RESEARCH PAPER

MicroRNA-302 Replacement Therapy Sensitizes Breast Cancer Cells to Ionizing Radiation

Zhongxing Liang • Jeffrey Ahn • Donna Guo • John R. Votaw • Hyunsuk Shim

ABSTRACT

Purpose Solid tumors can be resistant or develop resistance to radiotherapy. The purpose of this study is to explore whether microRNA-302 is involved in radioresistance and can be exploited as a sensitizer to enhance sensitivity of breast cancer cells to radiation therapy.
Methods MiR-302 expression levels in radioresistant cell lines were analyzed in comparison with their parent cell lines. Furthermore, we investigated whether enforced expression of miR-302 sensitized radioresistant breast cancer cells to ionizing radiation *in vitro* and *in vivo*.
Results MiR-302 was downregulated in irradiated breast cancer cells. Additionally, the expression levels of miR-302a were inversely correlated with those of AKT1 and RAD52, two critical regulators of radioresistance. More promisingly, miR-302a sensitized radioresistant breast cancer cells to radiation therapy *in vitro* and *in vivo* and reduced the expression of AKT1 and RAD52.
Conclusion Our findings demonstrated that decreased expression of miR-302 confers radioresistance and restoration of miR-302 baseline expression sensitizes breast cancer cells to radiotherapy. These data suggest that miR-302 is a potential sensitizer to radiotherapy.

KEY WORDS AKT · breast cancer · microRNA · radioresistance

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ABBREVIATIONS

AKT	protein kinase B
Gy	gray
miRNA	microRNA
RAD52	a protein, encoded by the *RAD52* gene, important for DNA double-strand break repair and homologous recombination
RT-PCR	reverse transcription polymerase chain reaction.
siRNA	small interfering RNA

INTRODUCTION

Radiotherapy is a common treatment strategy for many solid tumors, including breast cancer, with 40% to 60% of all cancer patients receiving radiation treatment [1]. However, solid tumors are usually resistant or develop resistance to radiotherapy. Radioresistance (RR) has limited the ability of radiotherapy to kill tumor cells. Though many studies have been conducted to further elucidate the mechanisms of radioresistance, they remain largely obscure due to the complex genetic cellular response to radiation and involvement of a large number of genes. Additionally, this suggests that modulators capable of simultaneously regulating a large number of target genes may be required by radioresistance. MicroRNA (miRNA) is one of such class of potential regulators. MicroRNAs are a class of recently-discovered, functional, non-coding, and small RNA. They have been shown to function as regulatory molecules by inhibiting protein translation and to play an important role in development, differentiation, cell proliferation, and apoptosis [2–4]. Several studies have suggested that the downregulation of miR-NAs may play a critical role in cancer progression [5–8]. A single microRNA can target a large number of genes and regulate several signal pathways simultaneously. Thus, some

miRNAs may be regulators of complex radioresistance response of tumor cells and directly exploited to sensitize radioresistant breast cancer cells to radiation therapy. Currently, a few investigations have successfully targeted oncogenes with artificial synthetic miRNAs (9,10). The overexpression of miRNA let-7 has been shown to sensitize lung cancer cells to radiation therapy *in vitro* (11). More recently, miR-521 has been shown to modulate the expression levels of cockayne syndrome A, a DNA repair protein, in the radio-sensitivity of prostate cancer cell lines (12). MiR-101 has been demonstrated to target DNA-PKcs and ATM to sensitize tumors to radiation (13). However, relatively few studies have been conducted specifically to investigate the involvement of microRNA in radioresistance so far. In the present study, we show that miR-302 expression was decreased in radioresistant breast cancer cells and restoration of miR-302 expression levels sensitized radioresistant breast cancer cells to irradiation *in vitro* and *in vivo*.

MATERIALS AND METHODS

Cell Lines and Cell Culture

The human breast cancer cell lines MDA-MB-231 and SKBR3 were grown respectively in RPMI1640 and DMEM media supplemented with 10% FBS, 100 U/ml of penicillin sodium, and 100 µg/ml of streptomycin sulfate at 37°C in a humidified atmosphere of 5% CO_2.

Induction of Radioresistant Breast Cancer Cell Lines

MDA-MB-231 and SKBR3 cells were irradiated with 2 Gy per day for 20 days. Surviving cells were cultured for the experiments. Radioresistant MDA-MB-231 and SKBR3 cells were designated as MDA-MB-231RR and SKBR3RR respectively

RT-PCR and Quantitative RT-PCR

Total RNA was isolated from cultured MDA-MB-231, SKBR3 and their radioresistant MDA-MB-231RR and SKBR3RR cells by using Trizol Reagent following the instructions provided by the manufacturer (Invitrogen, Grand Island, NY). Primer sequences of *U6 snRNA* and *β-actin* were described in previous publications (14,15). *U6 snRNA* was used as internal control for miR-302a amplification and *β-actin* as an internal control for AKT1 and RAD52. Primer sequences of *miR-302 members* are as follows: *miR-302a* (GeneBank Accession number NR_029835), 5′- CGTGGATGTACTTGCTTTGAA-3′ and 5′-TCACCAAAACATGGAAGCAC-3′; *miR-302b* (NR_029857), 5′- GCTCCCTTCAACTTTAACATGG-3′ and 5′-CTCCTACTAAAACATGGAAGCAC; *miR-302c*

(NR_029858), 5′- GGGGTACCTGCTGTGTGAA-3′ and 5′-TCCACTGAAACATGGAAGCA-3′; *miR-302d* (NR_029859), 5′-TAACATGGAGGCACTTGCTG-3′ and 5′-CACACTCAAACATGGAAGCAC-3′; and *miR-302e* (NR_031683), 5′- GCTTCCATGCTTCAGTTTCC-3′ and 5′-GGTGCTATTACTACATCCATCTTACC-3′. For *AKT1* (NM_001014432), the primer sequences are 5′-CATCACACCACCTGACCAAG-3′ and 5′-CTCAAATG-CACCCGAGAAAT -3′. For RAD52 (BC114954), the primer sequences are 5′- AGTTTTGGGAATGCACTTGG-3′ and 5′-TCGGCAGCTGTTGTATCTTG-3′. Quantitative and regular RT-PCR were performed following our previous descriptions (16). Five hundred nanograms of total RNA were reverse-transcribed into cDNA in a 20 µl of reaction volume at 42°C for 45 min with a GeneAmp Gold RNA PCR Reagent kit (Applied Biosystems, Foster City, CA). The thermal profile for cDNA PCR was 95°C for 10 min followed by 40 cycles of 95°C for 20s, and 60°C for 45 s. For regular RT-PCR, reactions were carried out in 20 µl of reaction volume with using a GeneAmp Gold RNA PCR Reagent kit (Applied Biosystems). SYBR Green quantitative PCR reaction was carried out in a 20 µl reaction volume containing 10 µl of 2× SYBR Green PCR Master Mix (Applied Biosystems). The cycling conditions were the same as those for regular PCR. The relative expression levels of each sample were measured using the $2^{-\Delta\Delta Ct}$ method (17). *U6 snRNA* and *β-actin* were used to normalize. ΔΔCt was expressed by the difference of ΔCts of two groups to be compared. The fold change was calculated using $2^{-\Delta\Delta Ct}$.

The results are presented as fold change of expression levels in the treated cells relative to the parental breast cancer cells.

Western Blotting Analyses

Equal amounts of protein were separated by SDS-PAGE and transferred to PVDF membranes (Bio-Rad, Hercules, CA). The membranes were blocked for 0.5 h in a blocking solution (5% milk in TBS-T [Tris-buffered saline containing Tween-20]) and incubated overnight at 4°C with polyclonal antibodies against AKT1, phosphorylated AKT1, and RAD52 (Cellsignaling, Danvers, MA), and monoclonal antibody against β-actin (Sigma–Aldrich, St. Louis, MO). All antibodies were used in the blocking solution following the working concentration recommended by the manufacturers. Detection by enzyme-linked chemiluminescence was performed. Quantification of protein bands was measured using the ImageJ software.

Luciferase Reporter Assay

Two pGL3-AKT1-3′-UTR recombinants, which contained a 107 bp fragment of 3′-untranslated region (UTR)

fragments of *AKT1* gene with or without the putative binding site for miR-302a, were constructed as reported (18,19). The pGL3-AKT1-3′-UTR recombinant without complementary sequence of miR-302a was used as a control. The resulting luciferase UTR-reporter vectors and increasing concentrations of miR-302a mimic were co-transfected into MDA-MB-231RR cells using LipofectAMINE 2000 reagent according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). Twenty-four hours after co-transfection, luciferase activity assays were performed using Steady-Glo Luciferase Assay System (Promega) following the manufacturer's instructions. Similarly, two pGL3-RAD52 recombinants contained a 148 bp 3′-UTR fragment of *RAD52* gene with or without the putative binding sites for miR-302a were constructed. Luciferase reporter assay was performed.

Construction of the Vector Expressing miR-302a and Stable Transfection

MiR-302a gene double-strands (Fig. 3a) were ligated with Block-iT Pol II miR RNAi Expression Vector (Invitrogen). Then, the vector with miR-302a or control miRNA plasmids was transfected into the MDA-MB-231RR and SKBR3RR cells using Lipofectamine-2000 (Invitrogen) according to the manufacturer's instruction. The microRNA expression construct contains the Blasticidin resistance gene to allow for Blasticidin selection of mammalian cells that are stably transfected with the construct. To establish stable cell lines that constitutively express miR-302a, the cells were selected by fresh RPMI1640 medium plus 15 µg/ml of Blasticidin (Invitrogen) every 3 to 4 days until Blasticidin-resistant colonies could be identified.

Cell Radiosensitivity Assay

Cell sensitivity to radiation was determined by the loss of colony-forming ability. MiR-302a-transfected and control oligo-transfected MDA-MB231RR and SKBR3RR cells were irradiated at an increasing dose (0–10 Gy) by using an x-ray machine (X-RAD 320, N. Branford, CT, USA) at 320 kV, 10 mA, with a filtration of 2-mm aluminum. After irradiation, a cell colony formation assay was performed. The cells were collected and plated, aiming at a density of 20–100 colonies per dish. Two replicate dishes were prepared for each datum point, and cells were incubated for 2 weeks. Colonies were stained with 0.5% of crystal violet in methanol solution. The cell survival fractions were calculated.

Cell Proliferation Assay

The cells at 48 h post transfection with miR-302a and control vectors were seeded in triplicate in 96-well plates (3,000 cells per well). Twenty-four hours later, the cell proliferation was measured by the Cell Titer 96 AQ (Promega, Madison, WI) according to the manufacturer's instruction. These experiments were repeated twice.

Animal Experiments

For generation of xenograft tumors, briefly, both hind legs of each athymic female nude mouse (nu/nu) (6–8 weeks of age) were subcutaneously injected with MDA-MB-231RR cells transfected with vector alone or with the vector encoding miR-302a, 5 mice for each group. Two weeks later, when the xenograft tumors formed in both hind legs, the right hind leg of each mouse was exposed to 5 Gy of x-ray and the left one was used as the mock-irradiation control. The radiation was performed by the same x-ray machine at a dose rate of 1 Gy/min. Tumor volume was measured on two perpendicular axes using a Vernier caliper once every other day and calculated using the following formula: $tumor\ volume = (width)^2 \times length/2$. At 14 days post irradiation, the mice were sacrificed and the tumors were removed and weighted. Data of tumor volume and weight were analyzed and compared.

Statistical Analysis

Real-time RT-PCR reaction was run in triplicate for each sample and repeated at least 2 times and the data were statistically analyzed with a Student's *T*-test.

RESULTS

Levels of miR-302 are Downregulated in Radioresistant Cells and Inversely Correlated with AKT1 and RAD52

Quantitative RT-PCR results showed that expression levels of all members of miR-302 were downregulated in irradiated cells compared to their parental cells (Table I). The miR-302 family members share the same seed sequence, which is a binding sequence to targets. The seed sequence is a critical factor for microRNA function. One of the miR-302 family members, miR-302a, showed consistently higher differential expression in all three irradiated cell lines compared to their parental cell lines (Table I). Thus, we selected miR-302a as a representative of the miR-302 family for our further experiment analyses. Furthermore, we analyzed expression levels of miR-302a, AKT1 and RAD52 in two types of breast cancer cell lines, triple-negative MDA-MB-231, Her2/neu positive SKBR3, and their radioresistant sublines, MDA-MB231RR, and SKBR3RR. RT-PCR results showed that miR-302a expression levels were decreased in radioresistant breast cancer cells while mRNA expression levels of AKT1 and RAD52 were

Table I Change Fold of *miR-302* Expression Levels After the Irradiation of Tumor Cells

Gy	MDA-MB-231			MCF-7			SKBR3		
	0	4	8	0	4	8	0	4	8
hsa-mir-302a	1	−3.70	−3.65	1	−5.2	−5.3	1	−3.5	−4.8
hsa-mir-302b	1	−1.08	−1.14	1	−18	−19	1	−4.1	−10.3
hsa-mir-302c	1	−3.05	−1.99	1	−305	−199	1	−25	−228
hsa-mir-302d	1	−2.75	−2.31	1	−43	−36	1	−3.6	−33.4
hsa-mir-302e	1	−2.75	−2.31	1	−43	−36	1	−2.4	−33.4

upregulated compared to their parental cells (Fig. 1a). Furthermore, protein expression levels of total AKT1, RAD52 and phosphorylated AKT1 were all upregulated in radioresistant breast cancer cells (Fig. 1b). These results demonstrated that expression levels of miR-302a are downregulated in radioresistant cells compared to their parental cells and are inversely correlated with the levels of AKT1 and RAD52.

MicroRNA-302 Directly Targets *AKT1* and *RAD52*

A bioinformatic analysis by TargetScan algorithms identified *AKT1* and *RAD52* as predicted target genes (Fig. 2a). To verify a direct interaction between miR-302 and its binding sites within *AKT1*, a luciferase reporter assay was performed. Briefly, a segment of AKT1 3′-UTR containing a miR-302 binding site was inserted into a luciferase reporter vector. Similarly, we cloned a fragment of RAD52 3′-UTR containing a miR-302 binding site to a luciferase reporter system. The resulting reporter vectors were co-transfected into the MDA-MB-231 cells with miR-302a mimics. MDA-MB-231 cells transfected with the vectors containing miR-302 binding sites showed a significant decrease of the luciferase activity when co-transfected with AKT1 3′-UTR (Fig. 2b) or RAD52 3′-UTR (Fig. 2c) with miR-302a binding sites. In addition, control vectors were generated by inserting AKT1 or RAD52 3′-

UTR fragments with a mutagenized miR-302a seed sequence. No significant change of the luciferase activity was observed following the co-transfection of these two mutagenized vectors with miR-302a (Fig. 2b–c). Taken together, these findings showed that miR-302 directly targets *AKT1* and *RAD52*.

MiR-302a Inhibits Expression of AKT1 and RAD52 in Radioresistant Cells

To show that miR-302 participates in the regulation of AKT1 and RAD52 expression, an *in vitro* functional analysis was performed by overexpressing miR-302a levels in radioresistant cells. A miR-302a expression vector was constructed by inserting pre-miR-302a sequence (Fig. 3a) into a microRNA expression vector and the constructed plasmids were stably transfected into MDA-MB-231RR cells (Fig. 3b). MiR-302a overexpression in microRNA-transfected radioresistant cells was confirmed by qRT-PCR (Fig. 3c). As shown in Fig. 3d, AKT1 and RAD52 expression levels were decreased by enforced expression of miR-302a in MDA-MB-231RR cells. To investigate the potential effect of the restored expression of miR-302a to AKT1 activity, we analyzed the phosphorylation of AKT1. Results show that phosphorylated-AKT1 protein was downregulated with miR-302a transfection in MDA-MB-231RR cells (Fig. 3d).
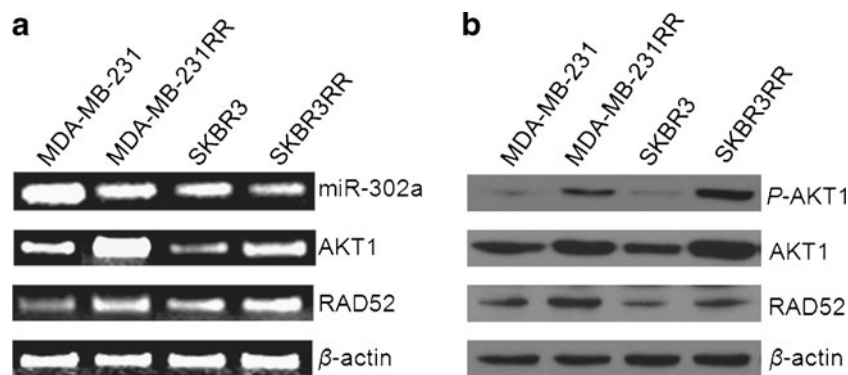
Fig. 1 Expression levels of miR-302a are inversely correlated with the levels of AKT1 and RAD52 in radioresistant breast cancer cell lines. (**a**) Expression levels of miR-302a determined by RT-PCR are decreased in the radioresistant breast cancer cell lines MDA-MB-231RR and SKBR3RR. Conversely, mRNA expression levels of *AKT1* and *RAD52* were upregulated in radioresistant cell lines compared to their parental cells. *β*-actin was used as a loading control. (**b**) Western blot analysis shows that *p*-AKT1, total AKT1, and RAD52 proteins are elevated in radioresistant breast cancer cells compared to their counterparts. *β*-actin was used as a loading control.

Fig. 2 Predicted target sites of miR-302 in 3′ UTRs of *AKT1* and *RAD52* genes. (**a**) The putative targeted sites in the 3′ UTRs of *AKT1* and *RAD52*. (**b**, **c**) Luciferase reporter assay results. MDA-MB-231 cells were transfected with the firefly luciferase reporter plasmid containing partial 3′-UTR of *AKT1* (**b**) or *RAD52* (**c**) with (3′UTR) or without (Mut 3′UTR) the putative miR-302 binding site. Blank vector was used as mock control (Mock). Luciferase activity was measured at 48 h post the co-transfection of luciferase reporter vector with miR-302a mimics. *$P < 0.01$ compared to Mut UTR.
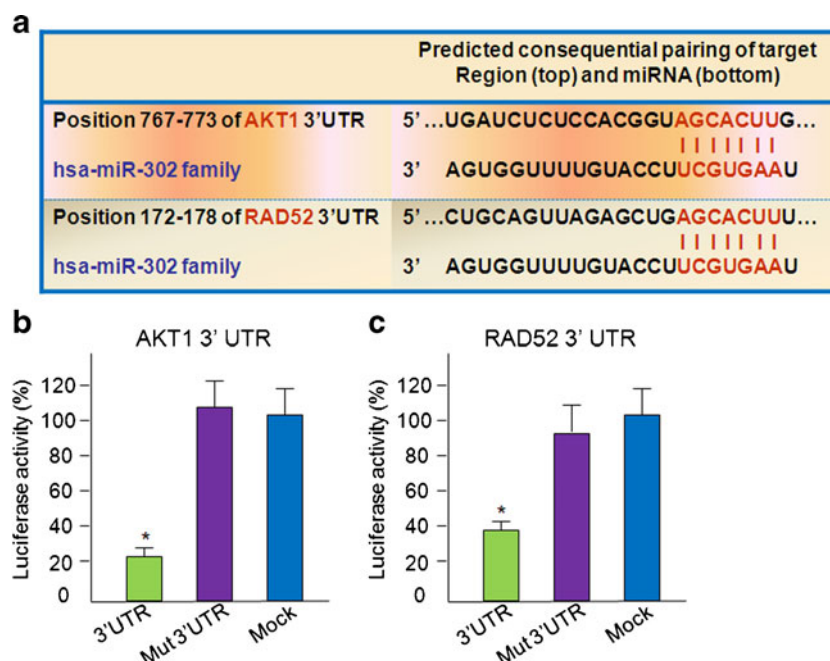

Enforced MiR-302a Expression Restores Sensitivity of Radioresistant Tumor Cells to Irradiation

To investigate whether overexpression of miR-302 sensitizes the radioresistant cells to irradiation treatment, we stably transfected miR-302a vector to the radioresistant cells MDA-MB-231RR and SKBR3RR, and determined sensitivity of these miR-302a-overexpressed cells to irradiation. The miR-302a-transfected cells were treated with increasing radiation dose (Gy). At 24 h post the radiation of the cells, a cell colony formation assay was performed and cell survival fractions were calculated. The data show that significantly lower survival fractions were observed for the miR-302a-transfected MDA-MB-231RR (Fig. 4a) and SKBR3RR (Fig. 4b) cells compared to their parental cell lines after irradiation. These findings suggest that the miR-302a-transfected MDA-MB-231RR and SKBR3RR cells were significantly sensitive to radiation therapy compared to control vector-transfected cells.
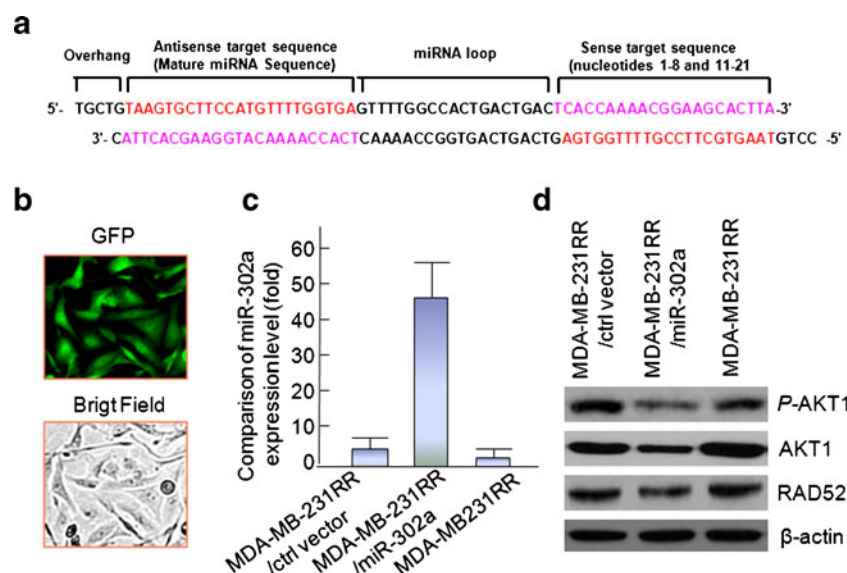
Fig. 3 Overexpression of miR-302 reduced the expression of AKT1 and RAD52. (**a**) Pre-miRNA double-strand oligo sequence inserted into a miRNA expression plasmid, Block-iT Pol II miR RNAi Expression Vector (Invitrogen). (**b**) Representative image (GFP) shows the transfection efficiency of the vectors after selection. (**c**) Levels of miR-302a were increased in miR-302a plasmid-transfected MDA-MB-231RR cells compared to mock MDA-MB-231RR cells. Levels of miR-302a were not significantly increased in control vector-trasfected MDA-MB-231 RR cells compared to mock MDA-MB-231RR cells. (**d**) Levels of phosphorylated AKT1, total AKT1 and RAD52 proteins were reduced in miR-302a plasmid-transfected MDA-MB-231RR cells compared to control vector-transfected or mock MDA-MB-231RR cells. *β*-actin was used as a loading control.
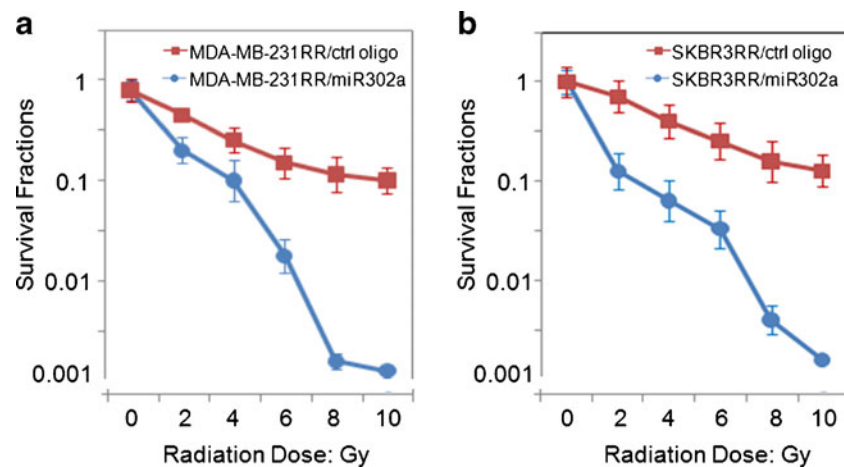
Fig. 4 MiR-302 sensitized radioresistant tumor cells to irradiation *in vitro*. (**a**) miR-302a sensitized MDA-MB-231RR cells to irradiation *in vitro*. MiR-302a vectors or control vectors were transfected into MDA-MB-231RR cells. Cells were irradiated with an increasing dose of X-ray. A clonogenic assay was performed. Survival fractions shown are the mean and SE from three independent experiments. (**b**) Similarly, the overexpression of miR-302a increased radiosensitivity of SKBR3RR cells compared to their counterparts *in vitro*.

To exclude effects of miR-302a overexpression on proliferation or cell survival of the cells, a cell proliferation assay was performed by using the Cell Titer AQ96 Assay kit (Promega). The cells were reseeded in a 96-well plate, 48 h post transfection. The growth of miR-302a transfected MDA-MB-231RR cells was $83.6\% \pm 16.2\%$ ($P=0.0982$) of control cell growth over 24 h post reseeded, indicating a non-significant effect of miR-302a on radioresistant cell growth.

MiR-302a Sensitizes Xenograft Tumors Derived from MDA-MB-231RR Cells to Radiation Therapy

To investigate *in vivo* effects of miR-302a on the sensitivity of radioresistant cells to radiotherapy, we assessed the effects of miR-302a on sensitizing the tumors derived from MDA-MB-231 radioresistant breast cancer cells to radiotherapy. The *in vivo* efficacy of miR-302a was assessed in two groups of mice bearing xenografts derived from miR-302a-transfected MDA-MB-231RR and control vector-transfected MDA-MB-231RR cells. Two weeks after the injection of tumor cells, the right hind leg of each mouse was irradiated with 5 Gy of x-ray and the left one was used as the mock-irradiation control. The radiation was performed by the same x-ray machine at a dose rate of 1 Gy. The volumes of xenograft tumors from four different treated groups were measured and evaluated at different times after irradiation (Fig. 5a). Representatives of xenograft tumors from two groups irradiated with 5 Gy were shown in Fig. 5b. As shown in Fig. 5a–b, enforced expression of miR-302a significantly sensitized MDA-MB-231RR xenograft tumors to irradiation compared to three control groups. The volume of the tumors with miR-302a was significantly reduced compared to the control at day 6, 8, 10, and 12 after the irradiation (Fig. 5a) ($p<0.05$). The comparison of xenograft tumor weight from two groups confirmed the data of tumor volume (Fig. 5c). To check

if overexpression of miR-302a modulates the expression of AKT1 and RAD52 *in vivo*, we measured the expression levels of miR-302a, AKT1, and RAD52 in the xenograft tumors from the miR-302a transfected group and control group with 5 Gy irradiation. MiR-302a was significantly overexpressed in the tumor tissues from the miR-302a transfected group compared to control group (Fig. 5d). The downregulation of AKT1 and RAD52 was observed in the miR-302a transfected group compared to control group (Fig. 5d).

These results showed that enforced expression of miR-302a was efficient for sensitizing breast cancer cells to radiotherapy through targeting AKT1 and RAD52.

DISCUSSION

The role of miRNA in cancer radioresistance remains largely unexplored, though strong evidence suggested that miRNAs can play a critical role in cancer (20). In the present study, our findings demonstrate that miR-302 is involved in radioresistance of breast cancer *via* the modulation of AKT1 and RAD52 and enforced expression of miR-302a sensitizes resistant cancer cells to irradiation *in vitro* and *in vivo*. This study provides new evidence in radioresistant mechanisms of breast cancer cells and suggests that miR-302 may be a potential sensitizer for radiation therapy.

The present study shows for the first time that MiR-302 is involved in radioresistance *via* directly targeting AKT1 and RAD52, two critical regulators to radioresistance. Radioresistance is a complex cell response which involves many genes and signaling pathways. A single miRNA can target multiple function genes to regulate a signaling network (21) through binding to the 3′-UTR of a gene in a partial complementary manner (22,23). Our bioinformatic studies found that *AKT1*
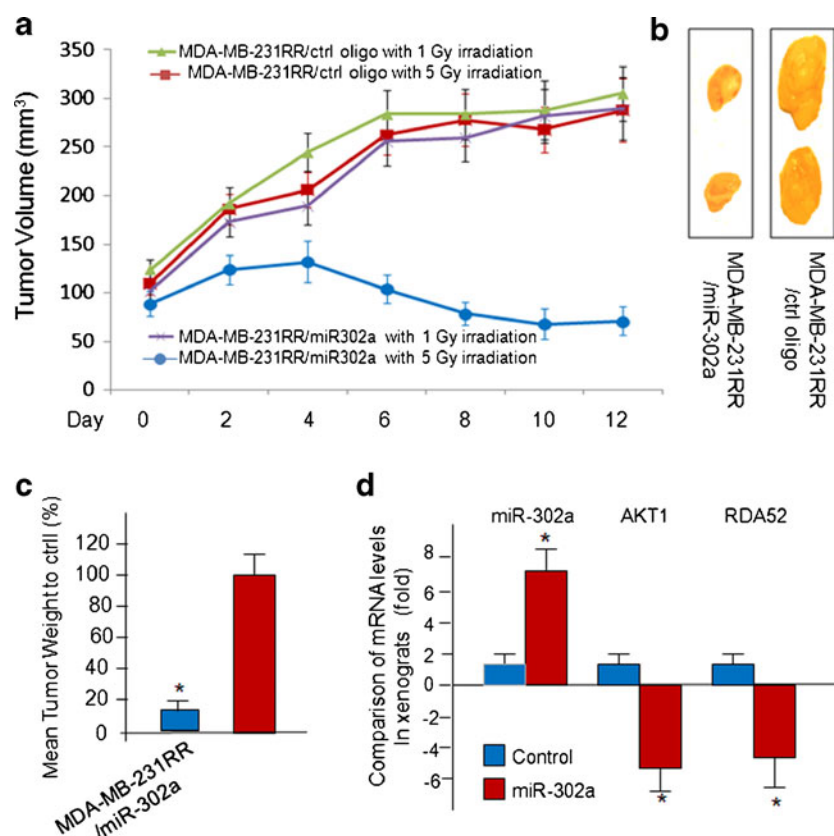
Fig. 5 Effects of miR-302 overexpression to radiosensitivity *in vivo*. (**a**) Mean xenograft tumor volume of each group at each time point. The day, when mice were irradiated, was designated as Day 0. Bars mean standard errors. Both hind legs of mice in two groups were injected with MDA-MB-231RR cells with or without the transfection of miR-302a vectors. The left hind leg that bore the developed tumor was irradiated at 5 Gy and the right hind legs were used as the mock-irradiated (1 Gy) control. The mice were sacrificed at 12 days after irradiation and the tumors were removed for weight comparison. $P < 0.05$. (**b**) Representatives of xenograft tumors from two groups irradiated with 5 Gy at final time point. (**c**) Comparison of xenograft tumor weight from two groups irradiated with 5 Gy. *$P < 0.01$. (**d**) Comparison of mRNA expression levels of miR-302a, AKT-1, and RAD52 in xenografts from two groups irradiated with 5 Gy, *$P < 0.01$.

and *RAD52* are the predicted targets of miR-302. More importantly, our data have demonstrated that miR-302a sensitized radioresistant MDA-MB-231RR cells to radiation therapy *in vitro* and *in vivo* and reduced the expression of AKT1 and RAD52. The PI3K/AKT pathway has been shown to play a critical role in three main mechanisms of radioresistance (24–26). RAD52 is an important protein for DNA double-strand break repair and homologous recombination (27–29). It was also found to interact with DNA recombination protein RAD51 (30,31), which suggested its role in RAD51-related DNA recombination and repair. Collectively, loss of miR-302s may be involved in the radioresistance of breast cells, mainly through the release of suppression on AKT1 and RAD52 expression.

The role of miRNAs in cancer is further supported by the fact that more than 50% of the human miRNA genes are located in cancer-associated genomic regions or at fragile sites (32,33). Many properties of miRNAs make them attractive candidates as sensitizers for radioresistance in comparison to other kinds of nucleic acids or proteins despite the challenges. Although the significant focus in this area has been directed

toward antisense-mediated inhibition of oncogenic miRNAs (34,35), several lines of evidence suggest that miRNA replacement represents an equally viable option. Some specific miRNAs are often overexpressed in cancer cells, but most miRNAs are downregulated in tumors (5,36). Global miRNA repression enhances cellular transformation and tumorigenesis in both *in vitro* and *in vivo* models (37), underscoring the protumorigenic effects of miRNA loss-of-function. In the present studies, our data have demonstrated that miR-302a restored sensitivity of radioresistant MDA-MB-231 breast cells to irradiation *in vitro* and *in vivo*. Two recent reports demonstrated that viral delivery of let-7 miRNAs suppressed tumor growth in a mouse model of lung adenocarcinoma (38,39). These observations suggest that re-expression of even a single miRNA in tumor cells could provide significant therapeutic benefits.

The advantage of miRNAs over siRNA/shRNA is their ability to affect multiple targets with a single hit, thus regulating a whole network of interacting molecules. However, this advantage, like many targeted therapies, leads to a major clinical concern, off-target effect and toxicity. In fact, a previous study

has shown that the level of repression achieved is dependent on both the amount of mRNA and the amount of available miRNA complexes (40). Our investigation has demonstrated that miR-302s are downregulated in radioresistant breast cancer cells compared to their parental cells while expression levels of AKT1 and RAD52 are upregulated. MiR-302 replacement therapy simply restores the depleted endogenous miRNAs, targeting the same genes that are also affected by the naturally occurring miRNA, so they may not result in "off-target" effects and toxicity. Actually, our *in vivo* data did not show any off-target effect. Therefore, the studies suggest that miR-302 replacement therapy as a sensitizer for radioresistance of breast cancer may be safe. However, it will be critical to design an appropriate carrier to deliver miR-302 specifically to the tumor cells in order to achieve the optimal therapeutic efficacy and decrease toxicity in our future research.

Taken together, our results show for the first time that the enforced expression of miR-302a efficiently sensitizes radioresistant tumor cells to irradiation through directly downregulating expression of both AKT1 and RAD52. We believe that this approach will open a broader path toward an efficient radiotherapy strategy for breast cancer.

ACKNOWLEDGMENTS AND DISCLOSURES

REFERENCES

1. Overgaard M. Radiotherapy as part of a multidisciplinary treatment strategy in early breast cancer. Eur J Cancer. 2001;37 Suppl 7:S33–43.
2. Croce CM, Calin GA. miRNAs, cancer, and stem cell division. Cell. 2005;122(1):6–7.
3. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell. 2004;116(2):281–97.
4. Ambros V. microRNAs: tiny regulators with great potential. Cell. 2001;107(7):823–6.
5. Lu J, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D, *et al*. MicroRNA expression profiles classify human cancers. Nature. 2005;435(7043):834–8.
6. Slack FJ, Weidhaas JB. MicroRNAs as a potential magic bullet in cancer. Future Oncol. 2006;2(1):73–82.
7. Cimmino A, Calin GA, Fabbri M, Iorio MV, Ferracin M, Shimizu M, *et al*. miR-15 and miR-16 induce apoptosis by targeting BCL2. Proc Natl Acad Sci U S A. 2005;102(39):13944–9.
8. Jiang J, Lee EJ, Gusev Y, Schmittgen TD. Real-time expression profiling of microRNA precursors in human cancer cell lines. Nucleic Acids Res. 2005;33(17):5394–403.
9. Dickins RA, Hemann MT, Zilfou JT, Simpson DR, Ibarra I, Hannon GJ, *et al*. Probing tumor phenotypes using stable and regulated synthetic microRNA precursors. Nat Genet. 2005;37 (11):1289–95.
10. Tsuda N, Kawano K, Efferson CL, Ioannides CG. Synthetic microRNA and double-stranded RNA targeting the 3′-untranslated region of HER-2/neu mRNA inhibit HER-2 protein expression in ovarian cancer cells. Int J Oncol. 2005;27 (5):1299–306.
11. Weidhaas JB, Babar I, Nallur SM, Trang P, Roush S, Boehm M, *et al*. MicroRNAs as potential agents to alter resistance to cytotoxic anticancer therapy. Cancer Res. 2007;67(23):11111–6.
12. Josson S, Sung SY, Lao K, Chung LW, Johnstone PA. Radiation modulation of microRNA in prostate cancer cell lines. Prostate. 2008;68(15):1599–606.
13. Yan D, Ng WL, Zhang X, Wang P, Zhang Z, Mo YY, *et al*. Targeting DNA-PKcs and ATM with miR-101 sensitizes tumors to radiation. PLoS One. 2010;5(7):e11397.
14. Liang Z, Wu H, Xia J, Li Y, Zhang Y, Huang K, *et al*. Involvement of miR-326 in chemotherapy resistance of breast cancer through modulating expression of multidrug resistance-associated protein 1. Biochem Pharmacol. 2010;79(6):817–24.
15. Liang Z, Wu T, Lou H, Yu X, Taichman RS, Lau SK, *et al*. Inhibition of breast cancer metastasis by selective synthetic polypeptide against CXCR4. Cancer Res. 2004;64(12):4302–8.
16. Liang Z, Brooks J, Willard M, Liang K, Yoon Y, Kang S, *et al*. CXCR4/CXCL12 axis promotes VEGF-mediated tumor angiogenesis through Akt signaling pathway. Biochem Biophys Res Commun. 2007;359(3):716–22.
17. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C (T)) Method. Methods. 2001;25(4):402–8.
18. Zhu H, Wu H, Liu X, Evans BR, Medina DJ, Liu CG, *et al*. Role of MicroRNA miR-27a and miR-451 in the regulation of MDR1/P-glycoprotein expression in human cancer cells. Biochem Pharmacol. 2008;76(5):582–8.
19. Kovalchuk O, Filkowski J, Meservy J, Ilnytskyy Y, Tryndyak VP, Chekhun VF, *et al*. Involvement of microRNA-451 in resistance of the MCF-7 breast cancer cells to chemotherapeutic drug doxorubicin. Mol Cancer Ther. 2008;7(7):2152–9.
20. Calin GA, Croce CM. MicroRNA signatures in human cancers. Nat Rev Cancer. 2006;6(11):857–66.
21. Griffiths-Jones S, Grocock RJ, van Dongen S, Bateman A, Enright AJ. miRBase: microRNA sequences, targets and gene nomenclature. Nucleic Acids Res. 2006;34(Database issue):D140–4.
22. Jackson RJ, Standart N. How do microRNAs regulate gene expression? Sci STKE. 2007;2007(367):re1.
23. Djuranovic S, Nahvi A, Green R. A parsimonious model for gene regulation by miRNAs. Science. 2011;331(6017):550–3.
24. Schuurbiers OC, Kaanders JH, van der Heijden HF, Dekhuijzen RP, Oyen WJ, Bussink J. The PI3-K/AKT-pathway and radiation resistance mechanisms in non-small cell lung cancer. J Thorac Oncol. 2009;4(6):761–7.
25. Bussink J, van der Kogel AJ, Kaanders JH. Activation of the PI3-K/AKT pathway and implications for radioresistance mechanisms in head and neck cancer. Lancet Oncol. 2008;9(3):288–96.
26. Jameel JK, Rao VS, Cawkwell L, Drew PJ. Radioresistance in carcinoma of the breast. Breast. 2004;13(6):452–60.
27. Mortensen UH, Lisby M, Rothstein R. Rad52. Curr Biol. 2009;19 (16):R676–7.
28. Van Dyck E, Stasiak AZ, Stasiak A, West SC. Binding of double-strand breaks in DNA by human Rad52 protein. Nature. 1999;398 (6729):728–31.
29. Symington LS. Role of RAD52 epistasis group genes in homologous recombination and double-strand break repair. Microbiol Mol Biol Rev. 2002;66(4):630–70.

30. Benson FE, Baumann P, West SC. Synergistic actions of Rad51 and Rad52 in recombination and DNA repair. Nature. 1998;391 (6665):401–4.

31. Shinohara A, Ogawa T. Stimulation by Rad52 of yeast Rad51-mediated recombination. Nature. 1998;391(6665):404–7.

32. Zhang B, Pan X, Cobb GP, Anderson TA. microRNAs as oncogenes and tumor suppressors. Dev Biol. 2007;302(1):1–12.

33. Manikandan J, Aarthi JJ, Kumar SD, Pushparaj PN. Oncomirs: the potential role of non-coding microRNAs in understanding cancer. Bioinformation. 2008;2(8):330–4.

34. Stenvang J, Silahtaroglu AN, Lindow M, Elmen J, Kauppinen S. The utility of LNA in microRNA-based cancer diagnostics and therapeutics. Semin Cancer Biol. 2008;18(2):89–102.

35. Love TM, Moffett HF, Novina CD. Not miR-ly small RNAs: big potential for microRNAs in therapy. J Allergy Clin Immunol. 2008;121(2):309–19.

36. Gaur A, Jewell DA, Liang Y, Ridzon D, Moore JH, Chen C, *et al.* Characterization of microRNA expression levels and their biological correlates in human cancer cell lines. Cancer Res. 2007;67 (6):2456–68.

37. Kumar MS, Lu J, Mercer KL, Golub TR, Jacks T. Impaired microRNA processing enhances cellular transformation and tumorigenesis. Nat Genet. 2007;39(5):673–7.

38. Esquela-Kerscher A, Trang P, Wiggins JF, Patrawala L, Cheng A, Ford L, *et al.* The let-7 microRNA reduces tumor growth in mouse models of lung cancer. Cell Cycle. 2008;7(6):759–64.

39. Kumar MS, Erkeland SJ, Pester RE, Chen CY, Ebert MS, Sharp PA, *et al.* Suppression of non-small cell lung tumor development by the let-7 microRNA family. Proc Natl Acad Sci U S A. 2008;105 (10):3903–8.

40. Doench JG, Sharp PA. Specificity of microRNA target selection in translational repression. Genes Dev. 2004;18(5):504–11.