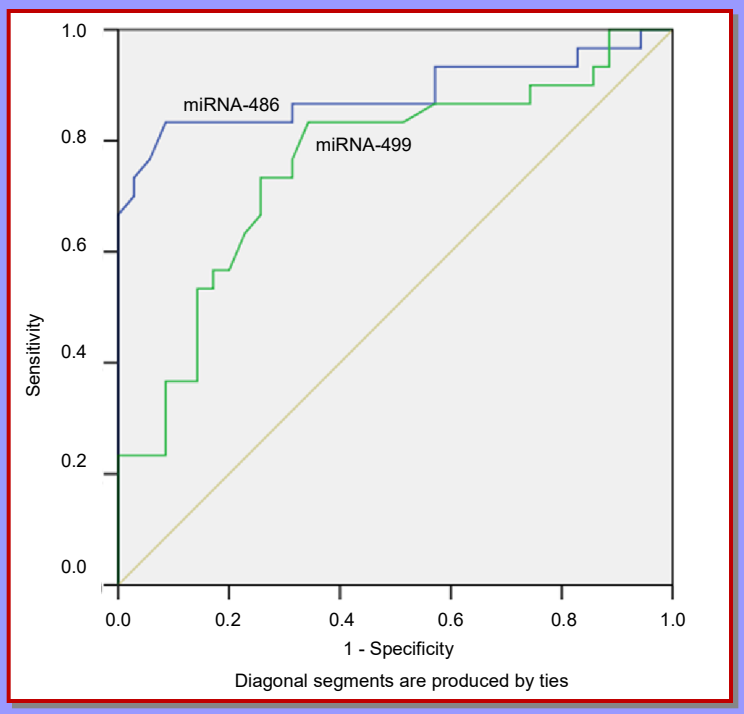

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miRNA-486 and miRNA-499 in human plasma evaluate the clinical stages of lung cancer and play a role as a tumor suppressor in lung tumorigeneisis not pathogenesis

Youchao Jia[1], Aimin Zang[1], Yanguang Feng[2], Xiao-Fang Li[1], Ke Zhang[3], Hefei Li[3], Ruiyao Wang[3], Yaning Wei[1] and Ran Huo[1]

[1]Hebei Key Laboratory of Cancer Radiotherapy and Chemotherapy, Department of Medical Oncology, Affiliated Hospital of Hebei University, Baoding, Hebei 071000, P. R. China; [2]Department of Internal Medicine, People's Hospital of Qing Yuan District, Hebei, Baoding 071 100, P. R. China; [3]Department of Thoracic surgery, Affiliated Hospital of Hebei University, Hebei, Baoding 071 000, P. R. China.

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Abstract

It was aimed to explore the expression level of miRNA-486 and miRNA-499 in the plasma of lung cancer patients and analysis their differences in expression. The expression level of both miRNA-486 and miRNA-499 in the plasma of non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC) were lower than that of the control group ($p<0.05$) and the decrease was more obvious in NSCLC. Compare with the miRNA-499, expression quantity in NSCLC patients plasma. There was statistical significance difference ($p<0.05$) between III～Ⅳstage and I～II stage. The expression quantity of miRNA in plasma of patients with extensive-stage SCLC was lower than that of patients with limited-stage SCLC ($p<0.05$). The sensitivity and specificity of plasms miRNA-486 respectively were 88.5% and 83.3%. The expression of miRNA-499 and miRNA-486 in lung cancer patients were up-regulated, and might be closely related to the occurrence and prognosis of lung cancer, and might be used as potential screening and prognosis index for lung cancer.

Introduction

miRNAs are tiny regulatory RNA that function to modulate the activity of specific mRNA and play important roles physiologic and pathologic processes, including tumorigenesis (Stefani and Slack, 2008). miRNAs are protected from endogenous RNase activity which is in a observably stable state in human plasma (Mitchell et al., 2008; Chen et al., 2008). miRNAs can be preserved stably from plasma in the ultra external environment (strong acids and alkalies, high temperature), that is the essential condition to serve as a useful biomarker for cancer diagnosis (Ho et al., 2010; Taylor and Gercel-Taylor, 2008; Huang et al., 2010). miR-486 is one of the most down-regulated micro RNAs in lung cancer (Wang et al., 2014). miR-486-5p may act as a tumor-suppressor, would provide potential diagnostic and therapeutic targets for the disease, contributing to the progression and metastasis of non-small cell lung cancer (NSCLC) by targeting ARHGAP5 (Wang et al., 2014).

miR-486 directly targets components of insulin growth factor (IGF) signaling including insulin-like growth factor 1 (IGF1), IGF1 receptor (IGF1R), and phosphoinositide-3-kinase, regulatory subunit 1 (alpha) (PIK3R1, or p85a) and functions as a potent tumor suppressor of lung cancer both *in vitro* and *in vivo* (Peng et al., 2013). miR-499 could contribute to poor prognosis by modulating cancer-related genes' expression and thus involve tumorigenesis and anti-chemotherapy (Qiu et al., 2015). It may influence the expression of a

series cancer-related genes that might be annotated to immunity and defense, cell growth, tumor invasion and metastasis, cancer stem cell, and cell death, although no available evidences supported any genes to be known or predicted target genes of miR-499. We show here that miR-486 and miR-499 are down-regulated in patients' plasma with lung cancer. Plasma samples were obtained from newly diagnosed lung cancer patients and non-cancer controls. The expression level of both miRNA-486 and miRNA-499 in the plasma of NSCLC and small cell lung cance (SCLC) is lower than that of the control group ($p<0.05$), and the decrease is more obvious in NSCLC. Compared with expression of miRNA-499 with NSCLC, there is statistical significance difference ($p<0.05$) between III～Ⅳstage and I～II stage. Nevertheless, the expression of miRNA-486 in plasma of patients with extensive-stage SCLC is lower than that of patients with limited-stage SCLC ($p<0.05$). The sensitivity and specificity of miR-486 is more precise than miR-499. Furthermore, the study provided novel evidence that the origination of lung cancer independent of miRNA-486 & 499's function.

Materials and Methods

Cohorts

Plasma samples for lung cancer patients and controls were collected and archived between January 2014 and January 2015. There were 35 lung cancer (NSCLC 21, SCLC14) and 30 control cases (pneumonia and pulmonary tuberculosis). All samples were collected before any treatment, and miR-486 and miR-499 levels were analyzed respectively from these groups.

Plasma extraction

Plasma was extracted by centrifuging whole blood at 2,000 rpm for 10 min at 4°C in 2 hours after whole blood was obtained from each patient and then frozen as separate aliquots at −80°C for storage. 400 µL of thawed plasma was boiled for 10 min at 100°C and then centrifuged at 13,000 xg for 2 min at 4°C.

RNA isolation and cDNA synthesis

miRNA was extracted directly from plasma by QIAzol Lysis Reagent and reverse-transcribed to complementary DNA. The RNA concentration was determined by measuring the absorbance at 260 nm using the NanoDrop ND1000 spectrophotometer and the purity of the RNA was estimated using the OD260/280 ratio. The RNA integrity was assessed by standard denaturing agarose gel electrophoresis, then the RNAs were used for labeling and array hybridi-zation. miR-486 & 499 and Caenorhabditis elegans miR-39 (cel-miR-39) were then measured using quantitative reverse transcription polymerase chain reaction. the RNA was converted into cDNA using All-in-One™ miRNA qRT-PCR Detection kit. Reaction system: Total RNA 2 uL，2.5 U/µL l Poly A Polymerase 1 µL，RTase Mix 1 µL，5X Reaction Buffer 5 µL，UTR-primer (50 µM) 1 µL, ddH_2O (RNase/DNase free) to 25µL. Hsa-miR-486: GATCAATCCTG TACTGAGCTGC. Hsa-miR-499: GGACGTCGTTA AGCTTGCAGTGA. cel-miR-39: GTACTCACCGGGT GTAAATCAG.

Reverse transcription-PCR

RT-PCR was performed by an Applied Biosystems 7500 real-time PCR system, with miR-486 & 499 and cel-miR-54 quantified using 2 × All-in-One qPCR Mix. 25 µL dH_2O was mixed with 2 × All-in-One qPCR Mix 10 µL, qPCR Forward Primer (2 µM) 2 µL, Universal PCR primer (2 µM) 1 µL, First strand cDNA 2 µL，ddH2O 5 µL. RT-PCR was then performed at 95°C for 10 min at 95°C for 10 sec at 60°C for 20 sec and at 72°C for 10 sec with the last three steps repeated for a total of 40 cycles. Each reaction was performed in triplicate.

Statistical treatment

A known quantity of synthetic cel-miR-39 was added for normalization after miR-486 & 499 and cel-miR-54 were then measured using quantitative reverse transcription polymerase chain reaction. Data was analyzed using the $2^{-\Delta\Delta CT}$ method. Analyze expression of microRNAs with clinical cases data by non-parametric test for non-normal distribution. Wilcoxon test is performed on two groups comparing and Kruskall-Wallis test is performed on multiunit groups.

Results

The expression level of both miRNA-486 and miRNA-499 in the plasma of NSCLC and SCLC is lower than that of the control group ($p<0.05$), and the decrease is more obviously NSCLC. There was a statistical significance on every comparing but the expression of miR-499 in between SCLC with controls, or SCLC with NSCLC (Figure 1).

There was no statistical significance between the expression of miR-486 with gender, age, smoking, pathologic types, differentiation degree, extent of the primary tumor (T) and regional lymph nodes (N) ($p>0.05$), but distant metastases (M) ($p = 0.015$) (Edge and Compton, 2010) in SCLC. The expression of miR-499 don't have any statistical significance on clinicopathologic features in SCLC (Table I).

There was no statistical significance between the expression of miR-486 with clinicopathologic features in NSCLC. There was no statistical significance between the expression of miR-499 with gender, age, smoking, pathologic types, differentiation degree, extent of the primary tumor (T) and regional lymph nodes (N) ($p>0.05$), but III/IV stage with I/II stage ($p = 0.073$) and

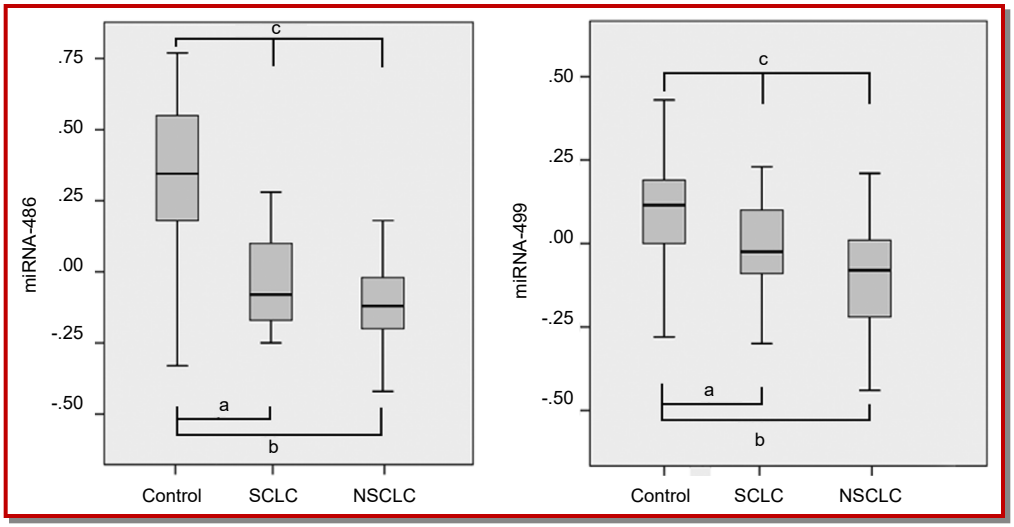

Figure 1: The expression of miR-486 and miR-499 of control group, SCLC and NSCLC. miRNA-486 was down-regulated in SCLC ([a]p<0.01) and the decrease is more obviously NSCLC ([b]p<0.01). There were significant differences among the three groups ([c]H = 28.712, p<0.001). The level of miR486 in SCLC rank above it in NSCLC (p<0.05). miRNA-489 was down-regulated in SCLC ([a]p>0.05) and the decrease is more obviously NSCLC ([b]p<0.05). There were significant differences among the three groups. ([c]H = 14.256, p = 0.001). The level of miR486 in SCLC rank above it in NSCLC (p<0.05). There was not statistically significant between SCLC with NSCLC (p>0.05)

Table I

Relationship between the expression of the miRNA-486 and miRNA-499 in the plasma of SCLC with clinico-pathologic features of lung cancer

		miRNA-486		miRNA-499	
		P_{50} (P_{25}~P_{75})[a]	p	P_{50} (P_{25}~P_{75})	p
Gender	Man	-0.12 (-0.19~0.02)	0.161	-0.04 (-0.1~-0.02)	0.051
	Women	0.1 (-0.05~0.15)		0.15 (0.07~0.20)	
Age	≤60 years	-0.05 (-0.19~0.04)	0.894	-0.09 (-0.25~0.10)	0.161
	>60 years	-0.11 (-0.18~0.13)		-0.02 (-0.04~0.13)	
Smoking	No	0.11(-0.01~0.13)	0.054	0.19 (0.09~0.23)	0.057
	Yes	-0.14 (-0.2~-0.04)		-0.04 (-0.13~-0.02)	
Lymph node metastasis	No	0.13 (0.1~0.25)	0.065	0.19 (0.11~0.23)	0.068
	Yes	-0.04 (-0.1~-0.05)		0.04 (0.13~-0.02)	
Tumor size	≤4 cm	0.11 (-0.18~0.18)	0.142	0.13 (0.04~0.22)	0.129
	>4 cm	0.02 (0.09~-0.05)		0.04 (0.17~-0.02)	
Stage	Limited	0.13 (0.10~0.25)	0.015	0.09 (0.05~0.23)	0.073
	Extensive	-0.14 (-0.20~-0.05)		-0.04 (-0.13~-0.02)	

low differentiation group with high differentiation group (p<0.05) (Table II).

There had more diagnostic value of miR-486 (0.790~0.979) than miR-499 (0.635~0.878) by receiver operating characteristic curve (p<0.05) (Figure 2).

Discussion

Several miRNAs were found to be altered more than 5-fold between longer-survival and shorter-survival groups, and levels of miR-486 and miR-499 were significantly associated with overall survival. The microRNA signature also was consistently an independent predictor of overall survival (Hu et al., 2010). The microRNA signature from the serum may serve as a non-invasive predictor for the overall survival of NSCLC.

miR-486 loss might be important in lung cancer development. miR-486 displayed lower expression levels in lung tumor tissues compared with the paired normal lung tissues (Shen et al., 2011). The findings in both surgical tissues and plasma specimens suggest that miR-486 down-regulation might play a role as a tumor suppressor in lung tumorigeneisis (Shen et al., 2011). We are pursuing a study to investigate possible mechanism of down-regulation of miR-486 in the develop-

Table II

Relationship between the expression of the miRNA-486 and miRNA-499 in the plasma of NSCLC with clinicopathologic features of lung cancer

		miRNA-486		miRNA-499	
		P_{50} (P_{25}~P_{75})[a]	p	P_{50} (P_{25}~P_{75})	p
Gender	Man	-0.13 (-0.3~0.03)	0.999	-0.04 (-0.1~-0.02)	0.831
	Women	-0.12 (-0.2~-0.04)		-0.1 (-0.16~-0.01)	
Age	≤60 years	-0.15 (-0.30~0.10)	0.972	-0.07 (-0.31~0.07)	0.803
	>60 years	-0.11 (-0.20~-0.07)		-0.10 (-0.16-0)	
Smoking	No	-0.15 (-0.32~-0.05)	0.307	-0.08 (-0.22~-0.01)	0.778
	Yes	-0.1 (-0.21~-0.04))		-0.08 (-0.25~-0.01))	
pathologic types	squamous carcinoma	-0.1 (-0.15~0.11)	0.232	-0.05 (-0.22~0.08)	0.681
	adenocarcinoma	-0.17 (-0.26~-0.04)		-0.09 (-0.23~0.01)	
Lymph node metastasis	No	-0.02 (-0.14~0.08)	0.149	0.01 (-0.04~0.08)	0.121
	Yes	-0.04 (-0.03~-0.06)		-0.07 (-0.10~-0.03)	
Tumor size	≤4 cm	-0.1 (-0.18~0.05)	0.169	-0.06 (-0.11~0.02)	0.158
	>4 cm	-0.18 (-0.32~-0.04)		-0.09 (-0.18~-0.03)	
TNM	I/II stage	0.07 (-0.11~0.13)	0.059	0.05 (-0.01~0.17)	0.003
	III/IV stage	-0.03 (0.02~-0.1)		-0.14 (-0.26~-0.05)	

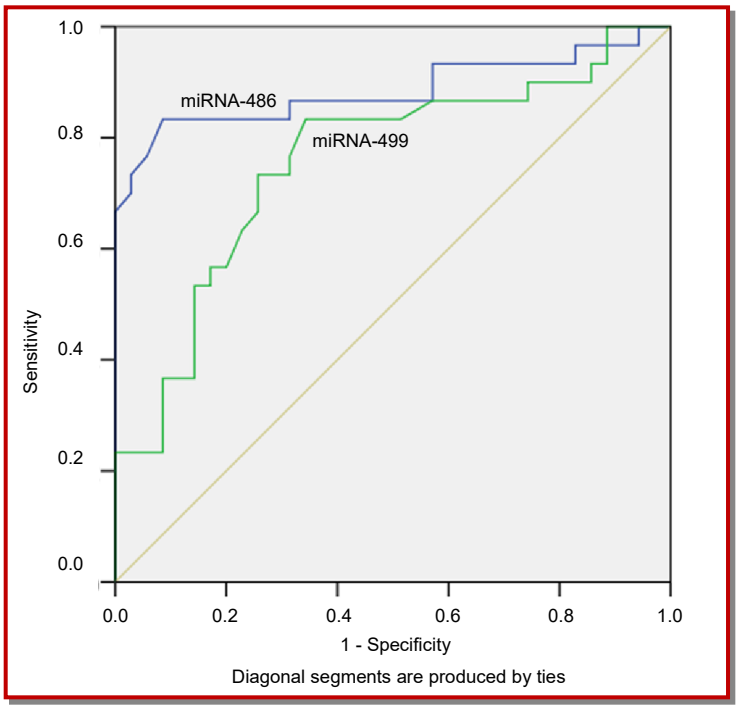

Figure 2: The evaluation of miR-486 and miR-499 on diagnosis of lung cancer by receiver operating characteristic curve. ROC curve of miR-486 and miR-499 for the diagnosis of lung cancer. The area under the ROC curve was 0.885 and 0.757 respectively ($p<0.05$). Diagonal segments are produced by ties

ment and progress of lung cancer. In our study, the expression level of both miRNA-486 in the plasma of NSCLC and SCLC is lower than that of the control group, and the decrease is more obviously NSCLC (Figure 1). As the one part of microRNA groups, miR-486 remains maintain expressing on finite concentration, it down-regulated as the suppressor with tumor formation. So, it could not down-regulated enough in SCLC which is more invasive than NSCLC. It just explained the result in above paragraphs that no statistical significance between the expression of miR-486 with clinicopathologic features which associated with pathogenesis and risk in NSCLC and SCLC but extensive-stage SCLC is lower than that of patients with limited-stage SCLC. At the same time, miR-486 can be a predictive biomarker for prognosis of lung cancer to

monitor the neoplasm recurrence.

miR-499 levels are higher in patients with stable chronic obstructive pulmonary diseases (COPD) compared with controls (Donaldson et al., 2013) and there was none association between miR-499 with risk of NSCLC (Vinci et al., 2011). We got the similar data that the expression of miR-499 don't have any statistical significance on clinicopathologic features in SCLC and NSCLC, but I/II stage with III/IV stage and differentiation degree in NSCLC. miR-499 could contribute to poor prognosis by modulating cancer-related genes' expression and thus involve tumorigenesis and anti-chemotherapy, which maybe a useful biomarker to predict lung cancer patients' prognosis (Qiu et al., 2015).

Conclusion

miR-486 and miR-499, as a tumor suppressor in lung tumorigeneisis, might be evaluate the clinical stages of lung cancer and predict lung cancer patients' prognosis. But, they were not directly associated with risk and pathogenesis for lung cancer.

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Conflict of Interest

Authors declare no conflict of interest

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Author Info
Xiao-Fang Li (Principal contact)
e-mail: xiaofang_li33@sina.com

First two authors contributed equally