MicroRNAs as Potential Agents to Alter Resistance to Cytotoxic Anticancer Therapy

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Abstract

Tumor cells use preexisting prosurvival signaling pathways to evade the damaging and cytotoxic effects of anticancer agents. Radiation therapy is a primary form of cytotoxic anticancer treatment, but agents that successfully modify the radiation response in vivo are lacking. MicroRNAs (miRNA) are global gene regulators that play critical roles in oncogenesis and have been found to regulate prosurvival pathways. However, there is little understanding of how cellular miRNA expression affects the response of a cancer to cytotoxic therapy and ultimately outcome. The let-7 family of miRNAs regulates expression of oncogenes, such as RAS, and is specifically down-regulated in many cancer subtypes. In fact, low levels of let-7 predict a poor outcome in lung cancer. Here, we report that the let-7 family of miRNAs is overrepresented in a class of miRNAs exhibiting altered expression in response to radiation. More strikingly, we also can create a radiosensitive state when the select let-7 family of miRNAs is overexpressed in vitro in lung cancer cells and in vivo in a Caenorhabditis elegans model of radiation-induced cell death, whereas decreasing their levels causes radioresistance. In C. elegans, we show that this is partly through control of the proto-oncogene homologue let-60/RAS and genes in the DNA damage response pathway. These findings are the first direct evidence that miRNAs can suppress resistance to anticancer cytotoxic therapy, a common feature of cancer cells, and suggest that miRNAs may be a viable tool to augment current cancer therapies. [Cancer Res 2007;67(23):11111–6]

Introduction

Radiation therapy is one of the three primary modalities used in cancer treatment. Although radiation has been in practice for over a century, the global genetic response necessary for tissues to survive radiation-induced injury remains largely unknown. This has limited the ability to develop meaningful routes to minimize normal tissue toxicity while enhancing tumor eradication. Although single-protein targeting strategies have not successfully altered the primary form of radiation-induced target cell death (11), the primary form of radiation-induced target cell death (11). Unfortunately, strategies directly targeting RAS or its upstream and/or downstream effectors have not successfully altered the radiation response in vivo (12). Because miRNAs target RAS as well as hundreds of additional genes (13), we hypothesized that their manipulation might more successfully alter the radiation response.

Materials and Methods

miRNA microarrays. Total RNA was collected from cells using the mirVana kit from Ambion (per manufacturer's instructions). A total of 10 μg was used for miRNA microarray by LC Sciences. To confirm the quality of the RNA, a UV test was performed and the samples were enriched for miRNAs by using a cutoff filter (um100 from Microcon-modified procedure). The microRNAs were then labeled and hybridized to a microarray chip with multiple repeat regions and a miRNA probe region, which detects miRNA transcripts listed in Sanger miRBase release 8.2. This consists of 440 human miRNA sequences. Multiple control probes were included in each chip. The control probes were used for quality controls of chip production, sample labeling, and assay conditions. For the in-depth data analysis of our time point experiments, LC Sciences performed multiarray normalization, ANOVA, and clustering analysis. The ANOVA and clustering analysis were performed on ratio data of individual arrays (with the multiarray normalization) instead of the often used intensity data of individual samples. They found this necessary to reveal the rather small miRNA variations among the samples of different time.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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©2007 American Association for Cancer Research. doi:10.1158/0008-5472.CAN-07-2858
Because there was only one sample for each time point, they used repeating probe sets of the arrays to have constructed "groups" that were needed for ANOVA analysis.

**Real-time PCR.** Quantification of levels of let-7a, let-7b, and let-7g was performed using the Taqman microRNA PCR system (ABI, per standard protocol). Levels were normalized to the 0 time point to determine changes in expression levels after irradiation.

**Clonogenic assays.** A549 cells were transfected with 90 nmol/L of the pre-let-7 or control pre-miR. The transfection method was optimized using a luciferase reporter construct sensitive to let-7 levels (lac fused to the

Figure 1. Relative levels of the let-7 microRNAs change significantly after irradiation. A, miRNA microarrays were performed on total RNA collected from irradiated A549 cells before and 2, 8, and 24 h after 2.5 Gy. The level of each member of the let-7 family changed significantly over this time. B, levels of let-7 miRNAs after irradiation in a normal lung epithelial line, CRL2741. C, real-time PCR results at 8 and 24 h of let-7a, let-7b, and let-7g from a separate A549 replicate and two additional lung cancer cell lines (A427, carcinoma; H441, adenocarcinoma). let-7a and let-7b levels are reduced and let-7g levels are increased after irradiation. Ratio of let-7 levels with the unirradiated 0 time point as the baseline. Bars, SD.
NRAS 3′-untranslated region), and we chose the method with the least toxicity and most efficient transfection (X-tremeGENE, Roche; data not shown). Twenty-four hours after transfection, cells were treated with increasing doses of radiation and then plated at different dilutions and grown without being disturbed. Colonies were counted after 2 weeks. Experiments were done in quadruplicate for each dose and each miRNA, and all experiments were repeated at a minimum of two times. Stratified t tests were performed to analyze significance for all cases. The P value was based on a two-tailed evaluation of the data that were pooled for each experiment across all doses as is standard procedure and is shown in the figures.

C. elegans work. Methods for culturing, handling, and genetic manipulation of C. elegans were as described by Brenner unless otherwise indicated. The animals referred to here as wild-type (WT) C. elegans correspond to the Bristol strain N2. Strains used in this study were obtained from the C. elegans Genetics Center unless otherwise noted. let-7–overexpressing strains were generated as described (4, 7, 14). For synchronization, gravid hermaphrodites were treated and isolated embryos were treated with radiation as described previously (15). Dose-response curves were generated at the first S-phase radioresistance peak as described previously (15). For each dose, a minimum of 100 animals was treated and scored per experiment, and experiments were repeated two to four times.

For statistical analysis, each of the mutant strains was compared against the WT using a stratified two-sample Wilcoxon rank sum test. Stratified t tests were performed to analyze significance for all cases. The P value was based on a two-tailed evaluation of the data and is shown in the figures or in the figure legends.

Determining the genetic basis of the let-7 response. RNA interference (RNAi): after synchronization, animals were placed on plates with the appropriate bacterial strain containing the plasmid that overexpresses double-stranded RNA (dsRNA) from the gene of interest and grown until the appropriate time for radiation. After irradiation, animals were placed on plates with the same bacterial strain and grown until the phenotypic analysis was performed.

For analysis of alteration of mammalian gene levels in response to changing let-7 levels, let-7 downstream genes were analyzed by Western blot analysis using standard protocols.

Results and Discussion

Despite strong evidence that miRNAs are associated with cancer and are potential biomarkers for outcome, little is known about how they affect the response of a tumor to cytotoxic treatment. To determine whether miRNAs are involved in the cellular response to cytotoxic therapy, we used miRNA microarrays to compare the relative levels of cellular miRNAs before and after radiation. We irradiated a lung cancer cell line, A549, in which let-7 levels are low (7) and RAS is activated (16). The levels of 81 miRNAs significantly changed after irradiation (Supplementary Figs. S1 and S2).

Significant changes in expression of most miRNAs were observed as early as 2 h after irradiation, with most of these early affected miRNAs returning to their baseline expression levels by 24 h. The same microarray analysis was performed in a normal lung epithelial cell line, CLR2741. The levels of most miRNAs, including

![Figure 2](image-url)
all members of the let-7 family, were significantly different between these two cell types before radiation (data not shown). However, both the normal and tumor cells exhibited similar patterns of miRNA expression changes in response to radiation (Supplementary Figs. S1 and S2).

Interestingly, each member of the let-7 family of miRNAs, barring one (let-7g), decreased significantly by 2 h after irradiation in both cancerous and normal lung epithelium (Fig. 1A and B). Of the 23 miRNAs with decreased expression after irradiation, 7 (30%) were members of the let-7 family, a 17-fold enrichment over their representation on the array (1.8%; 8 of 440 miRNAs on the array). Real-time PCR confirmed the microarray findings for representation on the array (1.8%; 8 of 440 miRNAs on the array). In addition, the radiation experiments were repeated in a separate replicate of A549 cells as well as in two additional lung cancer cell lines, A427 (carcinoma) and H4441 (adenocarcinoma). Levels of let-7a, let-7b, and let-7g were evaluated by real-time PCR, and consistent with the microarray findings, let-7a and let-7b were significantly down compared with their baseline at 8 h after irradiation, whereas let-7g was significantly elevated by 24 h after irradiation in all of the lung cell lines studied (Fig. 1C).

The similarity between the miRNA response after irradiation in multiple cancerous and a normal lung epithelial cell lines suggests that a highly conserved global miRNA response exists in lung cells after irradiation (Fig. 1C; Supplementary Fig. S2). In addition, these findings suggest that perhaps miRNAs are components of the cellular response to cytotoxic insult. While this would indicate that miRNA expression patterns might be a biomarker for radiosensitivity, it further prompted us to test the hypothesis that altering miRNA levels could be an efficacious approach to alter the cellular radiation response.

let-7 overexpression in A549 cell lines causes defects in proliferation (8) but does not cause apoptosis in these cells (17). Clonogenic cell survival assays measure all forms of cell death and are the recognized standard for radiation sensitivity assays. Therefore, we used this assay to test the effect of altering let-7 levels on the radiation response and cell survival. Specifically, we chose to evaluate the effect of let-7b, let-7a, and let-7g on the radiation response because (a) let-7b drops the most significantly after irradiation in A549 cells (Fig. 1); (b) let-7a levels have been implicated in predicting outcome in certain cancers, most notably lung cancer (8, 18); and (c) let-7g levels are up-regulated after irradiation and significantly changed only in the lung cancer cell lines (Fig. 1). To overexpress each of the let-7 homologues of interest, A549 cells were transfected with synthetic pre-miRNA containing scrambled sequences (Ambion) and clonogenic assays were performed. Significant radiosensitization was found in cells treated with pre-let-7b or pre-let-7a compared with control pre-miRNA (Fig. 2A; Supplementary Fig. S3). This effect on the radiosensitivity of cultured cells is comparable with other studies of transfected small interfering RNA–based radiosensitizers (19). In parallel experiments, anti-miRs were delivered to A549 cells to specifically decrease let-7 miRNA activity (7). As expected from the effects of let-7b overexpression, anti-let-7b caused significant radioprotection (Fig. 2C). Consistent with the opposite direction of altered expression levels of let-7g after irradiation, a unique role for let-7g in the radiation response was identified; let-7g overexpression protected A549 cells from radiation (Fig. 2B), whereas anti-let-7g caused radiosensitization of A549 cells (Fig. 2D). We hypothesize that overexpression of let-7a and let-7b causes radiosensitization and overexpression of let-7g radioprotection in part by overcoming the innate requirement of the cell to down-regulate or up-regulate these miRNAs as part of the response to cytotoxic insult. However, the molecular mechanisms of miRNA function in the radiation response may also be related to alteration in the levels of their targets, such as components of the DNA damage response (DDR) pathway and RAS (see below; refs. 7, 17). The exact differences in the roles of let-7g and the other tested let-7 family of miRNAs in the radiation response remain to be determined.

To confirm the ability of let-7 miRNAs to alter the radiation response in vivo, we turned to a powerful C. elegans–based in vivo model of radiation-induced reproductive cell death ["Radelegans" (15)]. The tissue studied in Radelegans is the developing C. elegans vulva, in which multipotential vulval precursor cells (VPC) undergo three rounds of cell division and differentiate into the mature vulva following RAS signaling (20). VPCs represent tissue clonogens, considered the critical and determinant targets of radiation in tumors that die via reproductive cell death (15), and thus, this model is considered an in vivo representation of clonogenic assays and radiation-induced tumor target cell death. In addition, because VPCs are synchronized, and all experiments are performed at the same point in the cell cycle (15), this model eliminates cell cycle
alterations as a mechanism for radiosensitization. Previously, radiation resistance in VPCs was shown to depend on RAS signaling and a normal DDR pathway (10). VPCs show specific expression of three let-7 paralogues, let-7, mir-48, and mir-84 (7, 14), which repress RAS expression in this tissue but do not alter the VPC cell cycle (4, 7, 21). On irradiation, VPCs in strains that overexpress either let-7 or mir-84 were significantly radiosensitive compared with WT animals (Fig. 3A), consistent with results of the in vitro analysis of let-7a and let-7b in lung cancer cells (Fig. 2; Supplementary Fig. S3).

let-7 loss-of-function mutants could not be analyzed for radiosensitivity due to gross defects in vulval development (4). Instead, animals harboring a mir-84 or a mir-48 deletion were analyzed because they develop without obvious vulval abnormalities (7, 21). In dose-response experiments, mir-84(tm1304) and mir-48(n4097) animals exhibited significant radioresistance across all radiation doses (Fig. 3B) compared with a WT strain, also consistent with the results of the in vitro analysis of let-7b (Fig. 2). These findings in Radelegans are therefore an in vivo confirmation of the in vitro cell line studies, supporting the potential of alteration of let-7 levels as a tool to change cell survival after irradiation.

To understand how let-7 alteration affects the radiation response, we tested the hypothesis that radioresistance in the mir-84 mutant was due to overexpression of some of its recently identified targets, including the let-60/RAS oncogene (7) as well as genes of the DDR pathway (17), which are also known to be critical in the radiation response (10). RNAi was performed through feeding in the mir-84 mutant for genes of interest (Fig. 4A). Indeed, let-60/RAS(RNAi), rad-51/RAD51(RNAi), coh-1/RAD21(RNAi), fcd-2/FANCD2(RNAi), and cdc-25.3/CDC25(RNAi) each significantly suppressed the radioresistance in mir-84(tm1304) animals across the doses studied (Fig. 4B). These findings support the hypothesis that the let-7 family of miRNAs modulates the response to cytotoxic anticancer therapy in an in vivo model by altering levels of

Figure 4. RNAi against RAS and DDR genes reverses the mir-84 radiation-resistant phenotype. A, a depiction of how the C. elegans RNAi experiments were performed. Synchronized embryos are placed on plates with the appropriate bacterial strain containing the plasmid that overexpresses dsRNA from the gene of interest and grown until appropriate time for radiation. Radiation is delivered at the previously defined appropriate point in the life cycle of the animal. B, RNAi for RAS and DDR genes leads to reversal of the radiation-resistant phenotype of the mir-84 (KO) strain. Results are normalized to mir-84. Bars, SD. Stratified t tests were performed to evaluate significance, individual dose points that were significant are marked with an asterisk, and P values based on a two-tailed evaluation of the data across both doses were significant for all strains and are as follows: let-60/RAS(RNAi), P = 0.05; rad-51/RAD51(RNAi), P = 0.05; coh-1/RAD21(RNAi), P = 0.01; fcd-2/FANCD2(RNAi), P = 0.02; and cdc-25.3/CDC25(RNAi), P = 0.03.
prosurvival and DDR pathways. In addition, we evaluated the level of several of these genes after irradiation in A549 cells by Western blot analysis and also saw an increase in their levels within the time frame of let-7 alteration, further validating this mechanism in mammalian systems (Supplementary Fig. S4).

Our work reveals a role for miRNAs in the immediate cellular response to cytotoxic anticancer agents, and we show that alteration of cellular miRNA levels, specifically the let-7 family, is able to affect cellular survival after cytotoxic therapy. Many miRNAs change levels significantly after irradiation, including mir-34, as shown by others as well (22). The mechanism of mir-34 up-regulation was shown to be due to altered transcription from a known radiation response gene, p53 (22). Our work instead shows down-regulation of most members of the let-7 family, and we further prove the significance of the let-7 family in the radiation response by showing that altering let-7 levels before radiation affects cellular survival. One could speculate that one possible mechanism is alteration in the levels of miRNA processing components (23), although this cannot explain how numerous miRNAs increase whereas others decrease.

Although the mechanism of let-7 on cell survival could result from its effect on cell proliferation and cell cycle (17), this is unlikely based on its effect in Radelegans, where cell cycle is removed as a variable. We hypothesize that the effects of let-7 are genetically more direct based on the reversal of the radiation resistance in C. elegans by genetically decreasing levels of prosurvival and DDR genes that are let-7 targets and the increased levels of some of these targets in mammalian cells after irradiation. It is tempting to speculate that miRNAs, which are often misexpressed in cancer, can now be used as a novel tool to help battle cancer in conjunction with current cytotoxic therapies. Future work will attempt to gain an understanding of the role miRNAs play in response to other cytotoxic therapies, such as chemotherapy.

Acknowledgments

Received 7/30/2007; revised 9/25/2007; accepted 10/15/2007.

Grant support: Breast Cancer Alliance grant and Asuragen. F.J. Slack was supported by grants from the NIH, National Science Foundation, and Asuragen.

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We thank the C. elegans Japanese KO consortium for the mir-84(tm1304) strain, the C. elegans Genetics Center for strain mir-86(mir907), Drs. Sarah Rockwell for advice, and Drs. Joann Sweasy and Peter Glazer for critical reading of this manuscript.

References

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