Boosting Influences From circ_0048856 On Non-Small Cell Lung Cancer Advancement Via Aiming At miR-1287-5p

Yinzai He

Inner Mongolia People's Hospital

Yanheng Liu

Inner Mongolia People's Hospital

Nier Cha

Inner Mongolia People's Hospital

Yanwei Gao

Inner Mongolia People's Hospital

Feng Li

Inner Mongolia People's Hospital

Meng Zhang

Inner Mongolia People's Hospital

Wei Luan (✉ okdhnd@126.com)

Inner Mongolia People's Hospital <https://orcid.org/0000-0002-7327-7567>

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Abstract

Background: Non small cell lung cancer (NSCLC) is one of the main causes of death in human diseases. In this study, we aimed to explore the prognostic value of serum *circ_0048856* in patients with NSCLC and its relationship with *miR-1287-5p*.

Methods: The expression levels of *circ_0048856* and *miR-1287-5p* in serum of NSCLC patients and healthy people were detected by qRT-PCR. The relationships between *circ_0048856* expression and clinical data were evaluated via χ^2 test. Overall survival analysis was carried out via Kaplan-Meier method with log rank test. Cox regression analysis was taken to evaluate the prognostic ability of *circ_0048856* in NSCLC. Luciferase reporter gene was used to detect the targeting relationship between *circ_0048856* and *miR-1287-5p*.

Results: The expression of *circ_0048856* in serum of NSCLC patients was significantly higher than that of healthy volunteers ($P < 0.001$). And its high expression was significantly correlated with TNM stage ($P = 0.002$) and lymph node metastasis ($P < 0.001$). In addition, Kaplan Meier log rank test analysis showed that the overall survival of patients with high expression of *circ_0048856* was significantly shorter than that of patients with low expression of *circ_0048856* (log rank test, $P = 0.002$). Serum *circ_0048856* expression is an independent prognostic biomarker in NSCLC patients. Furthermore, we found that *miR-1287-5p* is a direct downstream miRNA of *circ_0048856* through luciferase reporter gene analysis, and there is a negative correlation between *circ_0048856* and *miR-1287-5p*.

Conclusion: Serum *circ_0048856* is an independent prognostic biomarker in patients with NSCLC. *Circ_0048856* can participate in the progression of NSCLC by targeting *miR-1287-5p*.

Background

Lung cancer is a highly heterogeneous cancer [1]. Every year, about 1.8 million people are diagnosed with lung cancer and 1.6 million people die of lung cancer. The 5-year survival rate is very low, which is usually between 4–17% due to the stage and regional differences [2]. Lung cancer mainly includes non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC), accounting for 76% and 13% respectively [3]. Smoking is a major risk factor for lung cancer. Of course, the occurrence of lung cancer is also related to environmental and genetic factors [4, 5]. At present, surgical treatment is still the best treatment for early lung cancer [6]. In recent years, with the progress of medical technology, the treatment methods of NSCLC are diverse, and the mortality has been reduced [7]. However, NSCLC patients are prone to metastasis. Metastatic NSCLC is an advanced tumor that is difficult to cure, with low survival rate, almost no cure possibility and poor prognosis [8]. Therefore, it is urgent to find new prognostic markers and improve the treatment level of NSCLC.

Circular RNAs (circRNAs) are a class of covalently closed RNA loops widely expressed in eukaryotic cells. They are produced by a process called reverse splicing, and they are evolutionarily conserved [9, 10]. Studies have shown that circRNAs are microRNA (miRNA) sponges, protein regulators, transcription

regulators, protein templates, etc [11]. CircRNAs have a variety of biological functions and participate in a variety of physiological and pathological processes, including tumorigenesis and development [12–14]. The emergence of the circRNAs family provides a new entry point for the research and treatment of tumors. Studies have found that many circRNAs are involved in the progress of NSCLC. For example, Xu et al. pointed out that circ_0001869 promotes the progress of NSCLC through sponge miR-638 and promoting the expression of FOSL2 [15]. Sun and colleagues found circ_0000376 is highly expressed in NSCLC clinical specimens, which is related to adverse pathological parameters, and it can significantly promote the proliferation and metastasis of NSCLC cells, and enhance the drug resistance of cells [16]. Among them, *circ_0048856* is up-regulated in NSCLC samples, which may participate in the occurrence and development of NSCLC as a cancer promoting factor [17]. However, the prognostic value and molecular mechanism of *circ_0048856* in NSCLC are still unclear. Therefore, the study of serum *circ_0048856* as a prognostic marker of NSCLC has important clinical significance.

This study examined the prognostic significance of *circ_0048856* in serum of patients with NSCLC. Further verification found that *circ_0048856* and *miR-1287-5p* have a targeted relationship, and found that there is a negative correlation between them, which provides a new theoretical basis for the treatment of patients.

Methods

Subjects and samples

A total of 121 cases pathologically diagnosed with NSCLC and 113 healthy individuals were collected in this study. Before sampling, they did not receive any treatment including radiotherapy or chemotherapy. 5mL fasting blood specimen were obtained from all subjects, then serums were separated from whole blood via centrifugation. The supernate was immediately stored at -80°C until for use. All patients were followed up by telephone for 5 years. The clinicopathological features of the cases were shown in **Table 1**. This study was approved by the Ethic committee of Inner Mongolia People's Hospital. The written informed consent forms were provided from each participant in the present study prior to sampling.

Table 1. Relationship between *circ_0048856* expression and clinical features in NSCLC

Clinical Features	Cases (n=121)	<i>circ_0048856</i> expression		<i>P</i>
		Low (n=58)	High (n=63)	
Age (years)				0.446
≤60	52	27	25	
>60	69	31	38	
Gender				0.275
Male	83	37	46	
Female	38	21	17	
Smoking history				0.269
No	46	25	21	
Yes	75	33	42	
Tumor size(cm)				0.322
≤3	59	31	28	
>3	62	27	35	
TNM stage				0.002
I-II	47	31	16	
III-IV	74	27	47	
Lymph node metastasis				<0.001
No	79	48	31	
Yes	42	10	32	
Differentiation				0.274
Poor	81	36	45	
Well/moderate	40	22	18	

Cell culture

NSCLC cell lines A549 and H1299, as well as human bronchial epithelial cell (HBE) were purchased from American Type Culture Collection (ATCC). The cells were cultured in DMEM medium containing 10% fetal bovine serum in 37 °C and 5% CO₂ saturated humidity incubator for subculture once every two days. When the cells were covered with the bottom of the culture bottle and fused into a single layer, and the

density reached 70% ~ 80% fusion, the cells were digested with 0.25% trypsin and 1:3 ratio is used for subculture.

qRT-PCR

RNA Isolation Kit (Qiagen, Hilden, Germany) was used to isolate total RNA based on operation manual. The qualitative integrity of purified total RNA was tested by capillary electrophoresis using 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). RevertAidTM First Strand cDNA Synthesis Kit (Ferments, Vilnius, Lithuania) was adopted to perform the reverse transcription. The expression of gene in specimens were measured via quantitative real-time PCR (qRT-PCR). The reaction was carried out with SYBR Premix Ex TaqTM (TaKaRa, Dalian, China) kit in ABI 7500 instrument (ABI). The experiment was repeated at least 3 times. Finally, the levels of gene in NSCLC patients or healthy control group were calculated via the $2^{-\Delta\Delta C_t}$ method.

Cell transfection

Cell lines in exponential growth period were conducted cell transfection. The transfection vectors were si-*circ_0048856* and si-NC were purchased from Guangzhou RiboBio Co., Ltd.,(Guangzhou, China). They were transfected into NSCLC cells by LipofectamineTM2000 (Invitrogen, CA, USA) following the instruction of manufacturer, then cultured in 37°C humidified atmosphere with 5% CO₂ for 6h. The medium was replaced and they were transfected for 48h. Ultimately, qRT-PCR was employed to measure transfection efficiency.

Luciferase reporter assay

To verify the targeting relationship between *circ_0048856* and *miR-1287-5p*, we performed a luciferase reporter analysis. *Circ_0048856*-WT or *circ_0048856*-MUT (100 ng) were co-transfected with 100 nM *miR-1287-5p* mimics or *miR-1287-5p* mimics NC into NSCLC cells using Lipofectamine 2000 (Invitrogen, CA, USA) following the instruction of manufacture. 48 hours after transfection, the cells were harvested and detected by Dual-Luciferase Reporter Assay System Kit (Promega, Madison, WI, USA). The specific operation was carried out according to the instructions.

Statistical analysis

We used the software of SPSS 18.0 (SPSS. Chicago. IL, USA) was performed to analyze all data, and all graphs were plotted via GraphPad Prism (GraphPad, San Diego, CA, USA). The correlation between *circ_0048856* expression and clinicopathological features was assessed via χ^2 test. Difference between groups was compared by Student's t-test. Kaplan-Meier and cox regression analyses were conducted to assess the prognostic ability of *circ_0048856* in NSCLC. $P < 0.05$ was seen as obviously significant difference.

Results

Expression of *circ_0048856* is up-regulated in the serum of NSCLC patients

The relative expression of *circ_0048856* in serum samples of NSCLC subjects and healthy individuals were measred via qRT-PCR. All data revealed that the expression of *circ_0048856* in NSCLC serum samples was significantly up-regulated than that in healthy volunteers' serum specimens ($P<0.001$, **Figure 1**).

Relationship between *circ_0048856* expression and clinical features

The patients diagnosed with NSCLC were divided into low expression group (n=58) and high expression group (n=63), according to their median serum levels of *circ_0048856*. In addition, we examined the relationship between *circ_0048856* levels and the clinical features of NSCLC patients using chi-square test. All data proved that *circ_0048856* expression was significantly correlated with TNM stage ($P=0.002$) and lymphatic node metastasis ($P<0.001$). Nevertheless, *circ_0048856* expression was not found to be associated with other clinical features including age, gender, smoking history, tumor size and differentiation (all $P>0.05$, **Table 1**).

Prognostic value of *circ_0048856* expression in patients with NSCLC

To evaluate the prognostic ability of *circ_0048856* for NSCLC cases, we assessed the connection between *circ_0048856* levels and overall survival. The result of Kaplan-Meier with log rank test analysis revealed that the overall survival of patients with high *circ_0048856* expression was obviously shorter than those with low *circ_0048856* expression (log rank test, $P=0.002$, **Figure 2**).

Besides, we used the Cox regression analysis to assess the prognostic ability of *circ_0048856* in NSCLC patients. Both univariable and multivariable analysis results listed in **Table 2** showed that *circ_0048856* expression (HR=2.001, 95%CI: 1.258-3.184, $P=0.003$ and HR=1.669, 95% CI: 1.018-2.736, $P=0.042$) and lymphatic node metastasis (HR=2.118 95% CI: 1.340-3.348, $P=0.001$ and HR=1.766, 95% CI: 1.086-2.874, $P=0.022$) were useful prognostic indicators for overall survival in cases with NSCLC.

Table 2.

Cox regression analyses for *circ_0048856* in NSCLC patients

Factors	Univariate analysis		Multivariate analysis	
	HR(95% CI)	P values	HR(95% CI)	P values
<i>circ_0048856</i> expression	2.001 (1.258–3.184)	0.003	1.669 (1.018–2.736)	0.042
Age	-	0.332	-	-
Gender	-	0.999	-	-
Smoking history	-	0.935	-	-
Tumor size	-	0.666	-	-
TNM stage	-	0.072	-	-
Lymph node metastasis	2.118 (1.340–3.348)	0.001	1.766 (1.086–2.874)	0.022
Differentiation	-	0.554	-	-
Note: - indicated no data.				

Circ_0048856 directly targeted *miR-1287-5p*

We predicted that *miR-1287-5p* might be the direct downstream miRNA of *circ_0048856* through online software Starbase v2.0 (**Figure 3A**). To further verify the relationship between *circ_0048856* and *miR-1287-5p* was evaluated by luciferase reporter assay. *Circ_0048856*-3'UTR-WT or *circ_0048856*-3'UTR-MUT were co-transfected with 100 nM *miR-1287-5p* mimics or mimics NC into A549 and H1299 cells using Lipofectamine 2000 following the instruction of manufacture. The data revealed that in the cells transfected with *circ_0048856*-3'UTR-WT, *miR-1287-5p* mimics had significantly lower luciferase activity; when the binding site was mutated, the above inhibition was lost (**Figure 3B and C**). The experiment shows that *miR-1287-5p* is the direct downstream miRNA of *circ_0048856*.

Circ_0048856 has a negative correlation with *miR-1287*

To further explore the relationship between *miR-1287-5p* and *circ_0048856*, we detected the expression level of *miR-1287-5p* in serum of patients with NSCLC, and found that the expression level of *miR-1287-5p* was significantly decreased (**Figure 4A**), which was contrary to the expression pattern of *circ_0048856* in serum of patients with NSCLC. We also detected the expression of *miR-1287-5p* and *circ_0048856*, we found that the expression of *miR-1287-5p* in A549 and H1299 cells was lower than that in the control group, while the expression of *circ_0048856* in A549 and H1299 cells was higher than that in the control group (**Figure 4B**). Furthermore, knockdown of *circ_0048856* in NSCLC cells resulted in up regulation of *miR-1287-5p* expression (**Figure 4C**). Analysis of the correlation between *circ_0048856* and *miR-1287-5p* mRNA expression in serum of NSCLC showed that there was a negative correlation between them. (**Figure 4D**). Our results proved that *circ_0048856* is negatively correlated with *miR-1287-5p*.

Discussion

NSCLC is a kind of malignant tumor threatening the safety of human life, which has a high incidence and mortality. The disease is prone to metastasis and poor prognosis [18]. At present, many prognostic markers have been found, but the clinical application is less [19–21]. In this study, we confirmed that the expression of *circ_0048856* in serum of NSCLC was increased, and the expression of *circ_0048856* can be used as an independent prognostic biomarker for the overall survival rate of NSCLC patients, and it can also play a role by targeting *miR-1287-5p*.

In recent years, circRNAs have become a research hotspot. The role of circRNAs is to isolate or "sponge" other gene expression regulators, especially miRNAs. CircRNAs play an important role in a variety of biological functions and have broad application prospects [22, 23]. The accumulated data confirmed that circRNAs may become a new indicator for prognosis and diagnostic evaluation of lung cancer patients [24].

Previous studies have found that a large number of circRNAs are dysregulated in NSCLC. Although circRNAs are constantly found, their biological functions in NSCLC have just been explored [25–27]. We detected the expression level of *circ_0048856* in serum of NSCLC by qRT-PCR, and found that the expression level of *circ_0048856* was significantly up-regulated, which was consistent with previous studies [17]. The high expression of *circ_0048856* was significantly correlated with TNM stage and lymph node metastasis. Survival analysis showed that the overall survival of patients with high expression of *circ_0048856* was significantly shorter than that of patients with low expression of *circ_0048856*. Further analysis showed that *circ_0048856* expression and lymph node metastasis were independent prognostic biomarkers of overall survival in NSCLC patients. A study by Wang et al. found that *circ_0067934* was up-regulated in NSCLC tissues and cells, and its high expression was closely related to TNM stage, lymph node status and distant metastasis, indicating poor prognosis of NSCLC [28]. Geng et al. reported that the expression of *circ-MTHFD2* was up-regulated in NSCLC samples, and its high expression indicates poor prognosis of patients, which can be used as a diagnostic, prognostic marker and therapeutic target of NSCLC patients [29]. Liu et al. confirmed that the low expression of *circ_0001649* is associated with poor prognosis of NSCLC patients, and can be used as a prognostic marker of NSCLC [30].

In addition, we predicted the direct related miRNAs of *circ_0048856* by online prediction software Starbase V2.0, and found that *miR-1287-5p* may be the direct downstream miRNA of *circ_0048856*. Furthermore, we confirmed that *miR-1287-5p* is a direct downstream miRNA of *circ_0048856* by luciferase reporter gene analysis. Moreover, the expression of *miR-1287-5p* was significantly down regulated in NSCLC serum and cell lines, and the expression trend was opposite to *circ_0048856*, showing a negative correlation. We speculate that *circ_0048856* is involved in the progression of NSCLC by targeting *miR-1287-5p*.

Previous studies have shown that circRNAs can play a role in NSCLC by targeting miRNAs. Li et al. identified a new circRNA, *circ_0002483*, which can sponge multiple miRNAs and inhibit the progression of NSCLC through sponge miR-182-5p [31]. Another study found that *circ_0000735* had a targeting relationship with miR-1179/1182. *Circ_0000735* combined with miR-1179/1182 and participated in the

biological process of NSCLC cells [32]. Gao et al. confirmed that *circ_0074027* is carcinogenic in NSCLC, and its high expression indicates poor prognosis. *Circ_0074027* can promote the process of NSCLC cells as competitive endogenous RNA through *miR-185-3p/BRD4/MADD* pathway [33]. These studies have confirmed that circRNAs has a significant biological function in NSCLC, and can be used as a target for the diagnosis, prognosis and treatment of NSCLC.

There are several limitations in this study that cannot be ignored. Firstly, we recruit fewer patients and volunteers, which may affect the accuracy of the results. Secondly, we did not explore the specific mechanism of *circ_0048856* in NSCLC. The pathogenesis of NSCLC is very complex. In the next step, we will expand the sample size and further explore the molecular mechanism of *circ_0048856* in the occurrence and development of NSCLC, so as to bring new hope for the treatment of patients.

Conclusion

To sum up, *circ_0048856* may be an independent prognostic biomarker in patients with NSCLC. There is a negative correlation between *circ_0048856* and *miR-1287-5p*. *circ_0048856* may participate in the occurrence and development of NSCLC by targeting *miR-1287-5p*. Our study provides a new target for the treatment and prognosis of the disease, and provides a new theoretical basis for the prevention and treatment of the disease.

Abbreviations

Non small cell lung cancer (NSCLC)

Small cell lung cancer (SCLC)

Circular RNAs (circRNAs)

MicroRNA (miRNA)

Human bronchial epithelial cell (HBE)

American Type Culture Collection (ATCC)

Quantitative real-time PCR (qRT-PCR)

Declarations

Ethics approval and consent to participate

This study was supported by the Ethics Committee of Inner Mongolia People's Hospital and also has been carried out in accordance with the World Medical Association Declaration of Helsinki.

The subjects had been informed the objective. Certainly, written consents were signed by every subject in this study.

Consent for publication

We obtaining permission from participants to publish their data.

Availability of data and materials

All data generated or analysed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Y.H. and W.L. design of the work; Y.L. the acquisition, analysis, N.C. interpretation of data; Y.G. and F.L. the creation of new software used in the work; M.Z. have drafted the work or substantively revised it. All authors read and approved the final manuscript.

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Figures

Figure 1

Serum circ_0048856 expression levels detected by qRT-PCR in NSCLC patients and healthy controls. The results showed that the expression of circ_0048856 was significantly increased in NSCLC patients. $P < 0.001$.

Figure 2

Survival analysis. The overall survival of patients with high circ_0048856 expression was significantly shorter than those with low circ_0048856 expression (log rank test, $P=0.002$).

Figure 3

Circ_0048856 directly targeted miR-1287-5p. A, The direct target relationship predicted by starBase v2.0 between circ_0048856 and miR-1287-5p. B and C, Luciferase reporter assay to verify the targeting relationship between circ_0048856 and miR-1287-5p. *P<0.05.

Figure 4

Circ_0048856 was negatively correlated with miR-1287-5p. A, Expression of miR-1287-5p in serum of NSCLC, B, Expression of circ_0048856 and miR-1287-5p in NSCLC cell lines, C, Knockdown of circ_0048856 in NSCLC cells, miR-1287-5p expression was up-regulated, D, Correlation analysis of circ_0048856 and miR-1287-5p mRNA expression in serum of NSCLC. * $P < 0.05$.