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Let-7 g microRNA sensitizes fluorouracil-resistant human hepatoma cells

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Let-7 microRNA is expressed in lower lever in a variety of human tumors and is involved in tumorigenesis. This study investigated the inhibitory effect of the let-7 g microRNA on the expression of the HMGA2 gene in the fluorouracil (5-Fu)-resistant human hepatoma cell line Bel-7402/5-Fu, and the effect of let-7 g microRNA on drug sensitization in Bel-7402/5-Fu cells. Let-7 g microRNA and negative microRNA plasmids were constructed and transient transfected into Bel-7402/5-Fu cells. Expression levels of HMGA2 mRNA and protein in microRNA transient transfectants were clearly reduced as compared with negative microRNA transfectants and untreated cells. Flow cytometry revealed increased in S phase in let-7 g microRNA cells. dimethylthiazol-diphenyltetrazolium bromide (MTT) results indicated that microRNA transfectants had a higher cell inhibition rate than the negative vector or untreated cells after treatment with 0.13–13 μg/ml 5-Fu. In addition, cyclinA was down-regulated in the let-7 g transfectants cells.The results showed that let-7 g microRNA contributed to an increase of 5-Fu-induced cell cycle inhibit in human hepatoma cell and sensitized cells to 5-Fu, leading to increased the effectiveness of the drug in treating hepatoma cancer.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies and is responsible for 1,250,000 deaths per year worldwide. Multidrug resistance to chemotherapeutic agents is a major obstacle to potentially curative treatments for advanced HCCs. The mechanisms involved in drug resistance are complex and multifactorial and may be due to inadequate drug exposure or alterations in the cancer cell itself (Baird et al. 2003). Thus, new methods to improve the clinical response to chemotherapy are required. Gene therapy for malignant disease is a promising approach.

microRNAs (miRNAs) are a class of 18–24nt long small noncoding RNAs that regulate gene expression (Ambros et al. 2003). MicroRNAs are processed from nascent primary transcripts (pri-microRNA) by dsRNA-specific endonucleases, Drosha and Dicer, which are known to negatively regulate gene expression mainly through interactions with 3'-untranslated regions of their target genes (Ambros et al. 2003). Microarray-based measurements of microRNA levels between a large number of primary tumors and derived cell lines have shown that global expression levels of these noncoding RNAs are significantly reduced when compared with matched normal tissues (Lu et al. 2005; Johnson et al. 2005; Takamizawa et al. 2004). Specific over- or underexpression has been shown to correlate with particular tumor types (Volinia et al. 2006; Calin et al. 2004; He et al. 2005; Cummins et al. 2006). MicroRNA overexpression could result in down-regulation of tumor suppressor genes, accordingly, underexpression could lead to oncogene up-regulation.

High mobility group A2 (HMGA2) protein is a non-histone architectural transcription factor, which is a member of the HMGA family. This family is constituted by HMGA1a, HMGA1b, HMGA1c, and HMGA2 (Reeves et al. 2001; Boo et al. 2005). HMGA2 protein contains three DNA-binding domains, which have been named AT-Thooks, due to their ability to interact with the minor groove of AT-rich DNA sequences (Reeves et al. 2001). HMGA2 is expressed at very high levels during embryonic development whereas it is almost undetectable in differentiated cells (Chiappetta et al. 1996; Rogalla et al. 1996). Cyclin A gene as a cellular target for HMGA2 and suggest a mechanism for HMGA2-dependent cell cycle regulation (Tessari et al. 2003). Despite this evidence, the molecular events and the precise role played by HMGA2 in cell proliferation and tumorigenesis still need to be defined.

Here, we through MiRCURYTM LNA Arrays analyse confirmed that microRNAs expression differed between Bel-7402 and Bel-7402/5-Fu cell line. So The aim of this study was to gain insight into certain some miRNAs may regulate expression of HMGA2, accordingly lead to drug resistance in HCC.

2. Investigations and results

2.1. Part let-7 family members and some other microRNAs expressed in higher lever in Bel-7402/5-Fu than in Bel-7402 cell line

To investigate microRNAs expression differently between Bel-7402 and Bel-7402/5-Fu cell line MiRCURYTM LNA Arrays

Abbreviations: Bel-7402/5-Fu, fluorouracil-resistant human hepatoma cell line; 5-Fu, fluorouracil; HMGA2, high mobility group-A2; MTT, 3-[4,5-Dimethylthiazolzyl]-2,5-diphenyl tetrazolium bromide; Q-RT-PCR, quantitative real-time reverse transcription polymerase chain reaction.

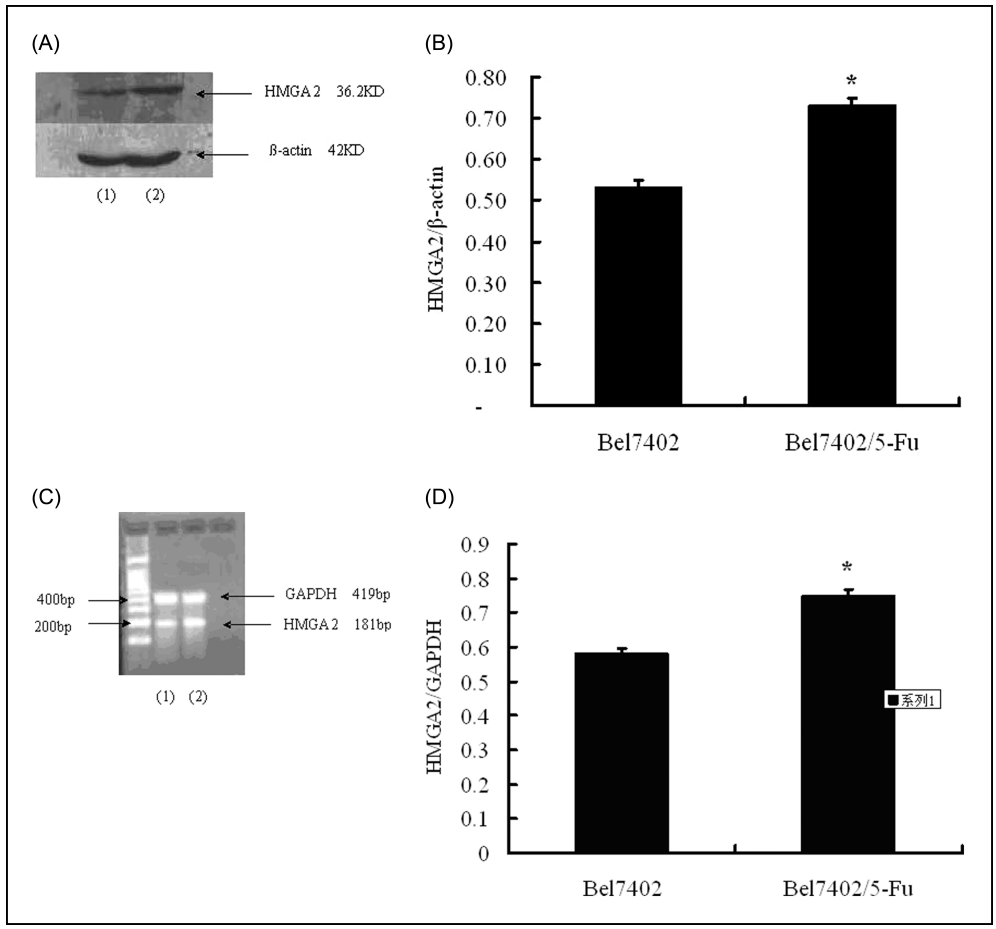

Fig. 1: Expression of HMGA2 in human hepatoma cell line Bel-7402 and fluorouracil (5-Fu)-resistant cell line Bel-7402/5-Fu. (A) Western blotting detected HMGA2 protein expression. 1, Bel-7402; 2, Bel-7402/5-Fu. (B) HMGA2 protein expression was increased in Bel-7402/5-Fu .HMGA2/β-actin ratio. *P < 0.05 vs. Bel-7402. (C) RT-PCR detected HMGA2 mRNA expression. 1, Bel-7402; 2, Bel-7402/5-Fu. (D) HMGA2 mRNA expression was increased in Bel-7402/5-Fu. HMGA2/GAPDH ratio.*$P < 0.05$ vs. Bel-7402.

were used for sensitive, specific, and comprehensive profiling of microRNAs. Every sample was repeated for four times to improve the miChip accuracy. Figure 1 shows an image of a microarray hybridized with Bel-7402 and Bel-7402/5-Fu RNAs. The profiles of microRNAs expression change were gained based on the data of the microarray. Compared with Bel-7402 and Bel-7402/5-Fu, there were 2 differentially up-regulated (>2) microRNAs and 390 down-regulated microRNAs (<0.5). Differentially up-regulated microRNAs included miR-192 and miR-194, while differentially down-regulated microRNAs included let-7d, let-7e, let-7f, let-7 g, miR-98, miR-195, miR-127-3p, miR-122 (Table) and so on.

2.2. Overexpression of HMGA2 in 5-Fu-resistant cells

To evaluate the underlying mechanisms of resistance to 5-Fu, Western blot and RT-PCR were used to evaluate HMGA2 expression. The results revealed elevated protein and mRNA levels in Bel-7402/5-Fu cells compared to Bel-7402 cells (Fig. 1A, B, C, D).

2.3. Transfect let-7 g microRNA into Bel-7402/5-Fu cells

Bel-7402/5-Fu cells were transfected with Lipofectamine 2000 *in vitro*. After 48 h, the fluorescence microscope detected transfection efficiency. Negative microRNA had the same transfection efficiency as let-7 g microRNA, with transfect efficiency > 35% (Fig. 2A, B).

Table: miRNA microarray expression data between Bel-7402 and Bel-7402/5-Fu cells

A. Down-regulated miRNAs		
miRNA name	Fold change	P-value
hsa-let-7d	0.3615	0.0085
hsa-let-7e	0.3271	0.0105
hsa-let-7f	0.1117	0.0031
hsa-let-7g	0.1406	0.0064
hsa-miR-98	0.1114	0.0003
hsa-miR-195	0.0818	0.0211
hsa-miR-127–3p	0.0841	0.0124
hsa-miR-122	0.2007	0.0027
B. Up-regulated miRNAs		
miRNA name	Fold change	P-value
hsa-miR-192	2.4632	0.0033
hsa-miR-194	2.0494	0.0045

Shown are all arrays in which the miRNAs change significantly, and the direction of change (A. down-expression or B. up-expression) in Bel-7402 and relative to Bel-7402/5-Fu cells. MiR-122 had a nearly 5-fold decreased in Bel-7402/5-Fu cells comparing with parental Bel-7402 cells.

2.4. Inhibition of HMGA2 protein expression by let-7 g microRNA

After transfected 48 h,HMGA2 protein level in Bel-7402/5-Fu cells transfected was analysed by western blot. The HMGA2 protein of Bel-7402/5-Fu cells transfected with let-7 g microRNA was significantly less than that of the negative or normal groups (Fig. 3A, B).

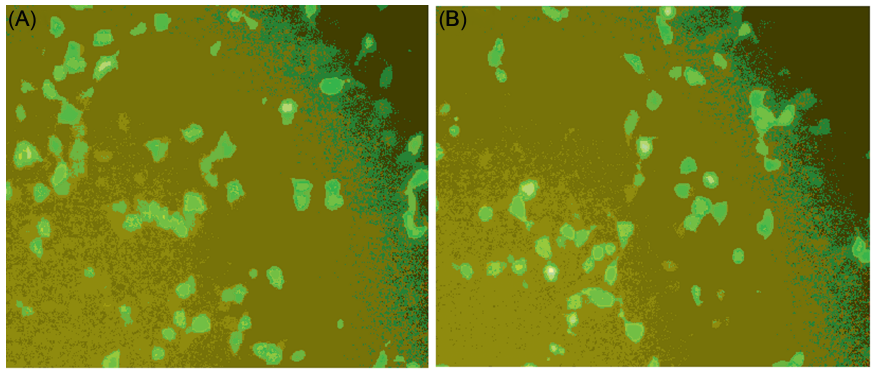

Fig. 2: Transfection efficiency of let-7 g and negative microRNA. After 48 h, fluorescence microscope detect transfection efficiency. Negative microRNA has the same transfect efficiency as let-7 g microRNA,the transfect efficiency > 35%. (A) Let-7 g microRNA. (B) Netagive microRNA.

2.5. Let-7 g microRNA influence on cyclinA protein expression through HMGA2

To examine whether the cyclinA protein was also silenced, its expression was measured by Western blotting. The negative vector had no significant effect on cyclinA expression. CyclinA protein was expressed at a higher level in normal cells and cells transfected with negative vector than in the cells transfected with let-7 g microRNA vector. Moreover, cyclinA protein expression was clearly decreased in cells transfected with let-7 g microRNA (Fig. 4A, B).

2.6. Growth inhibiting induced by microRNA

Flow cytometry showed that let-7 g microRNA cells had a markedly increased S phase population compared with negative microRNA or normal cells (Fig. 5A , B, C, D).

2.7. Influence of HMGA2 down-regulation on cell susceptibility to 5-Fu-induced death

MTT assay results showed that let-7 g transfectants had a lower cell viability and higher inhibition rate than negative control cells or untreated cells after treatment with various concentrations of 5-Fu: 0.013 μg/ml (12.38 ± 0.74 % *vs*. 1.81 ± 0.42 % or 2.24 ± 0.48 %); 0.13 μg/ml (12.68 ± 0.67 % *vs*. 8.14 ± 0.51 % or 7.72 ± 0.52 %); 1.3 μg/ml (34.36 ± 0.87 % *vs*. 11.78 ± 0.58 % or 12.90 ± 0.49 %); 13 μg/ml (37.04 ± 0.9 % *vs*. 12.50 ± 0.73 % or 13.18 ± 0.6 %). ($P < 0.05$ *vs*. Negative microRNA cells or normal cells). The inhibition rate showed a dose-dependent effect of 5-Fu (Fig. 6).

3. Disscussion

In this study, we show that microRNAs expression differend between Bel-7402/5-Fu cells and Bel-7402 cells. Some let-7 family members, such as let-7d, let-7e, let-7f, let-7 g, expressed miR-98 two times lower in Bel-7402/5-Fu cells than in Bel-7402 cells. The let-7 microRNAs, which are down-regulated in several cancers, can negatively regulate the oncogenes RAS and HMGA2 (Johnson et al. 2005; Boyerinas et al. 2008), providing a mechanism for the up-regulation of these oncogenes. There are two reasons why we choose HMGA2 as the target of let-7 in this study: (1) HMGA2 was the strongest let-7 target (Park et al. 2007); (2) HMGA2 is a more sensitive target of let-7 than RAS and RAS is not a major taeget of endogenous let-7 in some cancer cells and there is no inverse correlation between the expression of RAS and of either let-7 g, let-7d, let-7a/f (Doench et al. 2004). There are many variants of let-7 miRNA, let-7a to let-7i and miR-98. All the variants share an identical seed sequence that is critical for target recognition (Yang et al. 2008). So, we choose let-7 g as a deputy of the let-7 family. Members of the let-7 family function biologically as tumor suppressors and their loss is predicted to promote transformation and tumor progression.We transfect let-7 g into Bel-7402/5-Fu cells causes growth inhibition and cell cycle changes.

HMGA2 plays an important role in promoting cell proliferation and differentiation. CyclinA is a direct target gene of HMGA2 and is consistent with the role proposed for HMGA2 in cell transformation and cell growth during embryogengsis (Tessari et al. 2003). In this study, we also show higher HMGA2 expression in Bel-7402/5-Fu cells than in Bel-7402 cells. Transfection let-7 g into Bel-7402/5-Fu cells, reduced the expression

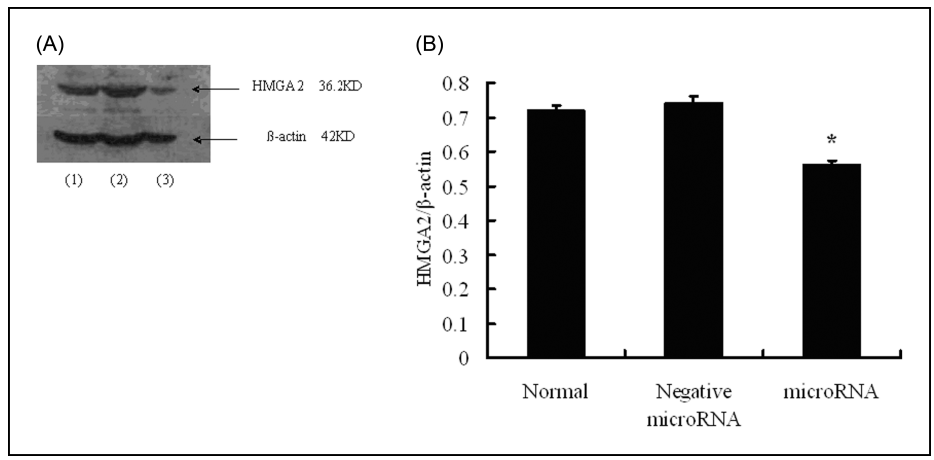

Fig. 3: Effects of let-7 g microRNA on HMGA2 protein expression. Western blot detected HMGA2 proteins expression. (A) HMGA2 protein: 1, Normal; 2, negative microRNA; 3, let-7 g microRNA transfected cells. HMGA2 protein expression was significantly reduced at let-7 g microRNA transfected cells. (B) HMGA2/β-actin ratio. *$P < 0.05$ vs. Negative microRNA or normal group.

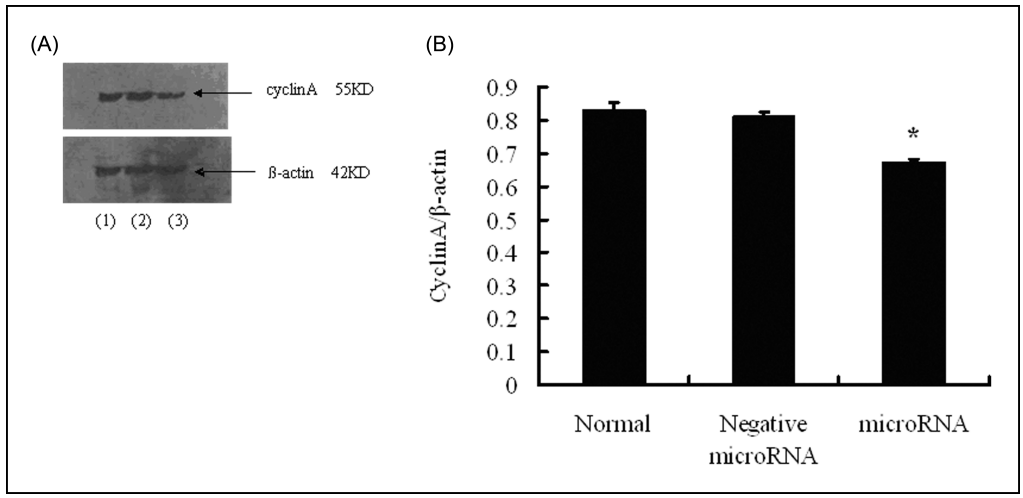

Fig. 4: Effects of let-7 g microRNA on cyclinA protein expression. Western blot detected cyclinA proteins expression. (A) cyclinA protein:1, Normal; 2, negative microRNA; 3, let-7 g microRNA transfected cells. cyclinA protein expression was significantly reduced at let-7 g microRNA transfected cells. (B) cyclinA/β-actin ratio. *$P < 0.05$ vs. Negative microRNA or normal group.

of HMGA2 and CyclinA. Flow cytometry showed that let-7 g microRNA cells had a markedly increased S phase population compared with negative microRNA or untreated cells, this predicted that let-7 g, mainly through suppressing HMGA2 and CyclinA expression, inhibits cell proliferation. We also found that let-7 g transfectants are more sensitive to 5-Fu than untransfectants and it was certified that 5-Fu is a drug specificity killing the cells in S phase. This may illustrate that reduced let-7 g plays an important role in 5-Fu-resistance by change of cell cycle.

The development of microRNA expression technology has made it possible to increase or decrease specific miRNA expression. This technology will be very useful in developing new treatments for cancer,because microRNA expression differs between normal and malignant cells (Yang et al. 2008 Wurdinger et al. 2007). Our study results on human hepatoma cells resistant to

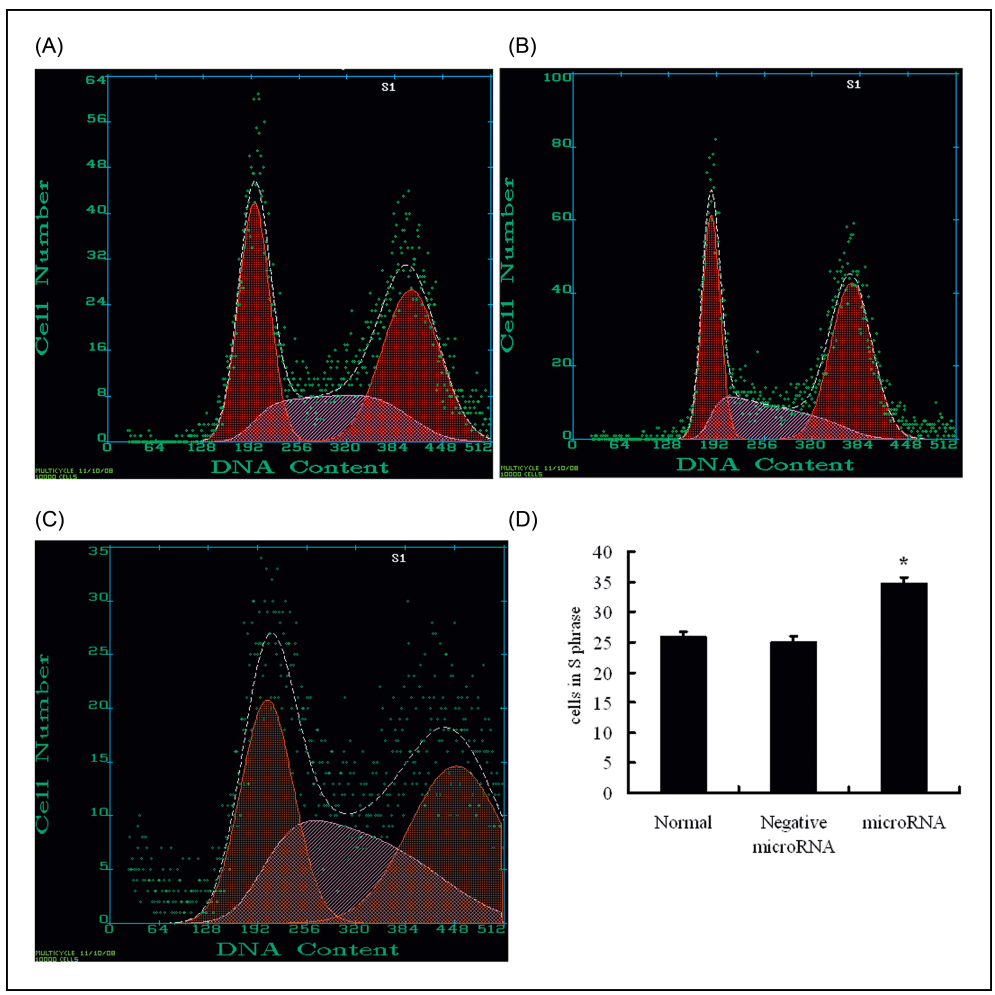

Fig. 5: Flow cytometry analysis let-7 g influenced on cell cycle. (A) Normal cells. (B) Negative microRNA transfected cells. (C) let-7 g microRNA transfected cells. (D) Flow cytometry showed that transfected with let-7 g microRNA was significantly increased in S-phase cell cycle than negative microRNA or normal groups. The mean ± SD of hypodiploid cells from three determinations are shown. ANOVA analysis is used. *$P < 0.05$ vs. Negative microRNA or normal group.

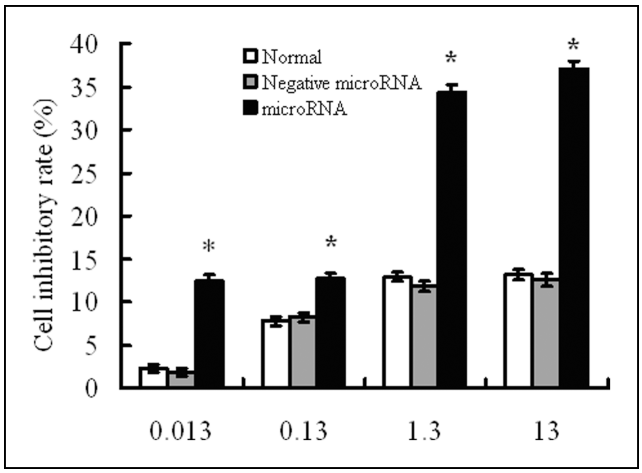

Fig. 6: Inhibition of let-7 g microRNA on fluorouracil (5-Fu)-resistant human hepatoma cell line Bel-7402/5-Fu. ANOVA analysis shows the mean difference of the let-7 g microRNA transfected group is significant at the concentration of 0.013, 0.13, 1.3 and 13 μg/ml of 5-FU. *$P < 0.05$ vs. Negative microRNA or normal group.

5-Fu suggest that let-7 g and HMGA2 proteins are good targets for cancer therapy (Malek et al. 2008), especially for cancers resistant to conventional chemotherapy. Nevertheless, *in vivo* delivery and tumor specificity are challenging issues for the use of microRNA as an anticancer therapeutic agent. Development of genetic vectors or formulations for *in vivo* delivery of miRNA will be necessary before miRNA can be used as a therapeutic agent.

4. Experimental

4.1. microRNA vector construction

pcDNATM6.2-GW/EmGFP-miR linear vector was purchased from GenePharma (Shanghai,China). Let-7 g insert sequence with the following sense: 5'-TGCTGTGAG GTAGTAGTTTGTACAGTTGTTTT GGCCACTGACTGACAACTGTACACTACTACCTCA-3' and antisense sequences were: 5'-CCTGTGAGGATGTAGTGTACAGTT GTCAGTCAGTGGCCAAAACAACTGTACAAACTACTACCTCA-3'. The sequence of transcript is 5'-TGAGGTAGTAGTTTGTAC AGTTGTTTTGGCCACTG ACTGACAACTGTACACTACTACCTCA-3' which can form a hairpin and processed into mature microRNA let-7 g (5'-TGAGGTAGTAGTTTGTACAGTT-3'). A negative control vector that expresses a hairpin shRNA with limited homology to any known sequences of human genome was commercially available (GenePharma). Plasmid DNA was purified by cesium chloride bromide gradient centrifugation. The purified DNA was diluted to 1 mg/ml and stored at − 20 °C until used.

4.2. Cell culture and transfection

The human hepatoma cell line Bel-7402 and the 5-fluorouracil-resistant cell line Bel-7402/5-Fu were purchased from Kaiji Cell Center (Nanjing,China). The cells were routinely maintained in phenol red-free 1640 (Gibco BRL, Grand Island, NY) containing 10 % fetal bovine serum (Hyclone, Logan, USA), at 37 °C in a humidified incubator containing 5 % CO_2, and for Bel-7402/5-Fu, 2 μg/ml 5-Fu was added. Transfection was carried out according to the manufacturer's protocol. Let-7 g and negative plasmid was diluted 4 μg with 250 μl OPTI-MEM (Invitrogen) and 5 μl Lipofectamine 2000 (Invitrogen) with 250 μl OPTI-MEM. After 5 min, the dilutions were mixed together and incubated at 37 °C for 25 min, then dispensed into each cell culture flask. Forty-eight hours after transfection, fluorescence microscope detect transfection efficiency.

4.3. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from the cells using TRIzol reagent (Invitrogen, USA) according to the manufacturer's protocol. Real-time quantitative PCR was performed using standard protocols on an Applied Biosystem's 7500 HT Sequence Detection System. Briefly, 5 μl of a 1/100 dilution of cDNA in water was added into 12.5 μl of the 2 × SYBR green PCR master mix (Ambion), with 800 nmol/L of each primer in a total volume of 25 μl. The sense and antisense primers for HMGA2 gene are 5'-CCAGGAAGCAGCAGCAAG-3' and 5'-CTTGGAGCTGGTTCTTGGT AG-3'. The sense and antisense primers for GAPDH gene are 5'-GACCACAGTCCATGCCATCAC-3' and 5'-CATACCAGGAAATGAGCTTGAC-3'. All reactions were run in trip-licate and included no template and no reverse tran-scription controls for each gene. The cycle number at which the reaction crossed an arbitrarily placed threshold (CT) was determined for each gene, and the relative amount of each mRNA to U6 RNA was calculated using the equation 2−ΔCT, where ΔCT = (CTmiRNA − CT5 s). Relative gene expression was multiplied by 106 to simplify the presentation of the data.

4.4. Western blotting

We homogenized cells in a lysis buffer containing 1 % NP40, 0.5 % sodium deoxycholate, 0.1 % SDS, 50 g/ml aprotinin, 100 g/ml phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 50 mM sodium fluoride and PBS (pH 7.4). Cell lysates were centrifuged at 10,000 g for 10 min at 4 °C. Protein content in the supernatants was determined by a BCA protein assay kit (Sijiqing Co. HangZhou). Equal amounts of lysate protein were run on 10 % SDS-PAGE and transferred to PVDF membranes (Amersham-Pharmacia Biotech). After blocking, we incubated PDVF membranes with mouse anti-HMGA2 primary monoclonal antibody at dilution of 2:1000 (Abnova Coporation), anti-cyclinA primary monoclonal antibody at dilution of 1:2000 (Cell Signaling Co.), anti-β-actin primary monoclonal antibody at dilution of 2:1000 (MultiSciences Biotech Co,.Ltd) for overnight at 4 °C and further incubated for 1 h with peroxidase conjugated Goat anti-mouse IgG secondary antibody at dilution of 1:1000. Bound antibodies were detected by Enhanced chemiluminescence (ECL) kit with a Lumino Image Analyzer (Taitec, Tokyo, Japan).

4.5. Flow cytometry detect the change of cell cycle

All cells washed twice with PBS and fixed with 70 % ethanol overnight at 4 °C. Then the cells were washed once with PBS and stained with 800 μl propidium iodide (50 mg/l, Sigma, St.Louis, USA) at room temperature for 30 min to determine the cell cycle diffusion of the cells by flow cytometry (EPICS-XL,Beckman Coulter, Fullerton, USA) and data were analyzed with CellQuest software version 3.3 (Becton Dickinson, San Jose, USA).

4.6. MTT assay

Cells from the above groups were seeded into a 96-well plate at 1×10^4 cells per well. After 24 h, transfect let-7 g micrpRNA and negative microRNA into Bel-7402/5-Fu cells. Various concentrations of 5-Fu were added 48 h after transfection. And cells were incubated for another 24 h. Then cells were treated with MTT (5 g/l, Sigma) for 4 h at 37 °C, and 200 μl dimethysl sulphoxide (DMSO) was added in each well for 10 min. The reaction was optically monitored at 570 nm (A_{570}) using a 96-well microtitre plate reader (Pharmacia, Piscataway, USA). All experiments were carried out in triplicate. The inhibitory rate(IR) of Bel-7402/5-Fu cells was calculated according to the following: equation IR(%) = [A570(control) − A570(drug)]/A570(control) × 100% where A_{570} (control) was the absorbance in let-7 g or negative vector transfected cells or untreaed cells, and A570 (drug) was the absorbance in the drug-treated group.

4.7. Statistical analysis

Statistical analysis was performed using SPSS software (Version 11.0,SPSS Incorporation,Chicago,USA).Data were expressed as mean ± SD and analyzed by one-way analysis of variance (ANOVA) and least significant difference (LSD) test, $P < 0.05$ was considered significant.

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