triggers cell cycle arrest and supresses cell colony growth abilty

HepG2 cells (a) and HL-7702cells (b) treated with Cyclin B1 siRNA or scramble RNA for 48 h and cell cycles were analyzed by the use of flow cytometry. Right panels: cell cycle measurements using flow cytometry. Left panels: Quantification of cell cycle distributions from right panels. The data were from three independent measurements ,*$P < 0.05$. □: Scramble RNA; ■: siRNA. (c) HepG2 cells transfected with Cyclin B1 siRNA or scramble RNA for 24 h were plated to 6 wells at the density of 1×10^3 and 0.02 μmol/L Daunorubicin was added to the cells. The cells were cultured for another 14 d, and colony numbers were scored with a microscope. The colony number of control cells without Daunorubicin was referred as 100% for calculation. The data were based on three independent colony-forming assays. *1*: Scramble RNA; *2*: siRNA-Cyclin B1; *3*: Scramble RNA+Dau; *4*: siRNA-Cyclin B1+Dau.

3 Discussion

Cyclin B1, the subunit of MPF, is aberrantly expressed in various primary tumors, and the expression of Cyclin B1 is showed to be positively regulated by c-MYC and H-ras [16-17] and negatively regulated by p53 and BRCA1 [18-19]. Overexpression of Cyclin B1 is believed to be a significant prognostic indicator in patients with NSCLC and squamous cell carcinoma [8]. Those data suggest that Cyclin B1 is implicated in tumorigenesis and suppression of kinase activity of MPF would be of a promising strategy for the inhibition of tumor cell proliferation. Indeed, Yuan, *et al* reported that siRNA induced depletion of Cyclin B1 greatly inhibited the kinase activity of cdc2/Cyclin B1 in HeLa cells, and that proliferation ability was reduced and apoptosis rate was increased by the suppression of Cyclin B1 in MCF-7, MDA-MB-435, BT-474 and HeLa cells [14]. The same group subsequently verified that targeting CyclinB1 knockdown render breast cancer cells susceptible to taxol, suggesting that targeting Cyclin B1 could be of an attractive strategy for the combination with anti-cancer agents in cancer therapy[20].

In this study, we for the first time provide evidence for combinational effect of CyclinB1 knockdown and Daunorubicin on apoptosis and proliferation in liver cancer cells. The CyclinB1 targeting siRNA could efficiently abolish the expression of CyclinB1 protein and induce obvious G2/M arrest in HepG2 cells, whereas only slight G2/M arrest was observed in CyclinB1 siRNA transfected HL-7702 cells, a normal human endothelial liver cancer cell line. The colony growth ability of HepG2 cells was also suppressed by the down regulation of CyclinB1, suggesting that tumorigenic growth ability of HepG2 cells is, at least in part, depended on the high expression of CyclinB1. The combination of 100 nmol/L CyclinB1 siRNA and different concentration of Daunorubicin is proved to trigger more apoptotic response and cell death in HepG2 cells compared to that treated with Daunorubicin alone, and this result was further confirmed by incubating HepG2 cells with a dose variation of CyclinB1 siRNA and 0.1 μmol/L Daunorubicin. To test selective combination effect of CyclinB1 siRNA and Daunorubicin, HL-7702 cell, a normal liver cell, was utilized, and it was proved that less apoptotic response was induced by the combination of CyclinB1 siRNA and Daunorubicin in HL-7702 cell compared to that in HepG2 cells. Those results support the notion that Daunorubicin combined with CyclinB1 knockdown would be an effective and selective strategy for liver cancer therapy.

Daunorubicin is widely used in the treatment of a wide range of cancers, including breast cancer, ovarian cancer, bladder cancer and liver cancer. Despite enhancing the life quality and overall survival of cancer patients, high dose of Daunorubicin also shows severe side effect, leading to severe life threatening heart damage. Therefore, new methods are needed to lower Daunorubicin dosage without losing its effectiveness to limit side-effect. The data from this work demonstrate that CyclinB1 siRNA could sensitize HepG2 cells to Daunorubicin, thereby reducing the side effect by lowing down the dosage of Daunorubicin in the treatment of liver cancer. Inhibiting CyclinB1 function combined with chemotherapeutical agents would be a promising approach to the treatments of cancers with high level of CyclinB1 expression.

In summary, this work provide further evidences for the notion that CyclinB1 could be an attractive target for cancer therapy, and CyclinB1 targeting knockdown in combination with anticancer chemotherapeutical agents would be of a potential effective strategy in which the side effects of chemotherapeutical agents could be significantly reduced in the treatment in liver cancer.

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siRNA 诱导 CyclinB1 沉默增加 HePG2 细胞对柔红霉素的敏感性 *

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摘要 探讨了 CyclinB1 在肝癌药物耐受形成中的作用．用 siRNA 技术沉默 CyclinB1 在细胞中的表达，使用流式细胞仪技术分析细胞的凋亡和周期分布，用细胞克隆形成能力分析和细胞毒性实验分析细胞的增殖能力．由 siRNA 诱导的 CyclinB1 下调导致 40%～50%肝癌细胞阻滞在 G2/M 期，并显著抑制肝癌细胞的单克隆形成能力；柔红霉素联合 CyclinB1 siRNA 较单独使用柔红霉素能更加有效地导致肝癌细胞凋亡，而在人正常肝细胞 HL-7702 中这种现象不明显．实验结果表明针对于 CyclinB1 的靶向性下调与肝癌药物的联合使用将有可能成为肝癌治疗的新策略．

关键词 CyclinB1，siRNA，柔红霉素，药物协同作用，耐药性

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