MicroRNA miR-374, a potential radiosensitizer for carbon ion beam radiotherapy

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Abstract. In this study, we compared the microRNA (miRNA) profiles of a control and X-ray- and carbon ion beam-resistant cells to identify miRNAs that can be used as radiosensitizers and biomarkers. Mouse squamous cell carcinoma line NR-S1, its X-ray-resistant derivative X60, and its carbon ion beam-resistant derivative C30 were subjected to miRNA microarray analysis. Expression of miRNAs shown to be upregulated or downregulated in the microarray analysis was confirmed by qRT-PCR. Downregulated miRNAs were overexpressed in human pancreatic cancer cell lines PANC1 and MIA PaCa-2, and the resulting cells were tested for radiosensitivity using colony-forming and sphere-forming assays. Of 1,265 miRNAs analyzed, 4 were downregulated and 11 were upregulated in X-ray-resistant and carbon ion beam-resistant cells. Two of the downregulated miRNAs, miR-196 and miR-374, were selected for overexpression in PANC1 and MIA PaCa-2 cells. Overexpression of miR-374 sensitized PANC-1 and MIA PaCa-2 cells toward carbon ion beam radiation. miRNA miR-374 has the potential to be a new radiosensitizer for carbon ion beam radiotherapy and a new biomarker to determine the optimal treatment for cancer.

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Introduction

Charged particle therapy, including carbon ion beam radiotherapy, is regarded as a promising treatment for various types of human cancer (1). Although clinical and pathological studies have shown that the mechanism by which densely ionizing radiation induces apoptosis in cancer cells involves redox regulation (2), a study has revealed that cancer stem cells and minor fractions of other cancer cells are able to survive radiation therapy (3). In contrast, charged particle therapy is generally supposed to exert its antitumor effects directly on cellular DNA, which is vulnerable to double-strand breaks (4). Because DNA double-strand breaks are lethal to cells (5), this may be a promising therapeutic mechanism for efficient eradication of cancer cells.

A growing body of evidence has suggested that small non-coding RNAs, known as microRNAs (miRNAs), are involved in the regulation of tumor initiation and progression (4,6-9). Several miRNAs have been found to be prognostic factors and clinical targets for cancer treatment (9-13). Zhang *et al* (9) demonstrated that miR-205, which is downregulated in radioresistant breast cancer cells, can be used as radiosensitizer in a preclinical model. Wang *et al* (12) found that miR-185 is downregulated in response to radiation and that elevation of miR-185 sensitizes renal cell carcinoma cells to X-ray irradiation. In this study, we focused on the expression of miRNA in carbon ion beam-resistant mouse squamous carcinoma cell lines (C30) to discover new radiosensitizers and prognostic factors. We demonstrated that miR-374, a miRNA suppressed in C30, can act as radiosensitizer when overexpressed in the human pancreatic cancer cell lines PANC1 and MIA PaCa-2. This result suggests that miR-374 is a potential prognostic factor for carbon ion beam radiotherapy and may be a promising new radiosensitizer.

Materials and methods

Cell lines. The NR-S1 (mouse squamous cell carcinoma; controls), X60 (radioresistant to X-rays), and C30 (radioresistant

to carbon ions) cell lines were used in this study. The NR-S1 line was kindly provided by Dr Koichi Ando of the Medicine and Biology Division, Gunma University Heavy Ion Medical Center. The X60 and C30 cell lines were kindly provided by Dr Katshutoshi Sato of the Advanced Radiation Biology Research Program of the Research Center for Charged Particle Therapy, National Institute of Radiological Sciences (Chiba, Japan). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, Tokyo, Japan) supplemented with 10% fetal bovine serum and penicillin/streptomycin and maintained at 37˚C in a 5% CO_2 incubator.

X-ray radioresistant cells. X60 cells were established by irradiating NR-S1 cells with 10 Gy of X-ray radiation once every 2 weeks. The cells were irradiated with a total dose of 60 Gy of X-ray radiation and cultured for 4 weeks after their final irradiation (14).

Carbon ion beam-resistant cells. C30 cells were established by irradiating NR-S1 cells with 5 Gy of carbon ion beam radiation once every 2 weeks. The cells were irradiated with a total dose of 30 Gy of carbon ion beam radiation and cultured for 4 weeks after their final irradiation.

miRNA microarray analysis. Nucleotides isolated from cell lines were analyzed by 3D-Gene microarrays (Toray Industries, Tokyo, Japan).

miRNA extraction and qRT-PCR. Total RNAs were extracted from the cultured cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription for miR-374 was performed with the TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems, Carlsbad, CA, USA). Quantitative reverse transcription PCR (qRT-PCR) was performed by using the TaqMan Universal PCR Master Mix (Applied Biosystems). Internal controls included snoRNA202, snoRNA234, and U6.

miRNA transfection. Transfection of 70-80% confluent cells was performed using Lipofectamine RNAiMAX (Invitrogen) following the manufacturer's instructions. The medium was replaced with new culture medium 24 h after transfection.

Colony formation assay. PANC-1 and MIA PaCa-2 underwent miRNA transfection and irradiated by gamma-ray or carbon ion beam. Gamma-ray irradiation was conducted by gamma cell (Best Theratronics Ltd., Ottawa, ON, Canada). Carbon ion beam irradiation was carried out at the Heavy Ion Medical Accelerator in Chiba (HIMAC) at the National Institute of Radiological Sciences, Japan. Irradiated cells were seeded into 6-cm dishes and incubated for 12 days. Colonies were then stained with crystal violet (0.5% w/v) and counted. The colony consisted of at least 50 cells. Survival curves were drawn by using Microsoft Excel software (Microsoft Corporation, Redmond, WA, USA).

Sphere formation assay. PANC-1 and MIA PaCa-2 cell lines were transfected with a negative control, miR-196 and miR-374. Cells (1,000, 500 and 100 per well) were seeded in a 96-well plate with serum-free medium (DMEM/F12) supplemented with N2 supplement (Invitrogen), epidermal growth factor (EGF; 20 ng/ml), and basic fibroblast growth factor (bFGF; 20 ng/ml) (both from R&D Systems, Inc., Minneapolis, MN, USA). After 14 days of incubation, spheres with diameters >100 μm were counted.

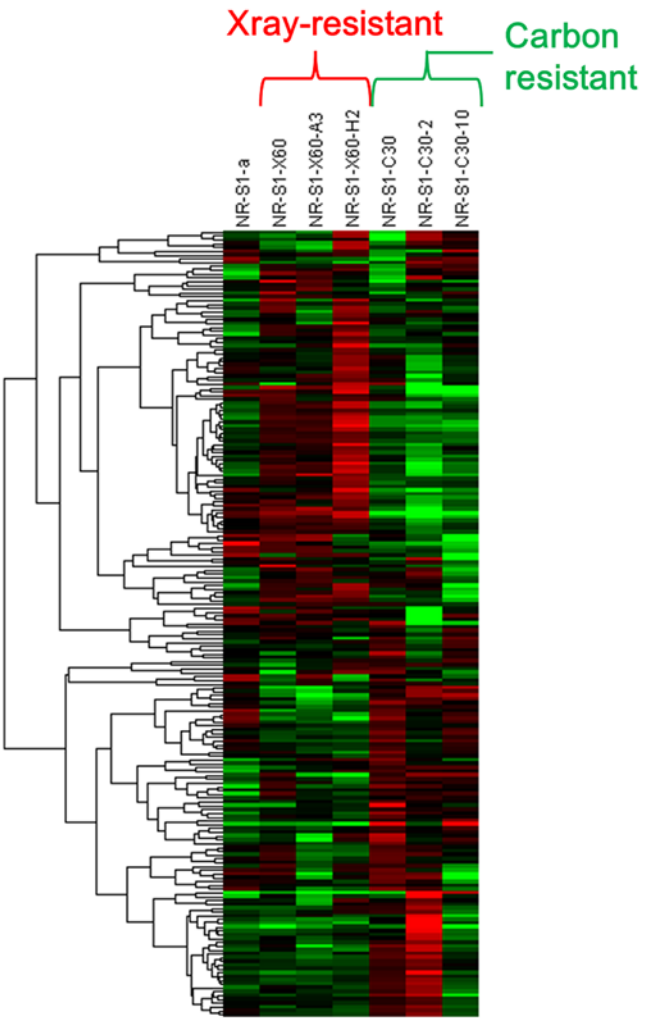

Figure 1. Cluster map from the microRNA microarray analysis. A total of 1,265 microRNAs were analyzed using microarray analysis. NR-S1-a is the control cell line. NR-S1-X60 is an X-ray-resistant cell line. NR-S1-X60-A3 and NR-S1-X60-H2 are single clones derived from NR-S1-X60. NR-S1-C30 is a carbon ion beam-resistant cell line. NR-S1-C30-2 and NR-S1-C30-10 are single clones derived from NR-S1-C30. MicroRNA expression was differentially changed in radioresistant cell lines.

Statistical analysis. Each experiment was repeated three times or more. Data are presented as the mean ± SD. Statistical significance was determined with Student's t-tests using Microsoft Excel software (Microsoft Corporation).

Results

miRNA expressions are differentially changed in response to carbon ion beam radiation. To identify miRNAs that may be involved in carbon ion beam resistance, radioresistant cell lines were produced from a mouse squamous cell carcinoma cell line, and then the miRNA profiles of the resistant cells were obtained using miRNA microarray analysis. The miRNA expression patterns of the radioresistant cell lines produced by X-ray and carbon ion beam irradiation were different (Fig. 1).

Table I. The miRNAs specifically expressed in C30 cell lines.

Name	NR-S1	X60	X60-A3	X60-H2	C30	C30-2	C30-10
mmu-miR-494-3p	-0.115	-0.219	-0.017	-0.222	0.358	0.08	0.047
mmu-miR-149-3p	-0.217	-0.015	-0.155	-0.008	0.193	0.026	0.132
mmu-miR-2861	-0.235	-0.159	-0.253	-0.244	0.222	0.464	0.036
mmu-miR-3068-3p	-0.083	-0.52	-0.784	-0.132	0.196	0.556	0.322
mmu-miR-18a-5p	0.03	0.017	0.276	0.493	-0.069	-1.064	-0.124
mmu-miR-205-5p	0.266	0.276	0.083	0.87	-0.136	-1.117	-1.603
mmu-miR-16-5p	0.007	0.174	0.099	0.539	-0.348	-0.616	-0.144
mmu-miR-23a-3p	0.062	0.127	0.168	0.529	-0.402	-0.395	-0.357
mmu-miR-677-3p	0.117	0.039	0.319	0.596	-0.283	-0.682	-0.547
mmu-miR-7a-5p	0.142	0.401	0.669	0.312	-0.459	-1.856	-0.431
mmu-miR-196a-5p	0.31	0.554	0.521	0.792	-1.085	-1.956	-1.334
mmu-miR-374c-5p	0.252	0.403	0.248	0.786	-0.576	-1.815	-0.688
mmu-miR-3968	0.012	0.226	0.147	0.299	-0.105	-0.569	-0.182
mmu-miR-6243	0.12	0.241	0.161	0.124	-0.019	-0.459	-0.307
mmu-miR-151-5p	0.42	0.295	0.314	0.042	-0.401	-0.303	-0.748

The selected miRNAs are shown. Numbers in the table represent average log2 ratio values from the microarray analysis. NR-S1 is a control cell line. X60 is an X-ray-resistant cell line. X60-A3 and X60-H2 are single clones of X60. C30 is a carbon ion beam-resistant cell line. C30-2 and C30-10 are single clones of C30. miRNA, microRNA.

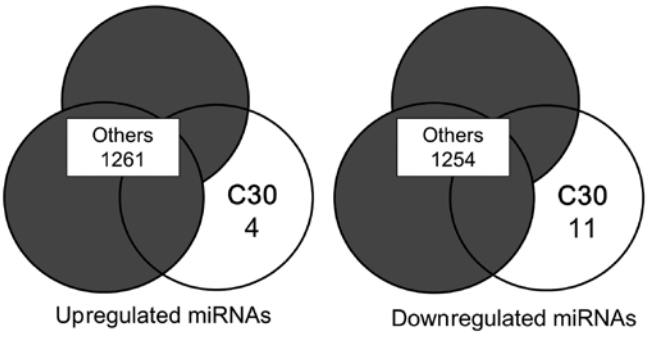

Figure 2. Target microRNAs (miRNAs). miRNAs specifically upregulated or downregulated in C30 cell lines were selected. There were 4 upregulated miRNAs and 11 downregulated miRNAs.

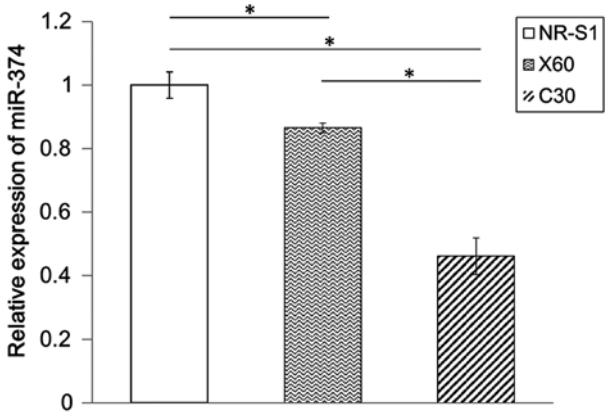

Figure 3. qRT-PCR of miR-374. TaqMan qRT-PCR was performed to evaluate miR-374 expression in each cell line. The results were consistent with the microarray analysis. *P<0.05 (Student's t-test; NR-S1 vs. X60, P<0.01; NR-S1 vs. C30, P<0.01; X60 vs. C30, P<0.01).

Among the 1,265 miRNAs analyzed, 4 were downregulated and 11 were upregulated (Fig. 2 and Table I). To evaluate which of these miRNAs were related to carbon ion beam resistance, miRNAs were screened using the NCBI Gene database and miRBase: the miRNA database. Each murine miRNA was searched to identify its function and determine whether its sequence matched that of a human miRNA. The miRNA mmu-miR-3068-3p was rejected because it does not exist in humans. Based on the average log2 ratio values from the microarray analysis, 3 upregulated miRNAs (mmu-miR-494-3p, mmu-miR-149-3p, and mmu-miR-2861) and 2 downregulated miRNAs (mmu-miR-196a-5p and mmu-miR-374c-5p) were selected for further study. To ensure that the cellular expression of the selected miRNAs was consistent with the microarray results, qRT-PCR was performed. Because the actual expression of miR-532 was not consistent with the microarray result, miR-532 was rejected (Fig. 3). Previous studies on the use of miRNAs, such as miR-205 (9) and miR-185 (12), as radiosensitizers focused on downregulated miRNAs. Therefore, we first focused on downregulated miRNAs, mmu-miR-196a-5p and mmu-miR-374c-5p.

Overexpression of miR-374 radiosensitized human pancreas cancer cell lines against carbon ion beam irradiation. Carbon ion beam radiotherapy is a promising treatment for pancreatic cell carcinoma, which is a therapy-resistant cancer, with respectable treatment outcomes in a clinical trial (15). We overexpressed miR-196 and miR-374 in human pancreatic cancer cell lines PANC1 and MIA PaCa-2 using lipofectamine RNAiMAX. The actual expression level was evaluated with qRT-PCR (Fig. 4A). A colony formation assay was performed with gamma-ray and carbon ion beam

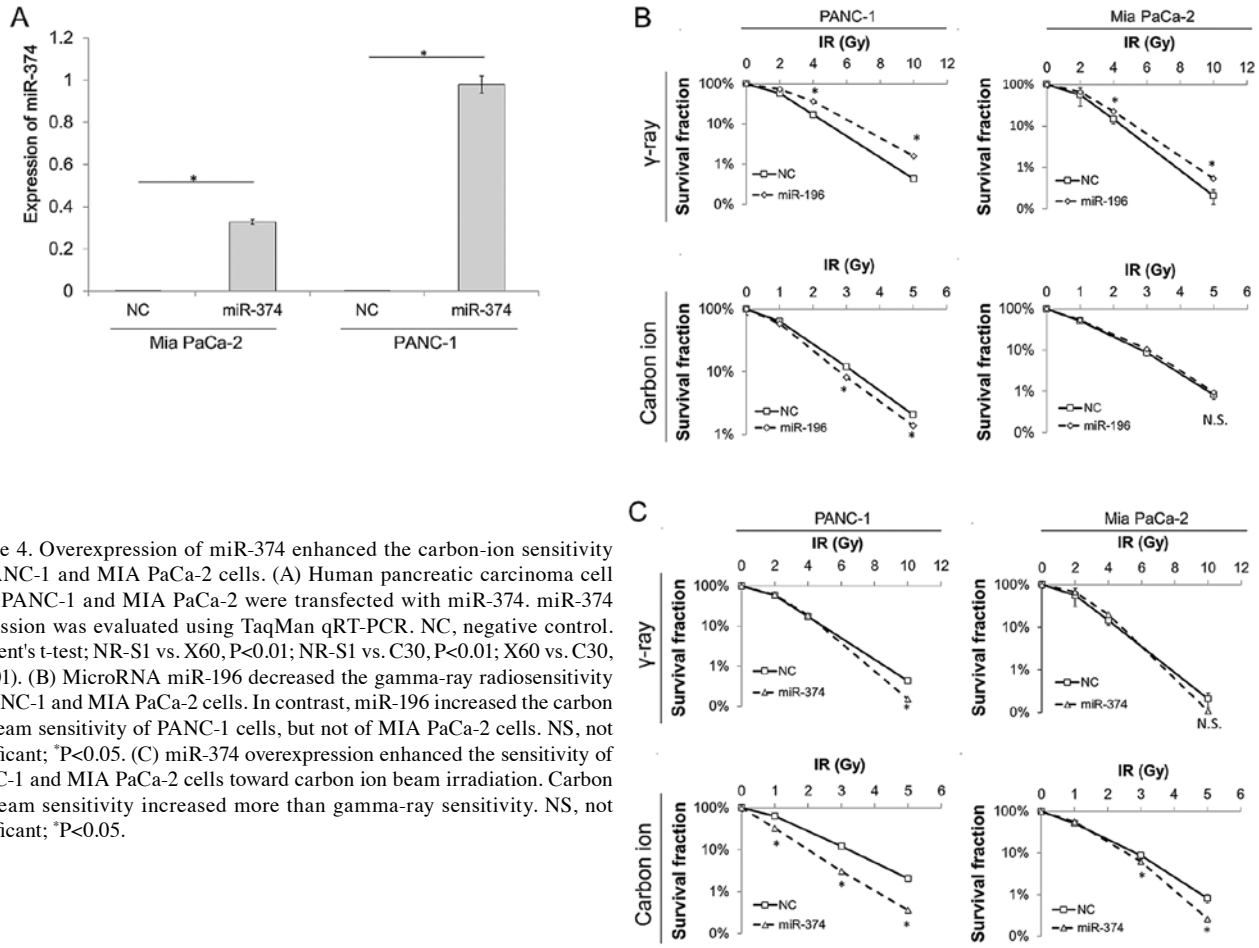

Figure 4. Overexpression of miR-374 enhanced the carbon-ion sensitivity of PANC-1 and MIA PaCa-2 cells. (A) Human pancreatic carcinoma cell lines PANC-1 and MIA PaCa-2 were transfected with miR-374. miR-374 expression was evaluated using TaqMan qRT-PCR. NC, negative control. (Student's t-test; NR-S1 vs. X60, P<0.01; NR-S1 vs. C30, P<0.01; X60 vs. C30, P<0.01). (B) MicroRNA miR-196 decreased the gamma-ray radiosensitivity of PANC-1 and MIA PaCa-2 cells. In contrast, miR-196 increased the carbon ion beam sensitivity of PANC-1 cells, but not of MIA PaCa-2 cells. NS, not significant; *P<0.05. (C) miR-374 overexpression enhanced the sensitivity of PANC-1 and MIA PaCa-2 cells toward carbon ion beam irradiation. Carbon ion beam sensitivity increased more than gamma-ray sensitivity. NS, not significant; *P<0.05.

irradiation to investigate whether miRNA expression changes radiosensitivity. Cell lines overexpressing miR-196 were more resistant to gamma-ray irradiation than the control. PANC-1 cells overexpressing miR-196 were a little more sensitive to carbon ion beam irradiation than the control; however, there was no change in carbon ion beam sensitivity in the MIA PaCa-2 cell line (Fig. 4B). Overexpression of miR-374 increased the sensitivity of both PANC-1 and MIA PaCa-2 cells to carbon ion beam irradiation. There was no change in gamma-ray sensitivity (Fig. 4C). This result suggests that miR-374 may be used as a new carbon ion radiosensitizer.

Discussion

The mechanisms of X-ray resistance have been studied and the DNA repair processes that occur through the ATR-Chk1 (12,16) and ZEB1-Chk1 pathways (9,17) are known to be involved. Carbon ion beam radiation is thought to be more effective than X-ray radiation in inducing double-strand DNA breaks; therefore, it should be more effective in treating X-ray-resistant cancer cells. However, the mechanisms leading to resistance to carbon ion beam radiation are not fully understood. Several studies have shown that radiation-induced changes in miR expression are often related to radioresistance. For example, miR-205 and miR-185, which are downregulated after X-ray irradiation, can radiosensitize cancer cells when overexpressed. We investigated miRNAs that are downregulated in carbon ion beam-irradiated cell lines, and evaluated whether overexpression of these downregulated miRNAs can radiosensitize the cell toward carbon ion beam radiation. In our study, miR-196 and miR-374 were downregulated in C30 cells. Overexpression of miR-374 enhanced the carbon ion beam radiosensitivity of human pancreatic cancer cell lines PANC-1 and MIA PaCa-2. Overexpression of miR-196 decreased the gamma-ray radiosensitivity of PANC-1 and MIA PaCa-2, and slightly enhanced the carbon ion sensitivity of PANC-1 (Fig. 4B). On the contrary, overexpression of miR-196 increased sphere formation in the MIA PaCa-2 cell line, while miR-374c suppressed it. The sphere formation assay is often used to evaluate cancer stem cell properties, and this result suggests that miR-374 has a potential to suppress cancer stem cells. Our findings suggest that miR-374 may be useful as a radiosensitizer for carbon ion beam radiotherapy. While several X-ray radiosensitizers have been studied to date, there are few studies describing carbon ion beam radiosensitizers. Although further investigations are required, including *in vivo* clinical trials, miR-374 showed high potential to be used as a carbon ion beam radiosensitizer or as a biomarker for carbon ion beam sensitivity.

The limitation of this study is that the mechanism through which miR-374 enhances carbon ion beam radiosensitivity is unclear. We searched for target DNAs involved in the regulation of carbon ion beam radiosensitivity using a target scan. None of the DNAs known to be involved in the regulation of X-ray radiosensitivity, such as ZEB19, ATR (12), were in the target DNA list of miR-374. This suggests that carbon ion

beam radiosensitivity is regulated by a pathway different from that of X-ray radiosensitivity.

In conclusion, we demonstrated the potential of miR-374 to become the first carbon ion beam radiosensitizer. We also showed that miR-374 can be used as a biomarker to determine the optimal treatment for cancer. In the future, further detailed studies of carbon ion beam radioresistance mechanisms and clinical trials for the human evaluation of miR-374 are expected.

Acknowledgements

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References

1. Kamada T, Tsujii H, Blakely EA, Debus J, De Neve W, Durante M, Jäkel O, Mayer R, Orecchia R, Pötter R, *et al*: Carbon ion radiotherapy in Japan: An assessment of 20 years of clinical experience. Lancet Oncol 16: e93-e100, 2015.
2. Yasueda A, Urushima H and Ito T: Efficacy and interaction of antioxidant supplements as adjuvant therapy in cancer treatment: A Systematic Review. Integr Cancer Ther 15: 17-39, 2016.
3. Skvortsova I, Debbage P, Kumar V and Skvortsov S: Radiation resistance: Cancer stem cells (CSCs) and their enigmatic pro-survival signaling. Semin Cancer Biol 35: 39-44, 2015.
4. Baek SJ, Ishii H, Tamari K, Hayashi K, Nishida N, Konno M, Kawamoto K, Koseki J, Fukusumi T, Hasegawa S, *et al*: Cancer stem cells: The potential of carbon ion beam radiation and new radiosensitizers (Review). Oncol Rep 34: 2233-2237, 2015.
5. Kunkel TA: DNA replication fidelity. J Biol Chem 279: 16895-16898, 2004.
6. Calin GA, Dumitru CD, Shimizu M, Bichi R, Zupo S, Noch E, Aldler H, Rattan S, Keating M, Rai K, *et al*: Frequent deletions and down-regulation of micro-RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. Proc Natl Acad Sci USA 99: 15524-15529, 2002.
7. He L, Thomson JM, Hemann MT, Hernando-Monge E, Mu D, Goodson S, Powers S, Cordon-Cardo C, Lowe SW, Hannon GJ, *et al*: A microRNA polycistron as a potential human oncogene. Nature 435: 828-833, 2005.
8. Ma L, Teruya-Feldstein J and Weinberg RA: Tumour invasion and metastasis initiated by microRNA-10b in breast cancer. Nature 449: 682-688, 2007.
9. Zhang P, Wang L, Rodriguez-Aguayo C, Yuan Y, Debeb BG, Chen D, Sun Y, You MJ, Liu Y, Dean DC, *et al*: miR-205 acts as a tumour radiosensitizer by targeting ZEB1 and Ubc13. Nat Commun 5: 5671, 2014.
10. Ma S, Tang KH, Chan YP, Lee TK, Kwan PS, Castilho A, Ng I, Man K, Wong N, To KF, *et al*: miR-130b Promotes CD133(+) liver tumor-initiating cell growth and self-renewal via tumor protein 53-induced nuclear protein 1. Cell Stem Cell 7: 694-707, 2010.
11. Liu C, Kelnar K, Liu B, Chen X, Calhoun-Davis T, Li H, Patrawala L, Yan H, Jeter C, Honorio S, *et al*: The microRNA miR-34a inhibits prostate cancer stem cells and metastasis by directly repressing CD44. Nat Med 17: 211-215, 2011.
12. Wang J, He J, Su F, Ding N, Hu W, Yao B, Wang W and Zhou G: Repression of ATR pathway by miR-185 enhances radiation-induced apoptosis and proliferation inhibition. Cell Death Dis 4: e699, 2013.
13. Huang F, Tang J, Zhuang X, Zhuang Y, Cheng W, Chen W, Yao H and Zhang S: MiR-196a promotes pancreatic cancer progression by targeting nuclear factor kappa-B-inhibitor alpha. PLoS One 9: e87897, 2014.
14. Sato K, Imai T, Okayasu R and Shimokawa T: Heterochromatin domain number correlates with X-ray and carbon-ion radiation resistance in cancer cells. Radiat Res 182: 408-419, 2014.
15. Tsujii H and Kamada T: A review of update clinical results of carbon ion radiotherapy. Jpn J Clin Oncol 42: 670-685, 2012.
16. Brezniceanu ML, Lau CJ, Godin N, Chénier I, Duclos A, Ethier J, Filep JG, Ingelfinger JR, Zhang SL and Chan JS: Reactive oxygen species promote caspase-12 expression and tubular apoptosis in diabetic nephropathy. J Am Soc Nephrol 21: 943-954, 2010.
17. Zhang P, Wei Y, Wang L, Debeb BG, Yuan Y, Zhang J, Yuan J, Wang M, Chen D, Sun Y, *et al*: ATM-mediated stabilization of ZEB1 promotes DNA damage response and radioresistance through CHK1. Nat Cell Biol 16: 864-875, 2014.