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Original Article

Study on the molecular mechanism of miR-31 in the invasion and metastasis of glioma cells

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Abstract: Objective: This study aims to explore the molecular mechanism of miR-31 in the invasion and metastasis of glioma cells. Methods: The different expression of miR31 in glioma patients and control were detected by Real-time PCR method. Human glioma cell line U87 was transfected with miR-31 mimic and miR-31 inhibitor was used to inhibit the expression of miR-31. The invasion and metastasis of cells were detected by transwell experiment. The target gene of hsa-miR-31 was predicted by bioinformatic technology. The expression levels of RhoA and ROCK-1 were detected by RT-PCR and western blotting methods. Results: The expression level of miR-31 was lower in glioma group than that of control group ($P < 0.05$), there were no significant differences between different age and gender. The invasion ability of U87 cells was significantly down regulated after they were transfected with miR-31 mimic ($P < 0.05$). However, the invasion ability of U87 cells increased after miR-31 was inhibited. The expression level of miR-31 in glioma cells showed a negative correlation with that of RhoA and ROCK-1. Conclusion: The expression level of miR-31 was low in glioma and displayed as anti-oncogene. It inhibited the invasion and metastasis of glioma cells by inhibiting the expression of RhoA and transmitted signal to the downstream molecules.

Keywords: Glioma, miR31, cell line U87, RhoA

Introduction

miRNAs is a kind of endogenous small RNA molecules including about 17-25 nucleotides, they play an important role in regulating process of modification after gene translation [1, 2]. Although the number of miRNAs in the human genome is far less than that of protein coding genes, miRNAs is thought to control more than half of the human mRNAs [3, 4]. miRNAs degraded mRNA or block its translation mainly through binding its target gene, which had a variety of roles in cells. Each miRNA can have multiple target genes and several miRNAs can regulate the same gene, so miRNAs have very complex biological effects. Recent studies showed that miRNAs played an important regulating role in the occurrence and development of tumor [5-7].

Glioma is one of the common primary tumors of the central nervous system, which almost account for more than half of all primary intracranial tumors. The heterogeneity of glioma is very high and its pathogenesis remains unclear. It is difficult to completely remove glioma by surgical operation because of its high degree of infiltration. At the same time, many anticancer drugs cannot reach the focus because of the blood-brain barrier, so the development of its clinical treatment is slow.

To understand the pathogenesis of glioma and identify the diagnostic and prognostic biomarker molecules is significant to improve the cure rate of glioma and prolong the life of patients [8, 9]. miR-31 located in p21.3 of human chromosome 9. Studies showed that it expressed abnormally in a variety of tumors and played an important role in the metastasis of tumor. However, the expressions of miR-31 are different in different tumors and their biological effects are also different.

Studies found low expression of miR-31 in the adult T cell leukemia [10], bladder cancer [11],

Table 1. Primers used in Real-time PCR

Gene	Accesion NO.	Primer (5'-3')
RhoA	NM_057750.4	F: CTGGTGATTGTTGGTGATGG
		R: GCGATCATAATCTTCCTGCC
ROCK-1	NM_001098209.1	F: AGTCTGTGGCAATGTGTGAG
		R: CTTCAAGCCGACTAACAGTG
GAPDH	NM_002046	F: GAAGGTGAAGGTCGGAGTC
		R: GAAGATGGTGATGGGATTTC

breast cancer [12], esophageal cancer and other tumors [13] and played a carcinostasis, while it showed abnormal high expression in colon cancer [14], lung cancer and osteosarcoma [15] and played the role of oncogene. There are few studies on miR31 in glioma. Therefore, we observed the expression of miR-31 in glioma and studied the molecular mechanism of miR-31 in the invasion and metastasis of glioma cells.

Materials and methods

Cells and samples

Human glioma cell line U87 was purchased from ATCC. They were cultured with DMEM medium containing 15% fetal bovine serum at 37°C with 5% CO_2. Peripheral blood samples were collected from definite glioma patients (20 male and 37 female; mean age 58.2 years old) in Zhangye hospital, healthy normal control blood samples (29 male and 20 female) were also collected. All subjects signed an informed consent form. This study was approved by the Ethics Committee of the Zhangye Hospital Affiliated to Hexi University of Gansu.

Real-time PCR

Total RNA was extracted from the blood samples using RiboPure-Blood Kit (Ambion, AM1928) according to the manual protocol. Total RNA was subjected to reverse transcription using MicroRNA Reverse Transcription Kit (Applied Biosystems, Cat. No. 4366597) according to the protocol. Real-time PCR were performed using SsoAdvanced SYBR Green Supermix (Bio-Rad, Cat. 172-5264). DAPDH gene was used as an internal control for normalization of RNA quantity. The primers were shown in **Table 1**. The data was analyzed using the $2^{-\Delta\Delta Ct}$ method [16].

Cell invasion related experiments

Matrigel was diluted with pre-cooling serum free DMEM medium to a final concentration of 1 mg/ml. 100 μl diluted Matrigel was added in the central bottom of transwell up-chamber and incubated at 37°C to gelatin, gelatin reconstruction was performed by adding 200 μl DMEM medium into each well. The culture medium was removed by centrifugation after cells were digested by trypsin. The cells were washed with PBS and suspended using the serum-free medium, they were cultured in transwell up-chamber, medium containing 10% FBS was added into transwell down-chamber. The transwell up-chamber were taken out after culture and fixed with 4% formaldehyde for 10 min and stained with 0.1% crystal violet. The cells through the microporous were calculated.

Western-blotting

Total proteins were extracted from cells using RIPA lysis buffer. The protein content was determined using Protein Assay Kit and analyzed with SDS-PAGE electrophoresis. Then they were electro-transferred to the PVDF membrane. The membrane was rinsed with TBS for 10 to 15 min and placed in TBS/T blocking buffer containing 5% (w/v) skimmed milk powder and shaked at room temperature for one hour. They were incubated at room temperature for two hours after added with appropriate dilution degree of primary antibody (diluted with TBST containing 1% (w/v) skimmed milk powder). Then the membrane was rinsed with TBST for three times (5 to 10 minutes one time). The membrane was incubated at room temperature for one hour with HRP labeled secondary antibody (1:10000) diluted with TBST containing 0.05% (w/v) skimmed milk powder and rinsed for three times with TBST (5 to 10 minutes at a time). The protein bands were scanned and quantified as a ratio to GAPDH using Quantity One software.

Statistical analysis

All statistical analysis was performed using SPSS version 11.5 statistical software. The results are expressed as mean ± SD. One-way ANOVA and t-test were used to evaluate the differences between groups. A value of $P < 0.05$ and $P < 0.01$ was taken to denote statistical significance.

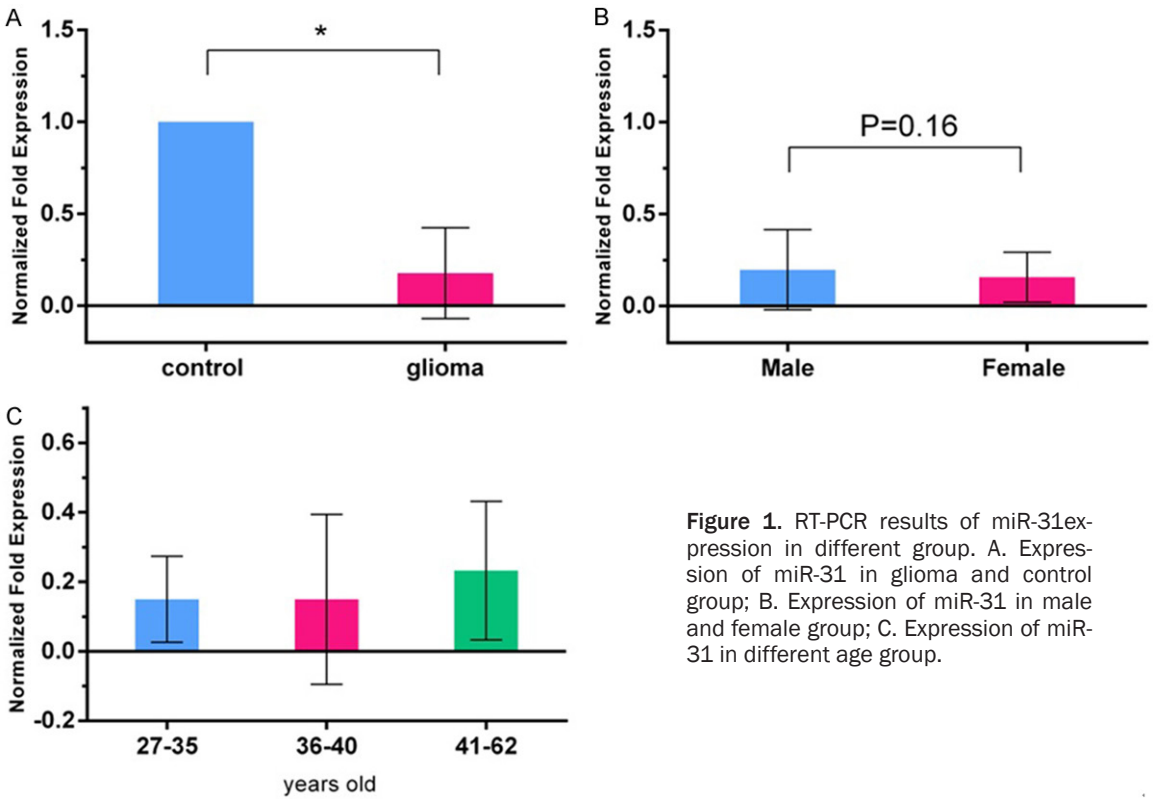

Figure 1. RT-PCR results of miR-31expression in different group. A. Expression of miR-31 in glioma and control group; B. Expression of miR-31 in male and female group; C. Expression of miR-31 in different age group.

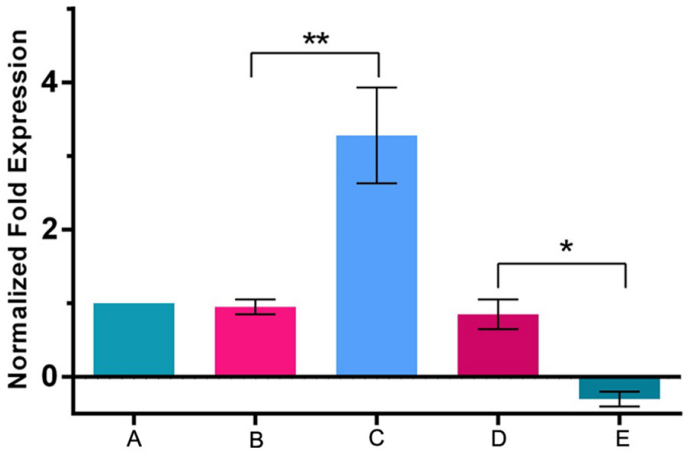

Figure 2. The expression levels of miR-31 in U87 cells transfected by miR-31 mimic and miR-31 inhibitor. A. Blank control; B. Mimic control; C. miR-31 mimic; D. Inhibitor control; E. miR-31 Inhibitor.

Results

Expression of miR-31

RT-PCR results showed that the expression level of miR-31 in glioma patients was lower than that of control group ($P < 0.05$), there was no significant difference among different ages and sex ($P > 0.05$). The results were shown in **Figure 1**.

The role of miR-31 in the process of invasion and metastasis of U87 cells

The expression levels of miR-31 in U87 cells transfected by miR-31 mimic and miR-31 inhibitor were detected respectively with RT-PCR method. We found that the expression level of miR-31 in U87 cells transfected by miR-31 mimic increased significantly while it decreased in U87 cells transfected by miR-31 inhibitor (**Figure 2**). The experiment results of cell invasion were shown in **Table 2**. We found that miR-31 inhibited the invasive ability of U87 cells. The invasive ability of U87 cells transfected by miR-31 mimic decreased significantly while it increased in U87 cells transfected by miR-31 inhibitor ($P < 0.05$).

The predicted target genes of miR-31

The target genes of miR-31 were predicted by bioinformatics, it was shown in **Table 3**.

Table 2. The result of cell invasion experiment

		miRNAs mimic		miRNAs inhibitor	
	Blank control	Control	miR-31	Control	miR-31
V1[a]	192[b]	199	98	184	257
V2	188	217	119	219	277
V3	204	211	108	202	282
	194.67 ± 6.80[c]	209.00 ± 7.48	108.33 ± 8.58**	201.67 ± 14.29	272.00 ± 10.80**

[a]: Field of vision; [b]: The cell numbers through the membrane; [c]: Mean ± SD; Compared with control group, *P < 0.05, **P < 0.01.

Table 3. The target gene prediction of hsa-miR-31

microRNA family	Target gene	Estimated false discovery rate	Target gene name	Target gene description
miR-31	ATN1	0.00096	Atrophin-1	Negative regulation of transcription
	CCL5	0.0017	C-C motif chemokine 5	Cytokine
	CEBPA	0.0018	CCAAT/enhancer-binding protein alph...	Transcription factor
	DACT3	0.0038	Dapper homolog 3	Negative regulation of Wnt signaling
	RHOA	0.091	Transforming protein RhoA	Gtpase
	FIH1	0.0039	Hypoxia-inducible factor 1-alpha in...	Hypoxia
	ITGA5	0.0042	Integrin alpha-5	Cell adhesion
	MAP3K14	0.006	Mitogen-activated protein kinase ki...	Protein kinase
	ITGAV	0.0074	Integrin alpha-V	Cell adhesion
	RDX	0.011	Radixin	Cytoskeleton maintenance and remodeling
	RHOBTB1	0.02	RHOBTB1 protein	Gtpase
	TIAM	0.021	T-lymphoma invasion and metastasis-...	Gtpase activation
	WAVE3	0.023	Tyrosine-protein kinase ABL1	Cytoskeleton maintenance and remodeling
	SLC26A3	0.028	Chloride anion exchanger	Transmembrane protein, ion transport
	PPP2R2A	0.028	Serine/threonine-protein phosphatas...	Serine/threonine phosphatase subunit
	MMS19	0.091	MMS19 nucleotide excision repair pr...	DNA repair
	FOXM1	0.091	Forkhead box protein M1	Transcription factor

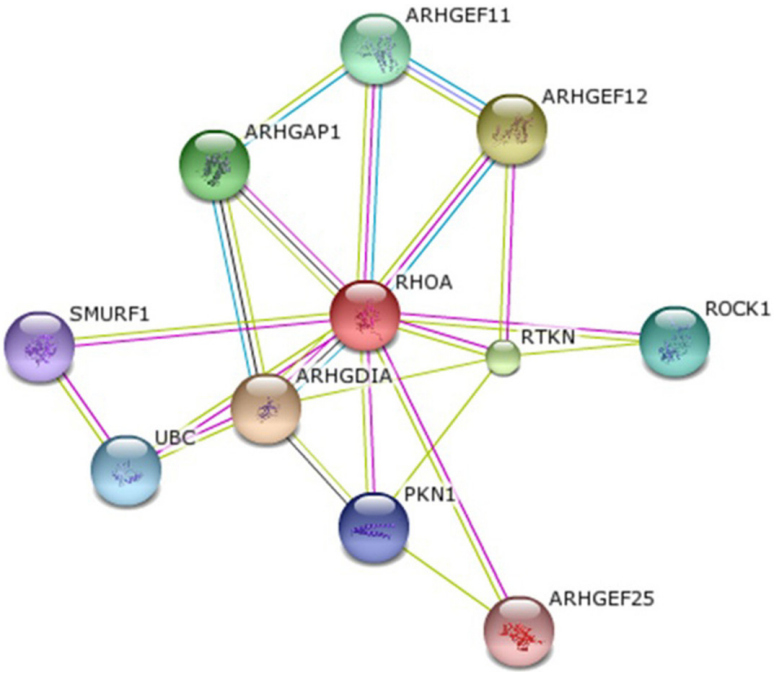

Figure 3. Proteins interacted with RhoA searched in String database.

The effects of miR-31 on RhoA signal pathway

Proteins interacted with RhoA were searched in String database, it was shown in **Figure 3**. We detected the expression levels of RhoA and ROCK-1 using RT-PCR and western blotting methods. They were shown in **Figures 4**, **5** respectively. We found that miR-31 inhibited the levels of RhoA and ROCK-1, there was a negative correlation between miR-31 and RhoA and ROCK-1.

Discussion

The discovery of miRNAs could be a milepost of life science. They had many biologi-

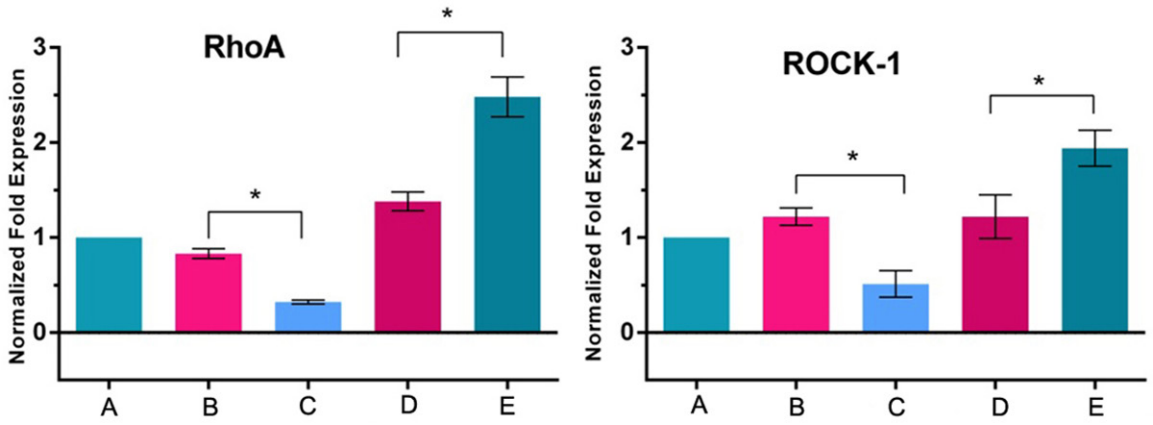

Figure 4. RT-PCR results of the expression levels of RhoA and ROCK-1 in U87 cells transfected by miR-31 mimic and miR-31 inhibitor. A. Blank control; B. Mimic control; C. miR-31 mimic; D. Inhibitor control; E. miR-31 inhibitor.

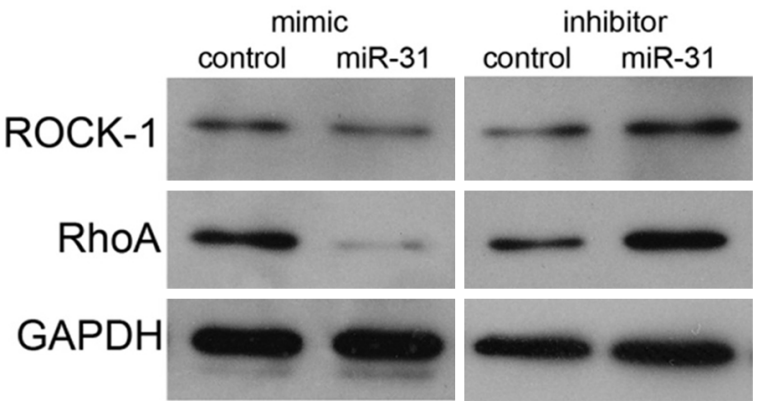

Figure 5. Western blotting results of the expression levels of RhoA and ROCK-1 in U87 cells transfected by miR-31 mimic and miR-31 inhibitor.

cal functions and the mechanism was complex. miRNAs formed a complex regulatory network and widely involved in the life process of cells such as proliferation and apoptosis [17, 18]. In this study, we choose the blood samples mainly because miRNAs is very stable in serum [19, 20]. We found that the expression level of miR-31 in glioma patients was significantly lower than that of the control group, which suggested that miR-31 may play as anti-oncogene in glioma. The results of RNAhybrid and TargetScan database analysis showed that many target genes of miR-31 were related with cellular invasion and metastasis. Glioma is an intracranial tumor very prone to metastasis, we found that the invasion ability of U87 cells transfected by hsa-mir-31 mimic decreased significantly, while the invasion ability increased when they were treated by hsa-mir-31 inhibitor.

RhoA gene was the target gene of miR-31 and was up-regulated in glioma [21-23]. Rho family belongs to the member of small G protein superfamily, it played a "switch" role by the transformation between active and inactive states. Rho family members are involved in regulating many biological processes, such as cell adhesion, cytoskeletal reorganization, cell transfer, cell cycle and gene transcription. Their diverse function was related with the diversity of their downstream effectors. The downstream effectors of RhoA include ROCK-1, Citron, PKN, PI-3K, etc [24]. RhoA was closely related with the development of tumor. Rho family members expressed in various tumor tissues and increased in malignancy tumors. They may play a very important role in the tumorigenesis and metastasis. In this study, we found that the cell invasion ability decreased and RhoA and ROCK-1 were down-regulated when they were transfected by hsa-mir-31 mimic, while RhoA and ROCK-1 were up- regulated when miR-31 was inhibited. There was positive correlation between RhoA and cell invasion ability. miR-31 may play as anti-oncogene in glioma by inhibiting RhoA, this result provides a good way for gene therapy of glioma.

The biological effect of miR-31 was different in different tumor cells, they were regulated by many factors. The expression and regulation of

genes were also a complex network. Therefore, although miRNAs play an important role in the process of gene expression and regulation, but the regulatory process is not by a single factor, it is controlled by a regulating system, which is an important problem of gene therapy.

Disclosure of conflict of interest

None.

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References

[1] Pritchard CC, Cheng HH and Tewari M. MicroRNA profiling: approaches and considerations. Nat Rev Genet 2012; 13: 358-369.

[2] Bartel DP. MicroRNAs: target recognition and regulatory functions. Cell 2009; 136: 215-233.

[3] Friedman RC, Farh KK, Burge CB and Bartel DP. Most mammalian mRNAs are conserved targets of microRNAs. Genome Res 2009; 19: 92-105.

[4] Laurila EM and Kallioniemi A. The diverse role of miR-31 in regulating cancer associated phenotypes. Genes Chromosomes Cancer 2013; 52: 1103-1113.

[5] Griffiths-Jones S, Grocock RJ, van Dongen S, Bateman A and Enright AJ. miRBase: microRNA sequences, targets and gene nomenclature. Nucleic Acids Res 2006; 34: D140-144.

[6] Wagner A, Mayr C, Bach D, Illig R, Plaetzer K, Berr F, Pichler M, Neureiter D and Kiesslich T. MicroRNAs associated with the efficacy of photodynamic therapy in biliary tract cancer cell lines. Int J Mol Sci 2014; 15: 20134-20157.

[7] Liu L, Chen L, Xu Y, Li R and Du X. microRNA-195 promotes apoptosis and suppresses tumorigenicity of human colorectal cancer cells. Biochem Biophys Res Commun 2010; 400: 236-240.

[8] Ohgaki H. Epidemiology of brain tumors. Methods Mol Biol 2009; 472: 323-342.

[9] Siegal T. Clinical impact of molecular biomarkers in gliomas. J Clin Neurosci 2015; 22: 437-444.

[10] Yamagishi M, Nakano K, Miyake A, Yamochi T, Kagami Y, Tsutsumi A, Matsuda Y, Sato-Otsubo A, Muto S, Utsunomiya A, Yamaguchi K, Uchimaru K, Ogawa S and Watanabe T. Polycomb-mediated loss of miR-31 activates NIK-dependent NF-kappaB pathway in adult T cell leukemia and other cancers. Cancer Cell 2012; 21: 121-135.

[11] Dai N, Zhong ZY, Cun YP, Qing Y, Chen C, Jiang P, Li MX and Wang D. Alteration of the microRNA expression profile in human osteosarcoma cells transfected with APE1 siRNA. Neoplasma 2013; 60: 384-394.

[12] Augoff K, Das M, Bialkowska K, McCue B, Plow EF and Sossey-Alaoui K. miR-31 is a broad regulator of beta1-integrin expression and function in cancer cells. Mol Cancer Res 2011; 9: 1500-1508.

[13] Lynam-Lennon N, Reynolds JV, Marignol L, Sheils OM, Pidgeon GP and Maher SG. MicroRNA-31 modulates tumour sensitivity to radiation in oesophageal adenocarcinoma. J Mol Med (Berl) 2012; 90: 1449-1458.

[14] Mosakhani N, Lahti L, Borze I, Karjalainen-Lindsberg ML, Sundstrom J, Ristamaki R, Osterlund P, Knuutila S and Sarhadi VK. MicroRNA profiling predicts survival in anti-EGFR treated chemorefractory metastatic colorectal cancer patients with wild-type KRAS and BRAF. Cancer Genet 2012; 205: 545-551.

[15] Liu X, Sempere LF, Ouyang H, Memoli VA, Andrew AS, Luo Y, Demidenko E, Korc M, Shi W, Preis M, Dragnev KH, Li H, Direnzo J, Bak M, Freemantle SJ, Kauppinen S and Dmitrovsky E. MicroRNA-31 functions as an oncogenic microRNA in mouse and human lung cancer cells by repressing specific tumor suppressors. J Clin Invest 2010; 120: 1298-1309.

[16] Livak KJ and Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001; 25: 402-408.

[17] Griffiths-Jones S, Grocock RJ, van Dongen S, Bateman A and Enright AJ. miRBase: microRNA sequences, targets and gene nomenclature. Nucleic Acids Res 2006; 34: D140-144.

[18] Wagner A, Mayr C, Bach D, Illig R, Plaetzer K, Berr F, Pichler M, Neureiter D and Kiesslich T. MicroRNAs associated with the efficacy of photodynamic therapy in biliary tract cancer cell lines. Int J Mol Sci 2014; 15: 20134-20157.

[19] Chen X, Ba Y, Ma L, Cai X, Yin Y, Wang K, Guo J, Zhang Y, Chen J, Guo X, Li Q, Li X, Wang W, Zhang Y, Wang J, Jiang X, Xiang Y, Xu C, Zheng P, Zhang J, Li R, Zhang H, Shang X, Gong T, Ning G, Wang J, Zen K, Zhang J and Zhang CY. Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. Cell Res 2008; 18: 997-1006.

[20] Chen X, Hu Z, Wang W, Ba Y, Ma L, Zhang C, Wang C, Ren Z, Zhao Y, Wu S, Zhuang R, Zhang Y, Hu H, Liu C, Xu L, Wang J, Shen H, Zhang J,

Zen K and Zhang CY. Identification of ten serum microRNAs from a genome-wide serum microRNA expression profile as novel noninvasive biomarkers for nonsmall cell lung cancer diagnosis. Int J Cancer 2012; 130: 1620-1628.

[21] Mizoguchi F, Murakami Y, Saito T, Miyasaka N and Kohsaka H. miR-31 controls osteoclast formation and bone resorption by targeting RhoA. Arthritis Res Ther 2013; 15: R102.

[22] Chang KW, Kao SY, Wu YH, Tsai MM, Tu HF, Liu CJ, Lui MT and Lin SC. Passenger strand miRNA miR-31* regulates the phenotypes of oral cancer cells by targeting RhoA. Oral Oncol 2013; 49: 27-33.

[23] Valastyan S, Chang A, Benaich N, Reinhardt F and Weinberg RA. Concurrent suppression of integrin alpha5, radixin, and RhoA phenocopies the effects of miR-31 on metastasis. Cancer Res 2010; 70: 5147-5154.

[24] Gajate C and Mollinedo F. Cytoskeleton-mediated death receptor and ligand concentration in lipid rafts forms apoptosis-promoting clusters in cancer chemotherapy. J Biol Chem 2005; 280: 11641-11647.