

miR-9 and let-7g enhance the sensitivity to ionizing radiation by suppression of NF κ B1

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Abbreviations: IR, ionizing radiation; miRNA, microRNA

Abstract

The activation of nuclear factor-kappa B1 (NF κ B1) in cancer cells may confer resistance to ionizing radiation (IR). To enhance the therapeutic efficiency of IR in lung cancer, we screened for microRNAs (miRNAs) that suppress NF κ B1 and observed their effects on radiosensitivity in a human lung cancer cell line. From time series data of miRNA expression in γ -irradiated H1299 human lung cancer cells, we found that the expression of miR-9 was inversely correlated with that of NF κ B1. Overexpression of miR-9 down-regulated the level of NF κ B1 in H1299 cells, and the surviving fraction of γ -irradiated cells was decreased. Interestingly, let-7g also suppressed the expression of NF κ B1, although there was no canonical target site for let-7g in the NF κ B1 3' untranslated region. From these results, we conclude that the expression of miR-9 and let-7g could enhance the efficiency of radiotherapy for lung cancer treatment through the inhibition of NF κ B1.

Keywords: ionizing radiation; let-7g; lung cancer; miR-9; NF κ B1

Introduction

When living cells are exposed to ionizing radiation (IR), a series of alterations occurs including transformation, cell cycle distress, mutations, sister-chromatid exchanges, chromosome aberrations, DNA repair, and apoptosis (Preston, 2005; Amundson, 2008). The final outcomes of IR-exposed cells are determined by the cellular gene expression pattern (Amundson *et al.*, 2003). Among the IR-responsive genes, the activation of nuclear factor-kappa B1 (NF κ B1) following genotoxic stress allows DNA damage repair and cell survival (Janssens *et al.*, 2005). The activation of NF κ B1 in cancer cells may induce radioresistance, which frequently prevents successful treatment (Aggarwal *et al.*, 2009).

Inhibition of NF κ B1 increases sensitivity of cancer cells to the apoptotic action of chemotherapeutic agents and radiation exposure (Li and Sethi, 2010). Various types of inhibitors targeting NF κ B1 have been actively investigated as potential adjuvant therapeutics for lung cancer together with radiotherapy (Kunnumakkara *et al.*, 2008). Radiosensitization induced by anti-inflammatory cytokines such as interleukin IL-4 and IL-10 in colorectal cancer was associated with NF κ B1 inhibition (Voboril and Weberova-Voborilova, 2007). An endogenous inhibitor of gene expression, microRNA (miRNA), plays a significant role at the post-transcriptional gene regulation based on the 3' untranslated region (UTR) sequences. The alteration of miRNA expression upon IR may affect the gene regulation in the cellular response to radiation exposure (Chaudhry *et al.*, 2010). It is important to find miRNAs targeting NF κ B1 as a potential therapeutic approach to overcome radioresistance in cancer treatment.

Many studies have analyzed the transcriptional regulation of mRNAs and miRNAs in γ -irradiated cells to understand cellular responses to IR (Park *et al.*, 2002; Weidhaas *et al.*, 2007; Jeong *et al.*, 2009). In this study, we screened the expression profiles of miRNA in γ -irradiated H1299 human lung cancer cell line to find miRNA targeting NF κ B1. We found that miR-9 and let-7g could increase the sensitivity of H1299 cells to IR *in vitro*. Thus, we propose that the suppression of NF κ B1 by miR-9 and let-7g may provide opportunities for both prevention and treatment of cancer.

Table 1. List of microRNA profiles targeting NF κ B1 in γ -irradiated H1299 cells

Genes	Expression of genes after γ -irradiation Log2 [Signal intensity]						Pearson's correlation coefficient
	0 h	2 h	4 h	8 h	12 h	24 h	
NF κ B1	8.95	9.01	9.31	9.43	9.36	9.00	
miR-424	6.32	6.00	6.29	4.23	4.98	5.44	-0.65
miR-9	5.80	5.95	5.90	4.29	4.46	4.40	-0.46
miR-195	7.84	7.38	8.32	6.87	7.09	7.20	-0.24
miR-183	4.05	4.11	3.41	5.57	5.40	5.35	0.33

Results

To identify miRNAs that suppress NF κ B1 expression, we profiled miRNA in H1299 lung cancer cells at 0, 2, 4, 8, 12, and 24 hours after 2 Gy-irradiation. We filtered the list of miRNAs in a time series profile based on the target prediction using TargetScan (Lewis *et al.*, 2005) and the correlation coefficient between NF κ B1 and miRNAs. Among 328 human miRNAs in the microarray, we found that expression of miR-9, miR-424, and miR-195 was inversely correlated with expression of NF κ B1 in γ -irradiated H1299 cells (Table 1). The expression patterns of these selected miRNAs are shown in a heatmap (Figure 1A). Among these selected miRNAs, we confirmed the expression of miR-9 and NF κ B1 in γ -irradiated H1299 cells using real-time RT-PCR (Figures 1B and 1C). We used let-7g and miR-26b as controls, because these two miRNAs do not have binding sites in NF κ B1 3'UTR. The expression of let-7g and miR-26b also decreased upon IR in H1299 cells, as did miR-9.

The miRNA miR-9 was previously found to be a candidate miRNA targeting NF κ B1, (Bazzoni *et al.*, 2009), but its effect on radiosensitivity was not examined. There is one defined miR-9 target site at position 29-35 of NF κ B1 3' UTR, as shown in Figure 2A. We introduced a miR-9-expression vector into H1299 cells and then measured the expression of miR-9 using real-time RT-PCR. We used let-7g and miR-26b as controls to check the specific effect of miR-9 (Figure 2B). miR-9 inhibited NF κ B1 expression in real-time RT-PCR as well as in western blot analysis (Figures 2C and 2D), indicating that a strong correlation between miR-9 and NF κ B1 exists. To confirm that 3' UTR of NF κ B1 has the binding site for miR-9 we cloned 3'UTR of the candidate gene downstream of the renilla luciferase reporter gene. MiR-9 target the NF κ B1 gene was confirmed by the decrement in the Renilla activity (Figure 2E). Interestingly, let-7g inhibited the expression of NF κ B1 as much as miR-9, while miR-26b did not.

To study whether miR-9 could sensitize H1299 cells to IR, we first overexpressed miR-9 and

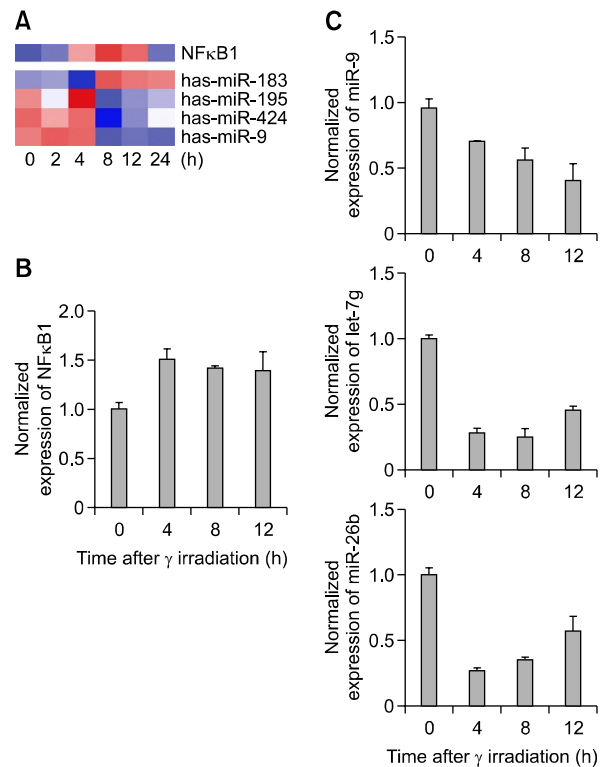


Figure 1. Expression patterns of NF κ B1 and microRNAs in γ -irradiated H1299 cells. (A) Heatmap analysis shows the expression of NF κ B1 and microRNAs targeting NF κ B1 in H1299 cells upon ionizing radiation at 0, 4, 8, and 12 h. (B) The expression of NF B1 mRNA was quantitated with real-time RT-PCR at the indicated time. The values were normalized with GAPDH mRNA. (C) The expressions of miR-9, let-7g, and miR-26b were measured by real-time RT-PCR in γ -irradiated H1299 cells at the indicated time. U6B mRNA was used as a normalization control for microRNAs. All values are presented as mean \pm SD from triplicate experiments.

measured cell survival upon γ -irradiation at 0, 2, 4, and 6 Gy using the MTT assay (Figure 3A). We used let-7g and miR-26b as controls, since there are no binding sites for these miRNAs in NF κ B1 3'UTR sequences. miR-9 suppressed the expression of NF κ B1 at both the mRNA and protein level (Figure 2D), and significantly sensitized H1299 cells to IR in a survival analysis. Interestingly, let-7g

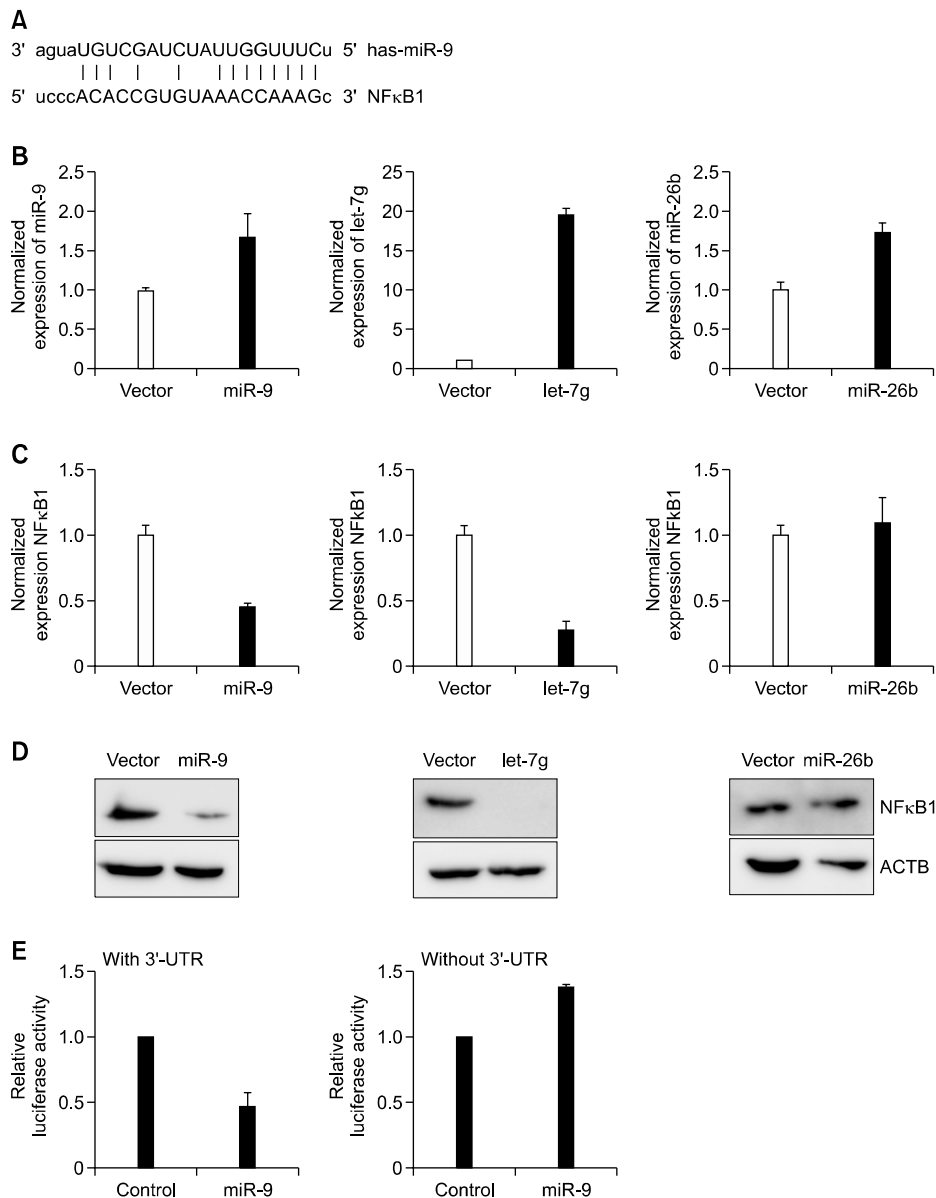


Figure 2. Suppression of NFκB1 by miR-9 and let-7g. (A) Target sequence of miR-9 in NF B1 3'UTR was predicted by TargetScan. (B) In miR-9, let-7g, and miR-26b transfected H1299 cells, the expression of each microRNA was confirmed by real-time RT-PCR. (C) The expression of NFκB1 mRNA in microRNA-transfected cells was measured by real-time RT-PCR. The relative NFκB1 expression levels were normalized against GAPDH and presented as mean ± SD from triplicate experiments. (D) The protein levels of NFκB1 were also examined by western blot in microRNA-transfected cells. (E) Cells were transfected with the empty renilla luciferase reporter gene (psiCHECK2) or the reporter gene fused to the NFκB1 3' UTR. In addition, the cells were co-transfected with miR-9 or without miR-9. Results are expressed as relative light units (RLU) and were normalized with the luciferase activity expressed constitutively by the psiCHECK2 vector.

also suppressed the expression of NFκB1 as much as miR-9 did, and also enhanced the sensitivity of H1299 cells to γ-irradiation. The expression of miR-26b did not alter the expression of NFκB1 or cell survival. In order to testify the microRNA mediated sensitization in other cell lines we selected A549 cells and performed the MTT assay by first over-

expressing miR-9 and then measuring the cell survival upon γ-irradiation at 0, 2, 4, and 6 Gy respectively. It was found that has-miR-9 sensitized A549 cells thereby further supporting our hypothesis (Figure 3C).

We also measured the expression patterns of NFκB1 in miR-9 and let-7g transfected cells upon

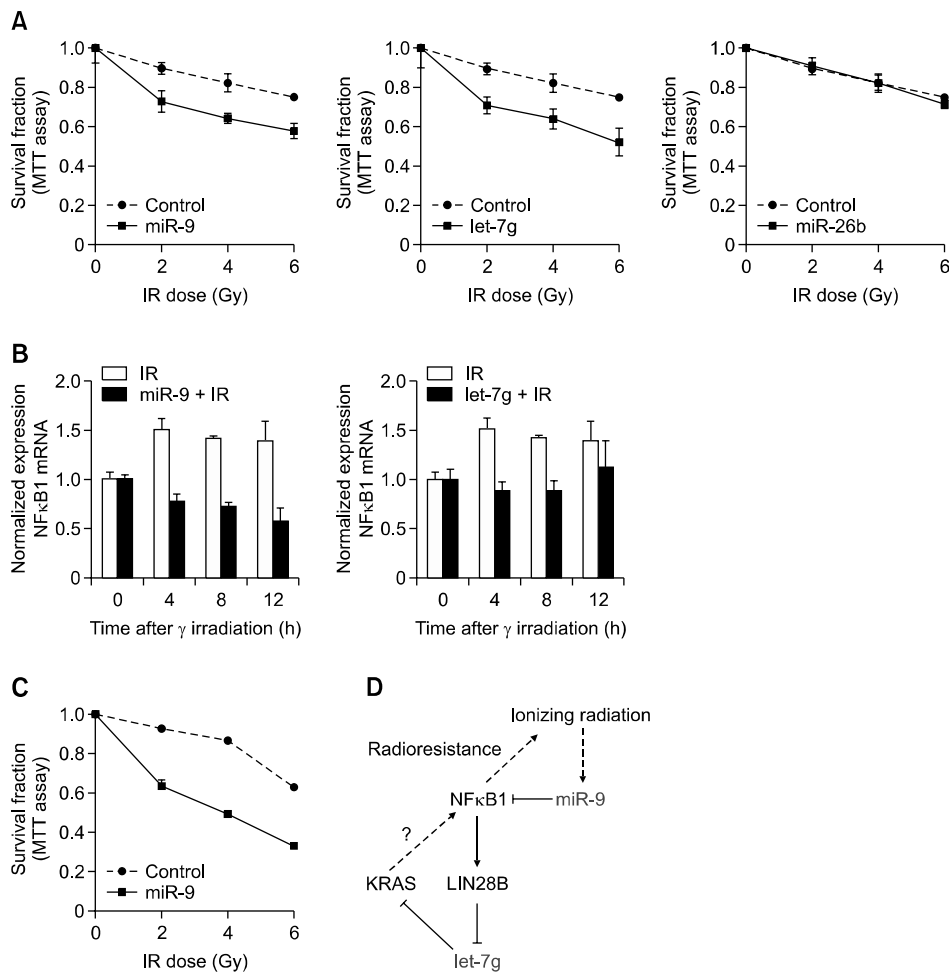


Figure 3. miR-9 and let-7g enhance the sensitivity to ionizing radiation in H1299 cells. (A) miR-9, let7g, and miR-26b were overexpressed and the cell survival fraction upon γ -irradiation was measured using MTT assay in H1299 cells. (B) The expression of NF κ B1 was measured in microRNA-transfected cells after γ -irradiation. (C) miR-9 was overexpressed and the cell survival fraction upon γ -irradiation was measured using MTT assay in A549 cells. (D) miR-9 can directly control the expression of NF κ B1, and the indirect regulation of NF κ B1 by let-7g may be proposed to control radiosensitivity.

IR (Figure 3B). The increase in NF κ B1 expression is inhibited in miR-9 and let-7g transfected cells, suggesting that the expression of NF κ B1 in γ -irradiated cells was regulated by the down-regulation of miR-9 and let-7g. These results demonstrate that miR-9 and let-7g suppress the expression of NF κ B1 and enhance the sensitivity to IR in H1299 lung cancer cells.

Discussion

In γ -irradiated H1299 human lung cancer cells, expression of miR-9 was down regulated while the expression of NF κ B1 was up regulated. Both miR-9 and let-7g suppressed the expression of NF κ B1, which resulted in increased sensitivity to

IR in the H1299 human lung cancer cell line. Given these results, we suggest that miR-9 and let-7g may play an important role in the response to ionizing radiation through the inhibition of NF κ B1. IR can induce DNA damage by inducing progressive changes in cell survival, growth, and proliferation by affecting gene expression (Amundson *et al.*, 2003). Previous reports suggest that radiation can change the expression pattern of some genes (Park *et al.*, 2002; Smirnov *et al.*, 2009). NF κ B1 has been previously suggested to alter the sensitivity to radiation and chemotherapeutic agents (Janssens and Tschopp, 2006; Perkins and Gilmore, 2006). Here we show that NF κ B1 is targeted by miR-9 and let-7g, which results in the increased sensitivity to IR. The let-7 family of miRNAs regulates expression of oncogenes, such as RAS, and is

specifically down regulated in many cancer subtypes. Low levels of let-7 predict a poor outcome in lung cancer. The let-7 family of miRNAs is over-represented in a class of miRNAs exhibiting altered expression in response to radiation. More strikingly, we can also create a radiosensitive state when the select let-7 family of miRNAs is overexpressed in lung cancer cells *in vitro*, whereas decreasing their levels causes radioresistance through control of the proto-oncogene homologue let-60/RAS and genes in the DNA damage response pathway (Weidhaas *et al.*, 2007). The let-7 family of miRNAs can suppress resistance to anticancer cytotoxic therapy, including radiotherapy. In the present study, let-7g sensitized the H1299 cells but also suppressed the expression of NF κ B1. Recently, Iliopoulos *et al.* (2009) reported that let-7g can suppress the expression of RAS, and ultimately suppress the expression of NF κ B1 (Iliopoulos *et al.*, 2009). In a previous report, we showed that increased let-7g suppressed KRAS in H1299 cells (Jeong *et al.*, 2009). This study examined miRNAs in radioresistance lung cancer cell lines and showed that miR-9 and let-7g can sensitize the H1299 cells by interacting with NF κ B1. Along with NF κ B1 there are other member genes like Lin28B, KRAS etc. (not included in the present study) which might be playing a crucial role in the overall regulation by interacting with each other and with microRNAs (Figure 3D). It is likely that miRNAs act in a similar dominant manner in radiation-induced DNA damage responses in other cancer types as well.

Methods

Cell culture and γ -irradiation

H1299 human lung cancer cell lines were maintained in RPMI 1640 medium and A549 cell lines were cultured in Dulbecco's Modified Eagle's medium (DMEM, Sigma Aldrich, St Louis, MO) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 2 mM L-glutamine. The cultured cells were either exposed to 0-10 Gy of radiation using a 4-MV linear accelerator (Clinac 4/100, Varian, Palo Alto, CA) or left irradiated as a negative control.

MicroRNA microarray and statistical analysis

miRNAs were extracted using the mirVanamiRNA isolation kit (Ambion, Austin, TX) according to the manufacturer's protocols. Purified miRNAs were labeled using the mirVanamiRNA Array Labeling Kit and coupled to the Cy5 Post-Labeling Reactive Dye (Amersham, GE Healthcare Bio-Sciences, Piscataway, NJ). The labeled samples were washed and hybridized in duplicate to mirVanamiRNABioarrays

(Ambion) using the mirVanamiRNABioarray Essentials Kit. Fluorescence intensities were processed and measured using the GeneChip scanner 3000 7G (Agilent Technologies, Santa Clara, CA). The levels of miRNA hybridization were determined using GenePix Pro 6.0 software as recommended by the manufacturer. The background-adjusted intensity for each miRNA was subjected to a global variance stabilization normalization (VSN) procedure (Huber *et al.*, 2002). The miRNAs targeting NF κ B1 were predicted using TargetScan, and Pearson's correlation coefficient was calculated between the expression levels of miRNA and NF κ B1 transcript (Sung *et al.*, 2010).

Constructs and transfection

The precursor of the hsa-miR-9 was amplified from the H1299 genomic DNA using the primers 5'-CCGGAATTCCTCAACTCCACTCGTGTCC-3' and 5'-ATTGCGGCCGCTGGGACTGTGACTCCTACCTG-3', and the resulting product was cloned into *EcoR1/Not1* restricted pcDNA3 (Invitrogen, Carlsbad, CA). The precursor of the has-miR-26b was amplified from the H1299 genomic DNA using the primers 5'CGGATGGGAATTGGATACAT3' and 5' AGCTACCTGACCACTGCTG 3' cloned into PGEMT easy vector (Promega, Madison, WI). The resulting product was sub-cloned into *EcoR1/Not1* restricted pcDNA3.

The expression vector for hsa-let-7g was provided S-H. Jeong (Jeong *et al.*, 2009), and the construct was transfected using FuGENE HD (Roche Applied Science, Mannheim, Germany) according to the manufacturer's protocols. Total RNA for real-time PCR, protein for western blot, and cells for MTT assay were collected after 48 h of transfection.

RNA preparation and quantitative real-time RT-PCR

Total RNA was extracted from cell lines using the TRIzol method according to the manufacturer's protocol (Kwak *et al.*, 2009). Total RNA was reverse transcribed to complementary DNA using Superscript II reverse transcriptase (Invitrogen) and oligonucleotide primers. Quantitative RT-PCR for genes was performed in a reaction mixture containing complementary DNA, SYBR Premix Ex Taq (Takara Bio Inc., Shiga, Japan), and primers for each gene.

Quantitation of miRNAs was carried out using TaqMan microRNA assays (Applied Biosystems, Foster City, CA). The PCR amplification was conducted in reaction mixture using the TaqMan Universal PCR Master Mixture according to the protocol supplied by the manufacturer. Samples were analyzed with the ABI PRISM 7000 sequence detection system (Applied BioSystems). All PCRs were performed in triplicate, and the specificity of the reaction was determined by melting curve analysis at the dissociation stage. We synthesized specific primers for NF κ B1 (forward 5'-CTGCTGACAATTTCCACAC-3'; reverse 5'-GCTCTCTGAGC ACCTTTGGA-3'). The relative quantitative method was used for the quantitative analysis. The calibrator was the averaged Δ Ct from the untreated cells and the endogenous controls were GAPDH for genes and U6B for miRNAs.

Western blotting

Cells were harvested, and lysed, and proteins were separated on a SDS/polyacrylamide gel and transferred to a PVDF membrane (Bio-Rad, Hercules, CA). After blocking, the membranes were incubated with the primary antibodies anti-ACTB and anti-NF κ B1 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were extensively washed and incubated with a horseradish peroxidase-conjugated secondary antibody (Bio-Rad). The antigen-antibody complexes were visualized by West-Q-Chemiluminescent Sub Kit Plus (BIOTANG, Waltham, MA).

Luciferase assay

The 3' UTRs of NF κ B1 were fused to the renilla gene using the *XhoI/NotI* restriction sites of the psiCHECK2vector (Promega). A total of 8×10^4 H1299 cells were co-transfected with 30 ng of the indicated vector and 90ng of the pcDNA3 cloned miR-9 using Fugene(HD) Roche for 48 h. Luciferase assays were performed using the Dual-Luciferase assay (Promega). Normalization of the Renilla expression was performed using the luciferase gene present on the psiCHECK2 vector.

MTT assay

H1299 cells were transfected with miR-9, miR26b, and let7g expression vector and A549 cells were transfected with miR-9 expression vector. Forty-eight hours after transfection, cells were seeded at 5,000/well into 96-well plates and exposed to various doses of IR at 0, 2, 4, and 6 Gy. After γ -irradiation, the cells were incubated for 5 days, MTT (3-(4, 5-dimethylthiazole)-2, 5-diphenyltetrazoliumbromide) assay reagents were added, and the absorbance was measured at 560 nm. All experiments were repeated at least three times, and data are presented as mean \pm standard deviation (SD). The Student's *t*-test was used to compare the means of the different groups. A *P*-value of < 0.05 was considered statistically significant.

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