siRNA-mediated MDM2 inhibition sensitizes human lung cancer A549 cells to radiation

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Abstract. MDM2 (murine double minute 2) is well-documented to play a key role in radiation response and tumor radiosensitivity, thus offering an attractive clinic drug target to enhance tumor sensitivity to anti-cancer radiotherapy. In this study, we designed and tested two siRNA fragments against human MDM2 in non-small cell human lung cancer A549 cells. Transfection of mammalian expression vector pUR/U6 containing either MDM2 siRNA1 or siRNA2 fragment was shown to reduce MDM2 mRNA levels by 72% and 31%, respectively. Western blotting detected a similar inhibition of MDM2 protein levels in cells transfected with MDM2 siRNA1. A549 cells transfected with the expression vector for siRNA1 significantly decreased cell proliferation and rendered cells more sensitive to radiation. The basal apoptotic and necrotic cells, 1% and 2%, respectively, detected among A549 cells were increased to 2.6% and 14.4% after γ-irradiation with 5 Gy. Transfection of MDM2 siRNA1 induced 30.1% apoptosis and 12.7% necrosis while combined treatment of siRNA1 and 5-Gy radiation increased apoptosis and necrosis to 45.9% and 15.2%, respectively. These data provide the first evidence that specific siRNA fragment (MDM2 siRNA1) targeting human MDM2 mRNA is able to enhance lung cancer radiosensitivity.

Introduction

Lung cancer is the leading cause of cancer-related deaths in the world, accounting for 1.2 million new cases annually. Non-small cell lung cancer (NSCLC) accounts for nearly 80% of all bronchogenic neoplasms and is characterized by a particularly poor prognosis with approximately 90% of patients dying within 5 years of diagnosis. Most NSCLC patients are inoperable at diagnosis and are treated with radiotherapy and/or chemotherapy. Currently, although radiotherapy is an important modality of lung cancer treatments, this therapeutic strategy alone could not improve efficiently the long-term survival rate. Therefore, to establish an effective treatment, novel drug compounds combined with radiotherapy are urgently needed. Specific molecular targets for enhancing radiation-mediated cell killing are heavily weighted toward the improvement of the cure rate for cancer (1-3).

It is now well established that, in addition to inducing nuclear DNA damage, therapeutic ionizing radiation (IR) can activate specific signaling transduction that can influence the overall cell survival (4,5). Accumulated evidence suggest a strong correlation between IR-induced gene expression and cellular radiosensitivity (4-7). Radiation-resistant breast cancer MCF+FIR cells that are derived from a long-term therapeutic regimen of radiation show elevated MDM2 expression (8). Using lung cancer cell lines with different radiosensitivities, we identified several potential candidate genes resistant to IR (9). Of particular interest among the up-regulated genes we have identified was MDM2 (murine double minute 2), a negative regulator of the p53 tumor suppressor, which was shown to be a critical component in response to IR (10). MDM2 expression levels were found to be increased in radioresistant non-small cell lung carcinoma A549 cells, indicating that MDM2 could be a suitable target for re-sensitization of radiation-resistant lung cancer cells.

MDM2 is an oncogene (11), and it promotes the growth of tumor cells (12). Amplification (13) or overexpression (14) of MDM2 has been found in a variety of human carcinomas, including NSCLC patients (15) with poor prognosis (16). Most importantly, MDM2 expression is found to affect cell response to radiotherapy and chemotherapy (10,17). Therefore, MDM2 may serve as a potential target to enhance the tumor radiosensitivity.

siRNA has been extensively studied as a means of attenuating the expression of specific gene transcripts both *in vitro* and *in vivo* (18,19). siRNAs are short, double-stranded RNA molecules that can target mRNAs with complementary sequence for degradation via a process termed RNAi (RNA interference). In this study, we applied vector-mediated delivery

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of two siRNAs to knock-down the expression of MDM2 gene in radioresistant human lung cancer A549 cells to investigate whether inactivating MDM2 can enhance the sensitivity to therapeutic dose of IR (5-Gy γ-ray). Our data show that MDM2 gene expression in A549 cells is specifically suppressed by MDM2 siRNA1 and MDM2 siRNA2 with the greater level of suppression by siRNA1. MDM2 siRNA1-transfected cells combined with 5-Gy IR significantly inhibited cell proliferation. Also, siRNA1 significantly increased IR-mediated cell death by increasing apoptosis as well as necrosis (45.9% and 15.2% compared to 2.6% and 14.4%, respectively, for IR alone). Therefore, a combination of MDM2 siRNA1 with radiotherapy would be a preferential strategy to enhance the therapeutic efficacy for radioresistant lung cancers.

Materials and methods

Construction of MDM2 siRNA plasmids. The sequences of the following two different MDM2-specific siRNAs were determined from the human MDM2 mRNA sequence (GenBank Accession no. XM_017531). MDM2 siRNA1: 5'-GTGCTGG GATTACAGGCATTTCAAGAGAATGCCTGTAATCCC AGCAC*TTTTTT*-3' (sense); 5'-*AATTAAAAAA*GTGCTGG GATTACAGGCATTCTCTTGAAATGCCTGTAATCCCA GCAC*GGCC*-3' (antisense). MDM2 siRNA2: 5'-TCACCTT GAAGGTGGGAGTTTCAAGAGAACTCCCACCTTCAA GGTGA*TTTTTT*-3' (sense); 5'-*AATTAAAAAA*TCACCTT GAAGGTGGGAGTTCTCTTGAAACTCCCACCTTCAAG GTGA*GGCC*-3' (antisense). To construct MDM2 siRNA plasmids, equal amounts of the sense and antisense templates were annealed, and the duplex oligonucleotides were inserted into the *Apa*I and *Eco*RI sites of pUR/U6 vector (a kind gift from Dr Z.Y. Shen, Cancer Institute of New Jersey UMDNJ Robert Wood Johnson Medical School, USA). All constructs were sequence-verified before use.

Cell culture and transient transfection. A549 lung cancer cells were maintained as adherent monolayer cultures in RPMI-1640 culture medium (HyClone, Logan, UT), supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified chamber at 37˚C in 5% CO_2. Cells grown in 6-well plates were transfected with 3 μg of MDM2 siRNA plasmids or pUR/U6 empty plasmid as a vector control, using Lipofectamine™ 2000 (Invitrogen, Long Island, NY, USA) according to the manufacturer's protocol. Cells were fed fresh medium 72 h post-transfection.

Irradiation. Cells grown in 6-well plates were irradiated with a single dose of 5 Gy under ambient conditions. All irradiations were performed at a ^{60}Co unit with a dose rate of ~1.51-1.68 Gy/min^{-1}. Plates containing control cells were taken to the radiation chamber but not those exposed to IR serving as the sham-IR control. The culture media of irradiated and sham-irradiated cells were replaced with fresh medium immediately after IR.

RT-PCR. Total RNA was extracted from A549 cells using TRIzol reagent (Invitrogen) at 72 h post-transfection with MDM2 siRNA plasmids. The concentration and purity of the RNA samples were determined spectroscopically. cDNA was synthesized according to the protocol of ImProm-II™ reverse transcription system (Promega, Madison, WI, USA). The newly synthesized cDNA was amplified by PCR. The primer sequences for MDM2 were forward, 5'-AAG ACT ATT CTC AGC CAT CA-3'; reverse, 5'-CAT ACT GGG CAG GGC ATT-3'. As internal standard, human endogenous GAPDH was amplified simultaneously. The primer sequences for GAPDH were forward, 5'-ATC CCA TCA CCA TCT TCC AG-3'; reverse, 5'-CCA TCA CGC CAC AGT TTC C-3'. The predicted size of the PCR products for MDM2 and GAPDH are 259 and 363 bp, respectively. Amplification cycles were: 94˚C for 5 min, then 30 cycles at 94˚C for 30 sec, 55˚C for 30 sec, 72˚C for 40 sec, followed by a final elongation at 72˚C for 5 min. Aliquots of PCR products were electrophoresed on a 2% agarose gel. PCR fragments were visualized by ethidium bromide staining and quantified with an image analyzer (Quantity One; Bio-Rad, Hercules, CA, USA).

Western blot analysis. Protein levels of MDM2 as well as normalization control ß-actin were determined with or without IR treatment. A549 cells were transfected with expression vector for MDM2 siRNA1 or empty vector (pUR/U6) for 72 h. The transfected cells were exposed to 5 Gy of γ-irradiation using a ^{60}Co irradiator and incubated for 48 h. Cells were then washed twice in ice-cold PBS, and lysed in mammalian cell lysis buffer (10 mM sodium phosphate pH 7.5, 100 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 1 mM phenylmethylsulfonyfluoride, and 15 mg/ml aprotinin). The lysates were transferred to new tubes and clarified by centrifugation at 5000 x g for 10 min at 4˚C. Protein concentration was determined using the BCA protein assay reagent kit (Pierce, Rockford, IL, USA). Identical amounts of protein were resolved by SDS-PAGE and blotted onto the polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). After electroblotting, the membranes were incubated in blocking buffer (phosphate-buffered saline containing 0.1% Tween-20 and 5% non-fat milk) for 1 h at room temperature, then immunoblotted with anti-MDM2 monoclonal antibody (1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4˚C. The membranes were washed, incubated with the sheep anti-mouse IgG horseradish peroxidase-conjugated secondary antibody (1:2500, Santa Cruz Biotechnology) for 1 h at room temperature, and washed again. The blotted proteins were detected by the Western Blotting Luminol Reagent (Santa Cruz Biotechnology) according to the manufacturer's directions. The membrane probed for MDM2 was stripped and reprobed for ß-actin (1:1000, Santa Cruz Biotechnology) to normalize for loading and/or quantification errors.

MTT assay. The effects on cell growth by the MDM2 siRNA1 were examined by MTT assay. Briefly, cells were seeded at a density of 5000 cells/well in 96-well plates and allowed to attach overnight. Cells were then transfected with pUR/U6 vector control or pUR/U6 containing MDM2 siRNA1, and irradiated 72 h post-transfection. After 48 h of irradiation, 20 μl of MTS solution (Sigma, St. Louis, MO, USA) was added to each well, and the cells were incubated for 90 min at 37˚C. The absorbance of each well was measured using a MR 600 Microplate reader (Wallac 1420 Victor 2 multilable

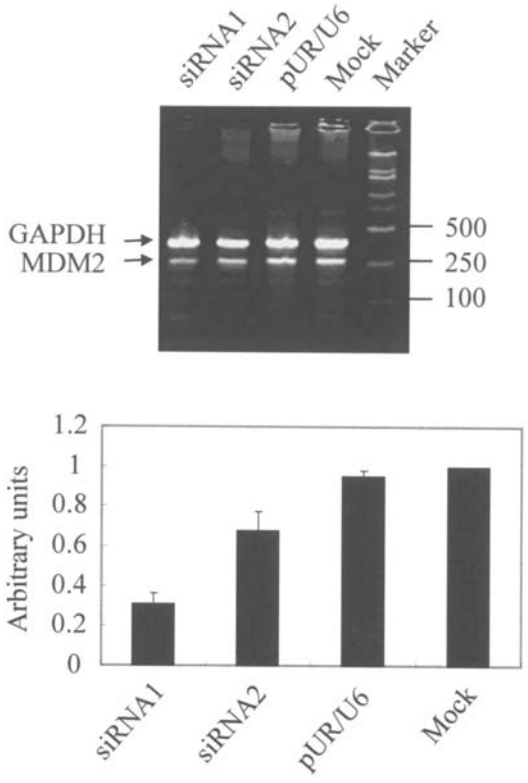

Figure 1. Effect of siRNAs on MDM2 mRNA in A549 cells. For the selection of an effective siRNA directed against MDM2, A549 cells were transfected with the mock (lipofectamine), pUR/U6 empty vector or MDM2-specific siRNA vectors. Seventy-two hours after transfection, the total RNA was extracted and mRNA expression was detected by semi-quantitative RT-PCR, as described in Materials and methods. Each PCR was run in triplicate. GAPDH mRNA was also amplified as an internal control. Lower panel shows the relative MDM2 mRNA expression levels estimated by densitometry and normalized to control GAPDH.

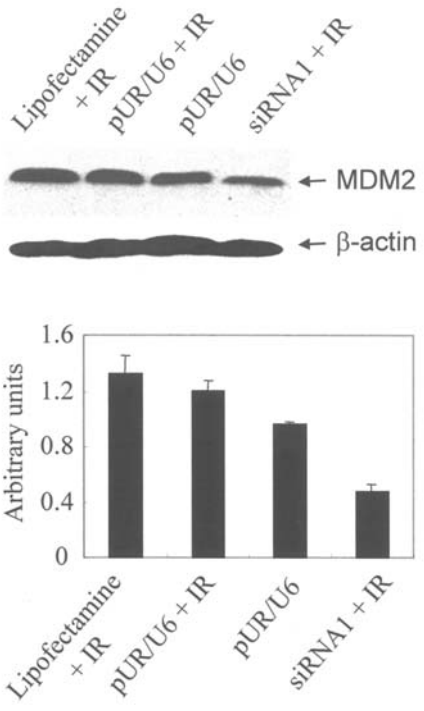

Figure 2. Effect of siRNA1 on MDM2 protein expression in A549 cells treated with IR. Cells were transfected with the mock, pUR/U6 or MDM2-specific siRNA1 vector before exposure to 5-Gy IR. Forty-eight hours post-radiation, the cell lysates were prepared and Western blot analysis was performed with anti-MDM2 antibody, and reprobed with ß-actin antibody as a control. Lower panel shows the relative MDM2 protein levels estimated using densitometry and normalized to control ß-actin.

counter system, Gaithersburg, MD, USA) at 490 nm. All samples were made in triplicate, and the data were analyzed using the Student's t-test.

Flow cytometry analysis. A549 cells were transfected with pUR/U6 expression vector for MDM2 siRNA1 or empty vector for 72 h before irradiation (5 Gy). Cells were harvested 48 h post-radiation, washed twice in PBS, and stained with Annexin V-FITC/PI (Baosai Reagent, P.R. China). The apoptotic and necrotic cells were assessed by flow cytometric detection using a FACSCalibur and analyzed with CellQuest software program (Becton Dickinson, San Jose, CA, USA).

Results

siRNA-mediated down-regulation of MDM2. We designed siRNA complementary to two different regions of the MDM2 mRNA as described in Materials and methods. Each siRNA was synthesized as complementary oligonucleotides and cloned into the pUR/U6 expression vector. The resulting constructs were then screened for their ability to down-regulate MDM2 expression. A549 cells were transfected with empty pUR/U6 vector or the vector for the expression of each siRNA, and RT-PCR was performed 72 h post-transfection. As shown in Fig. 1, siRNA1 and siRNA2 directed against MDM2 reduced MDM2 mRNA levels by 72% and 31%, respectively, compared to GAPDH, which was not affected by either siRNA. Although both siRNAs attenuated MDM2 expression significantly, the greatest level of suppression was observed by siRNA1. Therefore, MDM2 siRNA1 was used for further studies.

Next, Western blot analysis was performed to determine the protein levels of MDM2 in A549 cells transfected with expression vector for MDM2 siRNA1 followed by exposure to γ-irradiation (5 Gy). We found that, compared to ß-actin, IR-mediated induction of MDM2 expression (Fig. 2, lanes 1 and 2) was greatly reduced (69%) by siRNA1 directed against MDM2 (Fig. 2, lane 4). The reduction in protein levels is very similar to the reduction of mRNA levels shown in Fig. 1 (69% versus 72%). Also, compared to vector (pUR/U6)-transfected control cells (Fig. 2, lane 3), MDM2 level was much lower (52%) in A549 cells transfected with siRNA1 before exposure to 5-Gy IR (Fig. 2, lane 4). Together with Fig. 1, Fig. 2 clearly demonstrates that MDM2 siRNA1 was highly specific and efficient in MDM2 gene silencing at both the mRNA and protein levels in A549 cells.

siRNA-mediated MDM2 inhibition sensitizes A549 cells to IR. To determine if inhibition of MDM2 radiosensitizes A549 cells, cell proliferation was measured with MTT assay. Cells were transfected for 72 h with expression vector for MDM2 siRNA1 or vector alone before exposure to 5-Gy IR. Two days post-radiation, MTT assay was performed. As shown in Fig. 3, siRNA1-transfected A549 cells with 5-Gy

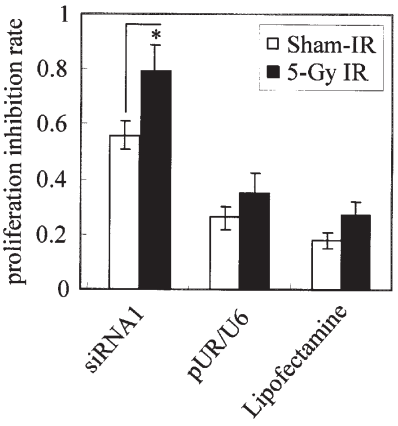

Figure 3. Effects of siRNA1-mediated silencing of MDM2 on the radiosensitivity of A549 cells. Cells were transfected with the mock, pUR/U6 or MDM2-specific siRNA1 vector before exposure to or without 5-Gy IR. The cell proliferation was determined 48 h post-radiation by MTT colorimetric assay. Each bar represents the relative cell growth inhibition (mean ± standard deviations) from three independent experiments. *p<0.01, compared to A549 cells transfected with vector for siRNA1 expression before sham-irradiation.

Table I. Flow cytometry analysis of the effects of MDM2 siRNA1 on cell death of A549 cells with or without 5-Gy IR.

Group	Apoptosis	Necrosis
A549	0.8±0.2	2.1±0.6
A549 + IR	2.6±0.6	14.4±3.5
A549 + pUR/U6	3.62±1.1	4.8±0.8
A549 + siRNA1	30.1±7.5[a]	12.7±4.8[a]
A549 + siRNA1 + IR	45.9±8.2[a]	15.2±6.3[a]

[a]p<0.01, compared to A549 + pUR/U6, by One-way ANOVA test for apoptosis and necrosis.

IR showed reduced proliferation compared to cells with exposure to sham-IR (p<0.01). The IR-induced inhibition of cell proliferation was not significant although cells appeared to be more inhibited when IR was combined with transfection of vector (pUR/U6) or mock (lipofectamine). These data suggest that siRNA1-mediated silencing of MDM2 significantly inhibits cell proliferation and renders A549 cells more sensitive to radiation.

To determine whether depletion of MDM2 by siRNA1 could promote the death of tumor cells, flow cytometry was performed. At 72 h after transfection with MDM2 siRNA1 expression vector, A549 cells were exposed to 5-Gy IR. Forty-eight hours post-radiation, cells were labeled with Annexin V-FITC/PI and analyzed with a FACSCalibur flow cytometer. The basal apoptotic and necrotic cells, 1% and 2%, respectively, detected in wild-type A549 cells were increased to 2.6% and 14.4% by 5-Gy γ-ray (Table I). MDM2 siRNA1 increased the number of apoptotic as well as necrotic cells by 30.1% and 12.7%, respectively, compared to 3.6% and 4.8%, respectively, observed in empty pUR/U6 vector-transfected A549 cells (p<0.01). Interestingly, IR further increased the number of both apoptotic and necrotic cells by 45.9% and 15.2%, respectively, in MDM2 siRNA1 transfected cells (p<0.01). These results suggest that the sensitivity of MDM2 siRNA1 expressing A549 cells to DNA damage caused by 5-Gy IR was significantly increased.

Discussion

Clinically, many non-small cell lung carcinomas (NSCLCs) are inoperable, and little tumor-controlling efficacy is achieved by radiotherapy or chemotherapy, or a combination of both therapies due to tumor resistance. Therefore, developing new therapies to improve the overall outcome of NSCLC patients is in urgent need (20). Although potential targets for therapeutic intervention in therapy-resistant lung cancers have been extensively studied in the last decade (21,22), no efficient molecular target has been identified.

Our present results demonstrate for the first time that inhibition of MDM2 with a specific human siRNA sequence (MDM2 siRNA1) is able to re-sensitize radiation-resistant lung cancer A549 cells. Oncogenes provide a potential target for cancer gene therapy (23). Overexpression of the oncogene MDM2 is a common event associated with the pathogenesis of most human cancers (24,25), including breast cancer, osteosarcomas and lung cancer (13,14,26-28). Activation of MDM2 increases the expression of genes that promote cell survival and block apoptosis (25). Transduction of MDM2-b (an alternative splice form of MDM2 most commonly observed in human cancers) into NIH3T3 cells can promote rapid cell proliferation (29). Interestingly, tumor regression was observed by the inactivation of MDM2 (30). Overexpression of MDM2 in various tumors is believed to inhibit p53, therefore favoring uncontrolled cell proliferation. We reported that MDM2 expression levels were increased in non-small cell lung carcinoma A549 cells in response to IR (9). Therefore, specific down-regulation of MDM2 may be a potential target to enhance radiosensitization.

Other groups have applied antisense to attenuate MDM2 expression resulting in the inhibition of proliferation of cancer cells *in vitro* (31-33). Our previous study also demonstrated that MDM2 ASODN (antisense oligodeoxynucleotide) enhanced radiosensitivity in A549 cells (9). Although antisense oligonucleotides are efficient to block MDM2, RNA interference is a powerful technique for the specific inhibition of expression of individual genes at the posttranscriptional level (34). siRNA has a number of advantages over antisense oligonucleotide strategy, in part from the greater resistance of siRNA to nuclease degradation. RNAi and antisense RNA have been compared and RNAi seemed to be quantitatively more efficient and durable in both cell culture and nude mice (35). Therefore, the feasibility of using siRNA in molecular cancer therapy and as a mediator of chemosensitization or radiosensitization has been shown by several groups (36,37).

In the present study, the functional consequences of siRNA-mediated suppression of MDM2, using expression pUR/U6 vectors for MDM2 siRNA1 and MDM2 siRNA2, was established in radioresistant A549 non-small cell lung cancer cells. Our results demonstrated that inhibition of MDM2 by siRNA1 enhanced response to radiation, which was

associated with significant reduced levels of cell proliferation (Fig. 3) as well as increased levels of apoptosis (45.9% and 2.6% for dual treatment of siRNA1 and IR, and IR alone, respectively; Table I). As a result, there was enhanced radiosensitivity in A549 cells. Although synthetic siRNAs can achieve effective and very rapid knockdown of target genes in mammalian cells, their effects are transient. To circumvent this problem, transfection of siRNA plasmid that stably integrates and expresses interfering double-strand RNA hairpins may be effective at knocking down target genes continually. In this study, although MDM2 siRNA was transfected transiently in puromycin-resistant A549 cells, we surprisingly found loss of long-term gene silencing (data not shown). Because of the loss of long-term gene silencing, the clonogenicity assay was not performed. However, in our previous studies, a correlation between clonogenic radiosensitivity and apoptotic cells was found (38,39).

In conclusion, we have tested two MDM2 siRNAs (i.e., siRNA1 and siRNA2), and found different inhibition efficiency as determined by both mRNA and protein levels (Figs. 1, 2 and data not shown). Accumulating evidence suggest that different regions of mRNA show a varied efficacy of gene inhibition by siRNA-mediated targeting. Therefore, it appears that the region of siRNA2 is not an efficient target for MDM2. While the manuscript of this article was under preparation, Yan Yu *et al* described increased resistance of human colorectal adenocarcinoma LoVo cells to the DNA damaging agent cisplatin after treatment with MDM2 siRNA (40), a result supportive to our conclusion. The most efficient MDM2 siRNA sequence (i.e., siRNA3) demonstrated by Yu *et al* (40) is different from the potential target sequence (i.e., siRNA1) observed in the current study, although they found 80% reduction of tumor growth in combination with cisplatin. Therefore, double siRNA treatment (i.e., MDM2 siRNA1 of this study and MDM2 siRNA3 of ref. 40) in combination with radiotherapy may provide a more efficient strategy in tumor radiosensitization. Overall, the data presented here provide evidence suggesting that MDM2 siRNA could be a promising and powerful method for sensitization of radioresistant cancer cells.

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