European Review for Medical and Pharmacological Sciences 2019; 23: 1513-1519

Influence of exosome-derived miR-21 on chemotherapy resistance of esophageal cancer

Y.-C. YANG[1], G.-J. LIU[2], D.-F. YUAN[3], C.-Q. LI[4], M. XUE[3], L.-J. CHEN[3]

[1]Queen Mary Institute, Nanchang University, Nanchang, China
[2]Department of Oncology, Henan Provincial People's Hospital, Zhengzhou, China
[3]Department of Radiotherapy, Xinxiang Central Hospital, Xinxiang, China
[4]Department of Oncology, Xinxiang Central Hospital, Xinxiang, China

Abstract. – OBJECTIVE: To explore the influence of exosome-derived micro-ribonucleic acid (miR)-21 on chemotherapy resistance of esophageal cancer and its mechanism.

MATERIALS AND METHODS: Human esophageal cancer TE-1 and Eca109/DDP cell lines and human normal esophageal Het-1A cells were cultured, and the exosomes were extracted from cells. After miR-21 was inhibited with an inhibitor and overexpressed with miRNA mimics combined with cisplatin, the cell viability was detected via cell counting kit-8 (CCK-8), the interaction between miR-21 and programmed cell death 4 (PDCD4) was detected via dual-luciferase reporter gene assay, and the changes in the protein level were detected via Western blotting.

RESULTS: The expression level of exosome-derived miR-21 in esophageal cancer cells was higher than that in normal esophageal cells, and it was the highest in cisplatin-resistant esophageal cancer cells. After treatment with cisplatin, miR-21 overexpression significantly reduced the invasion ability of esophageal cancer cells. After miR-21 overexpression, the sensitivity of esophageal cancer cells to cisplatin was lowered. MiR-21 interacted with the 3'-untranslated region (UTR) of PDCD4. Moreover, the miR-21 overexpression significantly down-regulated the mRNA and protein levels of PDCD4 in cells.

CONCLUSIONS: MiR-21 affects the sensitivity of esophageal cancer to cisplatin through targeting PDCD4.

Key Words:
MiR-21, PDCD4, Cisplatin, Esophageal cancer, Drug resistance.

Introduction

Esophageal cancer is the 8th most common cancer in the world and the 6th most common cause of cancer-related death [GLOBOCAN 2008 (IARC), Cancer Information (30/1/2012)]. The treatment of esophageal cancer has been greatly improved in recent years, but the prognosis of esophageal cancer is still unsatisfactory. Chemotherapy and/or therapeutic methods based on radiotherapy have been applied in many patients, and according to several meta-analyses, patients treated with neoadjuvant chemotherapy or combined chemoradiotherapy before esophageal cancer surgery have survival advantages over those treated with surgery alone[1]. However, the response to these treatments varies from individual to individual, and the treatment for patients with poor response to chemotherapy may be ineffective. It is necessary to determine individuals who are unlikely to benefit from the treatment before treatment so that the treatment can be suitable for patients who are most likely to benefit from it. New molecular biomarkers predicting the chemotherapy response need mining constantly. Moreover, the molecular biomarkers for chemotherapy response may also provide new therapeutic targets to overcome the potential chemotherapy resistance.

In this context, micro-ribonucleic acid (miRNA) is a potential biomarker[2]. Researchers[2,3] have demonstrated that the miRNA expression profile has differences among different esophageal tissues, which are correlated with the prognosis and clinicopathological features of esophageal cancer, directly affecting the tumor growth, proliferation, and invasion. In addition, evidence suggests that the miRNA expression has an influence on chemotherapy response in some other cancers[4]. However, there are very few data about the evaluation of esophageal cancer so far.

MiRNA is a kind of small non-coding RNA molecule existing in the free cells in the blood (including serum), which can regulate the protein production in messenger RNA (mRNA) and be definitely detected in frozen samples. Recently, the circulating miRNA level has been used in the identification of some cancers. 89 kinds of circulating

Corresponding Author: Yichen Yang, BM; e-mail: 1552773559@qq.com

miRNAs exist in a variety of forms in the serum, including extracellular vesicles called exosomes. Exosomes are shed from tumor cells and can come into the circulation. It is noteworthy that circulating miRNAs in exosomes are more stable than those in other forms, because they are protected by endogenous RNase degradation[5,6] and have great potential as specific biomarkers for diseases.

A unique marker for circulating exosome miRNA has been discovered in colon cancer recently[7,8]. However, there have been no researches on the evaluation of exosome-derived circulating miRNA in esophageal cancer yet. Therefore, the potential of miRNA in exosomes as a biomarker for esophageal cancer was evaluated in this study, and the correlation between miRNA expression and chemotherapy response in esophageal cancer was explored, so as to determine whether the chemotherapy-resistant esophageal cancer cells display the specific miRNA expression pattern, and whether the expression of potential chemotherapy resistance-related targets changes in chemotherapy-resistant cancer cells.

Materials and Methods

Cell Culture

The human esophageal cancer TE-1 and Eca109/DDP cell lines and human normal esophageal Het-1A cells used in this study were from the American Type Culture Collection (ATCC; Manassas, VA, USA), and cultured in the Dulbecco's Modified Eagle Medium (DMEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) in an incubator with 5% CO_2 at 37°C.

Extraction of Exosomes

The exosomes were extracted when 80% of TE-1 cells were fused. Then, the medium was collected, centrifuged at 2000×g for 20 min and filtered through a 0.22 μm filter to remove the cell debris. The supernatant (10 mL) was mixed with 2 mL ExosoQuick precipitation solution, followed by incubation at 4°C overnight and centrifugation at 500×g for 30 min. The exosomes in the sediment were re-suspended in 100 μL phosphate-buffered saline (PBS) and washed twice with it.

After different treatments, cells were collected to extract the total RNA using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the instructions. 1 μL RNA solution was taken on a microplate reader to determine the concentration and purity of total RNA. The optical density $(OD)_{260}/OD_{280}$ value should be 1.6-1.8. The remaining RNA solution was sub-packaged and stored at -80°C for standby application.

Complementary deoxyribonucleic acid (cDNA) was synthesized using a TaKaRa PrimeScript™ Kit (TaKaRa Inc., Otsu, Shiga, Japan) according to the instructions. First, 2 μg total RNA (the volume of RNA added was calculated based on the concentration), 1 μL Oligo (dT) primer (50 μM) and 1 μL dNTP Mixture (10 mM) were added, and the enzyme-free double-distilled water was also added till the total system volume up to 10 μL. The mixture was mixed evenly, heated in the PCR instrument at 65°C for 5 min, and quickly cooled on ice. The above reaction system was added with 4 μL 5×PrimeScript Buffer, 1 μL PrimeScript RTase, 0.5 μL RNase inhibitor and 4.5 μL enzyme-free water, and the mixture (20 μL in total) was mixed evenly, heated in the PCR instrument at 42°C for 45 min and then at 95°C for 5 min, and quickly cooled on ice. Finally, the single-stranded cDNA synthesized was stored at -20°C for PCR amplification.

Oligonucleotides and Cell Transfection

The antisense oligonucleotide sequence of miR-21 (anti-miR-21) is 5'-AGCUACAUUGUCUGCUGGGUUUC-3', and the scrambled oligonucleotide sequence (anti-miR-NC) is 5'-UCUACUCUUUCUAGGAGGUUGUGA-3'. The oligonucleotide was synthesized in a solid phase and purified using high-performance liquid chromatography (GenePharma Co., Ltd., Shanghai, China). The cells cultured in a 6-well plate with the serum-free medium were transfected with 200 pmoL oligonucleotides using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). At 6 h after transfection, the medium was replaced with the complete medium to culture cells under 5% CO_2 at 37°C.

Cell Proliferation and Apoptosis Assays

The cell viability and proliferation were evaluated *via* cell counting kit-8 (CCK-8) assay. First, the cells were inoculated into a 96-well plate and incubated with the CCK-8 solution (Dojindo, Kumamoto, Japan) for 4 h. The optical density (OD) value was detected at 450 nm using a plate reader to evaluate the cell viability. The apoptosis rate after cisplatin treatment was determined *via* flow cytometry. At 48 h after incubation with cisplatin, the cells were stained with propidium iodide, and the DNA fragmentation was detected *via* flow cytometry. The apoptosis rate was expressed as the ratio of cells containing fractional DNA content to living cells.

Dual-Luciferase Reporter Gene Assay

On the first day of experiment, the cells (appropriate cells were selected according to specific experiments) were digested and inoculated into a 35 mm cell culture dish, followed by incubation in the incubator with 5% CO_2 and saturated humidity at 37°C overnight. When the cell density was 70%, the cells were co-transfected with the luciferase reporter gene plasmid, LacZ expression plasmid, and other plasmids. After transfection for 24-36 h, the culture solution was discarded, and the cells were washed with pre-cooled PBS without calcium and magnesium ions. 350 μL pre-cooled harvest buffer was added into each dish to lyse the cells at 4°C or on ice for 10 min. During the lysis, a sufficient number of 1.5 mL microcentrifuge tubes were prepared, and ATP buffer and luciferin buffer were mixed (1:3.6) to be reaction solution and placed into tubes (100 μL/tube). An equal volume of cell lysis buffer (100 μL) was taken into the centrifuge tube in Step 5 and quickly mixed evenly. The OD value was read using a luminometer, followed by plotting *via* the corrected value and data analysis.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 (IBM, Armonk, NY, USA) was used for statistical analysis. The *t*-test was adopted for the difference between two groups, and one-way analysis of variance was adopted for the difference among groups. Bilateral 95% confidence interval (CI) was used in all tests. $p<0.05$ suggested that the difference was statistically significant.

Results

Expression of MiR-21 in Exosomes of Esophageal Cancer Cells, Cisplatin-Resistant Cells, and Normal Esophageal Cells

The exosomes were extracted from different cells, and the expression of miRNA in exosomes was detected (Figure 1). The results revealed that the expression level of exosome-derived miR-21 in esophageal cancer cells was higher than that in normal esophageal cells, and it was the highest in cisplatin-resistant esophageal cancer cells ($p<0.01$) (Figure 2).

MiR-21 in Exosomes Could be Ingested by Esophageal Cancer Cells

To determine whether miR-21 in exosomes can be ingested by esophageal cancer cells, the exosomes were isolated from Eca109/DD cells and incubated together with TE-1 cells. Then, the ingestion of miR-1246 by TE-1 cells was detected *via* qRT-PCR, and it was found that the number of miR-21 increased by 5 times in TE-1 cells ($p<0.001$) (Figure 3).

Sensitivity of Cells Treated in Different Ways to Cisplatin Detected Via CCK-8

The TE-1 cells and Eca109/DDP cell-derived exosomes were incubated and treated with 10 μmol/L cisplatin for 48 h. The results of CCK-8 assay manifested that the TE-1 cells had lower sensitivity to cisplatin, while such a trend was reversed after transfection with miR-21 inhibitor ($p<0.05$) (Figure 4).

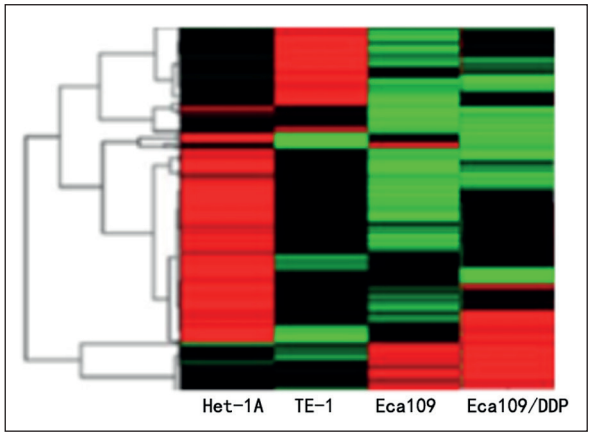

Figure 1. Cluster analysis of miRNA1 expression in exosomes of different cells.

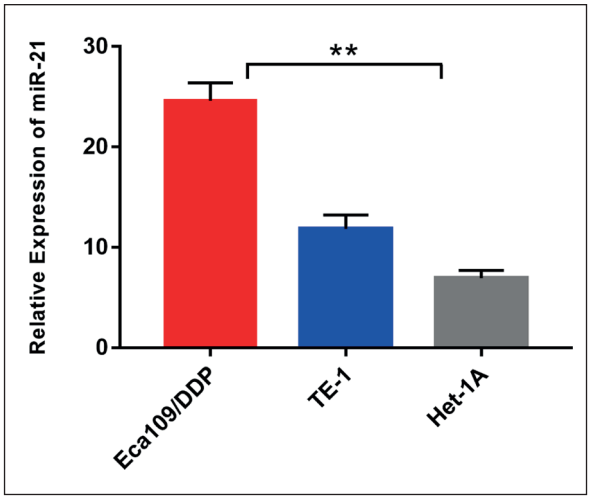

Figure 2. Expression level of miR-21 in exosomes of different cells.

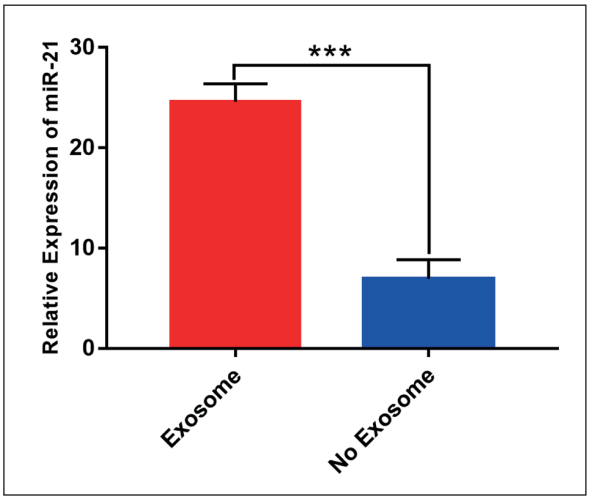

Figure 3. Expression level of miR-21 in cells after incubation of TE-1 cells and Eca109/DDP cell-derived exosomes.

Dual-Luciferase Reporter Gene Assay

The predicted targets of miR-21 were detected *via* bioinformatics analysis. The results showed that the 3'-UTR of PDCD4 bound to miR-21 in a highly-conserved way (Figure 5). The luciferase reporter gene assay for the binding site of mutant PDCD4 and miR-21 demonstrated that the miR-21 transfection could significantly inhibit the relative luciferase activity in wild-type cells, while there were no significant changes in the relative luciferase activity in mutant cells, indicating that miR-21 exerts an inhibitory effect on PDCD4 expression through the interaction with 3'-UTR of PDCD4 (Figure 6).

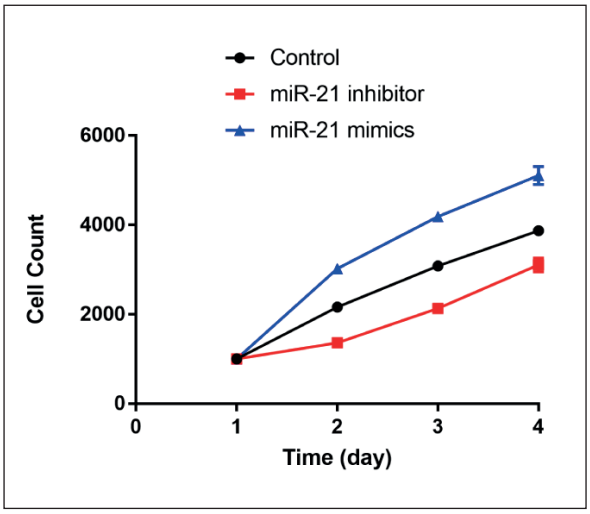

Figure 4. Cell proliferation detected *via* CCK-8.

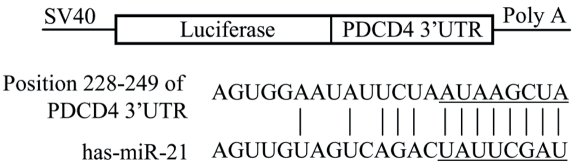

Figure 5. MiR-21 directly targets the 3'-UTR of PDCD4: Predicted binding site of miR-21 and 3'-UTR of PDCD4.

MiR-21 Overexpression Significantly Down-Regulated mRNA and Protein Levels of PDCD4 in Cells

After transfection with miR-21, the expression level of PDCD4 was detected in cells. It was found that the miR-21 overexpression significantly down-regulated the mRNA and protein levels of PDCD4 in cells compared with control group (Figure 7).

Discussion

This investigation clarified that miR-21 regulated the chemotherapy resistance of esophageal cancer, and also showed for the first time that the tumor-inhibiting factor PDCD4 played a role at the post-transcriptional level through binding to miR-21 in exosomes of esophageal squamous cell carcinoma. MiRNAs regulate various cellular pathways *via* regulating the expression of various target genes[9]. Therefore, miR-21 is considered to act as an oncogene, and studies[10-14] have revealed that miR-21 is overexpressed in a variety of malignant tumors, such

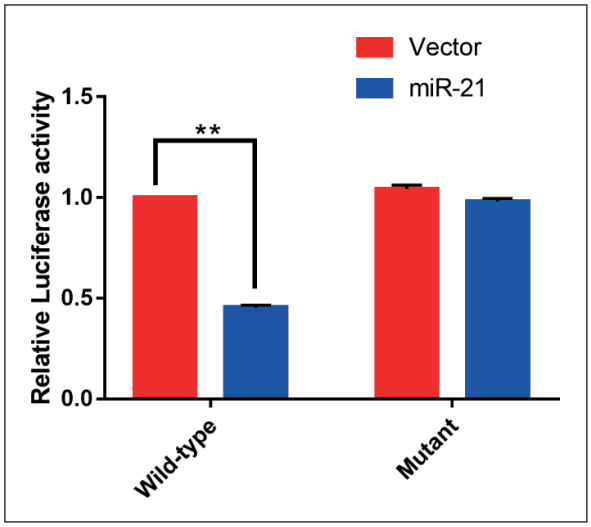

Figure 6. Interaction between miR-21 and PDCD4 detected via luciferase reporter assay.

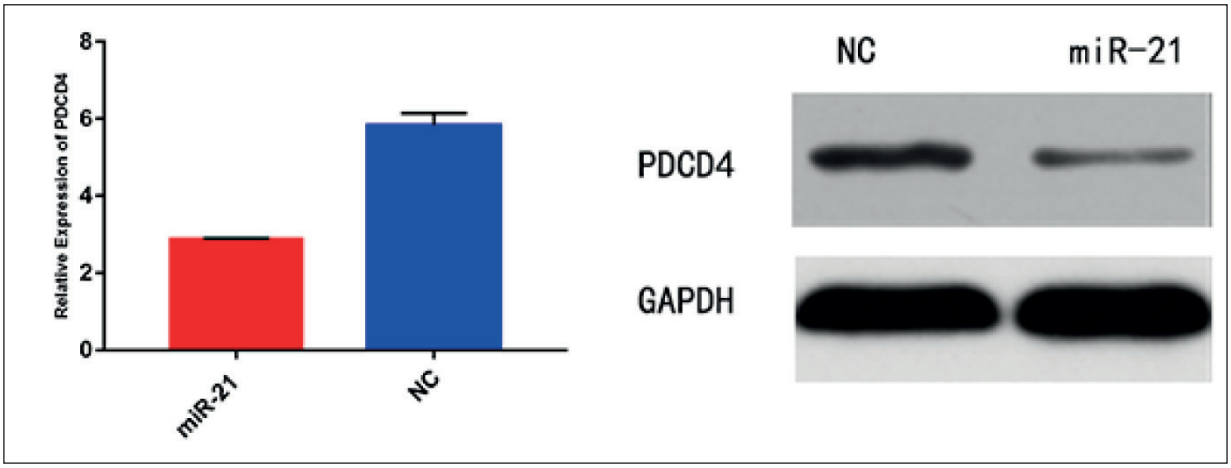

Figure 7. MiR-21 down-regulates mRNA and protein levels of PDCD4 in cells.

as breast cancer, brain tumor, lung cancer, prostate cancer, ovarian cancer, and pancreatic cancer. In addition, the correlation between miR-21 expression and prognosis has been proved in some cancers. Scholars have demonstrated that the high expression of miR-21 is correlated with the low survival rate of patients with colon cancer, pancreatic cancer, and non-small cell lung cancer[15]. In this work, the expression level of exosome-derived miR-21 in esophageal cancer cells was remarkably higher than that in normal esophageal cells, which is consistent with previous reports.

PDCD4 binds to and inhibits the translation initiation factor 4a at the molecular level, thereby affecting the protein translation[16]. Moreover, it has been found[17] that PDCD4 inhibits the activator protein-mediated trans-activation and induces the expression of cyclin-dependent kinase inhibitor p21. According to some reports[18,19], PDCD4 is a target for miR-21 that promotes the tumor progression, including cell proliferation, invasion, metastasis, and transformation of breast cancer. Therefore, it is assumed that PDCD4 is also an important target for miR-21 in esophageal squamous cell carcinoma. The results of this work manifested that there was a significant correlation between miR-21 expression and PDCD4 protein level, and the PDCD4 protein expression declined in cells transfected with miR-21. The reporter gene activity was significantly increased after transient transfection of cells with reporter gene plasmid containing 3'-UTR of PDCD4 mRNA and anti-miR-21 inhibitor. The above findings demonstrate that PDCD4 is negatively regulated by miR-21 at the post-translational level through binding to the 3'-UTR of PDCD4 mRNA.

At the same time, we observed that the functional oncogenic miRNA can be delivered from esophageal cancer cells through exosomes. It is noteworthy that miR-21 in exosomes induces the tumor progression, including increasing the cell proliferation, migration, and drug resistance. Moreover, miR-21 directly targeted PDCD4 expression through binding to its 3'-UTR. The importance of exosomes in the progression of cancer has been evaluated in many studies[20]. The exosomes secreted by highly-metastatic cells significantly facilitate the progression of poorly-metastatic cancer, which is in line with the view that cancer cells interact through exosomes. It was found that exosomes isolated from breast cancer cells can induce the chemotherapy resistance of breast cancer through increasing the cell growth. In this study, miR-21 was significantly enriched in exosomes, and the sensitivity of TE-1 cells to cisplatin declined after miR-21 overexpression. Therefore, further developing the exosome miR-21 in the serum as a predictor of chemotherapy resistance is conducive to the individualized treatment for patients. With the new progress in gene therapy, exosomes, as potential delivery vectors for miRNA can promote the development of anti-miR-21 therapeutic strategies. Besides, it was found that PDCD4 was a direct target of miR-21, which was directly targeted and down-regulated by miR-21. To sum up, the critical roles of cancer cell-derived exosomes and exosome miRNA in cancer progression were proved in this study. The oncogenic miRNAs secreted by esophageal

cancer cells, such as miR-21, can affect both adjacent and distant normal cells, thus promoting the tumor progression. Based on these results, it is speculated that targeting exosome miRNA can provide an alternative to esophageal cancer intervention.

Conclusions

We demonstrated that miR-21 was overexpressed in exosomes in esophageal cancer, and anti-miR-21 inhibited the chemotherapy resistance of esophageal cancer *in vitro*, which may be due to the down-regulation of tumor-inhibiting factor PDCD4 by miR-21. The findings of this study raise the possibility that anti-miR-21 has potential therapeutic value in patients with esophageal cancer. Anti-exosome miR-21 can act as a potentially useful target for cancer therapy.

Conflict of Interests

The authors declare that they have no conflict of interest.

References

1) Sjoquist KM, Burmeister BH, Smithers BM, Zalcberg JR, Simes RJ, Barbour A, Gebski V. Survival after neoadjuvant chemotherapy or chemoradiotherapy for resectable oesophageal carcinoma: an updated meta-analysis. Lancet Oncol 2011; 12: 681-692.
2) Hiyoshi Y, Kamohara H, Karashima R, Sato N, Imamura Y, Nagai Y, Yoshida N, Toyama E, Hayashi N, Watanabe M, Baba H. MicroRNA-21 regulates the proliferation and invasion in esophageal squamous cell carcinoma. Clin Cancer Res 2009; 15: 1915-1922.
3) Hummel R, Hussey DJ, Haier J. MicroRNAs: predictors and modifiers of chemo- and radiotherapy in different tumour types. Eur J Cancer 2010; 46: 298-311.
4) Cheng L, Sharples RA, Scicluna BJ, Hill AF. Exosomes provide a protective and enriched source of miRNA for biomarker profiling compared to intracellular and cell-free blood. J Extracell Vesicles 2014 Mar 26; 3. doi: 10.3402/jev.v3.23743. eCollection 2014.
5) Koberle V, Pleli T, Schmithals C, Augusto AE, Haupenthal J, Bonig H, Peveling-Oberhag J, Biondi RM, Zeuzem S, Kronenberger B, Waidmann O, Piiper A. Differential stability of cell-free circulating microRNAs: implications for their utilization as biomarkers. Plos One 2013; 8: e75184.
6) Hosseini M, Khatamianfar S, Hassanian SM, Nedaeinia R, Shafiee M, Maftouh M, Ghayour-Mobarhan M, ShahidSales S, Avan A. Exosome-encapsulated microRNAs as potential circulating biomarkers in colon cancer. Curr Pharm Des 2017; 23: 1705-1709.
7) Monzo M, Santasusagna S, Moreno I, Martinez F, Hernandez R, Munoz C, Castellano JJ, Moreno J, Navarro A. Exosomal microRNAs isolated from plasma of mesenteric veins linked to liver metastases in resected patients with colon cancer. Oncotarget 2017; 8: 30859-30869.
8) Feng ZY, Xu XH, Cen DZ, Luo CY, Wu SB. miR-590-3p promotes colon cancer cell proliferation via Wnt/beta-catenin signaling pathway by inhibiting WIF1 and DKK1. Eur Rev Med Pharmacol Sci 2017; 21: 4844-4852.
9) Feber A, Xi L, Luketich JD, Pennathur A, Landreneau RJ, Wu M, Swanson SJ, Godfrey TE, Litle VR. MicroRNA expression profiles of esophageal cancer. J Thorac Cardiovasc Surg 2008; 135: 255-260, 260.
10) Lawrie CH, Soneji S, Marafioti T, Cooper CD, Palazzo S, Paterson JC, Cattan H, Enver T, Mager R, Boultwood J, Wainscoat JS, Hatton CS. MicroRNA expression distinguishes between germinal center B cell-like and activated B cell-like subtypes of diffuse large B cell lymphoma. Int J Cancer 2007; 121: 1156-1161.
11) Tran N, McLean T, Zhang X, Zhao CJ, Thomson JM, O'Brien C, Rose B. MicroRNA expression profiles in head and neck cancer cell lines. Biochem Biophys Res Commun 2007; 358: 12-17.
12) Iorio MV, Visone R, Di Leva G, Donati V, Petrocca F, Casalini P, Taccioli C, Volinia S, Liu CG, Alder H, Calin GA, Menard S, Croce CM. MicroRNA signatures in human ovarian cancer. Cancer Res 2007; 67: 8699-8707.
13) Markou A, Tsaroucha EG, Kaklamanis L, Fotinou M, Georgoulias V, Lianidou ES. Prognostic value of mature microRNA-21 and microRNA-205 overexpression in non-small cell lung cancer by quantitative real-time RT-PCR. Clin Chem 2008; 54: 1696-1704.
14) Schetter AJ, Leung SY, Sohn JJ, Zanetti KA, Bowman ED, Yanaihara N, Yuen ST, Chan TL, Kwong DL, Au GK, Liu CG, Calin GA, Croce CM, Harris CC. MicroRNA expression profiles associated with prognosis and therapeutic outcome in colon adenocarcinoma. JAMA 2008; 299: 425-436.
15) Goke A, Goke R, Knolle A, Trusheim H, Schmidt H, Wilmen A, Carmody R, Goke B, Chen YH. DUG is a novel homologue of translation initiation factor 4G that binds eIF4A. Biochem Biophys Res Commun 2002; 297: 78-82.
16) Shiota M, Izumi H, Tanimoto A, Takahashi M, Miyamoto N, Kashiwagi E, Kidani A, Hirano G, Masubuchi D, Fukunaka Y, Yasuniwa Y, Naito S, Nishizawa S, Sasaguri Y, Kohno K. Programmed cell death protein 4 down-regulates Y-box binding protein-1 expression via a direct interaction with Twist1 to suppress cancer cell growth. Cancer Res 2009; 69: 3148-3156.
17) Li L, Zhou L, Li Y, Lin S, Tomuleasa C. MicroRNA-21 stimulates gastric cancer growth and invasion by inhibiting the tumor suppressor effects of pro-

grammed cell death protein 4 and phosphatase and tensin homolog. J BUON 2014; 19: 228-236.

18) Zhu S, Wu H, Wu F, Nie D, Sheng S, Mo YY. MicroRNA-21 targets tumor suppressor genes in invasion and metastasis. Cell Res 2008; 18: 350-359.

19) Zhang J, Zhang HD, Yao YF, Zhong SL, Zhao JH, Tang JH. β-Elemene reverses chemoresistance of breast cancer cells by reducing resistance transmission via exosomes. Cell Physiol Biochem 2015; 36: 2274-2286.

20) Nishida N, Mimori K, Fabbri M, Yokobori T, Sudo T, Tanaka F, Shibata K, Ishii H, Doki Y, Mori M. MicroRNA-125a-5p is an independent prognostic factor in gastric cancer and inhibits the proliferation of human gastric cancer cells in combination with trastuzumab. Clin Cancer Res 2011; 17: 2725-2733.