RNAi-mediated Targeting of Non-coding and Coding Sequences in DNA Repair Gene Messages Efficiently Radiosensitizes Human Tumor Cells

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Running Title: Combine amiRNA and siRNA to Target Gene

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Abstract

Human tumor cell death during radiotherapy is caused mainly by ionizing radiation (IR)-induced DNA double strand breaks (DSBs), which are repaired by either homologous recombination repair (HRR) or non-homologous 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 demonstrate that combining an artificial microRNA (amiR) engineered to target 3'-untranslated regions of XRCC2 (a HRR factor) or XRCC4 (a NHEJ factor) along with an siRNA to target the gene coding region can improve silencing efficiencies to achieve more robust radiosensitization than single approaches 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.

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 (DSBs) and, therefore, results in cell reproductive death (loss of the capacity to sustain proliferation) (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 non-homologous 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 (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.

MicroRNAs (miRNAs) are evolutionally conserved non-coding ~19-23 nucleotide RNAs that negatively regulate gene expression by binding to the 3’-untranslated region (UTR) of target genes (9, 10). It has been demonstrated that the artificial microRNA (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 XRCC4 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 demonstrated 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 non-tumor tissues and glioblastoma (GBM) samples were derived from brain surgeries at Emory University. Human brain tumor cell lines (U87MG and T98G) and
human lung cancer cell lines (A549 and H1299) were purchased from ATCC. A human normal bronchial epithelia cell line, HBEC-3KT, was obtained from Dr. John Minna’s laboratory at UT Southwestern (12). Human normal astrocyte cells and a brain tumor cell line, LN229, were obtained from Dr. Erwin Van Meir’s laboratory at Emory University (13). The human non-tumor 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 using 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 performed to avoid the designed sequence with substantial homology to other genes. The primers are shown in the Supplementary Table S1. The double strand oligos were generated by annealing equal amounts of each single strand oligo and cloned into a pcDNA™6.2-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, XRCC4-amiR or XRCC2-amiR with ViraPower™ Packaging Mix (Invitrogen) were formed into a complex with Lipofectamine™ 2000 (Invitrogen) and
transfected into the 293FT cells. The media containing the DNA-Lipofectamine™2000 complexes were removed and replaced with complete culture medium. The virus containing supernatant was collected and supplemented with polybrene. The mixture was added to the cell cultures. The transduced cells were harvested after 72–96 hr post-infection for further experiments. Some cells were treated with blasticidin and selected for amiR stably expressed cells.

**siRNA and mimic amiR transfection**

The control RNA and siRNA pool against human XRCC4 or XRCC2 were purchased from Santa Cruz Biotechnology Inc. The amiR mimic and single siRNA against human XRCC4 or XRCC2 were purchased from Qiagen. The sequence of siRNA against human XRCC4 (Cat. # SI00051905) is UUCUACUUGGUGCAAUACAGtt (for sense) and CUGAUAAUUGCACCAAGUAGAAtt (for antisense). The sequence of siRNA against human XRCC2 (Cat. # SI00077091) is UUAUAGUUUGUGUCGUUGCAAtt (for sense) and UUGCAACGACACAAACUAUAAtt (for antisense). U87MG and A549 cells were grown to 30-50% confluence and RNA transfections were carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. The transfection efficiency for the cells was checked by a Western blot assay at different times after transfection.

**Quantitative real-time PCR**

Total small RNA in the human tumor cells were extracted by using a Qiazol kit (Qiagen). RNA (1 μg) was used to synthesize cDNA by using a SuperScript III kit (Invitrogen) according to the manufacturer’s instructions. Real-time PCR was performed on a SDS 7500 Fast
Instrument (Applied Biosystems) with the System Software version 2.0.5 following the default cycling protocol (at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, and 60°C for 1 min). Fluorescence readings were taken during the 60°C step. Primers (Supplementary Table S1) for XRCC4, XRCC2 or β-actin (an endogenous control) mRNA were designed using Primer Express 3.0 software (Applied Biosystems). RQs were calculated using the ΔΔCt method. Quantitative RT-PCR was performed at least three times and a no-template control was included as a negative control.

**Luciferase assay**

Similar to the methods as described in our previous studies (14, 15), 293FT cells were transfected with the plasmids (encoding the 3’-UTR of XRCC2 or XRCC4 containing the potential amiR binding site or the site was deleted) with or without 100 nM amiR mimics (Qiagen) in 48-well plates. The primers used for the experiments are listed in the supplementary table S2. The cells were harvested 48 h after transfection, the cells were then lysed with a luciferase assay kit (Promega) according to the manufacturer’s protocol and were measured on a luminescence microplate reader LUMIstar Galaxy (BMG labtechnologies). β-galactosidase or renilla luciferase was used for normalization.

**Western blot**

Whole cell lyses for Western blot were prepared as described previously (14, 15). The antibodies against XRCC4, XRCC2 and β-actin were purchased from Santa Cruz Biotechnology. 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).

**Gamma H2AX (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 min, permeabilized for 5 min 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, Billerica, MA) for 1 h, washed in PBS, 1% BSA and incubated with an Alexa Fluor 488-conjugated goat anti-mouse secondary antibody (Invitrogen) for 1 h at room temperature. Cells were washed in PBS and mounted by using Vectashield mounting medium with 4,6 diamidino-2-phenylindole (Vector Laboratories). Fluorescent images were captured by using a CarlZeiss Axio Scope A1 with an Epi-Fluorescence microscope (Germany) equipped with a MRm Cooled Digital Camera with Axiovision software (version 4.8) for camera control, image acquisition, processing and a module for multi-channel display.

**Clonogenic surviving assay**

Cellular sensitivity to radiation was determined by the loss of colony-forming ability as described previously (14, 15). Briefly, 2 x 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 20-200 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 non-tumor tissues/cells

To study the effects of knock down DNA repair gene XRCC2 (for the HRR pathway) or XRCC4 (for the NHEJ pathway) on 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 since 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 glioblastoma multiforme (GBM) or lung tumor cell lines and their non-tumor counterparts by using Western blot. 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 cells (Fig. 1A, lane 5), the human lung cancer cell lines (Fig. 1A, lane 5, 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 non-tumor 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 non-tumor 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 benefit from the tumor radio-sensitization. Based on 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
two 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 6-8 seed nucleotides to match the sequence of the targeted gene 3’-UTR, therefore, one miRNA could target multiple mRNAs. To avoid the non-specific effects, we decided to choose the completely matched strategy (the amiR completely matched the target sequence). Based on 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). Based on 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 performed 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 amiR stably expressed cells that showed the positive GFP signals that reflected the amiR expression frequency of >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 co-infected with the two vectors (Fig. 3B). After we obtained the amiR stably 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 non-infected cells and the vector alone infected cells, the vector encoding amiR infected cells showed similar proliferation (Supplementary Fig. S1A) and clonogenic rates (Supplementary Fig. S1B), indicating that the amiR expression alone had less toxicity. In addition, when the cells that were sub-cultured for a relatively long time, the target genes were still inhibited (Supplementary Fig. S2A) and the cells still showed more sensitive to IR-induced killing than their vector alone infected cells (Supplementary Fig. S2B). These results suggest that although a lower level of the DNA repair gene does not affect normal survival of the cells, it does radio-sensitize the cells.

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 radio-sensitization 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 min), 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, 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, B). These results indicate the amiRs inhibited the DNA DSB repair, which is further supported by the survival data (using 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 demonstrate 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 on targeting the same gene in the tumor cells expressed with the amiR and study whether the combination approach could enhance the gene knock down efficiency and the human tumor cell radio-sensitization.

Combining amiR and siRNA to target XRCC2 or XRCC4 more efficiently knocked down the gene and radio-sensitized 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 72h after transfection in these tumor cells and inhibited more XRCC2 or XRCC4 expression in A549 or U87MG cells stably expressed with the amiR (against XRCC2 or XRCC4) (Fig. 5A). These results indicate that the combined approach is more efficient than the siRNA or amiR approach alone. We then examined the sensitivities of the
combined approach on cell radio-sensitivity. The results showed that the cell sensitivity levels depended on the XRCC2 or XRCC4 level: the siRNA against XRCC2 or XRCC4 more efficiently sensitized A549 or U87MG cells expressing the amiR-XRCC2 or amiR-XRCC4 than the cells containing the vector alone (Fig. 5B). These results further demonstrate that the combined approach is more efficient than the siRNA or amiR approach alone.

**Knocking down XRCC2 or XRCC4 by combining small RNAs to target both coding region and 3’-UTR is 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 ~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 coding region and 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 non-coding 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 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 amiR mimic with siRNA against XRCC2 or XRCC4 more efficiently sensitized the cells to IR-induced killing than using amiR or siRNA against XRCC2 or XRCC4 alone (Fig. 6B). These results provide strong evidence that using the small RNAs to target both the non-coding and coding regions of a gene could more efficiently knock down the targeted gene.

Since miRNA could either decrease the mRNA stability or block translation to reduce the targeted gene expression (19, 20), we were interested in elucidating how the amiR mimic decreases the targeted gene expression. For this purpose, we compared the effects of the amiR mimic or/siRNA on the mRNA level of the targeted gene by using a real-time PCR approach. The results showed that although the protein level of the targeted gene (XRCC2 or XRCC4) between the cells treated with the amiR mimic and the cells treated with the siRNA did not show any apparent difference (Fig. 6A), the mRNA level of the targeted gene (XRCC2 or XRCC4) was lower in the cells treated with siRNA (~30% of control level) than in the cells treated with amiR mimic (~65% of the control level) (Fig. 6C), suggesting that the effects of the amiR on reducing ~70% of the protein level of the targeted gene is through both decreasing the mRNA stability (~35%) and blocking translation (~35%). When compared with siRNA alone, combining the amiR and siRNA did not further decrease the mRNA stability (~30%) although the combined approach did further decrease the protein level, suggesting that the additional effect to reduce 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 demonstrate that the combined approach is specifically and efficiently for knocking down a gene. Since these small RNAs use different mechanisms to knock down a gene, the combined usage could reach significant addictive 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 up-regulating the miRNA could result in multiple un-expected effects on the cells’ fate. An amiR in a perfect complementary manner, however, could avoid the un-expected side effects by limiting its real target. The results showed that our designed amiRs could efficiently knock down the target genes without affecting the cells growth, suggesting that the amiRs specifically knocked down the target genes. Since most human genes are regulated by miRNA (24), it suggests that most human genes are available 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 (nt), 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 knock down 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 need 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 five matched nucleotides (data not shown), suggesting that these small RNAs could inter-bind each other and we should avoid the interaction among themselves.

Since 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 h after IR (Fig. 4A, B), indicating the inefficient NHEJ in the cells knocked down with XRCC4 since 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 h after IR, indicating the inefficient HRR in the irradiated cells knocked down with XRCC2. Interestingly, the radio-sensitizing 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 radio-sensitization by combining amiR and siRNA to target one repair gene could be comparable by using a single approach (amiR or siRNA) to target two repair genes. Efficiently targeting one
gene is more important than mildly targeting two genes because most human tumor cells are over-activated with one particular DNA repair pathway.

Taken together, our results show for the first time that combining amiR and siRNA to target both the non-coding 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.

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**References**


**Figure legends:**

Figure 1. XRCC2 and XRCC4 highly expressed in human tumor cell lines and tissues. A, The XRCC2 and XRCC4 levels were detected in different human tumor cell lines by Western blot. 1, normal human astrocyte cells; 2, U87MG cells; 3, T98G cells; 4, LN229 cells; 5, normal human bronchial epithelial cells: HBEC-3KT cells; 6, A549 cells; 7, H1299 cells. The red frames represent the U87MG and A549 cells were chosen for the following experiments described in this study. B, The XRCC2 or XRCC4 mRNA levels were detected in three samples from different human non-tumor brain tissues and five samples from different glioblastomas (GBM) by real-time PCR with the specific primers (Table S1).

Figure 2. Design the amiR targeting XRCC2 or XRCC4. A, The targeted sequence in the 3′-UTR of XRCC2 or XRCC4 gene: The total nucleotide number in different regions of the XRCC2 or
XRCC4 was indicated. The targeting sequence of the XRCC2 by the amiR starts from the nucleotide 1052 of the 3’-UTR and the targeting sequence of the XRCC4 by the amiR starts from nucleotide 85 of the 3’-UTR. The sequence in black represents the 3’-UTRs and the red represents the amiRs. B, Precursor-amiR (pre-amiR) double-strand oligos: The pre-amiR against XRCC2 or XRCC4 was designed by the BLOCK-iTTM RNAi Designer tool and purchased from Invitrogen. The sequence was cloned into a pcDNA™6.2-GW/EmGFP-miR vector as described in Materials and Methods. C, The effects of the amiR-binding site in the 3’-UTR of XRCC2 or XRCC4 on the luciferase activity: 293T cells were transfected with a firefly luciferase reporter plasmid containing partial 3’-UTR of XRCC2 or XRCC4 with the putative amiR binding site (WT) or without the binding site (DM). Luciferase activity was assayed 48 h after transfection with the amiR mimic (amiR-m) or without the mimic (with a control RNA).

Figure 3. The amiR against XRCC2 or XRCC4 decreased the targeted gene expression level. A, Detect the amiR infection efficiency in U87GM and A549 cells. The images reflect GFP signals, which represent the infection efficiencies of the lentivirus vectors. B, The effects of amiR on XRCC2 or XRCC4 expression in U87GM and A549 cells. β-actin was used as an internal loading control.

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 indicate standard deviations. C, The effects of the amiR on A549 and U87MG cell radio-sensitivity. The clonogenic assay was performed as described in Materials and Methods. Data shown are the mean and SE from three independent experiments.

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 blot 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 h 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 three independent experiments.

Figure 6. The effects of combining the small RNA (amiR mimic and siRNA) on knocking down the targeted gene and on radio-sensitizing the human tumor cells. A, The targeted gene expression levels in the cells at 72 h 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 radio-sensitivity.
At 72 h after the small RNA (including amiR mimic 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 three 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.
**Fig. 1**

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RNAi-mediated targeting of non-coding and coding sequences in DNA repair gene messages efficiently radiosensitizes human tumor cells

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