RNAi-Mediated Targeting of Noncoding and Coding Sequences in DNA Repair Gene Messages Efficiently Radiosensitizes Human Tumor Cells

Zhiming Zheng1,2, Wooi Loon Ng2, Xiangming Zhang2, Jeffrey J. Olson3, Chunhai Hao4, Walter J. Curran2, and Ya Wang2

Abstract

Human tumor cell death during radiotherapy is caused mainly by ionizing radiation (IR)--induced DNA double-strand breaks (DSB), which are repaired by either homologous recombination repair (HRR) or nonhomologous end-joining (NHEJ). Although siRNA-mediated knockdown of DNA DSB repair genes can sensitize tumor cells to IR, this approach is limited by inefficiencies of gene silencing. In this study, we show that combining an artificial miRNA (amiR) engineered to target 3′-untranslated regions of XRCC2 (an HRR factor) or XRCC4 (an NHEJ factor) along with an siRNA to target the gene coding region can improve silencing efficiencies to achieve more robust radiosensitization than a single approach alone. Mechanistically, the combinatorial knockdown decreased targeted gene expression through both a reduction in mRNA stability and a blockade to mRNA translation. Together, our findings establish a general method of gene silencing that is more efficient and particularly suited for suppressing genes that are difficult to downregulate by amiR- or siRNA-based methods alone. Cancer Res; 72(5); 1221–8. ©2012 AACR.

Introduction

Radiotherapy, one of the leading approaches in cancer therapy, contributes to more than 50% of cancer treatment. Ionizing radiation (IR) kills tumor cells mainly by inducing DNA double-strand breaks (DSB) and, therefore, results in cell reproductive death (loss of the capacity to sustain proliferation; refs. 1, 2). Therefore, inhibiting DNA DSB repair is an efficient way to sensitize human cells to IR-induced killing. In mammalian cells, there are two pathways to repair DNA DSB: homologous recombination repair (HRR) and nonhomologous end-joining (NHEJ). XRCC4 is an essential factor that stimulates DNA ligase IV in the NHEJ pathway (3, 4), and XRCC2 is an essential factor in the HRR pathway (5, 6). Targeting the XRCC4 coding region with siRNA has been reported to manipulate the NHEJ pathway (3, 4), and XRCC2 is an essential factor in the HRR pathway (5, 6). Targeting the XRCC4 coding region with siRNA has been reported to manipulate the NHEJ pathway (7) and targeting the XRCC2 coding region with siRNA has been reported to affect the HRR pathway (8). Many human tumors have a strong DNA DSB repair capacity; therefore, discovering a new approach that could target DNA repair genes more efficiently is essential to maximally sensitize human tumor cells to IR-induced killing.

miRNAs are evolutionally conserved noncoding RNA with 19 to 23 nucleotide that negatively regulate gene expression by binding to the 3′-untranslated region (UTR) of target genes (9, 10). It has been shown that the artificial miRNA (amiR) could be used for silencing gene expression by targeting its 3′-UTR (11). We were interested in testing the hypothesis that combining amiR to target the 3′-UTR of the DNA repair gene and siRNA to target the coding sequence of the same gene could maximally knock down the gene. In this study, we chose XRCCA4 and XRCC2 as the target genes to test whether we could use the combined approach to maximally knock down the genes and sensitize human tumor cells to IR-induced killing. Our data showed promising results and indicate the feasibility and efficiency of the combined approach for these repair genes. We believe that this combined approach is also very useful for many other gene targeting purposes, especially for some genes that are difficult to target using a single approach (either siRNA or amiR).

Materials and Methods

Human tissues, tumor cell lines, and irradiation

The frozen human brain nontumor tissues and glioblastoma multiforme (GBM) samples were derived from brain surgeries at Emory University, Atlanta, GA. Human brain tumor cell lines (U87MG and T98G) and human lung cancer cell lines (A549 and H1299) were purchased from American Type Culture Collection. A human normal bronchial epithelia cell line, HBEC-3KT, was obtained from the laboratory of Dr. John Minna at the University of Texas Southwestern, Dallas, TX (12). Human...
normal astrocyte cells and a brain tumor cell line, LN229, were obtained from the laboratory of Dr. Erwin Van Meir at Emory University (13). The human nontumor cell lines, HBEC-3KT and LN229, were cultured as described previously (12, 13) and verified by a soft agar colony-forming assay. IR was carried out with an X-ray machine (X-RAD 320, N. Branford 320 kV, 10 mA, the filtration with 2-mm aluminum) in our laboratory. The dose rates were about 2 Gy/min.

**Construction of amiR-expressing plasmid**

The XRCC4- or XRCC2-specific amiR was designed by a BLOCK-it RNAi Designer tool and purchased from Invitrogen. A BLAST analysis was conducted to avoid the designed sequence with substantial homology to other genes. The primers are shown in the Supplementary Table S1. The double-strand oligonucleotides were generated by annealing equal amounts of each single-strand oligonucleotide and cloned into a pcDNA6.2-GW/EmGFP-miR vector. After the sequence was verified, the amiR expression clone was inserted into the destination vector pLenti6/V5-DEST (Invitrogen) according to the manufacturer’s instructions.

**Lentivirus packaging and infection**

These experiments were similar to that described previously (14, 15). Briefly, approximately $4 \times 10^6$ 293FT cells were seeded in a 100-mm dish overnight. The pLenti6/V5-GW-miR expression plasmid Control-amiR, amiR-XRCC4, or amiR-XRCC2 with ViraPower Packaging Mix (Invitrogen) were formed into complexes with Lipofectamine 2000 (Invitrogen) and transfected into the 293FT cells. The media containing the DNA-a complex with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions.

Whole-cell lysates for Western blotting were prepared as described previously (14, 15). The antibodies against XRCC4, XRCC2, and β-actin were purchased from Santa Cruz Biotechnology Inc. and incubated overnight at 4°C. The membrane was scanned and analyzed using an Odyssey Infrared Imaging System (LI-COR Biosciences).

**γ-H2AX foci assay**

The assay followed the previously reported protocol (16, 17) with some modifications. Briefly, cells were fixed in 4% paraformaldehyde for 15 minutes, permeabilized for 5 minutes on ice in 0.2% Triton X-100, and blocked in 10% normal goat serum. The coverslips were incubated with an anti-γ-H2AX antibody (Millipore) for 1 hour, washed in PBS, 1% BSA, and incubated with an Alexa Fluor 488-conjugated goat anti-mouse secondary antibody (Invitrogen) for 1 hour at room temperature. Cells were washed in PBS and mounted by using Vectashield-mounting medium with 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories). Fluorescent images were captured by using a CarlZeiss Axio Scope A1 with an Epi-Fluorescence microscope (Germany) equipped with an RGB Cooled Digital Camera with Axivision software (version 4.8) for camera control, image acquisition, processing, and a module for multichannel display.
Clonogenic surviving assay
Cellular sensitivity to radiation was determined by the loss of colony-forming ability as described previously (14, 15). Briefly, \( 2 \times 10^5 \) cells were plated, per 60-mm dish with 3 mL of medium. The cells were irradiated 48 hours later. The cells were then collected and plated, aiming at 20 to 200 colonies per dish. Two replicates were prepared for each datum point and were incubated for 2 weeks to allow colonies to develop. Colonies were stained with crystal violet (100% methanol solution) before counting.

Results
XRCC2 or XRCC4 expressed higher in human tumor tissues/cells than in human nontumor tissues/cells
To study the effects of knocking down the DNA repair gene XRCC2 (for the HRR pathway) or XRCC4 (for the NHEJ pathway) for the sensitization of human tumor cells to radiation, we were interested in knowing the different expression of the repair genes in human normal tissues/cells and human tumor tissues/cells as such information would allow us to evaluate whether the sensitization approach could particularly benefit from killing more tumor cells. For this purpose, we compared the expression levels of XRCC2 or XRCC4 in the human GBM or lung tumor cell lines and their nontumor counterparts by using Western blot analysis. The results showed that when compared with the human normal astrocyte cells (Fig. 1A, lane 1), the human GBM cell lines (Fig. 1A, lane 2–4) showed higher levels of XRCC2 or XRCC4, particularly in the U87MG cells (Fig. 1A, lane 2). Similar to the GBM cell line results, when compared with the human normal bronchial epithelial cell lines (Fig. 1A, lane 5), the human lung cancer cell lines (Fig. 1A, lane 5 and 6) showed higher levels of XRCC2 or XRCC4, particularly in the A549 cells (Fig. 1A, lane 5). We then compared the expression levels of XRCC2 or XRCC4 in the frozen human GBM tissues and human nontumor tissues by using a real-time PCR assay. The results also showed higher expression levels of XRCC2 or XRCC4 in the human GBM tissues than in their nontumor counterpart tissues (Fig. 1B). These data suggest that it might be common that these repair genes highly express in human tumors and, therefore, knocking down these genes could be particularly beneficial for the tumor radiosensitization. On the basis of these data, we chose U87MG (human GBM cell line) and A549 (human lung cancer cell line) cells in our following experiments because the levels of XRCC2 and XRCC4 were relatively higher in these 2 cell lines (framed lanes in Fig. 1A) when compared with the normal human cells and other human brain and lung tumor cell lines tested. After choosing the candidate genes and cell lines, we designed the amiR that targets XRCC2 or XRCC4 and examined the effects of the amiR on knocking down the targeted genes because siRNA is a relatively mature approach for knocking down a gene.

The amiR against XRCC4 or XRCC2 efficiently inhibited the targeted gene expression
Natural mammalian miRNA only requires 6 to 8 seed nucleotides to match the sequence of the targeted gene 3'-UTR, therefore, one miRNA could target multiple mRNAs. To avoid the nonspecific effects, we decided to choose the completely matched strategy (the amiR completely matched the target sequence). On the basis of the sequence of XRCC2 or XRCC4 3'-UTR, we identified the potential-targeted sequences of 3'-UTR that could be bound by an amiR (Fig. 2A). On the basis of the information, we designed the construct encoding the precursor of amiR against XRCC4 or XRCC2 (Fig. 2B) and inserted the sequences into a lentiviral vector as we described previously (14, 15). To examine whether the sequence in the 3'-UTR of XRCC2 or XRCC4 could be bound by the amiR, we conducted the luciferase assay. The results showed that the amiR could bind well to the targeted sequence (Fig. 2C), suggesting the amiR could work as a miRNA to decrease the targeted gene expression. We then infected the plasmid encoding amiR-XRCC2, amiR-XRCC4, or both into U87MG or A549 cells. After using the antibiotic selection for 10 generations, we obtained the stable amiR expressed cells that showed the positive GFP signals that reflected the amiR expression frequency of more than 80% (Fig. 3A). We next examined the levels of the targeted protein XRCC2 and XRCC4 in these cells. The results showed that the miRs efficiently reduced their targeted gene expression in both U87MG and A549 cells (Fig. 3B), indicating that our designed amiRs worked well even in cells that were coinfected with the 2 vectors (Fig. 3B). After we obtained the stable amiR expressed cells, we examined the effects of the amiR on the proliferation (by growth curve) and clonogenic rate (plating efficiency) in these cells without any treatment. The results showed that when compared with noninfected cells and the vector alone–infected cells, the
The amiR against XRCC4 or XRCC2 efficiently sensitized human tumor cells to IR-induced killing

Next, we examined the effects of the amiR on the radiosensitization of the cells. We used a γ-H2AX assay to examine the DNA DSB repair efficiency because the signals of γ-H2AX reflect the amounts of DNA DSBs in irradiated cells (16, 18) and indirectly describe the efficiency of DNA DSB repair (17). The results showed that there was no apparent difference in the γ-H2AX–positive ratios among the examined cells immediately after IR (30 minutes), suggesting that there was not much difference in the induction of DNA DSB in these irradiated cells, however, the γ-H2AX–positive ratios clearly increased in the irradiated cells expressing amiR-XRCC2 or amiR-XRCC4 when compared with the irradiated cells transfected with the vector alone (Fig. 4A and B). The γ-H2AX–positive ratios left even more in the irradiated cells expressing both amiR-XRCC2 and amiR-XRCC4 than in the irradiated cells expressing amiR-XRCC2 or amiR-XRCC4 alone (Fig. 4A and B). These results indicate the amiRs inhibited the DNA DSB repair, which is further supported by the survival data (by a clonogenic assay to examine the reproductive death). The results showed that IR killed more cells expressing either amiR-XRCC4 or amiR-XRCC2 than the cells expressing the vector alone and killed even more of the cells expressing both amiR-XRCC2 and amiR-XRCC4 than the cells expressing amiR-XRCC2 or amiR-XRCC4 alone (Fig. 4C). These results show that the amiRs could be used to target DNA repair genes in human tumor cells and sensitize these tumor cells to IR-induced killing. Next, we were interested in detecting the effects of siRNA when targeting the same gene in the tumor cells expressed with the amiR and studying whether the combination approach could enhance the gene knock down efficiency and the human tumor cell radiosensitization.
Combining amiR and siRNA to target XRCC2 or XRCC4 more efficiently knocked down the gene and radiosensitized the human tumor cells

To find a better time for gene targeting, we examined the XRCC2 or XRCC4 levels in U87MG or A549 cells expressed with the amiR-XRCC2 or amiR-XRCC4 at different times after the cells were treated with siRNA against XRCC2 or XRCC4. The results showed that the control RNA did not affect the targeted gene expression at all time points but the siRNA maximally inhibited the targeted gene at 72 hours after transfection in these tumor cells and inhibited more XRCC2 or XRCC4 expression in A549 or U87MG cell radiosensitivity. The clonogenic assay was conducted as described in Materials and Methods. Data shown are the mean and SE from 3 independent experiments.

Knocking down XRCC2 or XRCC4 by combining small RNAs to target both the coding region and the 3'-UTR through increasing the mRNA degradation and inhibiting the translation

Some commercially available siRNA pools (for targeting a small gene that contains a short coding region) include a small RNA that targets the 3'-UTR of the gene. The small RNA that targets the 3'-UTR of the gene looks like a miRNA mimic and is a stable double-strand RNA with approximately 22 nucleotides. However, there are no commercially available siRNA pools that include small RNAs to target 3'-UTR of XRCC2 or XRCC4. Also, there is no report to compare the efficiency of any such combined approach (targeting both the coding region and the 3'-UTR of a gene) with the single approach (targeting coding region or 3'-UTR of a gene) on knocking down a gene. In addition, there is no mechanism study for elucidating the advantage of the combined approach if it does have some advantages on the gene knock down. Therefore, we next investigated the effects of the small RNA combined approach (targeting both noncoding and coding regions of XRCC2 or XRCC4) by mixing the amiR mimic and siRNAs against XRCC2 or XRCC4. We also tried to elucidate how the targeted gene

Figure 4. The amiR targeting the 3'-UTR of the XRCC2 or XRCC4 sensitized human tumor cells to IR-induced killing. A, incidence of γ-H2AX foci in irradiated U87MG cells. Cells were irradiated with 2 Gy, incubated at 37°C at different times and then processed for γ-H2AX foci counting. Values are the average numbers of γ-H2AX foci per cell (FPC). 50 cells were counted for each slide. B, the effect of the amiR on γ-H2AX foci A549 and U87MG cells at different times after 2 Gy exposure. Error bars, SDs. C, the effects of the amiR on A549 and U87MG cell radiosensitivity. The clonogenic assay was conducted as described in Materials and Methods. Data shown are the mean and SE from 3 independent experiments.
was knocked down by the combined approach if the combined approach did show a better effect on knocking down the repair gene than the siRNA or amiR mimic alone. The results showed that an amiR mimic specifically knocked down XRCC2 or XRCC4 (Fig. 6A) and mixing it with a siRNA more efficiently decreased the XRCC2 or XRCC4 expression level (Fig. 6A). As expected, mixing the amiR mimic with siRNA against XRCC2 or XRCC4 more efficiently sensitized the cells to IR-induced killing than using amiR alone, although the combined approach did further decrease the protein level by the combined approach is due to the two mechanisms that generate mRNA instability and block translation (Fig. 6C). These results further indicate that the combined approach is specifically

Figure 5. Combining amiR and siRNA to target both the 3' UTR and coding region of XRCC2 or XRCC4 efficiently inhibited the targeted gene and sensitized the human tumor cells to IR-induced killing. A, the Western blotting results showed the levels of XRCC2 or XRCC4 in A549 or U87MG cells containing amiR or vector alone at different times after transfected with the siRNA. β-Actin was used as an internal loading control. Vector, the cells containing the vector alone; amiR, the cells containing the vector encoding the XRCC2 or XRCC4 amiR; vc, the cells containing the vector alone treated with the control RNA; ac, the cells containing the vector encoding the XRCC2 or XRCC4 amiR treated with the control RNA. B, the effect of siRNA against XRCC2 or XRCC4 on sensitizing A549 or U87MG cells containing vector alone or containing vector encoding XRCC2 or XRCC4 amiR. The cells were treated with control RNA (cRNA) or siRNA against XRCC2 or XRCC4. At 72 hours after transfection, the cells were exposed to 4 Gy. The cells were collected and plated in new dishes for colony forming as described in Materials and Methods. Data shown are the mean and SE from 3 independent experiments.

Figure 6. The effects of combining the small RNA (amiR mimic and siRNA) on knocking down the targeted gene and on radiosensitizing the human tumor cells. A, the targeted gene expression levels in the cells at 72 hours after transfection with amiR mimic and/or siRNA. The amiR mimic was labeled as amiR-m. β-Actin was used as an internal loading control. B, the effect of the combined small RNA (including amiR mimic and siRNA) on cell radiosensitivity. At 72 hours after the small RNA (including amiR and siRNA) transfection, the cells were exposed to 4 Gy and then were collected for colony forming as described in Materials and Methods. Data shown are the mean and SE from 3 independent experiments. C, the mRNA levels of XRCC2 or XRCC4 in the cells treated with amiR mimic, siRNA, or both were measured by real-time PCR with the proper primers (Supplementary Table S1) as described in Materials and Methods. The independent experiments were done twice with triple samples.
and efficiently for knocking down a gene. Because these small RNAs use different mechanisms to knock down a gene, the combined usage could reach significant additive inhibition of the targeted gene expression.

Discussion

In this study, we show for the first time that our designed amiRs could efficiently knock down DNA repair gene XRCC2 or XRCC4, sensitize human tumor cells to radiation-induced killing, and combining with siRNA could more efficiently knock down the gene and radiosensitize human tumor cells.

A natural miRNA binding to the 3′-UTR of a gene is in a partial complementary manner (21, 22) and one miRNA could target multiple genes (23); so, delivering a miRNA into cells and upregulating the miRNA could result in multiple unexpected effects on the fate of cell. An amiR in a perfect complementary manner, however, could avoid the unexpected side effects by limiting its real target. The results showed that our designed amiRs could efficiently knock down the target genes without affecting the cell growth, suggesting that the amiRs specifically knocked down the target genes. Because most human genes are regulated by miRNA (24), it suggests that most human genes available are to be targeted by a designed amiR. It was reported that the manner of imperfect-complementary binding to 3′-UTR might protect the miRNA from an RNase degradation in Drosophila (25), suggesting that the perfect-complementary binding manner (to 3′-UTR of the target gene) of amiRs might affect the stability of the amiRs. To examine the possibility, we designed the XRCC4 amiRs with imperfect-complementary binding sequences (containing 7 mismatched nucleotides, which is similar to a natural miRNA) to the 3′-UTR (Supplementary Fig. S3A). We transfected the different XRCC4 amiRs to A549 cells and compared the targeting efficiency as well as the stability of the amiRs. We did not find differences in XRCC4 knockdown efficiency (Supplementary Fig. S3B) or stability of the amiRs (data not shown), suggesting that different from Drosophila miRNA stability, the human miRNA stability depends less on the match sequence to a targeted 3′-UTR, which might be due to the relatively short 3′-UTR and a simpler structure of the genome in Drosophila when compared with that in humans (26, 27). However, the real mechanism underlying the difference in miRNA degradation between humans and Drosophila needs more studies to provide direct evidence. In this study, we found that the efficiency of knocking down the targeted gene dramatically reduced when we mixed an amiR mimic with a siRNA that had more than 5 matched nucleotides (data not shown), suggesting that these small RNAs could interbind each other, and we should avoid the interaction among themselves.

Because NHEJ is a relatively fast process and is not affected by cell-cycle distribution (28, 29), The γ-H2AX-positive ratios in the irradiated cells containing XRCC4 amiR was higher than that in the irradiated cells containing the vector alone, especially at 6 hours after IR (Fig. 4A and B), indicating the inefficient NHEJ in the cells knocked down with XRCC4 as NHEJ is a relatively fast process and is not affected by cell-cycle distribution (28, 29). In addition, because HRR is a relatively slow process and occurs only during the S- and G2 phases (29, 30), the γ-H2AX-positive ratios in irradiated cells containing XRCC2 amiR was higher than that in the irradiated cells containing the vector alone, especially at 24 hours after IR, indicating the inefficient HRR in the irradiated cells knocked down with XRCC2. Interestingly, the radiosensitizing level by the siRNA against XRCC2 or XRCC4 in A549 or U87MG cells encoding the amiR XRCC2 or XRCC4 reached a similar level in the cells encoding both XRCC2 and XRCC4 amiRs (comparing the data shown in Fig. 4C and in Fig. 5B), suggesting that the tumor cell radiosensitization by combining amiR and siRNA to target one repair gene could be comparable by using a single approach (amiR or siRNA) to target 2 repair genes. Efficiently targeting one gene is more important than mildly targeting 2 genes because most human tumor cells are overactivated with one particular DNA repair pathway.

Taken together, our results show for the first time that combining amiR and siRNA to target both the noncoding and coding regions of XRCC2 or XRCC4 could more efficiently knock down the DNA DSB repair genes and sensitize human tumor cells to IR-induced killing. We believe that this combined approach will provide a broader use for knocking down any other genes (only if the gene contains more than 100 bp of the 3′-UTR) especially for those genes that are difficult to knock down by using only one approach amiR or siRNA.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

The authors thank Drs. John Minna and Erwin Van Meir for providing the cell lines and Doreen Theune for editing the manuscript.

Grant Support

This work is supported by NIH grant (GM080771), NASA grant (NNX11AC30G), and Emory Start-up fund (to Y. Wang). Z. Zheng is partially supported by the China Scholarship Council (No. 200962099).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received August 16, 2011; revised December 20, 2011; accepted December 26, 2011; published OnlineFirst January 11, 2012.

References


RNAi-Mediated Targeting of Noncoding and Coding Sequences in DNA Repair Gene Messages Efficiently Radiosensitizes Human Tumor Cells

Zhiming Zheng, Wooi Loon Ng, Xiangming Zhang, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-11-2785

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2012/01/11/0008-5472.CAN-11-2785.DC1

Cited articles
This article cites 28 articles, 14 of which you can access for free at:
http://cancerres.aacrjournals.org/content/72/5/1221.full.html#ref-list-1

Citing articles
This article has been cited by 2 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/72/5/1221.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.