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LIN28B confers radio-resistance through the posttranscriptional control of KRAS

Sun-Hye Jeong[1], Hong-Gyun Wu[2,4] and Woong-Yang Park[1,3,5]

[1]Departments of Biomedical Sciences,
Biochemistry and Molecular Biology
[2]Radiation Oncology
[3]Genomic Medicine Institute, MRC
[4]Cancer Research Institute
Seoul National University College of Medicine
Seoul 110-799, Korea
[5]Corresponding author: Tel, 82-2-740-8241;
Fax, 82-2-744-4534; E-mail, wypark@snu.ac.kr
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Abbreviations: *KRAS*, v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog; LIN28B, lin-28 homolog B (*C. elegans*); MTS, 3-(4,5-dimethylthiazol-2-yl))-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl) -2H-tetrazolium; qRT-PCR, quantitative real-time reverse transcriptase polymerase chain reaction

Abstract

To screen the differentially expressed microRNAs related to radio-resistance, we compared the microRNA profiles of lung cancer cells with different responses to ionizing radiation (IR). Of 328 microRNAs in microarray, 27 microRNAs were differentially expressed in NCI-H460 (H460) and NCI-H1299 (H1299) cells. Among them, *let-7g* was down-regulated in radio-resistant H1299 cells, and the level of *let-7g* was higher in radio-sensitive cells like Caski, H460, and ME180 in qRT-PCR analysis than in radio-resistant cells like A549, H1299, DLD1, and HeLa. Over-expression of *let-7g* in H1299 cells could suppress the translation of KRAS, and increase the sensitivity to IR. When we knockdown the expression of LIN28B, an upstream regulator of *let-7g*, the level of mature *let-7g* was increased in H1299 cells and the sensitivity to IR was also enhanced in LIN28B knockdown cells. From these data, we suggest that LIN28B plays an important role in radiation responses of lung cancer cells through inhibiting *let-7g* processing and increasing translation of KRAS.

Keywords: gene expression profiling; KRAS protein, human; LIN28B protein, human; lung neoplasms; microRNAs; mirnlet7 microRNA, human; radiation, ionizing

Introduction

Together with surgery and chemotherapy, ionizing radiation (IR) plays a central role in the treatment of human cancers. Approximately half of all cancer patients can be treated with radiotherapy, but the therapeutic strategy must be carefully adjusted to achieve an effective therapeutic dose while minimizing side effects (Wu *et al*., 2002). Nonetheless, a sizable proportion of patients do not respond to radiotherapy. There have been a lot of efforts to improve the delivery of high doses to tumors while saving normal tissues during last decades. Such efforts are mainly focused on the physical aspects such as planning and delivery, rather than the biological parameters determining the radio-sensitivity of each patient.

Radio-resistance has been shown to be related to the expression of several genes such as *TP53* (Biard *et al*., 1994), *BCL2* (Lee *et al*., 1999), and *BIRC5* (Asanuma *et al*., 2000). Over-expression of RAS (Sklar, 1988) confers radio-resistance, while ATM increases sensitivity to ionizing radiation (Westphal *et al*., 1997). Although such findings have helped establish models for the molecular mechanisms responsible for radio-sensitivity, the whole process is still poorly understood. Microarray technology permits simultaneous analysis of the expression levels of multiple genes at the whole genome level (Park *et al*., 2002). Genome-wide analysis can lead to the identification of gene regulatory pathways that result in the development of resistance to therapeutic procedures. Differential gene expression profiles of radio-resistant breast cancer, esophageal cancer, and uterine cervical cancer cell lines have been examined using microarray analysis (Hanna and Shrieve, 2001; Kitahara *et al*., 2002).

Translational suppression by microRNA is one of the major regulatory mechanisms in carcinogenesis, development, and immune system function (He *et al*., 2007; Flynt and Lai, 2008). The microRNA *let-7* has been reported to regulate the radiation response in human cancers (He *et al*., 2007; Weidhaas *et al*., 2007). In this study, we

compared the microRNA expression profiles of two lung cancer cells with different responses to IR and listed 27 microRNA candidates related to the differential responses to IR. *let-7g* was down-regulated in radio-resistant H1299 cells and could not suppress the translation of KRAS. When we

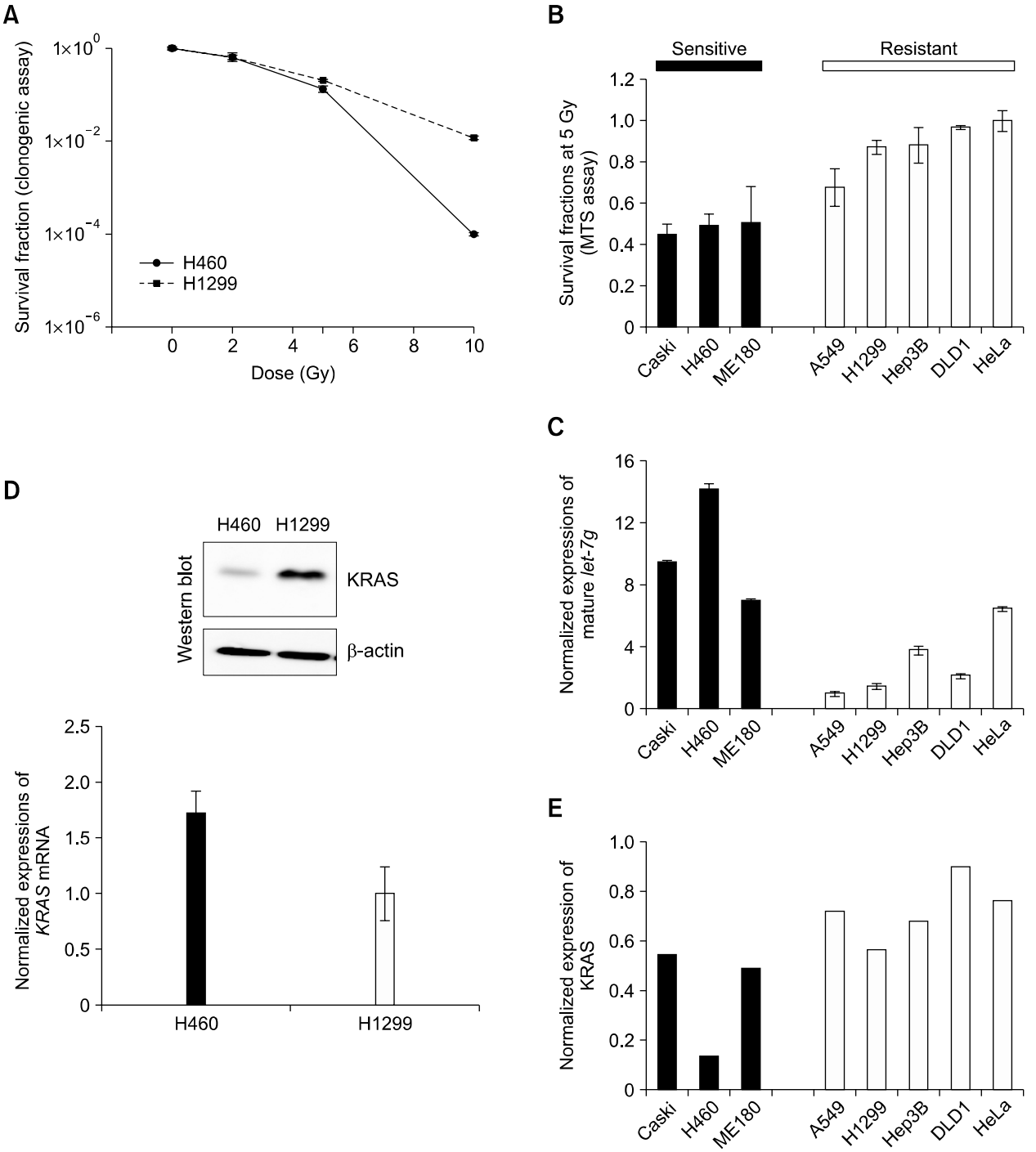

Figure 1. Differential expression of *let-7g* and post-transcriptional regulation of KRAS. Following γ-irradiation at 0, 2, 5, and 10 Gy, the numbers of foci were scored to measure survival faction in H460 and H1299 lung cancer cells (A). Sensitivity of eight cancer cell lines (Caski, H460, ME180, A549, H1299, Hep3B, DLD1, and HeLa) to ionizing radiation at 5 Gy was determined by MTS assay (B). Determination of the level of mature *let-7g* by qRT-PCR (C). Expression of *KRAS* mRNA and protein in H460 and H1299 cells was measured qRT-PCR and western blot analysis (D). KRAS protein levels in eight cancer cells were also quantified from the western blot and densitometric measurement (E).

knockdown *LIN28B* by siRNA, the maturation of *let-7g* was restored and the radio-sensitivity of H1299 cells was increased.

Results

Differential expression of microRNAs in lung cancer cells

To understand the post-transcriptional regulation related to radio-resistance, we chose H460 and H1299 human non-small cell lung cancer cells. H460 cells showed more sensitive response to IR than H1299 (Figure 1A). Using these two cells, we examined the profiles of microRNA expression and selected differentially expressed microRNAs. Using microRNA microarray, we could compare the expression of 328 human microRNAs among 622 probes in the microarray. From eight-times replicated data from two independent experiments, we selected 27 differentially expressed microRNAs by statistical analysis (log2 (signal intensity) $>$ 2, log2 (activation fold ratio) $>$ 2 or $<$ -2 and *t*-test *P* $<$ 0.001), as listed in Table 1.

Nine microRNAs were down-regulated in radio-resistant H1299 cell, and we selected *let-7g* to check its expression in eight cancer cells with different radio-sensitivity. We could classify them into two groups according to their survival fractions after irradiating 5 Gy (Figure 1B). Caski, H460, and ME180 cells were more sensitive to IR than A549, H1299, Hep3B, DLD1, and HeLa cells. Using qRT-PCR against mature *let-7g*, we checked the level of mature *let-7g* to find the up-regulation of *let-7g* in radio-sensitive cells (Figure 1C).

Post-transcriptional control of KRAS by *let-7g*

Oncogenic activation of KRAS is closely related to clinical course of lung cancer including the responsiveness to radiotherapy. As a target of *let-7g*, the protein level of KRAS was examined in H460 and

Table 1. List of differentially expressed microRNAs in radio-resistant H1299 cells.

microRNA	Log_2[Signal Intensity][1] (mean ± SD)		Log_2 [Activation Fold Ratio][2]	*P* value[3] (t-test)
	NCI-H460	NCI-H1299		
Down-regulated in NCI-H1299 cells				
let_7g	7.429 ± 0.334	10.686 ± 0.140	-3.257	1.91E-08
let_7i	5.851 ± 0.081	12.725 ± 0.292	-6.874	2.65E-11
miR_138	2.450 ± 0.571	6.813 ± 1.527	-4.363	0.00017
miR_152	2.688 ± 0.630	4.939 ± 1.441	-2.251	0.00158
miR_193b	3.037 ± 0.672	6.364 ± 0.482	-3.327	4.32E-07
miR_34a	3.100 ± 0.733	8.422 ± 0.229	-5.321	4.27E-08
miR_363_AS	6.840 ± 0.763	9.613 ± 0.467	-2.773	0.00006
miR_367	2.043 ± 0.696	4.854 ± 1.431	-2.811	0.00110
miR_491	8.377 ± 0.052	10.763 ± 0.763	-2.387	0.00002
Up-regulated in NCI-H1299 cells				
miR_100	12.105 ± 0.439	8.594 ± 0.339	3.511	1.20E-06
miR_10a	10.069 ± 0.142	7.407 ± 0.350	2.662	1.13E-08
miR_125b	10.030 ± 0.156	7.269 ± 0.836	2.760	0.00002
miR_136	5.480 ± 0.093	3.303 ± 0.976	2.177	0.00024
miR_181c	5.578 ± 0.158	3.274 ± 1.399	2.304	0.00161
miR_195	7.844 ± 0.264	3.680 ± 1.258	4.164	0.00001
miR_19a	7.192 ± 0.117	4.698 ± 1.596	2.493	0.00165
miR_28	7.720 ± 0.181	5.189 ± 1.918	2.530	0.00318
miR_301	7.158 ± 0.197	3.537 ± 1.165	3.621	0.00005
miR_30a_3p	5.608 ± 0.159	2.440 ± 0.869	3.167	0.00001
miR_30e_3p	6.051 ± 0.089	2.757 ± 1.009	3.294	0.00002
miR_331	5.250 ± 0.150	3.116 ± 0.920	2.135	0.00008
miR_362	5.804 ± 0.217	3.531 ± 1.885	2.273	0.00530
miR_365	5.076 ± 0.141	2.846 ± 1.282	2.231	0.00085
miR_374	6.208 ± 0.222	2.412 ± 1.260	3.796	0.00005
miR_501	5.053 ± 0.332	2.199 ± 1.808	2.853	0.00062
miR_9_AS	5.595 ± 0.170	3.355 ± 0.828	2.240	0.00006
miR_98	6.526 ± 0.096	3.831 ± 1.274	2.695	0.00038

H1299 cells to find the down-regulation of KRAS in H460 cells (Figure 1D). In addition, we also examined KRAS expression in eight cancer cells to find the reverse correlation with the level of *let-7g* (Figure 1E). These data suggest that KRAS expression is regulated at the post-transcriptional level in H460 cells.

Control of radio-sensitivity by *let-7g*

To further investigate the role of *let-7g* in radio-resistance, we over-expressed *let-7g* in radio-resistant H1299 cells to determine whether radio-sensitivity was affected by suppression of KRAS translation. Ectopically expressed *let-7g* could down-regulate KRAS expression in H1299 cells at both the protein and mRNA level (Figures 2A and 2B). In addition, the *let-7g* could enhance the radio-sensitivity (Figure 2C).

LIN28B regulates *let-7g* and KRAS expression

Because LIN28B can selectively block the processing of primary let-7 microRNA, we investigated the level of LIN28B in H460 and H1299 lung cancer cells (Figure 3A). To test whether the suppression of *let-7g* maturation by LIN28B in H1299 cells, we introduced siRNA against LIN28B into H1299 cells (Figure 3B). Knockdown of LIN28B by siRNA induced the decreased level of pre-*let-7g* (Figure 3B) and increased the level of mature *let-7g* (Figure 3C). KRAS protein levels were also decreased in siLIN28B-transfected cells as well (Figure 3B). Moreover, knockdown of LIN28B led H1299 cells to increase their sensitivity to IR (Figure 3D).

Discussion

To understand the post-transcriptional control of radio-sensitivity by microRNA, we analyzed microRNA profiles from two lung cancer cells with different responses to IR. Among 27 differentially expressed microRNAs, we focused on *let-7g* because it was known to suppress the translation of KRAS previously (Johnson *et al*., 2005). To evaluate the role of *let-7g* in radio-sensitivity, we examined the level of mature *let-7g* in eight cancer cells. An increased level of *let-7g* was confirmed in

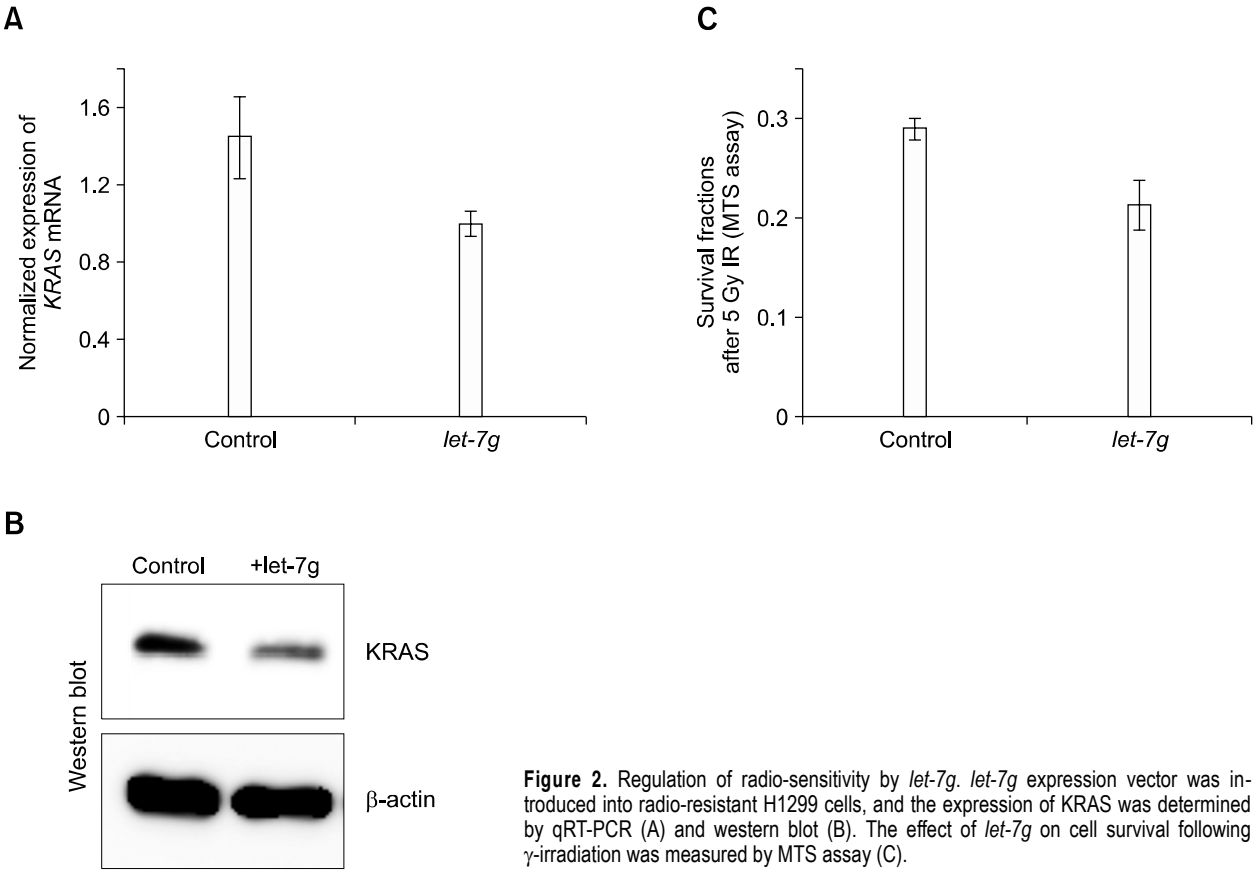

Figure 2. Regulation of radio-sensitivity by *let-7g*. *let-7g* expression vector was introduced into radio-resistant H1299 cells, and the expression of KRAS was determined by qRT-PCR (A) and western blot (B). The effect of *let-7g* on cell survival following γ-irradiation was measured by MTS assay (C).

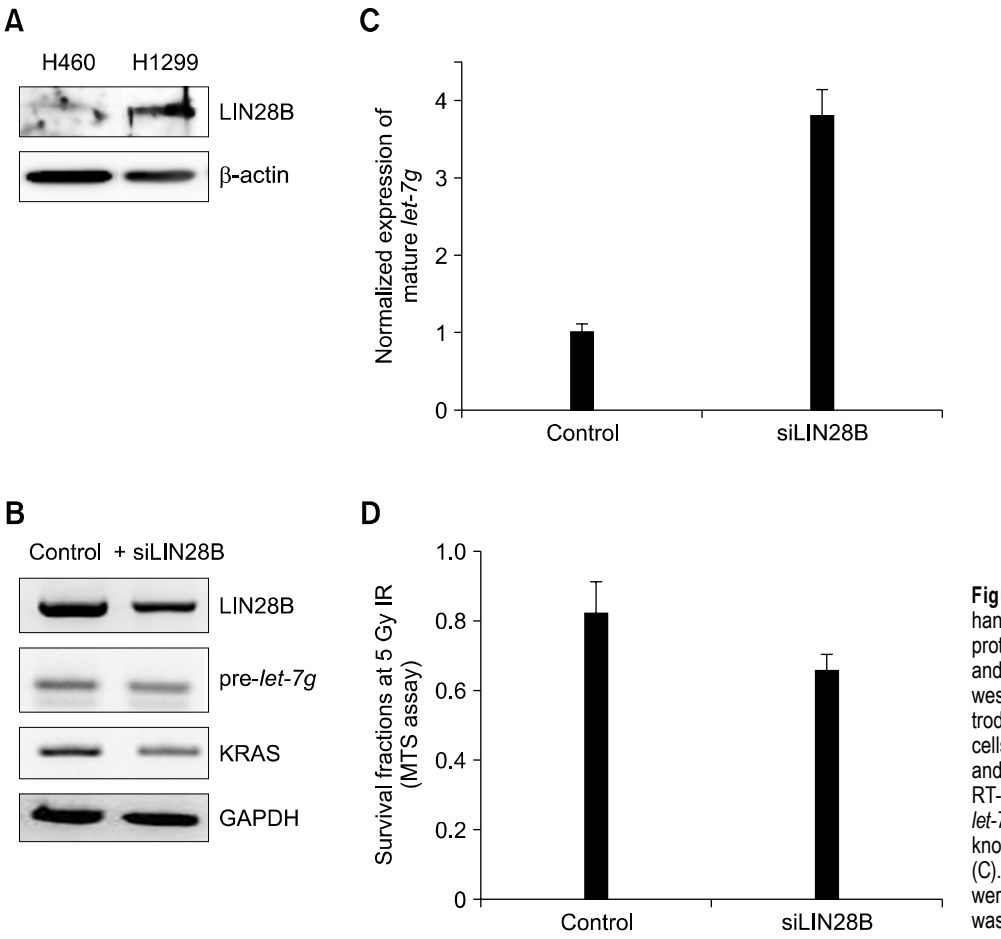

Figure 3. Knockdown of LIN28B enhances the radio-sensitivity. The protein levels of LIN28B in H460 and H1299 cells were measured by western blot (A). After the introduction of siLIN28B into H1299 cells, the level of LIN28B, pre-*let-7g* and KRAS were measured by RT-PCR (B). The level of mature *let-7g* was also measured in LIN28B-knockdown H1299 cells by qRT-PCR (C). LIN28B-knockdown H1299 cells were irradiated at 5 Gy and survival was determined by MTS assay (D).

other radio-sensitive cells like ME180 and Caski. Although we need to validate the correlation of *let-7g* and outcome of radiotherapy in patients, we can suggest that the level of *let-7g* was correlated with radio-sensitivity.

Direct control of microRNA processing by LIN28B or LIN28 may determine the level of mature *let-7g* in cancer cells (Viswanathan *et al*., 2008; Chang *et al*., 2009). LIN28 and LIN28B RNA binding proteins were recently demonstrated to negatively regulate *let-7* biogenesis. We tested hypothesis on the regulation of radio-sensitivity by LIN28B by knockdown experiment.

We also reconstituted the effect of *let-7g* by the over-expression of *let-7g* in a radio-resistant H1299 cells to enhance the radio-sensitivity. Over-expression of *let-7* can suppress many target mRNAs including *KRAS* and *HMGA2* (Johnson *et al*., 2005; Pillai *et al*., 2005; Dahiya *et al*., 2008). We selected *KRAS* as a candidate gene downstream of *let-7g* that is involved in radiation response. Oncogenic activation of KRAS is a candidate marker for determination of prognosis, and there are many reports suggesting that mutations in KRAS might play an important role in patient outcome (Guerrero *et al*., 1984; Bengala *et al*., 2009). The findings of this study suggest that post-transcriptional activation of KRAS may also regulate the response to radiotherapy.

Methods

Cell culture

H460, H1299, ME180, and Hep3B cells were maintained in Roswell Park Memorial Institute medium 1640 (RPMI 1640) enriched with 10% FBS (Hyclone, Logan, UT), 1% penicillin/streptomycin (Gibco-BRL, Carlsbad, CA), and 2 mM L-glutamine (Gibco-BRL, Carlsbad, CA). HeLa, A549, DLD1 and Caski cells were maintained in DMEM enriched with 10% FBS, 1% penicillin/streptomycin, and 2 mM L-glutamine.

Clonogenic assay and MTS assay

Cells were seeded into 6-well plates at a density of 200-500 cells/well and incubated overnight. After exposure to ionizing radiation from an accelerator (Clinac 4/100, Varian, Palo Alto, CA), cells were cultured for 10-15 days.

Colonies were fixed with 100% chilled methanol and stained with crystal violet (Sigma, St. Louis, MO).

The MTS assay was performed using the CellTiter Aqueous OneSolution kit (Promega, Madison, WI). Cells were seeded into 96-well plates at a density of 5,000 cells/well and incubated overnight. After exposure to ionizing radiation, cells were incubated for 4-5 days, MTS (3-(4,5-dimethylthiazol-2-yl))-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) was added to each well and incubated for 1 h, and absorbance was measured at 490 nm.

MicroRNA microarray and data analysis

Small RNAs from H460 and H1299 cells were isolated using mirVana microRNA isolation kit (Applied Biosystems/Ambion, Austin, TX) as described by the manufacturer. The purity and integrity of total RNA were checked using Bioanalyzer (Agilent, Santa Clara, CA). Synthesis of probes from total RNA samples, hybridization, detection, and scanning were performed according to standard protocols from Ambion (Applied Biosystems/Ambion, Austin, TX). We used samples from duplicated experiments and a mirVana microRNA microarray containing 2,000 spots with quaduplicates for 600 microRNAs from human, mouse, and rat. For data analysis, fluorescence intensity was processed and measured using a Exon scanner or GeneChip scanner 3,000. The raw intensity values were corrected and normalized with GeneSpring program (Agilent, Santa Clara, CA)(Park *et al*., 2002).

Western blot analysis

Whole cell extracts were prepared using RIPA lysis buffer containing protease inhibitor cocktail (Roche, Basel, Switzerland). Protein extracts were separated in a 12% SDS-PAGE gel and transferred to a nitrocellulose membrane. Membranes were blocked with 5% BSA and incubated with mouse anti-KRAS (Oncogene Research Products, La Jolla, CA), rabbit anti-LIN28B (Abcam, Cambridge, MA), or mouse anti-beta-actin (Sigma, St. Louis, MO) antibody. Membranes were then incubated with HRP-conjugated anti-mouse IgG (Santa Cruz Biotechniques, Santa Cruz, CA) and anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) and visualized with SuperSignal West Pico Cheminoluminescent Substrate (Pierce, Rockford, IL).

qRT-PCR

The relative Ct method with SYBR green was performed with the 7,000 Real-Time PCR System (Applied Biosystems, Austin, TX). The following primers were used: KRAS, 5'-CGTAGGCAAGATGCCTTGA-3' and 5'-CCTCTTGACCTGCTGTGTCG-3'; LIN28B, 5'-TGCACTTCAACTCTCCCTCG-3' and 5'-GAACTGAAGGCCCCTTTTTG-3'. Levels of mature microRNA species were measured by quantitative PCR using commercially available TaqMan probes (Applied Biosystems, Austin, TX) according to the manufacturer's instructions using U6B small RNA as an internal standard for normalization.

Cloning

Primary let-7g sequence was amplified from HeLa genomic DNA and cloned into the pCR-8-Topo vector (Invitrogen, Carlsbad, CA) for direct use in *in vitro* transcription. Cloned pri-miRNAs were subcloned into pcDNA-Dest47 for expression study. Full-length KRAS was cloned into the pCEP4 vector (Invitrogen, Carlsbad, CA).

Knockdown with siRNA

siRNA targeting LIN28B (siLIN28B) was purchased from Dharmacon (Lafayette, CO). siLIN28B or non-targeting siRNA was transfected to H460 and H1299 cells at 100nM. Total RNA was collected at 24 h after transfection to quantify LIN28B mRNA by quantitative RT-PCR with GAPDH as a normalization control. The protein levels were measured by western blot analysis using anti-LIN28B antibody (Abcam, Cambridge, MA).

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