Increased miRNA-22 expression sensitizes esophageal squamous cell carcinoma to irradiation

Xiao-chun WANG*, Zhu-Bo ZHANG, Yue-Ying WANG, Hong-Ying WU, De-Guan LI, Ai-Min MENG and Fei-Yue FAN

Tianjin Key Laboratory of Molecular Nuclear Medicine, Institute of Radiation Medicine, Chinese Academy of Medical Science, Tianjin, 300192, China

*Corresponding author. Tel: 86-22-8568-3043; Fax: +86-22-8568-3043; Email: wxc3188@126.com

(Received 7 August 2012; revised 26 October 2012; accepted 29 October 2012)

miRNA-22 was previously reported to be a tumor suppressor. The aim of this study was to explore the expression and function of miRNA-22 in esophageal squamous cell carcinoma (ESCC). Expression of miRNA-22 in 100 ESCC tissues was examined by q-PCR. The correlation between miRNA-22 level and clinicopathological features was analyzed using SPSS16.0 statistical software. Moreover, the effect of miRNA-22 expression on radiosensitivity of ESCC cells was examined. miRNA-22 expression decreased in ESCC tissues, and statistical analyses showed that the expression of miRNA-22 was associated with the stage of clinical classification. No correlation was found between miRNA-22 expression and the overall survival of ESCC patients. However, significant positive correlation was found between miRNA-22 expression and the survival of patients who received radiotherapy ($P < 0.05$). Increased expression of miRNA-22 sensitized ESCC cells to $\gamma$-ray radiation and promoted the apoptosis of ESCC cells induced by $\gamma$-ray radiation. Increased expression level of miRNA-22 had effects on Rad51 expression after irradiation. These results demonstrate for the first time that decreased miRNA-22 expression correlates with increased radiotherapy resistance of ESCC, and that this effect is mediated, at least in part, by the Rad51 pathway.

**Keywords:** miRNA-22; ESCC; $\gamma$-ray; radiosensitivity; Rad51

**INTRODUCTION**

Esophageal carcinoma (EC) is a highly malignant disease with an overall 5-year survival rate of less than 10%. The worldwide incidence of EC is increasing, particularly for adenocarcinoma located in the lower esophagus and gastroesophageal junction. In contrast to that of western countries, esophageal squamous cell carcinoma (ESCC) is the most prevalent type of EC in China. Radiotherapy has the widest application to ESCC patients, and plays a central role in contemporary treatment concepts [1, 2]. Unfortunately, local failure remains a major concern, with persistent or recurrent disease being reported in around 40–60% of patients [3, 4]. So, the radiosensitization is a promising approach to improving the therapeutic ratio for irradiation.

miRNAs are conserved 19–22 nucleotide non-coding RNAs. They repress protein expression at the post-transcriptional level, mainly by annealing with the 3’ UTR of the target mRNA and interfering with its translation and/or stability [5]. The precise number of miRNA genes in the human genome is still unknown, but estimates of the total number of miRNA precursors are as high as 25,000. These molecules are thought to regulate approximately 30% of the genes in the human genome [6, 7]. Studies have shown that some miRNAs regulate cellular differentiation, proliferation and apoptotic processes that are important in cancer aggravation. miRNA profiling across multiple human cancers has suggested that miRNAs are potential indicators of the developmental origin of poorly differentiated cancers. In the present study, we found that miRNA-22 expression decreased in ESCC tissues, and that its expression was positively associated with the survival of patients who received radiotherapy.

In addition, increased miRNA-22 expression promoted radioresistance of ESCC cells.
MATERIALS AND METHODS

Tissue specimens
One hundred fresh tissue samples, which contained ESCC and the adjacent histologically normal tissue, were procured from surgical resection specimens collected by the Department of Tumor Medicine, Shandong Provincial Chest Hospital from 2001 to 2007. Primary tumor regions and the corresponding histologically normal tissues from the same patients were separated by experienced pathologists and immediately stored at –70°C until use. None of the patients received treatment before surgery, and all signed informed consent forms for sample collection. Of these 100 patients, 58 received concurrent chemotherapy with radiation therapy after surgery. Radiation therapy was administered in daily divided doses with linear accelerators, and all patients received thoracic irradiation with daily fraction doses of 2.0 Gy for a total dose 50 Gy. The chemotherapy program was Cisplatin (CDDP) + 5-fluorouracil (5-FU). The other 42 patients were treated with chemotherapy only. Use of patient samples of tumors and adjacent histologically normal tissues was approved by the institutional Ethics Committee.

RNA extraction
Total RNA was extracted from ESCC tissue and the corresponding normal tissue using the Absolutely RNA™ Miniprep kit (Stratagene, Santa Clara, CA) according to the manufacturer’s instructions. RNA quantification was performed with a DUVR 800 UV/Vis Spectrophotometer (Beckman Coulter, Fullerton, CA).

Quantitative reverse transcription-polymerase chain reaction
TaqMan miRNA assays (ABI PRISM, Carisbad, CA) were used to detect the expression levels of mature miRNA-22. For the reverse transcription (RT) reactions, 10 ng of total RNA was used in each reaction and mixed with the RT primer. RT reactions were performed at 16°C for 30 min, 42°C for 30 min, 85°C for 5 min and then maintained at 4°C. Following the RT reactions, 1.5 µl of cDNA was used for a polymerase chain reaction (PCR) using 2 µl of the TaqMan primers. The PCR was conducted at 95°C for 10 min followed by 40 cycles of 95°C for 15 sec and 60°C for 60 sec in an ABI 7500 real-time PCR system. The real-time PCR results were analyzed and expressed as the relative miRNA level using the U6 snRNA for normalization purposes. The RT and PCR primers for miRNA-22 were purchased from ABI PRISM (ABI PRISM, Carisbad, CA). The fold change in the miRNA expression in each tumor sample relative to the average expression in the non-cancerous control was calculated based on the threshold cycle (CT) value using the following formula:

Relative gene expression = 2^{−ΔΔCt}, where −ΔΔCt = (Ct gene of interest − Ct internal control gene) Treated − (Ct gene of interest − Ct internal control gene) Untreated [8]. A 2-fold change in either direction was considered to be significant.

Cell lines
The ESCC cell lines EC9706 and KYSE150 were purchased from the tumor cell bank of the Chinese Academy of Medical Science. These cells were cultured in RPMI 1640 (Invitrogen) supplemented with 10% FBS.

Ionizing radiation
KYSE150 or EC9706 cells were exposed to different doses of irradiation in a JL Shepherd Model 143 and 137Cesium γ-irradiated at a rate of 2.4 Gy/min.

miRNA northern blots
For miRNA northern blots, 15 µg of total RNA was separated on a 15% denaturing polyacrylamide gel, electro-transferred onto a GeneScreen Plus hybridization transfer membrane (PerkinElmer, Waltham, Mass) and hybridized using ULTRAhyb-Oligo buffer (Ambion, Austin, TX). Oligonucleotides complementary to the mature miRNA-22 were end-labeled with T4 polynucleotide kinase (Invitrogen, Carlsbad, CA) and used as probes. Hybridization was performed at 42°C overnight, and the membranes were washed twice in 0.1 × SSPE and 0.1% SDS at 42°C for 15 min each. The membranes were then exposed to a storage phosphor screen (GE Healthcare Bio-Sciences, Piscataway, NJ) for 8 h and imaged using a Typhoon 9410 Variable Mode Imager (GE Healthcare Bio-Sciences, Piscataway, NJ). Saved images were cropped using Photoshop 6.0 (Adobe Systems Inc., San Jose, CA).

Western blot analysis
Whole cell extracts were prepared from ESCC tissues and cultured cells by homogenizing cells in a lysis buffer (10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% NP40) containing a cocktail of protease inhibitors. After centrifugation at 15 000 rpm for 30 min at 4°C, supernatants were recovered and used for immunoblot analysis. The proteins were separated by SDS-PAGE and then transferred to polyvinylidene difluoride membranes (Millipore). Blots were blocked and then probed with Rad51 (1:500, Santa Cruz), Ku70 (1:500, Santa Cruz) and β-actin (1:5000, Abcam). After washing, the blots were incubated with horseradish peroxidase-conjugated secondary antibodies and visualized by super ECL detection reagent (Applygen, Beijing, China).

Plasmid construction and cell transfection
The miRNA-22 expression vector was constructed by cloning of annealed oligonucleotides that contained the pre-miRNA-22 sequence into the pSuppressorNeo expression vector. A scrambled sequence without significant
homology to any rat, mouse or human gene was used as a negative control (vector group) (ABI PRISM, Carisbad, CA). Anti-miR™ miRNA-22 inhibitor (ABI PRISM, Carisbad, CA) was used to knockdown miRNA-22 expression. The expression constructs or inhibitor were transfected into the KYSE150 or EC9706 cell line, respectively, using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. A northern blot was used to confirm the overexpression of miRNA-22.

**Colony formation assay**
A total of 400 cells were aliquoted into each well of a 6-well plate in triplicate and exposed to γ-rays with different irradiation dose. After 10 days of incubation, the colonies were stained with Giemsa stain, and a minimum of 50 viable cells were counted. Quantity One software (version 4.6.2) was used to analyze the results.

**γ-H2AX foci formation assay**
Briefly, cells were grown in cover slips kept in 35 mm petri plates and irradiated with different dosages (0, 2, 4 and 6 Gy) of γ-rays. Cell layers were washed with PBS and fixed for 20 min in 4% paraformaldehyde at room temperature; afterwards the cells were washed twice in PBS. For immunofluorescence staining, cells were permeabilized for 3 min in 0.25% Triton X-100 in PBS, washed two times in PBS and blocked for 1 h with 5% BSA in PBS. Antibody was diluted (1:200) in 1% BSA in PBS. Cells were incubated with primary antibody for 1 h at room temperature, washed three times in PBS and incubated with secondary antibody for 1 h at room temperature. Finally, cells were rinsed and mounted with ProLong Gold antifade with DAPI mounting media (Molecular Probe, USA). Images were captured using a Carl Zeiss confocal microscope. Acquisition settings were optimized to obtain maximal signal in immunostained cells with minimal background. A total of 200 nuclei were counted. All experiments were carried out in triplicates, independently from each other. The primary antibodies used were rabbit anti-γH2AX (Cell Signaling, USA). Secondary antibodies used were Alexa Fluor 488 goat anti-rabbit IgG (Molecular Probe, USA).

**Flow cytometry**
Cells were harvested 8 h post-irradiation with a dose of 0, 2, 4 and 6 Gy for apoptosis detection using the annexin V-FITC apoptosis detection kit (Sigma), and subsequently analyzed by flow cytometry.

**Statistical analysis**
All statistical analyses were performed using SPSS16.0 software. We statistically evaluated experimental results using a χ² test and an ANOVA test. Survival curves were constructed using the Kaplan–Meier method. P < 0.05 was considered statistically significant.

**RESULTS**

The expression of miRNA-22 in ESCC tissue and its correlation with clinicopathological features
Expression of miRNA-22 was detected in 100 ESCC samples and the adjacent histologically normal tissues using RT-qPCR. miRNA-22 expression was normalized to the control U6B small nuclear RNA gene (RNU6B). The results showed that miRNA-22 expression levels were significantly lower in ESCC tissues compared with the corresponding non-cancerous tissues (Fig. 1).

Statistical analyses showed that the expression of miRNA-22 was only associated with the stage of clinical classification (Table 1). To determine if miRNA-22 expression was association with prognosis, a Kaplan–Meier survival analysis and a log-rank test were performed. No correlation was found between miRNA-22 expression and overall survival (P = 0.237, Fig. 2A). In the patients who did not receive radiotherapy (42 out of 100), no difference was found between miRNA-22 high-expression and low-expression patients (P = 0.425, Fig. 2B). In contrast, in patients who received concurrent chemotherapy with radiation therapy (58 out of 100), the survival rate of miRNA-22 high-expression patients was higher than that of miRNA-22 low-expression patients (P = 0.042, Fig. 2C). These results showed that the miRNA-22 expression status may have effects on the radiotherapy outcome of ESCC patients.
Effects of miRNA-22 expression on the radiosensitivity of ESCC cells

Expression of miRNA-22 in four ESCC cell lines was measured using RT-qPCR. As shown in Fig. 3, the sequence of miRNA-22 level was EC9706 > KYSE510 > KYSE450 > KYSE150. In the next experiments, EC9706 and KYSE150 were selected as cell models to study.

A colony formation assay was performed to examine the effects of miRNA-22 expression on the radiosensitivity of ESCC cells. The survival fraction of KYSE150 cells with forced miRNA-22 expression was lower than that of control groups (Fig. 4A and B). Conversely, the survival fraction of miRNA-22 knockdown EC9706 cells was significantly higher than that of control groups (Fig. 4C and D). These results indicate a positive correlation between miRNA-22 expression and radiosensitivity of ESCC cells to gamma radiation.

We also examined the effects of miRNA-22 expression on the proliferation of ESCC cells using MTT experiments. The results obtained indicated that the proliferative ability of cells increased in low miRNA-22 level cells and decreased in miRNA-22 high-expression cells (Fig. 5A and B).

Expression of miRNA-22 had an effect on the repair of DNA double-strand breaks induced by irradiation

To investigate whether miRNA-22 expression has an effect on the repair ability of DNA damage induced by γ-rays, we performed γ-H2AX foci formation assays 1 h post-irradiation at different dosages. The number of γ-H2X foci...

Table 1. Relationship between miRNA-22 expression and tumor clinicopathological features

| Clinicopathological features | Number of cases | miRNA-22 expression | P  |
|-----------------------------|-----------------|--------------------|----|
|                             | Low | High |    |
| Age, years                  | 35  | 24   | 11 | 0.226 |
| ≥60                         | 65  | 52   | 13 |        |
| TNM classification pT       |     |      |    |
| pT1                         | 12  | 9    | 3  | 0.843  |
| pT2                         | 33  | 24   | 9  |        |
| pT3                         | 55  | 43   | 12 |        |
| Lymph node metastasis       |     |      |    |
| N0                          | 45  | 35   | 10 | 0.815  |
| N1                          | 55  | 41   | 14 |        |
| Stage                       |     |      |    |
| I                           | 10  | 10   | 0  | 0.005  |
| II                          | 12  | 5    | 7  |        |
| III                         | 36  | 31   | 5  |        |
| IV                          | 42  | 30   | 12 |        |
| Grade                       |     |      |    |
| G1                          | 25  | 20   | 5  | 0.689  |
| G2                          | 30  | 21   | 9  |        |
| G3                          | 45  | 35   | 10 |        |
in miRNA-22 knockdown cells was significantly lower than in control cells after irradiation ($P < 0.01$) (Fig. 6A). In accord with this result, the percentage of apoptotic cells with a low miRNA-22 level was significantly lower than that of control cells ($P < 0.05$) (Fig. 6B). Conversely, the number of γ-H2X foci in forced miRNA-22 expression cells was significantly increased (Fig. 6C). The percentage of apoptotic cells with a high miRNA-22 level was significantly higher than that of control cells ($P < 0.05$) (Fig. 6D). These data show that miRNA-22 expression can affect the repair ability of DSBs induced by irradiation.

We next examined the expression of two key proteins Rad51 and Ku70, which were correlated with DNA double-strand breaks (DSBs) repair. As shown in Fig. 7A, Rad51 expression increased after miRNA-22 knockdown after irradiation, but the Ku70 level did not change. Forced expression of miRNA-22 decreased Rad51 expression after irradiation and the Ku70 expression was not affected (Fig. 7B).

**DISCUSSION**

Studies have shown that miRNA expression fingerprints correlate with the clinical and biological characteristics of tumors, including tissue type, differentiation, aggression, response to therapy and prognosis [9]. Thus, a large amount of diagnostic information is encoded in a relatively small amount of miRNA. Liu et al. found that the miRNA-143 and miRNA-145 clusters were statistically different between esophageal cancer tissues and matched controls. The combined expression of miRNA-143 and miRNA-145 was significantly associated with a risk for EC [10]. miRNA-25 was found to be upregulated in 60 ESCC tissues compared with matched adjacent non-cancerous tissues. The upregulation of miRNA-25 was significantly correlated with status of lymph node metastasis and TNM stage, and markedly promoted migration and invasion of ESCC cells [11]. Kurashiqe et al. reported that the serum concentration of miRNA-21 in ESCC patients was...
significantly higher than that in healthy controls. A significant reduction in serum miRNA-21 levels was observed in post-operative samples vs pre-operative samples [12]. Other miRNA, such as miRNA-223 [13], miR-142-3p [14] and let-7 [15] were all found to have effects on ESCC progress. MiRNA-22 was previously reported to be a tumor suppressor. The relative expression level of miRNA-22 in lung cancer tissues was lower than that in normal tissues. Overexpression of miRNA-22 was shown to significantly inhibit the proliferation and invasion of lung cancer cell lines [16]. miRNA-22 expression was downregulated in hepatic cell cancer (HCC), and low miRNA-22 expression was predictive of poor survival in HCC patients. Overexpression of miRNA-22 significantly inhibited HCC cell proliferation and tumorigenicity [17]. However, the expression status of miRNA-22 in ESCC has not been reported until recently. Here, we detected the expression levels of miRNA-22 in ESCC patients, and a statistical analysis showed that the expression of miRNA-22 was negatively associated with the stage of clinical classification. Because some patients in our study received radiotherapy treatment and others did not, moreover, the chemotherapy project for all 100 patients was the same, we want to know if the miRNA-22 expression has an effect on the radiotherapy outcome for ESCC patients. Our results showed that the survival time of patients with high miRNA-22 expression was longer than that of patients with low miRNA-22 expression following radiotherapy, but miRNA-22 had no effect on survival time in patients who did not receive radiotherapy. This is the first report of a correlation between miRNA-22 expression and radiotherapy outcome.

To confirm the clinical data, we explored the correlation between miRNA-22 expression and radiosensitivity in ESCC cells. The colony formation and MTT assay proved that an increased miRNA-22 level prompted radiation sensitization of EC9706 cells. The radiosensitivity of cells is influenced by many factors. One of the most important factors is the repair ability of DNA DSBs. If the damage induced by radiation can be efficiently repaired, the cell will survive, otherwise the cell will undergo apoptosis. Given that our data proved overexpression of miRNA-22 increased the radiation sensitivity of ESCC cells, we hypothesize that this is due to miRNA-22 affecting the repair ability of DSBs induced by radiation in ESCC cells. The results of our γH2X foci experiment proved our hypothesis. There are two pathways for repairing DSBs, homologous recombination (HR) and non-homologous end-joining (NHEJ). Rad51 is a key protein in the HR DNA repair pathway. It is the major strand-transferase required for mitotic recombination [18]. The NHEJ pathway repairs the DNA by a homology-independent mechanism, rejoining broken ends irrespective of the sequence. Upon exposure to different genotoxic insults, the Ku70/80 heterodimer (Ku) binds to the broken DNA ends and recruits the catalytic subunit of the DNA-dependent protein kinase (DNA-PKcs). The formation of this complex results in the subsequent recruitment and phosphorylation of other proteins, such as XRCC4, DNA Ligase IV, Cernunnos/XLF and Artemis, so as to ligate the broken DNA ends [19]. To further explore the molecular mechanism of miRNA-22 affecting DSBs repair, we then measured the expression levels of Rad51 and Ku70 in miRNA-22 knockdown cells by forced expression after irradiation. The results indicated that miRNA-22 only affected the expression of Rad51. Overexpression of the Rad51 protein has been reported to stimulate homologous recombination and increase resistance of mammalian cells to ionizing radiation [20]. This result, combined with our data, suggests that downregulation of miRNA-22 can promote the repair ability of DSBs induced by γ-ray irradiation in ESCC cells, and that this effect is mediated, at least in part, by the Rad51 pathway.

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![Fig. 5](image_url) Effects of miRNA-22 expression on the proliferation of ESCC cells. MTT assay was performed after three groups of cells were radiated using γ-ray with a dose of 6 Gy. A, Proliferation ability of EC9706 cells with decreased miRNA-22 level was significant higher than that of control cells (P < 0.05). B, Proliferation ability of KYSE150 cells with increased miRNA-22 level was significant lower than that of control cells (P < 0.05).
apoptosis by repressing PTEN expression. This UV stress response may inhibit the acute activation of the caspase signaling cascade and provide a time window for cells to repair the DNA damage induced by UV [21]. These various results show that miRNA-22 can play different roles in the context of different malignancies and different types of irradiation. Further studies should be implemented to explore the potential mechanism.

CONCLUSION

In conclusion, we have found, for the first time, that miRNA-22 expression is correlated with the prognosis of ESCC patients who receive radiotherapy. An increased miRNA-22 level inhibited the DSBs repair ability of ESCC cells, at least in part through the HR pathway, thereby improving the radiation sensitivity of ESCC cells.

SUPPLEMENTARY DATA

Supplementary data is available at the Journal of Radiation Research online.
ACKNOWLEDGEMENTS

We thank Dr Tian Jing for the collection of tumor samples.

FUNDING

Grant support was received from the National Natural Science Foundation of China (30901723, 81272511), the Natural Science Foundation of Tianjin (11JCYBJC13700) and the Institute Fund of Radiation Medicine (SF1103, SF1106).

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