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Original Article

Early diagnostic and prognostic value of serum exosomal miR-1246 in non-small cell lung cancer

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Abstract: Lung cancer is one of the most common malignant tumors. A growing body of evidence has demonstrated that circulating microRNAs (miRNAs) have great potential for the diagnosis and prognosis of lung cancer. In this study, we aimed to determine the clinical significance of serum exosomal miR-1246 in non-small cell lung cancer (NSCLC). Real-time PCR was performed to measure the expression level of serum exosomal miR-1246 in NSCLC patients. The correlations between the serum exosomal miR-1246 level and prognosis of NSCLC were then investigated. The expression of serum exosomal miR-1246 was significantly increased in NSCLC patients. Receiver operating characteristic (ROC) analysis showed that serum exosomal miR-1246 showed good performance for discriminating NSCLC patients from healthy controls and patients with non-malignant respiratory diseases. The level of serum exosomal miR-1246 was decreased following treatments, but increased in the cases with recurrence. In addition, serum exosomal miR-1246 level was strongly associated with lymph node metastasis and TNM stage. Survival analysis showed that the patients in the high serum exosomal miR-1246 group had poorer overall survival and disease-free survival. Multivariate analysis showed that serum exosomal miR-1246 level was an independent prognostic factor for NSCLC. In conclusion, serum exosomal miR-1246 might be a useful diagnostic and prognostic biomarker for NSCLC.

Keywords: Serum exosomal miR-1246, non-small cell lung cancer, prognosis, biomarker, early diagnosis

Introduction

Lung cancer is the leading cause of cancer-related mortality. In 2018, there were approximately 2,093,876 new cases of lung cancer and 1,761,007 deaths globally [1]. Non-small cell lung cancer (NSCLC) is the major type of lung cancer, accounting for approximately 85% of all cases [2]. The lung cancer cells tend to infiltrate into surrounding tissue and metastasize to distant organs, resulting in cancer progression and the unfavorable clinical outcome of NSCLC. Despite great advances in the therapies such as surgery, radiotherapy, chemotherapy and targeted therapy during the past decades, the 5-year survival rate for advanced-stage NSCLC remains very low. Therefore, it is important to explore novel biomarkers for early detection and prognosis of NSCLC [3, 4].

MicroRNAs (miRNAs) are a class of highly conserved, short and small (18-24 nucleotides) non-coding RNAs that play important roles in the regulation of translation and degradation of mRNAs through complimentary binding with the 3'-untranslated regions (UTRs) of target genes [5, 6]. Notably, previous studies have provided strong evidence that more than 1500 miRNAs might influence the expression of over 60% of human genes [7, 8]. MiRNAs have been found to play crucial roles in many biologic processes such as proliferation, migration, invasion, differentiation, and apoptosis [9]. In addition, aberrant expression of miRNAs is associated with various diseases including cancer. These small molecules can act as oncogenes and/or tumor suppressors, depending on their targeted genes and tumor microenvironment [10].

Exosomes are small vesicles and typically 30-150 nm in diameter. They carry a variety of substances such as proteins, short-chain peptides, lipids, mRNAs, and non-coding RNAs [11, 12]. MiRNAs are particularly abundant in the exosomes since they are protected by the dou-

Table 1. Correlation between serum exosomal miR-1246 expression and clinicopathologic findings in NSCLC

Finding	Number	Relative serum exosomal miR-1246		
		Low	High	*P*
Age				0.616
<60	47	22	25	
≥60	58	30	28	
Gender				0.727
Female	34	16	18	
Male	71	36	35	
Smoking				0.205
No	21	13	8	
Yes	84	39	45	
Primary location				0.766
Left lung	50	24	26	
Right lung	55	28	27	
Differentiation				0.121
Well and moderate	67	37	30	
Poor	38	15	23	
Lymph node metastasis				<0.001
No	65	42	23	
Yes	40	10	30	
TNM stage				<0.001
I-II	56	40	16	
III-IV	49	12	37	

ble lipid layer. Abnormal expression of circulating exosomal miRNAs has been demonstrated as an important biomarker for the diagnosis and prognosis of NSCLC. For instance, the expression levels of plasma exosomal miR-23b-3p, miR-10b-5p and miR-21-5p were increased in NSCLC, and upregulation of these plasma exosomal miRNAs was associated with unfavorable prognosis of NSCLC [13].

MiR-1246 has been shown to play a critical role in the initiation and progression of NSCLC [14, 15]. However, whether serum exosomal miR-1246 is aberrantly expressed in NSCLC and its potential clinical significance were unknown. Therefore, the present study aimed to elucidate the diagnostic and prognostic values of serum exosomal miR-1246 in NSCLC.

Materials and methods

Patients and serum samples

A total of 105 patients with NSCLC, 50 patients with non-malignant respiratory diseases (NMRD), and 50 healthy volunteers were enrolled in the study. Patients who had received any anti-cancer treatment prior to diagnosis of NSCLC were excluded. All the NSCLC cases were pathologically confirmed by two independent pathologists. Staging of NSCLC was based on the American Joint Committee on Cancer (AJCC) staging system. All clinical data were retrospectively collected from medical records and summarized in **Table 1**. For the patients with NMRD, 15 patients had asthma, 21 had bronchiectasis, and 14 patients had chronic obstructive pulmonary disease. This study was approved by the Ethics Committee of The Second Affiliated Hospital of Harbin Medical University, and conducted in compliance with the provisions of Declaration of Helsinki. All the participants provided written informed consent. The blood samples were taken from all the participants and subjected to centrifugation at 3,000 g for 15 min. The serum supernatant was collected and stored at -80°C for further use.

Quantitative reverse transcription PCR (qRT-PCR)

Total RNA was extracted from the serum samples with mirVana™ miRNA Isolation Kit (Ambion Inc., Austin, TX, USA) following the manufacturer's protocol. The concentration and quality of total RNA was measured using a NanoDrop 2000 (Thermo Fisher Scientific, Wilmington, DE, USA). The total RNA was reversed transcribed to cDNA using the PrimeScript RT Reagent Kit (Takara, Dalian, China). The amplification of cDNA was carried out through the miScript SYBR-Green PCR Kit (QIAGEN Inc, Valencia, CA, USA) on the ABI 7500 Real-Time PCR System (Applied Biosystems, Foster, CA, USA). The PCR conditions were 95°C for 5 min, followed by 40 cycles of 94°C for 15 sec, 55°C for 30 sec. The Caenorhabditis elegans miRNA cel-miR-39 was used as a synthetic spike-in control RNA. The $2^{-\Delta\Delta Ct}$ method of relative quantification was used for comparing the expres-

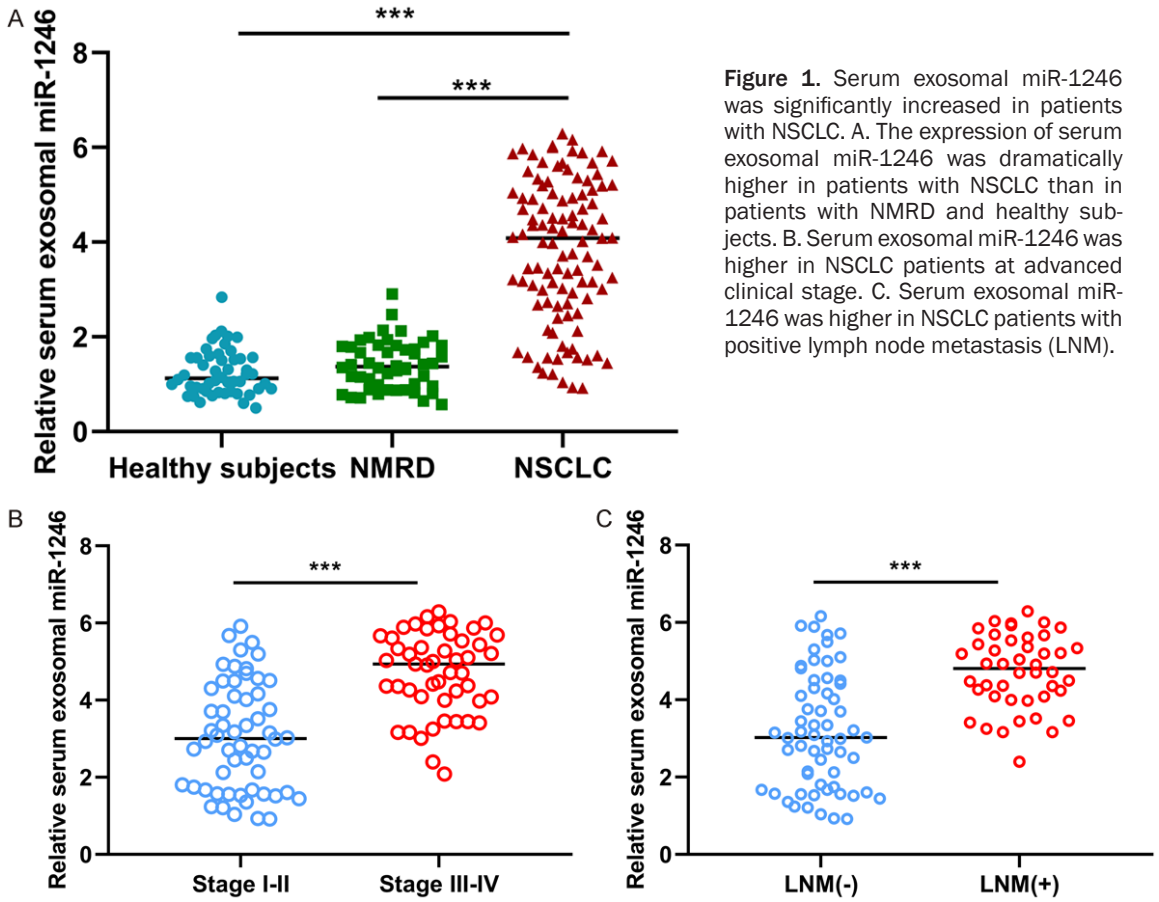

Figure 1. Serum exosomal miR-1246 was significantly increased in patients with NSCLC. A. The expression of serum exosomal miR-1246 was dramatically higher in patients with NSCLC than in patients with NMRD and healthy subjects. B. Serum exosomal miR-1246 was higher in NSCLC patients at advanced clinical stage. C. Serum exosomal miR-1246 was higher in NSCLC patients with positive lymph node metastasis (LNM).

sion of serum exosomal miR-1246 in different groups.

Enzyme-linked immunosorbent assay

The expression of carcinoembryonic antigen (CEA) in NSCLC patients and healthy subjects was examined with the Human Carcino-Embryonic Antigen ELISA Kit (Abcam, Cambridge, MA, USA).

Statistical analysis

All the statistical analyses were performed with GraphPad Prism version 6 (GraphPad Software, San Diego, CA, USA). The Mann-Whitney U test or Kruskal-Wallis test was used to compare the serum exosomal miR-1246 level in different groups. Associations between serum exosomal miR-1246 expression and clinicopathologic values were analyzed using the chi-square test. The diagnostic performance of serum exosomal miR-1246 was analyzed by receiver operating characteristic (ROC) curve and the area under the curve (AUC). Survival curve was performed using Kaplan-Meier method and log-rank test. Cox proportional hazards model was used for multivariate analysis. $P<0.05$ was considered significant.

Results

Serum exosomal miR-1246 is significantly upregulated in NSCLC

The relative levels of serum exosomal miR-1246 in patients with NSCLC, patients with NMRD, and healthy controls were compared by qRT-PCR. Our results showed that the expression of serum exosomal miR-1246 was markedly higher in patients with NSCLC than in patients with NMRD and healthy controls (***$P<0.001$) (**Figure 1A**). In addition, the NSCLC patients at advanced clinical stage or with lymph node metastasis had higher levels of serum exosomal miR-1246 compared to their respective controls (***$P<0.001$) (**Figure 1B**, **1C**).

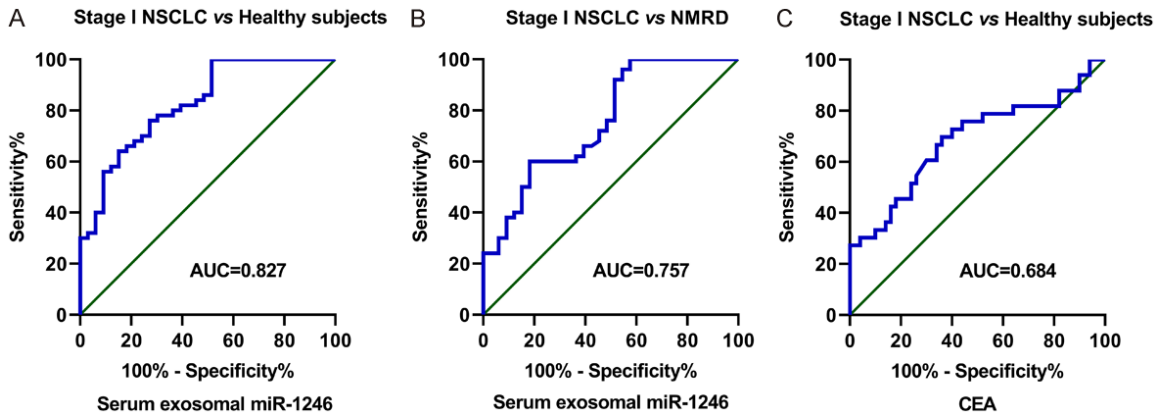

Figure 2. Diagnostic value of serum exosomal miR-1246 for NSCLC. A. The AUC value of serum exosomal miR-1246 for discriminating stage I NSCLC patients from healthy controls was 0.827. B. The AUC value of serum exosomal miR-1246 for discriminating stage I NSCLC patients from patients with NMRD was 0.757. C. The AUC value of CEA for discriminating stage I NSCLC patients from healthy controls was 0.684.

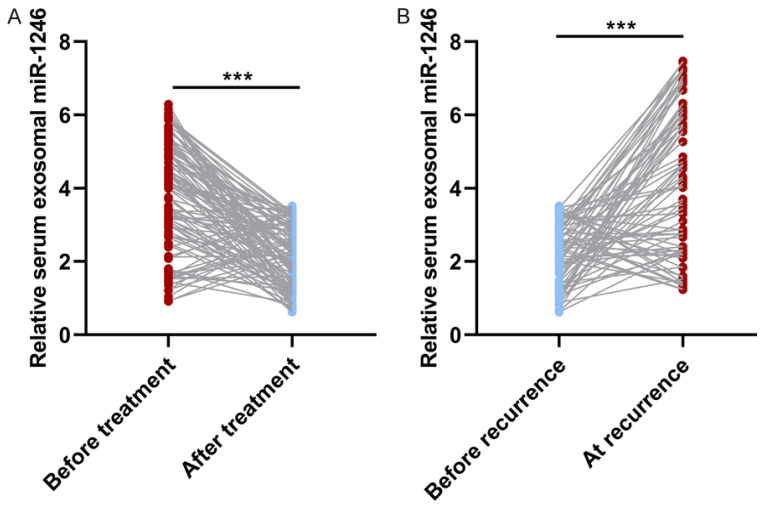

Figure 3. Association between serum exosomal miR-1246 and therapeutic response and recurrence. A. The expression level of serum exosomal miR-1246 was significantly decreased following treatment. B. Serum exosomal miR-1246 was increased in cases with recurrence.

Diagnostic value of serum exosomal miR-1246 for early stage NSCLC

A total of 33 NSCLC cases were at stage I. We evaluated the diagnostic value of serum exosomal miR-1246 for detecting the NSCLC at an early stage. ROC analysis revealed that serum exosomal miR-1246 discriminated early stage NSCLC patients from healthy subjects, with an AUC value of 0.827 (**Figure 2A**). The AUC value of serum exosomal miR-1246 that discriminated early stage NSCLC patients from patients with NMRD was 0.757 (**Figure 2B**). The AUC value of traditional tumor marker CEA that discriminated early stage NSCLC patients from healthy controls was 0.684 (**Figure 2C**).

Serum exosomal miR-1246 deceased following treatments and increased with recurrence

We compared the expression levels of serum exosomal miR-1246 before and after treatments. Our results showed that the serum exosomal miR-1246 was significantly decreased following treatments (***P< 0.001) (**Figure 3A**). A total of 74 cases suffered recurrence, and the expression of serum exosomal miR-1246 was significantly upregulated at the time of recurrence (***P<0.001) (**Figure 3B**).

Correlation between serum exosomal miR-1246 and clinical outcome of NSCLC

Based on the median level of serum exosomal miR-1246 in NSCLC patients, all NSCLC cases were divided into a high serum exosomal miR-1246 group and a low serum exosomal miR-1246 group. High serum exosomal miR-1246 expression was positively associated with advanced TNM stage (P<0.001) and lymph node metastasis (P<0.001) (**Table 1**). Survival analysis showed that the NSCLC patients in the high serum exosomal miR-1246 group had worse overall survival (OS) (P=0.0002) and disease-

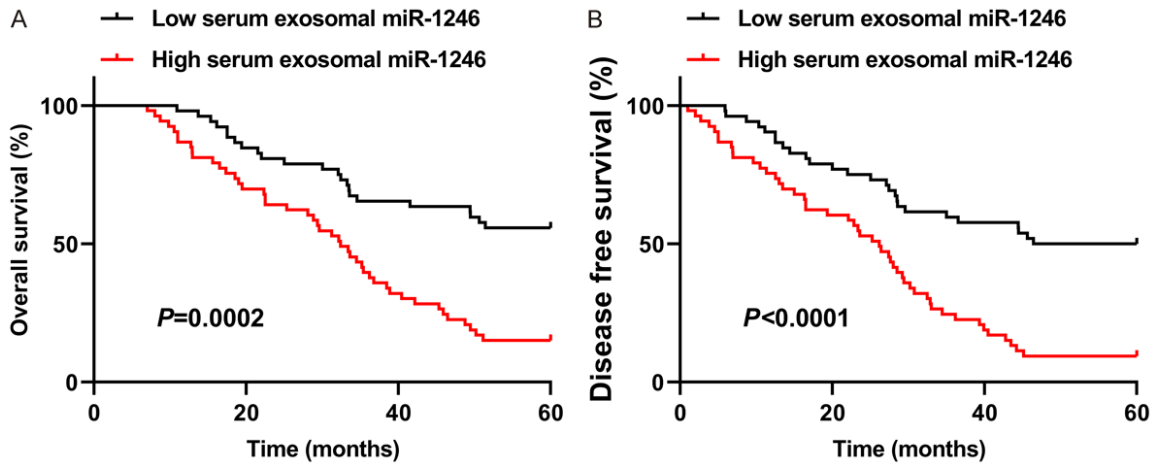

Figure 4. Association between serum exosomal miR-1246 and survival in NSCLC. A. NSCLC patients in the high serum exosomal miR-1246 group had worse OS than those in the low serum exosomal miR-1246 group. B. NSCLC patients in the high serum exosomal miR-1246 group had shorter disease-free survival than those in the low serum exosomal miR-1246 group.

Table 2. Multivariate analysis of factors significantly associated with OS of NSCLC

Factor	Hazard Ratio	95% confidence interval	*P*
Age	1.127	0.741-1.729	0.521
Gender	1.318	0.785-2.244	0.305
Smoking	1.206	0.712-1.960	0.443
Primary location	0.852	0.458-1.426	0.812
Differentiation	1.684	0.974-3.218	0.149
Lymph node metastasis	2.658	1.414-5.713	0.007
TNM stage	3.841	1.891-8.230	<0.001
Serum exosomal miR-1246	2.540	1.281-4.686	0.012

free survival ($P<0.0001$) than patients in the low serum exosomal miR-1246 group (**Figure 4A**, **4B**). The multivariate analysis indicated that TNM stage ($P<0.001$), lymph node metastasis ($P=0.007$), and serum exosomal miR-1246 ($P=0.012$) were independent prognostic factors for OS of NSCLC (**Table 2**).

Discussion

Early detection and prognosis of NSCLC is important for reducing mortality. Unfortunately, currently no reliable biomarker is available for NSCLC. In the present study, we demonstrated that the serum exosomal miR-1246 was significantly increased in patients with NSCLC. In addition, upregulation of serum exosomal miR-1246 discriminated early stage NSCLC from healthy controls and patients with NMRD. The expression of serum exosomal miR-1246 was decreased following treatments, and increased in those cases with recurrence. Moreover, strong correlations were observed between increased serum exosomal miR-1246 and aggressive clinicopathologic measures as well as poor OS/RFS. Multivariate analysis revealed that serum exosomal miR-1246 was an independent risk factor for NSCLC. These findings indicate that serum exosomal miR-1246 might be a biomarker for the early detection and prognosis of NSCLC.

Several studies have explored the role of miR-1246 in NSCLC. For example, downregulation of miR-1246 inhibited the expression of stemness markers and epithelial-mesenchymal transition markers in NSCLC. In addition, miR-1246 inhibition suppressed proliferation, sphere-formation, colony formation and invasion of NSCLC cells [16], indicating that miR-1246 plays an oncogenic role in tumorigenesis of NSCLC. Yang et al. demonstrated that the expression of circulating miR-1246 was increased in patients with NSCLC. Enforced expression of miR-1246 promoted the migration and invasion capacity of lung cancer cells [17]. MiR-1246 seems to be closely linked with radioresistance of lung cancer cells. The levels of intracellular and extracellular miR-1246 were both upregulated following irradiation, and increased miR-1246 was strongly correlated with radiore-

sistance of NSCLC cells [14]. MiR-1246 promoted lung cancer cell proliferation and enhanced radioresistance through targeting DR5, indicating that targeting miR-1246 might be a useful strategy to improve the therapeutic outcome of NSCLC [15].

MiR-1246 has also been identified as a tumor promoter in other types of cancer. For instance, Chen et al. found that miR-1246 promoted proliferation, migration, and invasion of cervical cancer cells by inhibiting thrombospondin 2 [18]. In addition, the expression of serum miR-1246 was significantly upregulated in cervical squamous cell carcinoma patients with lymph node metastasis compared to those without lymph node metastasis [19]. Similarly, the expression level of miR-1246 was significantly high in colorectal cancer (CRC) tissues and cell lines [20]. Interestingly, serum exosomal miR-1246 was also found to be increased in CRC patients compared to healthy controls. The level of serum exosomal miR-1246 was significantly down-regulated after surgical treatment [21]. Therefore, upregulation of serum exosomal miR-1246 is not specific for NSCLC. Combining serum exosomal miR-1246 and traditional tumor biomarkers as well as clinicopathologic data will improve early detection and prognosis.

MiR-1246 may also act as a tumor suppressor in cancer. Interestingly, miR-1246 was decreased in lung cancer cell lines. Ectopic expression of miR-1246 suppressed the invasion and epithelial mesenchymal transition of lung cancer cells *in vitro*, indicating that miR-1246 might play a tumor suppressive role in lung cancer [22]. These findings need further investigation. It is possible that the role of miR-1246 at different stages of lung cancer might be different. In addition, the *in vitro* findings might not reflect the actual situations *in vivo*.

In conclusion, for the first time, our findings have demonstrated that serum exosomal miR-1246 level is significantly increased in patients with NSCLC. In addition, upregulation of serum exosomal miR-1246 is closely correlated with diminished survival and poor prognosis of NSCLC. Therefore, serum exsomal miR-1246 is a promising biomarker for early detection and prognosis.

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Disclosure of conflict of interest

None.

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References

[1] Siegel RL, Miller KD and Jemal A. Cancer statistics, 2018. CA Cancer J Clin 2018; 68: 7-30.

[2] Molina JR, Yang P, Cassivi SD, Schild SE and Adjei AA. Non-small cell lung cancer: epidemiology, risk factors, treatment, and survivorship. Mayo Clin Proc 2008; 83: 584-594.

[3] Domvri K, Zarogoulidis P, Darwiche K, Browning RF, Li Q, Turner JF, Kioumis I, Spyratos D, Porpodis K, Papaiwannou A, Tsiouda T, Freitag L and Zarogoulidis K. Molecular targeted drugs and biomarkers in NSCLC, the evolving role of individualized therapy. J Cancer 2013; 4: 736-754.

[4] Wood SL, Pernemalm M, Crosbie PA and Whetton AD. Molecular histology of lung cancer: from targets to treatments. Cancer Treat Rev 2015; 41: 361-375.

[5] Han T, Mei Y, Wang Y and Feng Z. miR-5582-5p inhibits cell proliferation of non-small cell lung cancer through targeting FGF-10. Int J Clin Exp Pathol 2019; 12: 1087-1094.

[6] He L and Hannon GJ. MicroRNAs: small RNAs with a big role in gene regulation. Nat Rev Genet 2004; 5: 522-31.

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

[8] Kozomara A and Griffiths-Jones S. miRBase: integrating microRNA annotation and deep-sequencing data. Nucleic Acids Res 2011; 39: 152-7.

[9] Jiang R, Zhang C, Liu G, Gu R and Wu H. MicroRNA-101 inhibits proliferation, migration and invasion in osteosarcoma cells by targeting ROCK1. Am J Cancer Res 2017; 7: 88-97.

[10] Lan H, Lu H, Wang X and Jin H. MicroRNAs as potential biomarkers in cancer: opportunities

and challenges. Biomed Res Int 2015; 2015: 125094.
[11] Sun L, Liu X, Pan B, Hu X, Zhu Y, Su Y, Guo Z, Zhang G, Xu M, Xu X, Sun H and Wang S. Serum exosomal miR-122 as a potential diagnostic and prognostic biomarker of colorectal cancer with liver metastasis. J Cancer 2020; 11: 630-637.
[12] Zaborowski MP, Balaj L, Breakefield XO and Lai CP. Extracellular vesicles: composition, biological relevance, and methods of study. Bioscience 2015; 65: 783-797.
[13] Liu Q, Yu Z, Yuan S, Xie W, Li C, Hu Z, Xiang Y, Wu N, Wu L, Bai L and Li Y. Circulating exosomal microRNAs as prognostic biomarkers for non-small-cell lung cancer. Oncotarget 2017; 8: 13048-13058.
[14] Fan L, Wang J, Cao Q, Ding X and Li B. Aberrant miR-1246 expression promotes radioresistance in non-small cell lung cancer: a potential prognostic biomarker and radiotherapy sensitization target. Am J Cancer Res 2020; 10: 314-335.
[15] Yuan D, Xu J, Wang J, Pan Y, Fu J, Bai Y, Zhang J and Shao C. Extracellular miR-1246 promotes lung cancer cell proliferation and enhances radioresistance by directly targeting DR5. Oncotarget 2016; 7: 32707-32722.
[16] Kim G, An HJ, Lee MJ, Song JY, Jeong JY, Lee JH and Jeong HC. Hsa-miR-1246 and hsa-miR-1290 are associated with stemness and invasiveness of non-small cell lung cancer. Lung Cancer 2016; 91: 15-22.
[17] Yang F, Xiong H, Duan L, Li Q, Li X and Zhou Y. MiR-1246 promotes metastasis and invasion of A549 cells by targeting GSK-3β-mediated Wnt/β-Catenin pathway. Cancer Res Treat 2019; 51: 1420-1429.
[18] Chen J, Yao D, Zhao S, He C, Ding N, Li L and Long F. MiR-1246 promotes SiHa cervical cancer cell proliferation, invasion, and migration through suppression of its target gene thrombospondin 2. Arch Gynecol Obstet 2014; 290: 725-32.
[19] Chen J, Yao D, Li Y, Chen H, He C, Ding N, Lu Y, Ou T, Zhao S, Li L and Long F. Serum microRNA expression levels can predict lymph node metastasis in patients with early-stage cervical squamous cell carcinoma. Int J Mol Med 2013; 32: 557-67.
[20] Della Vittoria Scarpati G, Calura E, Di Marino M, Romualdi C, Beltrame L, Malapelle U, Troncone G, De Stefano A, Pepe S, De Placido S, D'Incalci M, Marchini S and Carlomagno C. Analysis of differential miRNA expression in primary tumor and stroma of colorectal cancer patient. Biomed Res Int 2015; 2114: 840921.
[21] Ogata-Kawata H, Izumiya M, Kurioka D, Honma Y, Yamada Y, Furuta K, Gunji T, Ohta H, Okamoto H, Sonoda H, Watanabe M, Nakagama H, Yokota J, Kohno T and Tsuchiya N. Circulating exosomal microRNAs as biomarkers of colon cancer. PLoS One 2014; 9: e92921.
[22] Yuan D, Xu J, Wang J, Pan Y, Fu J, Bai Y, Zhang J and Shao C. Extracellular miR-1246 promotes lung cancer cell proliferation and enhances radioresistance by directly targeting DR5. Oncotarget 2016; 7: 32707-22.