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Radiation modulation of microRNA in prostate cancer cell lines

Sajni Josson¹, Shian-Ying Sung², Kaiqin Lao³, Leland W.K. Chung⁴, and Peter A.S. Johnstone⁵

¹Radiation Oncology, Winship Cancer Institute, Emory University, Atlanta, Georgia

²China Medical University Hospital, Taichung, Taiwan

³Applied Biosystems, Foster City, California

⁴Department of Urology, Winship Cancer Institute, Emory University School of Medicine, Atlanta, Georgia

⁵Department of Radiation Oncology, Indiana University School of Medicine, Bloomington, Indiana

Abstract

Background—MicroRNAs (miRNA) are gene regulators and play an important role in response to cellular stress.

Methods—Using multiplexed quantitative real-time PCR we performed global miRNA screening of prostate cancer cells in response to radiation treatment.

Results—Several miRNA were significantly altered in response to radiation treatment. Significant changes were observed in miR-521 and miR-34c. To determine the role of miR-521 in radiation response we transiently overexpressed miR-521 using miR-521 mimic. The miR-521 mimic significantly sensitized prostate cancer cells to radiation treatment. Conversely, ectopic inhibition of miR-521 resulted in radiation resistance of prostate cancer cells. To determine the mechanism by which miR-521 modulates radiation sensitivity we measured the expression levels of one of its predicted targets, Cockayne syndrome protein A (CSA). CSA is a DNA repair protein, and its levels correlated inversely with the levels of miR-521. Radiation treatment downregulated the levels of miR-521 and upregulated CSA protein. Similarly, ectopic inhibition of miR-521 resulted in increased CSA protein levels. Therefore by altering the levels of CSA protein, miR-521 sensitized prostate cancer cells to radiation treatment.

Conclusion—miR-521 modulates the expression levels of DNA repair protein, CSA and plays an important role in the radiosensitivity of prostate cancer cell lines. Thus miR-521 can be a potential target for enhancing the effect of radiation treatment on prostate cancer cells.

Keywords

MicroRNA; radiation; profiling; prostate cancer

Introduction

MicroRNAs (miRNA) are short noncoding RNA that regulate gene expression. miRNAs primarily bind to the 3' UTR of an mRNA and either represses its translation or may result in the degradation of the mRNA (1). A single miRNA may have several mRNA targets. A few studies have been performed to determine the role of miRNAs in radiation response

(2-4). He et al. demonstrated the role of miR-34 family in response to DNA damage and cellular stress. Interestingly, they showed how p53 transcriptionally activated miR-34 family members in response to DNA damage. Thus miR-34 mediated p53 function and induced cell cycle arrest. miR-34 directly interacted with mRNA involved with cell cycle progression and downregulated their expression (2). Since p53 is the guardian of the genome, it plays a central role in response to radiation and activates proteins and miRNA involved in DNA repair. Another such study involved the role of let-7 family in enhancing radiation sensitivity in lung cancer cells (3). This study demonstrated that let-7 family of miRNAs are downregulated in response to radiation treatment and activates cell survival pathways for radio-resistance such as the Ras pathway (3).

Ionizing radiation (IR) activates various survival and death signaling pathways. Previous studies demonstrate that radiation induces changes in a large number of genes. Microarray studies using mouse brains treated with radiation resulted in alterations of around 855 genes (> 1.5 fold) within 4 h of treatment (5). These genes were mostly involved in DNA repair/synthesis, stress response and cell cycle control. However, the role of miRNA, and how they integrate into the radiation signaling pathways is largely unknown. In this study, global miRNA profiling is performed to determine important miRNA in stress response. Two cell lines were used to study radiation response, the LNCaP and C4-2 prostate cancer cells. The former is marginally tumorigenic and androgen dependent where as the later is highly tumorigenic, androgen independent and metastatic (6).

Materials and Methods

Cell Culture

The human prostate cancer cell lines LNCaP and C4-2 were used. Cells were grown in a 5% CO₂ incubator at 37 °C in media consisting of T-medium (Invitrogen) supplemented with 5% (v/v) fetal bovine serum and 1% Penicillin-Streptomycin.

Quantitative Real Time PCR

Cells were trypsinised and total miRNA was extracted using a mirVana miRNA isolation kit (Ambion, Inc., Austin TX). mRNA was then measured spectrophotometrically. miRNA analysis was performed as previously mentioned (7). Samples were reverse transcribed using 0.5 µl of 10x cDNA archiving kit buffer (Applied Biosystems, Foster City, CA), 1 µl MMLV reverse transcriptase (50U/µl), 0.25 µl of 100 mM dNTP, 0.0625 µl of AB RNase inhibitor (20 U/µl), 0.25 µl of 330-plex reverse primer (RP, 50 nM each), 2 µl of total RNA (10 ng/µl), 0.25 µl dH₂O and 0.685 µl of 100 mM MgCl₂. The incubation conditions used were 20 °C for 30 sec, 42 °C for 30 sec and 50 °C for 1 sec for 30 cycles. The enzyme was inactivated at 85 °C for 5 min. Further Pre-PCR amplification was performed. The reaction mixture contained 12.5 µl of 2x Universal Master Mix with no UNG (Applied Biosystems, Foster City, CA), 5 µl of RT sample, 2.5 µl of 330-plex Forward primer (FP, at 500 nM each), 1.25 µl of 100 µM of universal reverse primer (UR), 1.25 µl of 5 U/µl of AmpliTaq Gold, 0.5 µl of 100 mM of dNTP, 1 µl of 100 mM of MgCl₂ and 1 µl of dH₂O. The PCR reaction was as follows: 95°C for 10 min to activate Taq-GOLD, 55°C for 2 min., 18 cycles of 95°C for 1 sec and 65°C for 1 min. The Pre-PCR mixture was diluted by adding 75 µl of dH₂O. Real-Time: The probes for the Taqman reaction contained 18 nucleotides of RT-RP of each miRNA at the 3' end, with the fluorescence dye FAM at the 5' end and a minor groove binder with non-fluorescence quencher, MGB, on the 3' end. The reaction mixtures contained 5 µl of 2x Universal Master Mix with no UNG (Applied Biosystems, Foster City, CA), 2 µl of a 5 µM FP + 1 µM TaqMan probe mixture, 0.1 µl of 100 µM UR, 0.1 µl of 4x diluted pre-amplified RT-PCR sample, and 2.8 µl of dH₂O. The Real time reaction was as follows: 95 °C for 10 min., 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. An AB 7900

HT Sequence Detection System in a 96-well plate format was used. Each sample was run in duplicate. Three hundred and thirty miRNA's were tested. RNU6B (Applied Biosystems, Foster City, CA), was used as a control, and their values were not different between the groups tested (Suppl. 3). Additionally miR-16 (Applied Biosystems, Foster City, CA) was also used as an additional control; their values did not change in response to radiation treatment. The raw data is represented as a heatmap, and was analyzed for fold changes. The statistically significant fold changes are depicted on Table 1

Clonogenic assay

Cells were plated in low densities in 6-well plates for 24 h and then were radiated with the appropriate radiation dose. Twenty-four hours later the media were changed and incubated until they formed colonies having at least more than 50 cells. Seventeen days later colonies were counted. The colonies were rinsed with PBS and stained with methanol/crystal violet dye. The surviving fraction was calculated as a ratio of the number of colonies formed divided by the total number of cells plated times the plating efficiency. The surviving fraction was further plotted in log scale (Fig 1b).

Radiation

External beam radiation was delivered on a 600 Varian linear accelerator with a 6 MV photon beam. A 40×40 cm field size was utilized and Petri dishes were placed on 1.5 cm of superflab bolus. Monitor units (MU) were calculated to deliver the dose to a depth of $d_{\max}$ at a dose rate of 600 MU/minute.

Overexpression and inhibition of hsa-miR-521 and cell viability assay

The control miRNAs, hsa-miR-521 mimic and inhibitor was purchased from Dharmacon (Lafayette, CO). Cells were plated at 80% confluence at the time of transfection. Cells were transfected with the miR-521 mimic (100 nM), miR-521 inhibitor (100 nM) or control mimic (100 nM) and lipofectamine in serum free medium for 5 h. Twenty four hrs later cells were irradiated (6 Gy). Five days later cell viability was assayed using MTS assay and measured spectrophotometrically at 490 nm. Alternatively, cell viability was assayed using Trypan blue exclusion with 0.4% trypan blue 24 h after radiation treatment. Western analysis was performed 24 hr after radiation.

Western Analysis

Whole cell lysates were prepared and western analysis was performed as previously described (8). The primary antibodies for CSA was purchased from Santa cruz Biotechnology, MnSOD from Millipore and β -actin from Sigma. Densitometry analysis was performed using Quantity One. Protein levels were normalized to β -actin. Fold changes were determined and plotted.

Statistical analysis

All experiments were performed in triplicate, repeated at least two or three times, and representative findings are shown. Student's t-test was used to determine the statistical significance between groups.

Results

Differential expression of miRNA in prostate cancer in response to radiation treatment

The miRNA modulated by radiation treatment was determined in LNCaP and C4-2 prostate cancer cell lines. The miRNA profile was determined 4 h post radiation. miRNA levels were determined using quantitative real time PCR analysis. Only 55% of the miRNA were

detected in these prostate cancer cell lines. miRNA altered in response to radiation are represented as a heatmap (Fig. 1a, Suppl. 1). Forty eight miRNAs of the 330 miRNAs were differentially expressed in androgen dependent and androgen independent cell lines (Suppl. 2). To determine the radiation sensitivity of LNCaP and C4-2 cells we performed clonogenic assay. Both the cell lines had similar radiation sensitivity (Fig. 1b). In response to radiation several miRNA were altered, those which were altered more than 3 fold are listed in Table 1. Interestingly, certain miRNA changes were common to both the cells lines. For example, miR-521 was downregulated in both cell lines in response to radiation treatment. Additionally, miRNAs miR-196a and miR-133b, were decreased around 10 fold in both cell lines. miR-34c was found to be increased in LNCaP and C4-2 cells. miR-487, miR-122a and miR-145 had a 100-fold decrease in LNCaP cells, and miR-143 in C4-2 cells in response to radiation. miR-372 and miR-520c had more than 400-fold increases in LNCaP cells in response to radiation. These results demonstrate that several miRNA are significantly altered in response to ionizing radiation in prostate cancer cell lines. These important miRNA may play a concerted effect in determining the outcome of radiation treatment.

Modulation of miR-521 altered the radiation responsiveness of prostate cancer cells

Since miR-521 was found to be downregulated to a greater extent in response to radiation in both cells, we tested the hypothesis whether overexpression miR-521 could enhance radiation effects. Using transient transfection we overexpressed miR-521 mimic in LNCaP cells. miR-521 levels were determined using quantitative real time PCR analysis. miR-521 was increased in the miR-521 mimic group compared to control miRNA (Fig. 2a). Consistent with previous results we observed a decrease in miR-521 levels in response to radiation in the control group. However, due to very high levels of miR-521 mimic, we did not observe a decrease in miR-521 in this group in response to radiation. To determine if miR-521 mimic could increase radiation sensitivity of prostate cancer cells, we performed cell viability assays such as trypan blue exclusion (Fig. 2c) and MTS assay (Fig. 2c). In response to radiation treatment there was increased cell death in both the control and the miR-521 mimic transfected cells compared to their controls. However, miR-521 mimic radiated group had significantly decreased cell viability compared to control radiated group. These results are consistent with the hypothesis that miR-521 overexpression induces radiation sensitivity of prostate cancer cells. Similar results were observed by MTS assay. To further confirm the role of miR-521 in radiation sensitivity, we used miR-521 inhibitor to determine if it could induce radio-resistance in prostate cancer cells. Similar studies were performed as before where miR-521 was transiently inhibited using miR-521 inhibitor and cell viability was assayed using trypan blue exclusion and MTS assay. Additionally, clonogenic assay was performed. In all three assays, the miR-521 inhibitor treated group was significantly more resistant to radiation compared to control group with radiation (Fig. 3a, 3b, 3c). These results suggest that miR-521 plays an important role in radiation sensitivity of prostate cancer cells.

miR-521 modulates radiation response by altering the expression of CSA

Using the Sangers website, several proteins were listed which have been previously shown to play a role in cell stress response (Table 2)(9). The miR-521 target site on each of the protein listed was conserved in 3 or more different species. miR-521 was predicted to bind to 7 or more sites on the mRNA of each of the targets (9). Cockayne syndrome A (CSA) was one of the predicted targets of miR-521. Since CSA is a DNA repair protein, we determined if its levels were altered in response to radiation or in response to miR-521 inhibitor. CSA expression levels were increased several fold in response to radiation compared to control (Fig. 4a). In miR-521 inhibitor treated cells, CSA was significantly increased, suggesting that inhibition of miR-521 resulted in upregulation of CSA protein. Since CSA expression was higher in miR-521 inhibitor treated cells compared to control, the miR-521 inhibitor

treated cells were more resistance to radiation compared to control. Additionally we determined the levels of another antioxidant protein, Manganese superoxide dismutase (MnSOD) in these treatments. MnSOD is a mitochondrial anti-apoptotic and antioxidant enzyme that dismutates superoxide radicals (10). Increased levels of MnSOD are known to protect against radiation induced oxidative damage (11). MnSOD expression was induced in response to radiation in both control and miR-521 inhibitor treated groups (Fig. 4b). Inhibition of miR-521 resulted in a moderate increase in MnSOD. MnSOD may be an indirect target of miR-521. Taken together, these results suggest that miR-521 plays an important role in radiation response by altering the levels of DNA repair protein CSA and anti-oxidant protein, MnSOD in prostate cancer cells.

Discussion

The objectives of this study are: 1) to determine if certain specific miRNA species may be altered on radiation treatment of prostate cancer cells; and 2) if the radiation altered miRNA may have a regulatory function in augmenting radiation sensitivity. Several miRNA were altered in response to radiation. miR-521 was found to be most prominently decreased by ionizing radiation in both LNCaP and C4-2 cells (Table 1). These data suggest that the mRNA targets of this miRNA may be important for the radiation stress response in prostate cancer cells. When miR-521 was subsequently overexpressed in LNCaP cells, they were more sensitized to radiation treatment. Further, when miR-521 was inhibited the prostate cancer cells were more resistant to radiation treatment. Together these results confirm the role of miR-521 in modulating the radiation response of human prostate cancer cells.

To determine if miR-521 regulated proteins involved in radiation response we searched the Sangers website for the predicted targets of miR-521. CSA was one of the predicted targets of miR-521. CSA is a DNA repair protein, and Cockayne syndrome (CS) patients with a mutation in this gene have pronounced photosensitivity and neuro-developmental abnormalities (12). Also cells from CS patients have decreased transcription coupled repair. In response to radiation we observe that downregulation of miR-521 leads to the increase in CSA protein. Consistent with these results specific ectopic inhibition of miR-521 also results in increased CSA protein levels. Thus miR-521 may specifically target CSA and affect the radiosensitivity of the prostate cancer cells. We also determined if miR-521 could alter other proteins involved in radiation response such as MnSOD. Inhibition of miR-521 also resulted in moderate increases in MnSOD protein levels, thus protecting the cell from oxidative damage induced cell death. These data collectively suggest that miR-521 plays an important functional role in modulating the radiation response of LNCaP prostate cancer cells and might be used as a target for radio-sensitization.

Other miRNAs that decreased in both cell lines were miR-196a and miR-133b. miR-133b has been shown to be altered in cardiac stress (13). Several other miRNAs such as miR-487, miR-122a, and miR-145 were decreased in LNCaP cells, and miR-143 was decreased in C4-2 cells. Recent studies have demonstrated the role of Let-7 members in radiation response (3). Overexpression of let-7b resulted in radio-sensitization and inhibition of let-7g caused radio-resistance (3). Our results showed moderate decreases in let-7a in both LNCaP and C4-2 cells in response to radiation. Let-7f was decreased in LNCaP and increased in C4-2 cells. Let-7d was increased in LNCaP cells. These changes were not more than 2 fold.

miRNAs which were increased significantly in both cell lines were miR-34c. These results are consistent with recent studies which demonstrate that miR-34 family of miRNAs are targets of p53 (2). LNCaP cells express low levels of wild-type p53 (14), which may have been activated in response to radiation and activated miR-34c. We observed a 3-5 fold increase in miR-34c miRNA in response to radiation. Several-fold upregulation of other

miRNA such as, miR-372, miR-520c and miR-449 was observed in response to radiation only in LNCaP cells. C4-2 cells showed increases in miR-383, miR-154* and miR-488. Further studies need to be performed to validate the role of the above mentioned miRNA in radiation sensitivity of cancer cells.

In summary, we observed several miRNAs that were altered in response to radiation treatment and found that miR-521 confers radiation sensitivity to LNCaP prostate cancer cells by modulating repair proteins such as CSA and MnSOD. Thus miR-521 could be an attractive therapeutic target in combination with radiation therapy.

Conclusion

About sixty percent of cancer patients get radiation treatment. Although radiation treatment is very effective, it may be beneficial to identify radio-sensitizers. Using prostate cancer cell lines we identified several novel microRNA which maybe involved in radiation sensitivity. Of special interest is miR-521 which is shown to specifically sensitize prostate cancer cell line LNCaP against radiation treatment. Therefore, miR-521 could be a potentially good target to enhance the efficacy of radiation treatment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

miRNA	microRNA
UTR	untranslated region
RT	Reverse transcription
RP	Reverse primer
FP	Forward primer
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt
CSA	Cockayne syndrome protein A
MnSOD	Manganese superoxide dismutase

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Figure 1.

a. Unsupervised hierarchical clustering heat map of miRNA expression profile at 4 h in response to ionizing radiation. The groups are: LNCaP, LNCaP + 6 Gy, C4-2 cells and C4-2 + 6 Gy. Quantitative real time PCR analysis was used to measure miRNA levels. The cluster heat map was produced using expression levels (Ct value) of 182 miRNAs. Note that a higher Ct value means a lower expression level. If the values between 0-20, are in blue; if 20-35 in yellow and 35-40 is red. All experiments were performed in duplicates. b. Clonogenic survival of C4-2 and LNCaP prostate cancer cells in response to radiation treatment.

Figure 2.

Overexpression of miR-521 sensitizes LNCaP cell to radiation treatment. a. Quantitative real time PCR analysis of miR-521 levels in LNCaP cells treated with a miR-521 mimic and radiation. b. Cell viability of LNCaP cells overexpressing miR-521 in response to radiation treatment using trypan blue exclusion assay. c. Cell viability response of LNCaP cells overexpressing miR-521 mimic and their response to radiation using MTS assay, five days after treatment. Percent viability was determined. All experiments were performed in triplicate, repeated at least two to three times, and representative findings are shown. Student's t-test was used to determine the statistical significance.

Figure 3.

a. Cell viability (Trypan Blue exclusion) assay was used to determine the role of miR-521 inhibitor in radiation resistance in LNCaP prostate cancer cells. b. Cell viability (MTS) assay was used to determine the role of miR-521 inhibitor in radiation resistance in LNCaP prostate cancer cells. c. Clonogenic assay was performed to determine the role of miR-521 in radiation resistance in LNCaP prostate cancer cells.

Figure 4.

a. Quantified western analysis of miR-521 predicted target, CSA in response to miR-521 inhibitor and radiation treatment in LNCaP prostate cancer cells. b. Quantified western analysis of MnSOD in response to miR-521 inhibitor and radiation treatment in LNCaP prostate cancer cells. Representative western blots are attached below the graphs.

miRNA altered in radiation response in prostate cancer cells using quantitative real time PCR analysis. Analysis was done in duplicates and significance was analyzed using Student's t test. All values have a significance of $p < 0.05$.

Table 1

miRNA Decreased	LNCaP cells	LNCaP cells + 6 Gy Fold	miRNA Decreased	C4-2 cells	C4-2 cells + 6 Gy Fold
hsa-miR-521	1	absent	hsa-miR-521	1	absent
hsa-miR-196a	1	absent	hsa-miR-196a	1	.06
hsa-miR-133b	1	.39	hsa-miR-133b	1	.1
hsa-miR-487	1	.003	hsa-miR-143	1	.004
hsa-miR-122a	1	.004	hsa-miR-135b	1	.16
hsa-miR-145	1	.008	hsa-miR-218	1	.18
hsa-miR-100	1	.02			
hsa-miR-107	1	.02			
hsa-miR-187	1	.05			
hcmv-miR- UL22A-1	1	.19			
Upregulated miRNA	LNCaP cells	LNCaP cells + 6 Gy Fold	Upregulated miRNA	C4-2 cells	C4-2 cells + 6 Gy Fold
hsa-miR-34c	1	2.9	hsa-miR-34c	1	4
hsa-miR-372	1	556	hsa-miR-383	1	23
hsa-miR-520c	1	417	hsa-miR-154*	1	22
hsa-miR-449	1	21	hsa-miR-488	1	16
hsa-miR-520f	1	3	hsa-miR-379	1	5

Table 2

Predicted target proteins for miR-521 (9).

List of predicted targets for miR-521:

DNA Repair:

ERCC8 (CSA)

REV1L

MBD4

Cell repair:

GST-A4

MT3

Others:

BAD

NRP1

FGF4, FGFR1

AIG-1

ADAM9

HFE

CDH1