



Enhancing radiosensitivity of TE1, TE8, and TE 11 esophageal squamous carcinoma cell lines by Hdm2-siRNA targeted gene therapy in vitro

Jalil Pirayesh Islamian^{1*}, Mohsen Mohammadi², Behzad Baradaran³, Alireza Farajollahi¹, Seyed Mahmoud Reza Aghamiri⁴, Mohammad Asghari Jafarabadi⁵, Hadi Karami⁶, Amir Monfaredan⁷, Dariush Shanehbandi³

¹ Department of Medical Physics, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran

² Department of Medical Radiation Science, School of Paramedicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran

³ Immunology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran

⁴ Department of Radiation Medicine, Faculty of Nuclear Engineering, Shahid Beheshti University of Medical Sciences, Tehran, Iran

⁵ Department of Statistics and Epidemiology, Tabriz University of Medical Sciences, Tabriz, Iran

⁶ Department of Biotechnology, Faculty of Medicine, Arak University of Medical Sciences, Arak, Iran

⁷ Department of Hematology, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran

Article Info



Article Type:

Original Article

Article History:

Received: 25 Feb. 2016

Revised: 24 June 2016

Accepted: 26 June 2016

ePublished: 28 June 2016

Keywords:

Cell line
 Esophagus cancer
 Hdm2-siRNA
 Oncogene
 Radiosensitivity
 Squamous cell carcinoma

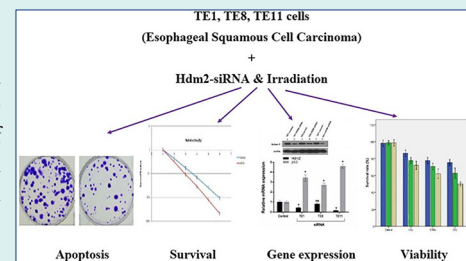
Abstract

Introduction: Human double minute2 (hdm2) level increases in most human malignancies. Therefore, inhibition of tumor growth and also induction of radiosensitivity may be provided by hdm2 inhibitors. The effects of hdm2-siRNA on hdm2 protein expression, cell apoptosis rate, and radiosensitivity of human esophageal squamous cell carcinoma (ESCC) were studied.

Methods: The hdm2 gene was silenced in TE1, TE8, and TE11 ESCC cell lines using 200nM siRNA by liposomal transfection method followed by irradiation with 0.5, 1, 2, 4, and 6 Gy γ -rays *in vitro*. The gene expression levels were evaluated by real time PCR and Western Blotting methods. MTT, TUNEL, and also colony forming assays were used to compare the radiosensitivity of the cell lines before and after the treatments.

Results: Hdm2-siRNA reduced the hdm2 protein as compared to the vehicle control and scrambled groups, and also increased the radiation-induced apoptosis especially in TE11 cells. The related dose reduction factors (DRFs) for the silenced TE1, TE8, and TE11 cells calculated to be 1.20, 1.30, and 2.75, respectively.

Conclusion: Increasing radiosensitivity of tumor cells may be provided by silencing the oncogenes.



Introduction

Radioreistance causes failure in the esophageal cancer treatment, also in neoadjuvant therapy.¹⁻³ Therefore, enhancing radiosensitivity may reduce radiation doses, needed in high dose therapy, and also related side effects on normal surrounding tissues.^{2,4} There was found an extended variation in the gene expression profiles of esophageal cancer.⁵⁻⁷ It was suggested that different responses of tumor cells to radiotherapy is related to variations in the gene expression patterns.⁴ Human double minute2 (hdm2) acts as inhibitor of the tumor suppressor,⁸⁻¹⁰ and is enhanced in human malignancies such as breast cancer,

melanoma, esophageal cancer, non-small cell lung cancer, non-Hodgkin lymphoma, leukemia, and sarcoma.¹⁰ Analysis of hdm2 level in relation to p53 gene expression has demonstrated direct relation of p53 missense mutations with overexpression of hdm2 protein,¹¹ so that mutation or deletion of the p53 gene has been found in about 50% of human malignancies.¹² Meanwhile in others, the function of p53 is effectively reduced by hdm2.¹²⁻¹⁶ The main activity of hdm2 is interfering with p53 induced apoptosis and growth inhibition of tumor.^{17,18} Furthermore, the hdm2 induces carcinogenesis by a p53 independent approach.¹⁹ Hdm2 also mediates export of p53 by either the nucle-



*Corresponding author: Jalil Pirayesh Islamian, Email: pirayeshj@gmail.com



© 2016 The Author(s). This work is published by BioImpacts as an open access article distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by-nc/4.0/>). Non-commercial uses of the work are permitted, provided the original work is properly cited.

ar export signal (NES) or the RING finger of hdm2 and the NES of p53.^{10,20} It was shown that hdm2-siRNA have induced MCF-7 cell apoptosis and reduced reproduction of the cell.²¹ Finally, the mechanism of hdm2-siRNA-induced apoptosis, as well as the effect of hdm2 inhibition on various p53-dependent and apoptosis-related genes, is not clearly known in human cancers.¹⁰

In the present study, the effects of silencing hdm2 gene on the radiosensitivity of TE1, TE8, and TE11 ESCC (human esophageal squamous cell carcinoma) cell lines were evaluated.

Materials and methods

Cell lines and cell culture

The ESCC cell lines TE1(RBRC- RCB1894), TE8 (RBRC-RCB2098), and TE11 (RBRC-RCB2100) were purchased from cell bank, the RIKEN BioResource Center through the National Bio-Resource Project of the MEXT (RIKEN; Tsukuba, Ibaraki, Japan). The cells were cultured in RPMI 1640 culture medium (Sigma-Aldrich, St. Louis, MO, USA), supplemented with 10% fetal bovine serum (FBS; (Sigma-Aldrich, St. Louis, MO, USA), 100 U/mL penicillin, and 100 µg/m streptomycin (Gibco Inc., Paisley, UK.), under the conditions of 5% CO₂ in a humidified incubator at 37°C.

Preparation of siRNA

Twenty one-nucleotide siRNAs were synthesized by Santa Cruz Biotechnology (Dallas, Texas, USA) manufacturer's protocol using 20-ACE protection chemistry. The siRNA sequence targeting hdm2 (GenBank: Accession No. NM_002392) was corresponded to the coding regions 59–80 after the start codon, and the mock siRNA sequence was 5'-AAUAGUGUAUACGGCAUGCdTdT-3'.

Transfection of cells

Transfection of hdm2-siRNA or mock siRNA was performed with Lipofectamine 2000 Plus reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Transfection efficiency was evaluated by a fluorescence microscopy.

Isolation of RNA

RNX-Plus reagent (Cinagen Co., Tehran, Iran) was used for extracting total RNA. Briefly, the cells were treated with RNX solution at 25°C for 5 min, and then centrifuged at 12000 RPM at 4°C for 15 min after adding 200 µL of chloroform. Then, the upper phase with an equal volume of isopropanol were centrifuged in high speed at 4°C for 15 min, the RNA-pellet was washed with 1 mL of 75% ethanol, and dissolved in diethylpyrocarbonate treated water. Optical densitometry in wavelengths of 260 and 280 nm was used for determining concentration of the purified RNA.

Preparation of cDNA

Reverse transcriptase reaction was performed using Revert Aid™ First Strand cDNA synthesis kit (MBI, Fer-

mentas, Lithuania). Briefly, RNA was treated with DNase I (Invitrogen, Carlsbad, CA, USA) before cDNA synthesis to avoid DNA contamination. The cDNA was retrotranscribed in 20 µL reaction solution containing 5 µg total RNA, reaction buffer, RNase inhibitor (20 unit), dNTP mix (20 nM), random hexamer primer, oligo (dt) primer, and 200 unit M-MuLV reverse transcriptase. Reverse transcription procedure was performed at 42°C for 60 min and terminated by heating at 70°C for 5 min.

Quantitative RT-Polymerase chain reaction (qRT-PCR)

Specific primer sequences were designed for p53 and hdm2 and β-actin ribosomal RNA using free online Primer BLAST software (fast prim6) and gene confirmation was done with GeneDetect® oligonucleotide gene probes (Oligo 3) in triplicates as follows: hdm2 primers: 5'-CAGAGCCAAGCGGCGGAGA-3'(forward primer) and 5'-AGAAGCTGCTGGTGGCGGG-3' (reverse primer), P53 primers: 5'-TGGGCGTGAGCGCTTCGAGA-3'(forward primer) and 5'-GGTGGCTGG AGTGAGCCCTGC-3' (reverse primer), and β-actin primers: 5'-TCCCTGGAGAAGAGCTACG-3' (forward primer) and 5'-GTAGTTTCGTGGATGCCACA-3' (reverse primer). Beta-actin rRNA was used as an internal control and the relative gene expression was measured by the Livak formula ($2^{-\Delta\Delta Ct}$). All real-time PCR reactions were executed in reaction tubes containing 2x SYBER GREEN PCR master mix reagent (A B I, Vernon, CA, USA), 190 nM primer and 1µg cDNA. RT- qPCR was performed using Corbett Rotor-Gene 6000 thermal cycler (Corbett Life Science, Sydney, Australia). The thermal cycle conditions were included one cycle of 95°C for 5 min followed by 42 cycles of 95°C for 20 s (denaturation) and 60°C for 20 s (annealing) and 72°C for 20 s (extension). Finally, accuracy of reaction was confirmed by the melting curve analysis with Corbett Rotor-Gene 6000 software (Corbett Life Science, Sydney, Australia).

Western blot analysis

Whole cell lysates were prepared as described previously by Lynam-Lennon et al.²² Protein samples (25 µg) were separated on 10% SDS PAGE gel, transferred to polyvinylidene difluoride (PVDF) membrane and probed with hdm-2 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, Code No. sc-29394). Membranes were processed by enhanced chemiluminescence method (Roche, Germany). Protein bands were visualized by autoradiography and were scanned using a Hewlett-Packard Scan jet 5470c (PaloAlto, CA, USA) scanner. Signal intensities were quantified using ImageJ software (1.45s version) (National Institute of Health, USA). Hdm2 expression values were normalized to β-actin and expressed as fold change compared to the control.

Irradiation

The Cells at a seeding density of 1.0×10^5 /well were cultured in 6-well culture plates and incubated for 16 h at 37°C prior to the infection. After the period, the cells

were treated as mock-infected or infected with *mdm2* siRNA, and irradiated with γ -rays of 60-Co machine under QC procedures, (Teletherapy Unit, THERATRON[®] 1000E, Canada) at a dose rate of 98.46 cGy/min working at SAD=100 cm, a field size of 15×15 cm and the doses of 0, 0.5, 1, 2, 4, and 6 Gy in a single fraction at room temperature (23 ± 2°C) 48 h after the infection. Then the cultured cells were counted to score the viable cells, 3 days after the irradiation.

MTT assay

A number of 8.0×10^3 cells/well were seeded in the 96-well plates. After attachment, the cells were treated with *hdm2*-siRNA and irradiated following 24 h incubation. The cell proliferation rate was measured according to Aghaee et al.²³ Briefly, 20 μ L of 5 mg/mL MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltrazolium bromide) (Sigma-Aldrich, St. Louis, MO, US), was added to each well. The cells were incubated in 5% CO₂ at 37°C for 4 h, treated with 200 μ L of dimethyl sulfoxide to solubilize the colored formazan product and solubilized by 25 μ L Sorensen's buffer. An ELISA plate reader (BioTeck, Bad Friedrichshall, Germany) was used to read the absorbance at the wavelength of 570 nm.

TUNEL assay

In situ cell death detection kit (Roche Diagnostics, Risch-Rotkreuz, Switzerland) was used for TUNEL assay. Briefly, cells were fixed with 4% paraformaldehyde and permeabilized in 0.1% Triton-100, 0.1% sodium citrate and incubated with TUNEL reaction mixture then with alkaline phosphatase-conjugated anti-fluorescein antibody, stained with nitro blue tetrazolium/ 5-bromo-4-chloro-3-Indolyl phosphate and counterstained with methyl green. A reaction without terminal transferase was used as a negative control. Twenty four hours after irradiation with 1, 1.50, and 2Gy γ -rays, the cells were assayed.

Clonogenic cell survival assay

The clonogenic survival assay was used to evaluate the radiosensitivity of the TE1, TE8, and TE11 cells infected with *hdm2*-siRNA. Briefly, the transfected cells were cultured in six-well plates with an appropriate cell density, allowed 24 h to attach and subsequently exposed to 60-Co γ -rays. After irradiation, the cells with renewed media were incubated for 14 days at 37°C in a humidified incubator. The produced colonies were counted after fixing with a mixture of methanol-acetic acid (3:1) followed by staining with 2% Giemsa (Merck, Germany). The colonies consisting of more than 50 cells were scored as the survivors. Then plating efficiency and survival fraction were calculated and the sensitizing enhancement ratio was defined as the radiation was required to obtain a specific surviving fraction (SF). Each data point was performed in triplicate. The obtained data were normalized to the plating efficiencies of the sham treated control and used to plot the survival curves by means of the GraphPad Prism 6 (GraphPad Software, Inc., San Diego, CA, USA).

Statistical analyses

Statistical analyses were performed by two-way ANOVA and unpaired two-tailed *t* test, using Prism (version 4.0) from Graph Pad. Data were expressed as mean ± SE and the *p* value less than 0.05 was considered significant.

Results

Expression of *hdm2* and *p53* in TE1, TE8, and TE11 cells

The mRNA expression profiles of *hdm2* and *P53* in the cells were analyzed by real-time PCR assay. The transiently knockdown efficiencies of 200 nM *hdm2*-siRNA in the cells were first evaluated using quantitative RT-PCR. Results are displayed in Fig. 1. Twenty-four hours after transfection of the cells with *hdm2*-siRNA, the relative levels of *hdm2* RNA were decreased compared to the controls (*p* < 0.05). The expression of *B-actin* mRNA was not affected in any of the groups (Fig. 1).

To ascertain whether the effect is *p53*-dependent or not, we employed three cell lines of the cancers with different *p53* statuses. The results showed a markedly down regulation of *hdm2* mRNA levels in TE1, TE8, and TE11 cells transfected with *hdm2* siRNA for 24 h, and increased *p53* mRNA levels. Western blotting analysis demonstrated that the transfection of siRNAs, directed against *hdm2* mRNA, resulted in a decrease in *hdm2* protein levels in TE1, TE8, and TE11 cells (Fig. 1). Densitometry analysis of the prepared western blot films showed that *hdm2* levels were decreased.

Cell viability and cell apoptosis

The *hdm2*-siRNA in combination with radiation decreased the cells viability (Fig. 2) and also increased the frequencies of apoptosis in TE1, TE8, and TE11 cancer

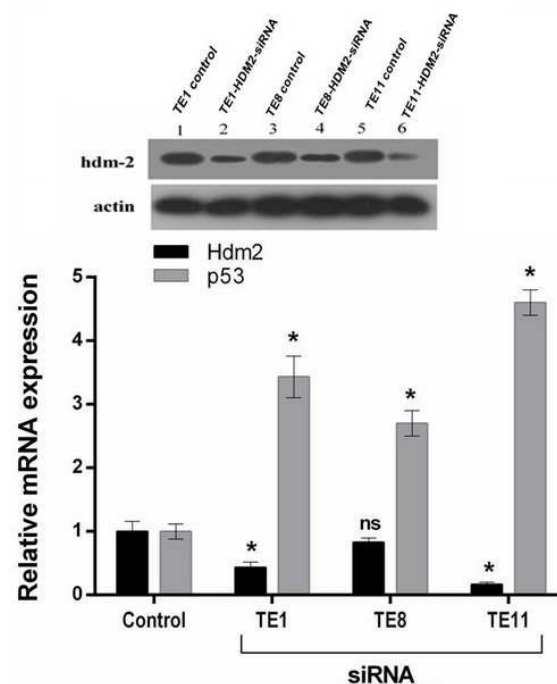


Fig. 1. Relative mRNA expression levels of *hdm2* and *p53* for TE1, TE8, and TE11 cells in the controls (Untreated TE1, TE8, TE11 cells) and knocked by *hdm2*-siRNA (* *p* < 0.01).

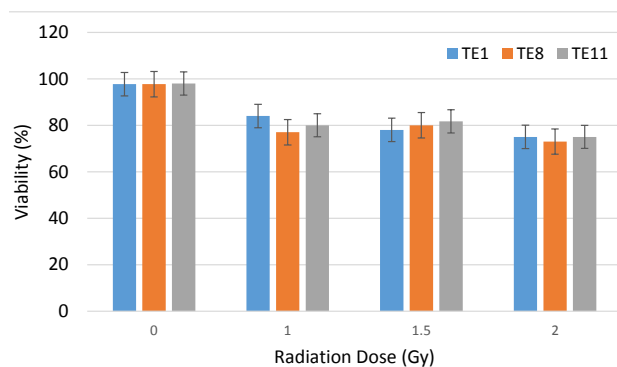


Fig. 2. Viability of TE1, TE8, and TE11 cells in the control and radiation groups that were determined by MTT assay.

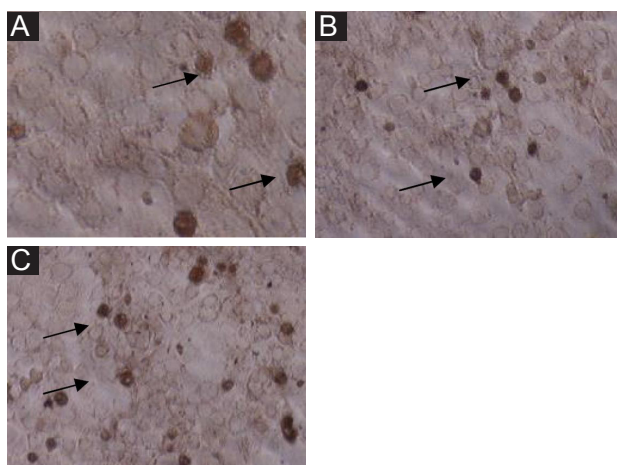


Fig. 3. Detection of apoptosis by TUNEL test. Effects of 200 nM of hdm2-siRNA followed by 200 cGy of gamma rays (IR) on cytotoxicity and proliferation of TE1 (A), TE8 (B), and TE11 (C) cells. The treated ESCC cells were evaluated by the cell death ELISA assay after 48 h. The apoptotic cells were shown by arrows on the images.

cells (Fig. 3). Terminal deoxytransferase (TdT) - mediated deoxyuridine triphosphate (dUTP) nick end labeling (TUNEL) assay was used to monitor the extent of DNA fragmentation due to apoptosis.

Cell radiosensitivity

The data on the radiosensitivities of the cells, ascertained by clonogenic assay, were presented in Fig. 4. The siRNA-mediated inhibition of the hdm2 resulted in an increased sensitivity to ionizing radiation in siRNA transfected cells compared to the untransfected cells. The increased radiosensitivity (i.e. DRF) was quantitatively about 1.2, 1.3, and 2.75 for TE1, TE8, and TE 11 cells, respectively.

Discussion

In present study, hdm2-siRNAs were used to transfect TE1, TE8, and TE11 ESCC cells before exposure to ionizing radiation, and a significant increase in sensitivity to ionizing radiation was found. Our study showed the using of hdm2-siRNA to augment radiation-mediated killing of ESCC and reconfirmed the potential using of siRNA as an

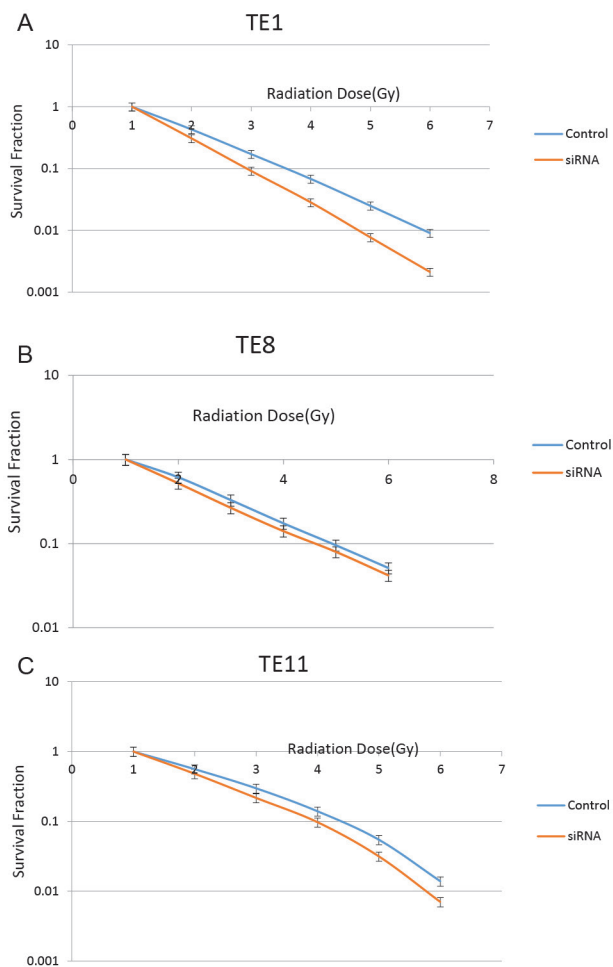


Fig. 4. Survival curves of TE1 (A), TE8 (B), and TE11(C) cell lines, with (red curve) and without (blue curve) hdm2-siRNA infection, were seeded onto 6-well plates and further irradiated with various doses (0-6 Gy). The cells were cultured for 14 days to allow colony formation..

adjuvant gene therapy strategy to radiotherapy.^{8,21,24} Radiotherapy is the common treatment approach that can potentially provide a cure for ESCC. The curability of radiation can be mediated by the radiosensitivity of the tumor target.²⁵

The decreased levels of mdm2, as a critical component of the responses to ionizing and UV radiation,²⁶ sensitize cells to the ionizing radiation. Thus, mdm2 is a potential target for therapeutic intervention because its inhibition may sensitize the subset of human tumors and make radiotherapy more effective.^{8,26} To identify genes involved in radiosensitivity and improve effectiveness of radiotherapy in esophageal cancer, we investigated the radiation sensitivities of three human esophageal cancer cell lines, namely TE1, TE8, and TE11 in presence and absence of hdm2-siRNA.

As TE1, TE8, and TE11 cells displayed different hdm2 gene expression levels, it could be considered to be an appropriate model based on molecular radiobiology techniques to study hdm2 gene silencing and possible increased radiation sensitivity of the cells. Ogawa et al performed a study on radiosensitivity of esophageal cancer cells in 13

categories.⁴ They found TE11 cells as the most sensitive cells to ionizing radiation. Our study also confirmed their results on radiosensitivity of TE11 cells, also when the cells were infected with hdm2-siRNA. Further, in some studies, microarray technique has shown to facilitate the simultaneous study of the expression of many genes.⁴ The results of oligonucleotide microarray have shown that 54 radiosensitive genes were up-regulated four folds and 17 genes were down-regulated ≥ 4 -fold in TE-11 ESCC cell lines when compared with other ESCC cell lines.⁴

Radiosensitivity is a complex product of cellular and tissue responses. Among the molecules, p53 was found to have a major role on the cellular responses to radiation, but there are inconsistencies in the reported mechanisms by which p53 affects cell survival.²⁴ When we compared the expression of p53 mRNA and protein in TE1, TE8, and TE11 cells, there were found no significant differences except in the hdm2-siRNA infected cells (Fig. 1). Our results indicated that the reduction in hdm2 expression and increase in p53 expression levels in the cells led to the cell radiosensitization that was probably related to the induction of apoptosis.

Initial screening of siRNA for its effectiveness showed that target proteins (hdm2) were downregulated from 24 h post-transfection (Fig. 1) with protein levels being comparable with the levels seen in untransfected cells. These findings are consistent with previous data reporting the half-lives of these proteins to be in the time range of 24 – 48 h.²¹ In TE1 and TE8 cells, we found an increased radiosensitivity when hdm2 up-regulated and p53 down-regulated. The results of clonogenic and MTT assays also confirmed these findings (Figs. 2 and 4). We found that the cell viability reduction following the treatments had different degrees in the TE1, TE8, and TE11 cells, however, survival of the TE11 cells was significantly changed compared to the control (Fig. 4A-C), so that the DRFs of TE1, TE8, and TE11 cells were calculated to be 1.2, 1.3, and 2.75, respectively.

Hdm2 overexpression can decrease the apoptosis in esophageal squamous cell carcinomas. Our obtained data on apoptosis indicated that the overexpression of hdm2 greatly decreased the apoptotic rate in response to radiation (Fig. 2), and confirmed the finding of Liu et al on MCF-7 breast cancer cells.²¹ We found that silencing of hdm2 mRNA directly enhanced apoptosis and decreased cell proliferation in TE1, TE8, and TE11 cells (Fig. 2). In addition, hdm2-siRNA transfection not only reduced the target gene expression specifically, but also induced changes in the expression of p53. Our results provided evidence that siRNA technology may be considered as an effective method for inhibition of oncogene expression and activate apoptotic and tumor suppressor genes.

Conclusion

Improved cancer treatment and increased radiosensitivity of esophageal tumor cells may be provided by silencing the related oncogenes.

Research Highlights

What is current knowledge?

✓ Radioresistance is a major complication in the esophageal carcinoma treatment.

What is new here?

✓ Treatment with Hdm2-siRNA could reduce the related radioresistance.

Ethical issue

The present study did not conflict with medical ethics due to its performance as an in vitro study.

Competing interests

The authors declare no conflict of interests.

Acknowledgements

This research was supported by Iran National Science Foundation (INSF) Grant (#90007980), and experimentally performed in the Departments of Medical Physics and Immunology in Tabriz University of Medical Sciences, Tabriz, Iran.

References

- Ishikura S, Nihei K, Ohtsu A, Boku N, Hironaka S, Mera K, et al. Long-term toxicity after definitive chemoradiotherapy for squamous cell carcinoma of the thoracic esophagus. *J Clin Oncol* **2003**; 21: 2697-702. doi: 10.1200/jco.2003.03.055.
- Lynam-Lennon N, Maher SG, Maguire A, Phelan J, Muldoon C, Reynolds JV, et al. Altered mitochondrial function and energy metabolism is associated with a radioresistant phenotype in oesophageal adenocarcinoma. *PLoS One* **2014**; 9: e100738. doi: 10.1371/journal.pone.0100738.
- Nguyen GH, Murph MM, Chang JY. Cancer stem cell radioresistance and enrichment: where frontline radiation therapy may fail in lung and esophageal cancers. *Cancers (Basel)* **2011**; 3: 1232-52. doi: 10.3390/cancers3011232.
- Ogawa R, Ishiguro H, Kuwabara Y, Kimura M, Mitsui A, Mori Y, et al. Identification of candidate genes involved in the radiosensitivity of esophageal cancer cells by microarray analysis. *Dis Esophagus* **2008**; 21: 288-97. doi: 10.1111/j.1442-2050.2007.00759.x.
- Frankel A, Armour N, Nancarrow D, Krause L, Hayward N, Lampe G, et al. Genome-wide analysis of esophageal adenocarcinoma yields specific copy number aberrations that correlate with prognosis. *Genes Chromosomes Cancer* **2014**; 53: 324-38. doi:10.1002/gcc.22143
- Greenawalt DM, Duong C, Smyth GK, Ciavarella ML, Thompson NJ, Tiang T, et al. Gene expression profiling of esophageal cancer: comparative analysis of Barrett's esophagus, adenocarcinoma, and squamous cell carcinoma. *Int J Cancer* **2007**; 120: 1914-21. doi: 10.1002/ijc.22501.
- Pandilla R, Kotapalli V, Gowrishankar S, Chigurupati M, Patnaik S, Uppin S, et al. Distinct genetic aberrations in oesophageal adenocarcinoma and squamous carcinoma. *Eur J Clin Invest* **2013**; 43: 1233-9. doi: 10.1111/eci.12163.
- Islamian JP, Mohammadi M, Baradaran B. Inhibition of human esophageal squamous cell carcinomas by targeted silencing of tumor enhancer genes: an overview. *Cancer Biol Med* **2014**; 11: 78-85. doi: 10.7497/j.issn.2095-3941.2014.02.002.
- Momand J, Jung D, Wilczynski S, Niland J. The MDM2 gene amplification database. *Nucleic Acids Res* **1998**; 26: 3453-9.
- Yuan Y, Liao YM, Hsueh CT, Mirshahidi HR. Novel targeted therapeutics: inhibitors of MDM2, ALK and PARP. *J Hematol Oncol* **2011**; 4: 16. doi: 10.1186/1756-8722-4-16.
- Goldar S, Khaniani MS, Derakhshan SM, Baradaran B. Molecular mechanisms of apoptosis and roles in cancer development and treatment. *Asian Pac J Cancer Prev* **2015**; 16: 2129-44.

12. Hainaut P, Hollstein M. p53 and human cancer: the first ten thousand mutations. *Adv Cancer Res* **1999**; 2000: 81-138.
13. Brooks CL, Gu W. p53 Ubiquitination: Mdm2 and Beyond. *Molecular cell* **2006**; 21: 307-15. doi: 10.1016/j.molcel.2006.01.020.
14. Eymen B, Gazzeri S, Brambilla C, Brambilla E. Mdm2 overexpression and p14(ARF) inactivation are two mutually exclusive events in primary human lung tumors. *Oncogene* **2002**; 21: 2750-61. doi: 10.1038/sj.onc.1205359.
15. Fakhrazadeh SS, Rosenblum-Vos L, Murphy M, Hoffman EK, George DL. Structure and organization of amplified DNA on double minutes containing the mdm2 oncogene. *Genomics* **1993**; 15: 283-90. doi: 10.1006/geno.1993.1058.
16. Freedman DA, Wu L, Levine AJ. Functions of the MDM2 oncoprotein. *Cell Mol Life Sci* **1999**; 55: 96-107.
17. de Rozieres S, Maya R, Oren M, Lozano G. The loss of mdm2 induces p53-mediated apoptosis. *Oncogene* **2000**; 19: 1691-7. doi: 10.1038/sj.onc.1203468.
18. Montes de Oca Luna R, Wagner DS, Lozano G. Rescue of early embryonic lethality in mdm2-deficient mice by deletion of p53. *Nature* **1995**; 378: 203-6. doi: 10.1038/378203a0.
19. Steinman HA, Burstein E, Lengner C, Gosselin J, Pihan G, Duckett CS, et al. An alternative splice form of Mdm2 induces p53-independent cell growth and tumorigenesis. *J Biol Chem* **2004**; 279: 4877-86. doi: 10.1074/jbc.M305966200.
20. Mandard A, Hainaut P, Hollstein M. Genetic steps in the development of squamous cell carcinoma of the esophagus. *Mutat Res/Rev Mutat Res* **2000**; 462: 335-42. doi:10.1016/S1383-5742(00)00019-3
21. Liu TG, Yin JQ, Shang BY, Min Z, He HW, Jiang JM, et al. Silencing of hdm2 oncogene by siRNA inhibits p53-dependent human breast cancer. *Cancer Gene Ther* **2004**; 11: 748-56.
22. Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* **1998**; 391: 806-11. doi: 10.1038/35888.
23. Aghaee F, Pirayesh Islamian J, Baradaran B. Enhanced Radiosensitivity and Chemosensitivity of Breast Cancer Cells by 2-Deoxy-D-Glucose in Combination Therapy. *J Breast Cancer* **2012**; 15: 141-7. doi: 10.4048/jbc.2012.15.2.141.
24. Okumura H, Natsugoe S, Matsumoto M, Mataka Y, Takatori H, Ishigami S, et al. The predictive value of p53, p53R2, and p21 for the effect of chemoradiation therapy on oesophageal squamous cell carcinoma. *British J Cancer* **2005**; 92: 284-9. doi: 10.1038/sj.bjc.6602322.
25. Walsh TN, Noonan N, Hollywood D, Kelly A, Keeling N, Hennessy TP. A comparison of multimodal therapy and surgery for esophageal adenocarcinoma. *N Engl J Med* **1996**; 335: 462-7. doi: 10.1056/nejm199608153350702.
26. Perry ME. Mdm2 in the response to radiation. *Mol Cancer Res* **2004**; 2: 9-19.