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Downregulation of MDM2 expression by RNAi inhibits LoVo human colorectal adenocarcinoma cells growth and the treatment of LoVo cells with *mdm2*siRNA3 enhances the sensitivity to cisplatin

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Abstract

To investigate the biological effect of *mdm2* in human colorectal adenocarcinoma LoVo cells, three *mdm2*siRNA constructions were recombinated and transient transfected into human colorectal adenocarcinoma LoVo cells with low differentiation character in vitro. The results showed that *mdm2*siRNA3 reduced mRNA level of *mdm2* and protein level of *mdm2*, leading to proliferation inhibition on LoVo cells, and reduced tumor growth in nude mice. It was found that depletion of MDM2 in this pattern promoted apoptosis of LoVo cells and Cisplatin (DDP) treated in the *mdm2*siRNA3 transfected cell population would result in a substantial decrease by MTT colorimetry. Decreasing the MDM2 protein level in LoVo cells by RNAi could significantly inhibit tumor growth both in vitro and in vivo, which indicated that *mdm2* gene played a definite role in the development and aggressiveness of human colon carcinoma. It also could be a therapeutic target in colorectal carcinoma. The synergistic activation of RNAi and cell toxicity agents indicated that the combination of chemotherapy and gene therapy will be a promising approach in the future.

Keywords: *mdm2*; Colonic carcinoma; LoVo; RNA interference

The mdm2 (murine double minute 2) gene and its protein MDM2 was originally found to be amplified and overexpressed in a mouse BALB/c 3T3 cell line [5]. MDM2 appears to play a role in many normal physiological [20] and pathological [23] pathways. It has been known that MDM2 as an oncogene [19] promotes tumor cells growth [13]. *mdm2* gene coded for a 491 amino acid residue protein [14] with a zinc-binding RING finger motif [12]. It is mapped [17] on human chromosome 12q13-14. MDM2 is believed to be involved in most aspects of cellular functions, including transcription [24], cell proliferation [10], differentiation [21], and apoptosis [6]. Amplification [28] or overexpression [7] of *mdm2* has been found in a variety of human carcinomas, and it has been correlated with a progression potential for malignancy [16] and resistance to chemotherapy [36]. The role of MDM2 in colon carcinoma [15] has been extensively examined in many studies for the past decade. Overexpression of MDM2 was found in most colon carcinoma patients [1] and was correlated with poor prognosis [41] in those patients.

In this study, we first investigated MDM2 in a colon carcinoma cell line in which this protein was overexpressed might result in the inhibition of cell proliferation in vitro and in vivo. We designed, synthesized, and utilized small interfering RNAs (siRNAs) that were selective for *mdm2* to investigate their effectiveness in altering the repair capacity of the cells to cisplatin-DNA damage as well as the resistance of the cells to cisplatin. Therefore, *mdm2* is an

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attractive target to confer increased cellular sensitivity to cisplatin-based chemotherapy.

Methods

Plasmid construction. To generate MDM2 knockdown construction, one annealed set of oligonucleotides encoding short hairpin transcripts corresponding to *mdm2* mRNA (GenBank Accession No. NM-010786) were cloned into p*Silencer* 2.1_U6 (Ambion; hereafter abbreviated to p*Silencer*). In brief, the short-hairpin-RNA-encoding complementary single-stranded oligonucleotides, which hybridized to give overhangs compatible with *Apa*I and *Eco*RI, were designed with a computer program available on the Internet http://www.ambion.com/techlib/misc/psilencer_converter.html. Oligonucleotides encoding short hairpin RNAs were then ligated into p*Silencer*. Bacterial colonies were pooled and used for plasmid preparation. The positive clones were confirmed by sequencing.

Cell line and cell culture. The human colorectal adenocarcinoma cell line LoVo was obtained from the Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. The cells were grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), 50 U/ml penicillin, and 50 μg/ml streptomycin. The LoVo cells were maintained in a humidified 37 °C incubator with 5% CO_2, fed every 3 days with complete medium (contain 10% FBS), and subcultured when confluence was reached.

mdm2siRNA. mdm2 siRNA1 is from nt 1828 to 1892 in *mdm2* mRNA (Fig. 1)

5′-GATCCGCCCTAGGAATTTAGACAACTTCAAGAGAGTTG
TCTAAATTCCTAGGGTTTTTTGGAAA-3′
5′-AGCTTTTCCAAAAAACCCTAGGAATTTAGACAACTCTC
TTGAAGTTGTCTAAATTCCTAGGGCG-3′

mdm2 siRNA2 is from nt 1918 to 1982 in *mdm2* mRNA (Fig. 1)

5′-GATCCGTTGACCTACTTTGGTAGTGTTCAAGAGACAC
TACCAAAGTAGGTCAATTTTTTGGAAA-3′
5′-AGCTTTTCCAAAAAATTGACCTACTTTGGTAGTGTCTCT
TGAACACTACCAAAGTAGGTCAA CG-3′

mdm2 siRNA3 is from nt 2208 to 2272 in *mdm2* mRNA (Fig. 1)

5′-GATCCGTTAGCTTGGCCTACAGTCATTCAAGAGATGACT
GTAGGCCAAGCTAATTTTTTGGAAA-3′
5′-AGCTTTTCCAAAAAATTAGCTTGGCCTACAGTCATCTCT
TGAATGACTGTAGGCCAAGCTAA CG-3′

Transfection of cells. A total of 2×10^5 cells were seeded into each well of a 6-well tissue culture plate (Costar). The next day (when the cells were 70–80% confluent), the culture medium was aspirated and the cell monolayer was washed with prewarmed sterile phosphate-buffered saline (PBS). Cells were transfected with the appropriate plasmids by using FuGENE6 Transfection Reagent (B.M.) in accordance with the manufacturer's protocol. The cells were harvested at different time points. Western blot analysis or other experiments were performed.

RT-PCR. Total RNA was extracted from cells with TRIzol reagent (Invitrogen Life Technologies) and quantified by UV absorbance spectroscopy. The reverse transcription reaction was performed using the Superscript First-Strand Synthesis System (Invitrogen Life Technologies) according to the protocol. After incubation at 42 °C for 80 min, the reverse transcription reaction was terminated by heating at 70 °C for 15 min. The newly synthesized cDNA was amplified by PCR. The reaction mixture contained 2 μl cDNA template, 1.5 mM $MgCl_2$, 2.5 U Tag polymerase, and 0.5 μM β-actin sense 5′-GACTACCTCATGAAGATCC TC-3′; anti-sense 5′-CGTCACACTTCATGATGG-3′ was used as an internal control. Amplication cycles were: 94 °C for 3 min, then 33 cycles at 94 °C for 1 min, 58 °C for 1 min, 72 °C for 1.5 min, followed by 72 °C for 15 min. Aliquots of PCR product were electrophoresed on 1.5% agarose gels and PCR fragments were visualized by ethidium bromide staining.

Fig. 1. Three *mdm2*siRNA's location in *mdm2*mRNA.

Western blot analysis. Cells were collected at different time points (24, 48, and 72 h) and lysed in mammalian cell lysis buffer, then western blot analysis was performed with the use of conventional protocols. Cells were washed thrice with PBS containing 1 mM phenylmethylsulfonyl fluoride, scraped off the dishes, and pelleted at 5000 *g* for 10 min. Cell pellets were then lysed in cold TNT buffer [20 mM Tris–HCl (pH 7.4), 200 mM NaCl, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and 1% aprotinin] for 30 min with occasional rocking. The lysates were transferred to new tubes and clarified by centrifugation at 20,000*g* for 20 min at 4 °C. Identical amounts (50 μg of protein) of cell lysates were resolved by 12% SDS–PAGE. The membranes were incubated in blocking solution consisting of 5% powdered milk-free fat in TBST [10 mM Tris–HCl (pH 8.0), 150 mM NaCl, and 0.1% Tween 20] at room temperature for 1 h, then immunoblotted with monoclonal antibodies. The antibodies and dilutions used included anti-MDM2 (9E10; 1:1000 dilution; Santa Cruz) and anti-β-actin (AC-15; 1:5000 dilution; Sigma), detection by enzyme-linked chemiluminescence was performed according to the manufacturer's protocol (ECL; Amersham Pharmacia Biotech, Piscataway, NJ). Protein expression was quantified by Molecular Analyst software (Bio-Rad Laboratories, Hercules, CA).

Cell growth assay. At 24 h after transfection, LoVo cells transfected with indicated plasmids were harvested and replated at a density of 50 cells/mm^2 in triplicate. The total cell number was quantified every day with a hematocytometer and an Olympus inverted microscope. Cell viability was assessed by using trypan blue.

Flow cytometry analysis. Flow cytometry analysis was used to determine apoptosis of the cells. In brief, LoVo cells were transfected with *mdm2*siRNA3; 24 h later, cells were deprived of serum for 48 h. Then cells were harvested, washed once in PBS, and stained with propidium iodide (BD Biosciences). The apoptotic cells were assessed by flow cytometric detection of sub-G_1 DNA content.

TUNEL assay. Apoptotic cells were confirmed with the in situ cell death detection kit, Alkaline Phosphatase (Invitrogen), in accordance with the manufacturer's instructions. In brief, LoVo cells were grown on coverslips. The next day, cells were transfected with *mdm2*siRNA3. At 48 h after transfection, cells were deprived of serum for 36 h. Coverslips with adherent cells were fixed in 4% paraformaldehyde for 1 h at room temperature and permeabilized with 0.1% Triton X-100 for 2 min on ice. DNA fragments were labeled with the TdT-mediated dUTP nick end labeling (TUNEL) reaction mixture for 60 min at 37 °C in a humidified atmosphere in the dark. The coverslips were then incubated with Converter alkaline phosphatase for 60 min at 37 °C in a humidified chamber, rinsed in PBS. Cells were mounted cell side downward on a microscope slide, and the apoptotic cells (dark blue staining) were counted under a microscope. Three fields were randomly counted for each sample.

Drug sensitivity assays. Cells were detached by trypsinization, seeded at 1.0×10^3 cells/well in a 96-well microtiter plate, and treated with various concentrations of DDP (1, 5, 10, 50, 100, and 1000 nM). Seventy-two hours later, the effects on cell growth were examined by MTT assay; 20 μl of MTT solution (5 mg/ml in PBS) was added to each well, and the cells were incubated for 4 h at 37 °C. The DMSO formed by metabolically viable cells was dissolved in 150 μl cell lysis buffer, and fluorescence was monitored using a microplate at a wavelength of 490 nm. The percentage of cell growth was calculated by defining the absorption of cells not treated with paclitaxel (control) as 100%.

Tumor growth in nude mice. Equal numbers (10^6 or 2×10^6) of LoVo cells transfected with control plasmids and *mdm2*siRNA3 were harvested by trypsinization 2 days after transfection, washed twice with $1 \times$ PBS, and resuspended in 0.2 ml saline. Five nude mice (4 to 6 weeks old, male) were given bilateral subcutaneous injections with

control cells or cells transfected with *mdm2*siRNA3. The mice were kept in pathogen-free environments and checked every 3 days. The date at which a palpable tumor first arose and the weight of the tumor were recorded.

Statistical analysis. SPSS10.0 for Windows (SPSS) was used to analyze the data and plot curves. A two-tailed unpaired *t* test was used to compare the statistical significance of the differences in data from the two groups.

Results

Suppression of MDM2 overexpression in LoVo cells by RNAi

We designed and synthesized three *mdm2*siRNA sequence, and ligated the sequence into p*Silencer* vector. The plasmid of p*Silencer* contains a U6 promoter that directs the synthesis of oligonucleotides in an inverted repeat with 9 nt for its loop, with six T bases added at the end to serve as a termination signal for RNA polymerase III. The RNA is expected to fold back to form a hairpin loop structure after being transcribed; the hairpin dsRNA can then be further cleaved by Dicer to generate a 21-nucleotide siRNA, the active form for the RNAi effect, which will form dsRNA–endonuclease complexes and will bind and destroy three *mdm2*mRNA transfected into cells. Three *mdm2*siRNA were, respectively, transfected into LoVo cells and its effects on MDM2 levels were determined by comparison with control plasmids transfected cells by Western blot at the time points indicated. Twenty-four and 48 h after transfecting three *mdm2*siRNA targeting the region of the *mdm2* transcript, both the *mdm2* mRNA (Fig. 2) and protein expression were significantly inhibited, whereas the β-actin or control siRNA had no effect.

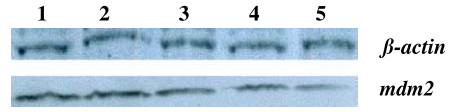

Fig. 3. Western blot analysis. Downregulation of MDM2 levels using RNA interference (RNAi). Western blot analysis of LoVo cells was done with *mdm2*siRNA1, *mdm2*siRNA2, and *mdm2*siRNA3, and reprobed with β-actin antibody as a control. 1, LoVo; 2, control; 3, *mdm2*siRNA1; 4, *mdm2*siRNA2; 5, *mdm2*siRNA3.

We found that the MDM2 expression levels were suppressed by up to 80% in LoVo cells at 5 days after transfection *mdm2*siRNA3 (Fig. 3). Actually the levels of MDM2 were decreased as early as 24 h after transfection. The inhibitory effect was shown to be specific because transfection with control siRNA did not alter MDM2 levels. These data indicated that vector-based RNAi could effectively suppress MDM2 overexpression and resulted in prolonged decreases in specific cellular gene expression without marked effects on other cellular proteins.

Decreased levels of MDM2 significantly inhibit the growth rate of LoVo cells

In our study, LoVo cells were transfected with *mdm2*siRNA3. The number of LoVo cells was then counted every 2 days after transfection. The results showed that RNAi directed against *mdm2* significantly decreased the growth rate of LoVo cells, with a 60–70% decrease at different time points repeatedly in three separate experiments. (Fig. 4) *mdm2*siRNA3 leads to a reduced cellular growth rate. LoVo cells were transfected with *mdm2*siRNA3 or control siRNA.

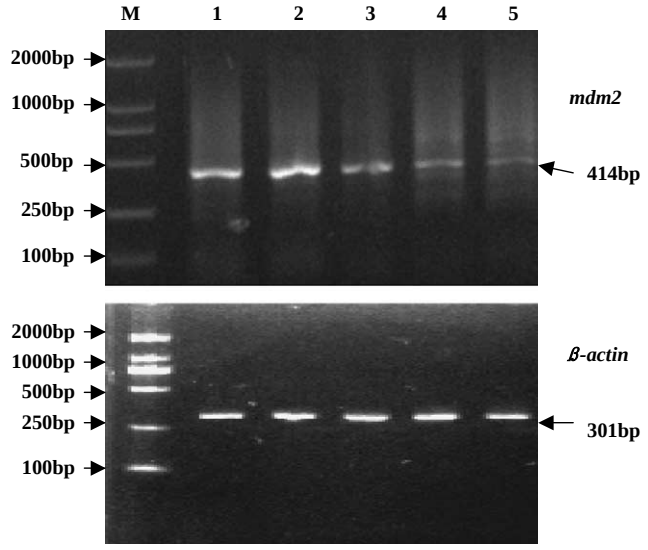

Fig. 2. RT-PCR analysis. RT-PCR analysis showed the lower *mdm2* mRNA level in LoVo cells transfected with *mdm2*siRNA. M, marker—DL2000; 1, LoVo; 2, control; 3, *mdm2*siRNA1; **4**, *mdm2*siRNA2; 5, *mdm2*siRNA3.

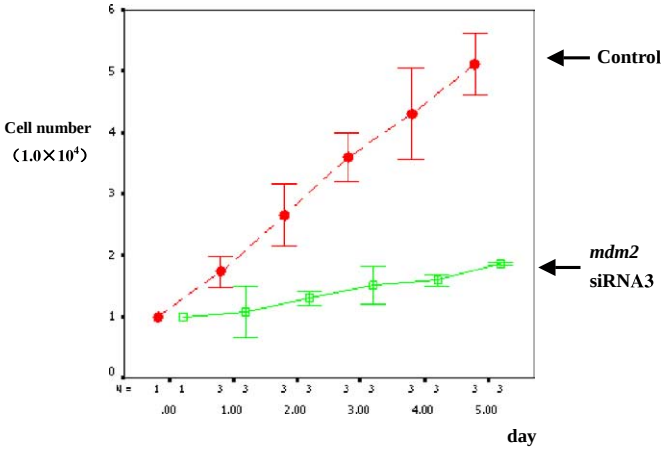

Fig. 4. Growth curve of LoVo cells after *mdm2*siRNA3 transfection. The *x* and *y* axes show time and cell number, respectively. The *mdm2*siRNA3 group (green) significantly inhibits LoVo cell proliferation in comparison with the control group (red). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

Induction of apoptosis in LoVo cells by mdm2siRNA3

The above data demonstrated that knockdown of MDM2 in LoVo cells could significantly inhibit the growth of tumor cells in vitro. To determine whether depletion of MDM2 could promote the death of tumor cells, flow cytometry and TUNEL assays were performed. At 48 h after transfection with *mdm2*siRNA3 or control, LoVo cells were deprived of serum for 36 h. These cells were then analyzed by flow cytometry and TUNEL assay. Significant sub-G1 (apoptotic) populations were observed in the flow cytometry assay (Table 1). Twenty-four hours later we found that 16.72% of LoVo cells transfected with *mdm2*siRNA3 underwent apoptosis after serum starvation, compared with 0% in the control group (Figs. 5A–C); 48 h later we found that 28.03% of LoVo cells transfected with *mdm2*siRNA3 underwent apoptosis, compared with 7.85% in the control group (Figs. 5D–F). We also confirmed the apoptosis of LoVo cells by TUNEL assay (Fig. 6). These data suggested that depletion of *mdm2* by RNAi in LoVo cells made the cells more sensitive to apoptosis after serum deprivation.

Effect of mdm2siRNA3 on chemosensitivity of LoVo cells to DDP

Following the initial characterization of *mdm2*siRNA3 knockdown methodology, we began a series of experiments to demonstrate that decreased MDM2 levels lead to enhanced colon carcinoma cell sensitization to DNA damaging agents. LoVo cells were transfected by *mdm2*siRNA3 recombinant and these cells were treated with chemotherapeutic agents. Cell survival/killing was assessed using the MTT assay (Fig. 7) (Table 2). Cells were transfected with *mdm2*siRNA3, then exposed to various doses of DDP for 48 h and viability was accessed. The percentage of cell growth was calculated by comparison of the LoVo from treated control cells. The IC_{50} value of LoVo cells to DDP and *mdm2*siRNA3 group (green) was 0.60 μg/ml. The IC_{50} value of LoVo cells to DDP group (red) was 3.20 μg/ml. When combined with DDP, *mdm2* siRNA3 significantly inhibited tumor growth with reductions in tumor cells of 80% as compared with that seen with *mdm2*siRNA3. Decreased levels of MDM2 clearly led to enhanced cell killing to all agents analyzed. These results suggest that the sensitivity of LoVo cells transfected *mdm2*siRNA3 to DNA damage caused by DDP is increased. However, MDM2 silencing only produced partial sensitization to DNA damage in cells.

Table 1
Ratio of cell cycle phase of every group after transfection by *mdm2*siRNA3

Group	G_0–G_1 (%)	G_2–M (%)	S (%)	Apoptosis (%)
24 h				
LoVo	51.65	11.41	36.94	0
Control	51.55	13.30	35.15	0
*mdm2*siRNA3	35.41	31.48	33.11	16.72
48 h				
LoVo	47.38	16.62	36.00	7.85
Control	44.55	25.34	30.11	12.00
*mdm2*siRNA3	31.91	23.53	44.56	28.03

Inhibition of tumor growth by mdm2siRNA3 in nude mice

Injections of LoVo cells in nude mice demonstrated relevant tumor biology. In this study, the LoVo cells were labeled through expression of a stable integrant of *mdm2* gene. Nude mice laden with an injection of LoVo cells were randomly selected for treatment with *mdm2*siRNA3. Tumor growth was inhibited by treatment with *mdm2*siRNA3. After 21 days following treatment, the side of mouse back treated with LoVo cells alone had a tumor vegetated, and no tumor volume was in the other side treated with *mdm2*siRNA3 (Table 3) (Fig. 8). Furthermore, the *mdm2*siRNA3 significantly inhibited the production of LoVo in the tumors. Therefore, the *mdm2*siRNA3 transfer is a significant novel method for inhibition of tumor growth in vivo.

Discussion

Cancer cells often show abnormal in the signal-transduction pathways [2], leading to proliferation in response to external signals [40]. Oncogene overexpression [29] is a common phenomenon in the development and progression of many human cancers. Oncogenes therefore provide a potential target [4] for cancer gene therapy.

The oncogene *mdm2* is expressed in a high proportion [31] of most human cancers, including colon carcinoma [1] gastrointestinal cancer [18], lymphoma [38], melanoma [3], and myeloid leukemia [11]. In its physiological role, MDM2 is broadly expressed during embryogenesis [9] and in tissue compartments [22] of the adult that possess high proliferative capacity [32]. Overexpression of MDM2 seems to define a common event associated with the pathogenesis of most human cancers [37]. Previous studies [30] demonstrated that the continued presence of MDM2 was required for cancer development [4] and not just for initiation [25], and inactivation of MDM2 [35] resulted in the sustained regression [26] of tumors.

Therefore, specific downregulation of MDM2 [8] might be a potential therapeutic strategy against human cancers, including colon carcinoma. In fact, the antagonists of MDM2, including full-length antisense mRNA [27] and oligonucleotides against *mdm2* mRNA [33], were previously reported to inhibit proliferation of cancer cell lines in vitro. However, it succeeded only in some situations; these technologies have been difficult to apply universally. Recently the advent of RNAi-directed knock-down [34] has sparked a revolution in somatic cell genetics, allowing the inexpensive, rapid analysis of gene function in

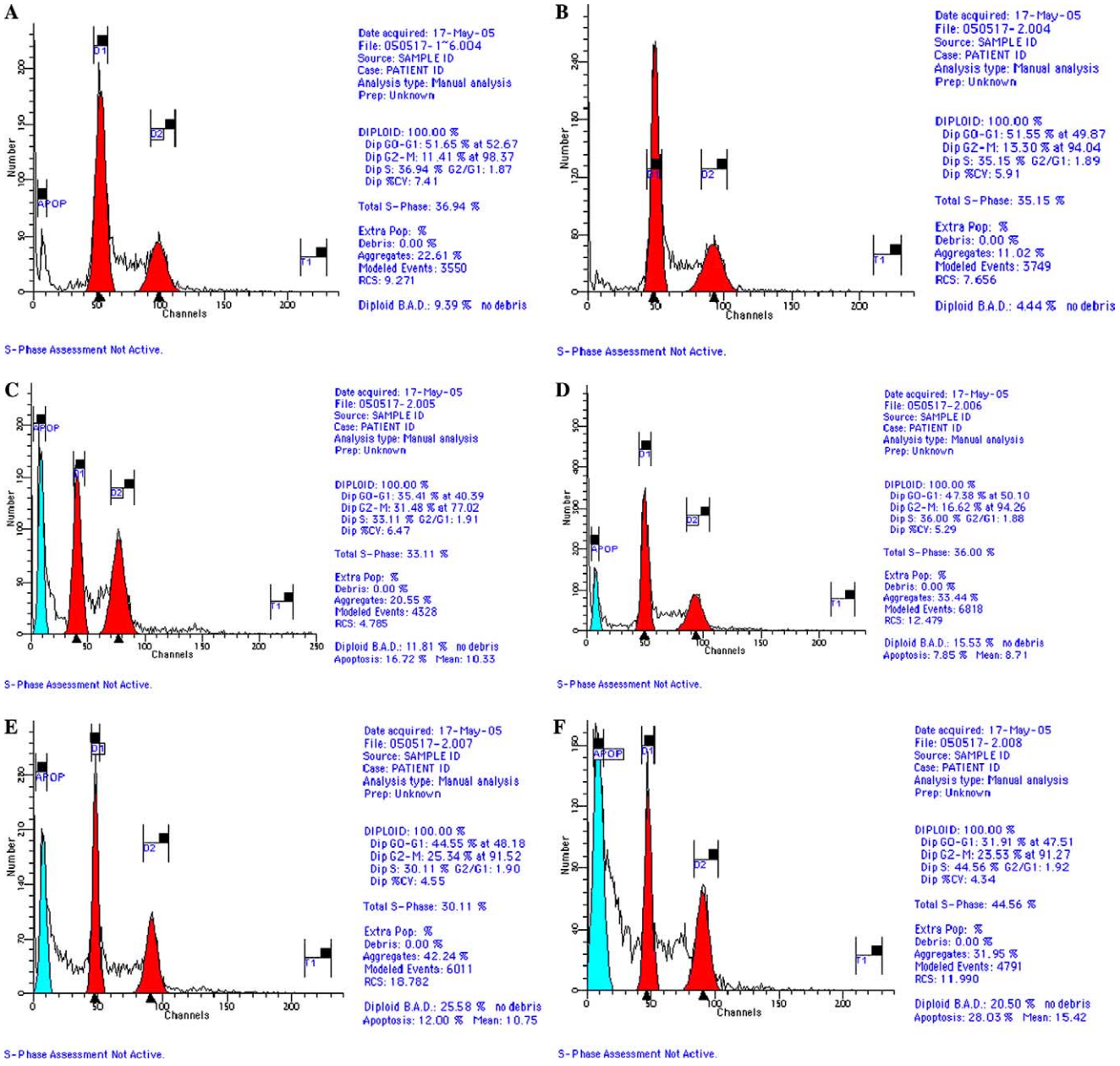

Fig. 5. Apoptosis of LoVo cells analyzed by flow cytometry. Downregulation of MDM2 promoted apoptosis of LoVo cells after serum deprivation. LoVo cells were transfected with *mdm2*siRNA3 or control siRNA. After 48 h, cells were deprived of serum for 36 h. Cells were then collected by trypsinization. The apoptotic cells were determined by flow cytometry. (A) Apoptosis at 24 h after LoVo transfection; (B) apoptosis at 24 h after control siRNA3 transfection; (C) apoptosis at 24 h after *mdm2*siRNA3 transfection; (D) apoptosis at 48 h after LoVo transfection; (E) apoptosis at 48 h after control siRNA3 transfection; (F) apoptosis at 48 h after *mdm2*siRNA3 transfection.

mammals, and might be exploited for gene therapy. Some studies [39] directly compared RNAi with antisense RNA and found that RNAi seemed to be quantitatively more efficient and durable in cell culture and in nude mice.

By means of the RNAi method, cellular growth assays, in vitro, were used to determine the functional consequences of RNAi-mediated decreases in of MDM2 in established colon carcinoma cells. Our results demonstrated that RNAi can effectively downregulate oncogene overexpression with great specificity. We showed that the plasmids endogenously expressing siRNA could successfully deplete up to 70% of MDM2 expression in LoVo cells at 5 days after transfection. Furthermore, the tumor inhibition effects persisted for at least 21 days after transfection in dishes and for 2 m in nude mice as shown by experiments in vitro and in vivo, even though the protein level of MDM2 in silenced clones expressing siRNA was back to almost the same level as in the control cells at 21 days after transfection. It was therefore not surprising for us to show here that a transient reduction of MDM2 protein level by *mdm2*siRNA3 could significantly inhibit the growth rate of LoVo cells. Additionally, the

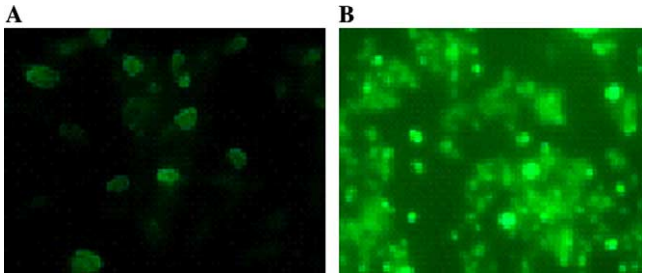

Fig. 6. TUNEL assay to detect apoptotic cells in situ. LoVo cells were grown on coverslips and transfected with *mdm2*siRNA3 or control siRNA. After 24 h, cells were deprived of serum for 36 h. Cells were then analyzed for apoptosis with the TUNEL assay.

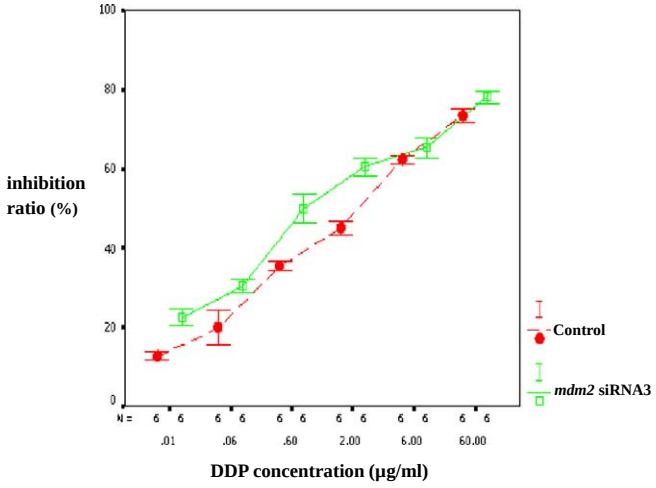

Fig. 7. Effect of *mdm2*siRNA3 on chemosensitivity of LoVo cells to DDP. Cell survival or apoptosis levels of LoVo cells treated with DDP and *mdm2*siRNA3. LoVo cells were treated with *mdm2*siRNA3 and DDP and cell survival/killing was analyzed by the MTT assay. The *x* and *y* axes show DDP concentration and cell inhibition ratio, respectively.

remarkable effect in nude mice supported the effectiveness of this treatment.

These studies have marked a new era in the genetic manipulation of human cancer development by allowing oncogenes to be downregulated by RNAi. Many anticancer agents induce apoptosis. When activated, MDM2 increases the expression of genes that promote cell survival and block apoptosis. Our data also suggested that knockdown of MDM2 by *mdm2*siRNA3 in LoVo cells could increase the sensitivity of these cells to apoptotic stimuli, such as serum starvation. This was most probably one of the reasons for the anti-tumor effects. A decrease in MDM2 levels by techniques brought about by, for example, an antisense approach might cause apoptosis of certain tumor cells or might increase the sensitivity of the cells to apoptotic stimuli. In LoVo cells, suppression of MDM2 expression in response to *mdm2*siRNA3 could induce these cells to apoptosis. However, the pathways that MDM2 controls and/or that are involved in the observed apoptosis upon serum deprivation remain obscure and need further study.

Table 3
*mdm2*siRNA3 significantly inhibits LoVo cell growth in nude mice

No. of cells (2×10^6)	Tumor weight (mg)	Tumors	Injections	Latency (day)
Control siRNA	856–1524	2	4	15–21
*mdm2*siRNA3	0	0	4	>21

Inducible chemotherapy resistance to DDP has been shown to be reversed by inhibiting MDM2. In the current study, the functional consequences of *mdm2*siRNA3-mediated decreases in MDM2 activation by DDP in an established colon cancer cell line were determined using assays of apoptosis, and in vitro assays of tumor growth. This analysis demonstrates that transfection of tumor cells with siRNA directed against the MDM2 subunit significantly decreases inducible MDM2 activation in response to treatment with DDP. These studies also demonstrated that the enhanced response to chemotherapy was associated with increased levels of apoptosis. As a result, there was enhanced chemosensitivity to DDP in vitro.

*mdm2*siRNA3 significantly inhibited tumor growth with reductions in tumor cells of 80% as compared with that seen with *mdm2*siRNA3. These xenograft studies demonstrated anti-tumor effects of *mdm2*siRNA3 administration in combination with DDP in an in vivo model of human colon cancer. Additional studies will determine whether *mdm2*siRNA3 will increase the therapeutic index of chemotherapy-induced cell death in cancer cells as compared with normal cells.

Conclusion

In summary, our study demonstrates the feasibility of utilizing *mdm2*siRNA3 to specifically reduce the *mdm2* expression level in human cancer cells and provides direct evidence for the potential use of *mdm2*siRNA3 as a chemotherapy-sensitizing agent.

Table 2
The results of cell inhibition ratio after treated by DDP in various concentration

Concentration (μg/ml)	0.006	0.06	0.6	2	6	60
Control	12.63 ± 1.06	19.87 ± 4.55	35.40 ± 1.31	45.10 ± 1.81	60.53 ± 0.80	69.90 ± 1.55
*mdm2*siRNA3	22.37 ± 2.10	30.37 ± 1.80	50.13 ± 3.75	60.57 ± 2.47	66.93 ± 1.31	78.17 ± 1.69
P	0.004	0.022	0.037	0.001	0.010	0.030

Fig. 8. *mdm2*siRNA3 significantly inhibits LoVo cell proliferation in nude mice. we investigated the effects of *mdm2* by in vivo subcutaneous injection into nude mice method. At 3 weeks after transfection there was no tumor in the side of which was transfected *mdm2*siRNA3.

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