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Clinical significance of circ-MTHFD2 in diagnosis, pathological staging and prognosis of NSCLC

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Abstract. – OBJECTIVE: To explore the clinical significance of circ-MTHFD2 in the diagnosis, pathological staging, and prognosis of non-small cell lung cancer (NSCLC).

PATIENTS AND METHODS: A total of 100 pairs of cancer tissues and adjacent tissues were surgically removed from NSCLC patients treated from July 2011 to January 2013 in our hospital were selected. All tissue samples were pathologically confirmed. Real Time-Polymerase Chain Reaction (qRT-PCR) was adopted to detect the expression of circ-MTHFD2 in NSCLC samples and its characteristic as a circular ribonucleic acid (circRNA). The receiver operating characteristic (ROC) curve was drawn to determine the diagnostic potential of circ-MTHFD2 in NSCLC. The relationship between circ-MTHFD2 expression and the clinical characteristics of NSCLC patients was analyzed by the χ^2-test. Kaplan-Meier survival curves were depicted to assess the prognostic potential of circ-MTHFD2 in NSCLC. The effect of circ-MTHFD2 on the overall survival rate of NSCLC patients was uncovered by introducing the Cox proportional hazards model.

RESULTS: The expression of circ-MTHFD2 in cancer tissues of NSCLC patients was higher than that in adjacent tissues ($p<0.05$), and there was no remarkable difference in the expression of circ-MTHFD2 before and after RNase R digestion ($p>0.05$). The area under the curve (AUC) of circ-MTHFD2 ROC was 0.701, with the cut-off value of 3.534, 90% sensitivity and 71% specificity. Circ-MTHFD2 expression was closely associated with smoking history, tumor size, tumor-node-metastasis (TNM) stage, lymph node metastasis, and recurrence in NSCLC patients ($p<0.05$). The prognosis of NSCLC patients with high expression of circ-MTHFD2 was evidently poorer than those with low expression. High expression of circ-MTHFD2 was an independent risk factor affecting the prognosis in NSCLC ($p<0.05$).

CONCLUSIONS: The high expression of circ-MTHFD2 has clinical significance in the diagnosis, pathological staging, and prognosis of NSCLC.

Key Words:
Circ-MTHFD2, Non-small cell lung cancer, Diagnosis, Staging, Prognosis.

Introduction

Lung cancer is a malignant tumor with the highest cancer-related morbidity and mortality rates in the world, which poses a serious threat to human life and health[1]. Non-small cell lung cancer (NSCLC) accounts for 85% of all lung cancer cases, including large cell lung cancer, squamous cell carcinoma, and adenocarcinoma. Although significant progress has been made in surgical treatment, radiotherapy, and chemotherapy, the prognosis of NSCLC patients is still poor, and their 5-year survival rate is as low as 15%[2]. Due to the lack of evident clinical symptoms and effective screening programs, most of the NSCLC patients are diagnosed in the middle or late stages, with unfavorable prognoses[3]. With the rapid development of molecular diagnosis in recent years, effective molecular diagnostic markers that contribute to early detection and timely treatment of NSCLC can improve the prognosis of affected patients.

Circular ribonucleic acids (circRNAs) are endogenous, non-coding RNAs (ncRNAs) formed by reverse splicing. They are featured with closed cyclic structures linked by covalent bonds without 5'- and 3'-ends[4]. CircRNAs are ubiquitous in human cells and more stable than linear RNAs.

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In addition, they have certain tissue specificity and cell development, which enable them to be utilized as diagnostic biomarkers for human diseases[5-7]. CircRNAs, interact with microRNAs (miRNAs), synergistically regulate protein translation, thus participating in post-transcriptional regulation of cancer. Abnormally expressed circRNAs are closely related to tumor occurrence and development, as well as malignant degree and prognosis[8,9]. It is reported that hsa-circ-007950 is significantly upregulated in NSCLC tissues and the area under the receiver operating characteristic (ROC) curve is 0.756, indicating its diagnostic potential in NSCLC. Moreover, other circRNAs can be employed as biomarkers for determining the staging and prognosis of NSCLC[10]. This study uncovered clinical significance of circ-MTHFD2 in the diagnosis, pathological staging, and prognosis of NSCLC.

Patients and Methods

General Information

A total of 100 pairs of cancer tissues and adjacent tissues (≥ 3 cm) were surgically removed in NSCLC patients treated in our hospital from July 2011 to January 2013. All patients were selected according to guidelines of the World Health Organization (WHO) and the International Association for the Study of Lung Cancer (IASLC)[11]. There were 59 males and 41 females recruited, aged 29 to 76 years old, with a median age of 59 years old. Based on histological classification, there were 35 cases of squamous cell carcinoma, 58 of adenocarcinoma, and 7 of others. Classified by tumor size: 66 cases were < 3 cm, and 34 were ≥ 3 cm. There were 25 stage I patients, 39 stage II, and 36 stage III according to the tumor-node-metastasis (TNM) staging. 64 cases had smoking history and 39 cases had lymphatic metastasis. This study was approved by the Ethics Committee of The First Affiliated Hospital of Xi'an Jiaotong University. Signed written informed consents were obtained from all participants before the study.

Methods

The medical records of the patients were collected, including age, gender, family history, smoking history, pathological type, pathological stage, and other information. Patients were followed up every 6 months by outpatient service and telephone call since the surgery was conducted until January 2019. The overall survival time was defined from the date of diagnosis to the date of death or the last follow-up. Patients who were suspected of relapse were diagnosed in time by biopsy.

Materials

Reagents: reverse transcription kit (from Promega Corporation, Madison, WI, USA), SYBR Green quantitative Polymerase Chain Reaction (qPCR) fluorescent mixture (Beijing BLKW Biotechnology Co., Ltd., Beijing, China), RNase R (Epicenter Science and Biotechnology Co., Ltd., Madison, WI, USA), TRIzol (Invitrogen, Carlsbad, CA, USA), and primers (Shanghai Biological Engineering Co., Ltd., Shanghai, China).

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

RNAs in tissues were extracted by phenol/chloroform method as follows. Tissues were lysed in 1 mL of TRIzol reagent at room temperature for 20 min, and then, incubated with 20 μL of chloroform for 10 s shaking. After letting stand at room temperature for 12 min, the mixture was subjected to centrifugation at 12000 rpm for 15 min at 4°C. The supernatant was transferred to a new Eppendorf (EP; Hamburg, Germany) tube, with the same amount of isopropyl alcohol added. It was left at room temperature for 10 min, and centrifuged at 12000 rpm for 10 min at 4°C. After washing twice with 75% ethanol and air dried, RNA was diluted in a proper amount of diethyl pyrocarbonate (DEPC)-treated water (Beyotime, Shanghai, China) and quantified. 1 μg of RNA was mixed with or without 1 μL of RNase R (20 U/μL) at 37°C for 20 min, and then, washed with RNeasy cleaning agent. Circ-MTHFD2 primer sequences were: forward: ATCGTCACTCAGACGGTTGT, reverse: TGTCATCGACGTGCGTGTTA; glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primer sequence: forward: GCACCGTCAAGGCTGAGAAC, reverse: TGGTGAAGACGCCAGTGGA. In accordance to the reverse transcription kit procedure, 1 μg of messenger RNA (mRNA) was reversely transcribed to complementary deoxyribonucleic acid (cDNA). Based on the operation steps of SYBR Green qPCR fluorescence mixture reagent kit, cDNA was taken as a template for real-time fluorescence qPCR under the reaction conditions at 95°C for 7 min, and 40 cycles at 95°C for 12 s and 60°C for 40

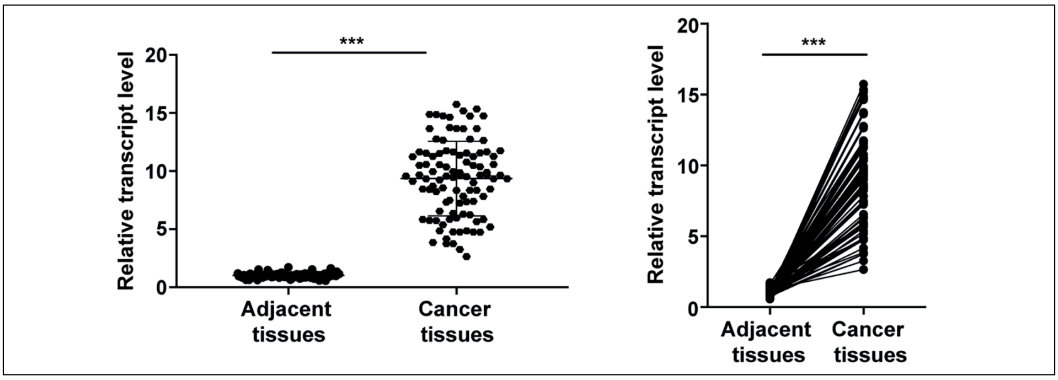

Figure 1. Expression of circ-MTHFD2 in cancer tissues and adjacent tissues.

s. The relative expression of circ-MTHFD2 gene was calculated *via* $2^{-\Delta\Delta Ct}$ method.

Evaluation Indicators

The score was determined by the relative expression of circ-MTHFD2: 1 point for 1-3, 2 points for 4-6, 3 points for 7-9, 4 points for 10-12, and 5 points for 13-15. According to the score, the ROC curve of the subjects was obtained to analyze the survival rate. Those below 9 were classified as low expression group, while those above 9 were classified as high expression group.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 20.0 software (IBM Corp., Armonk, NY, USA) was employed for analysis. The measurement data were expressed as mean ± standard deviation, and the counting data were expressed as percentage. The differences between groups was verified by the *t*-test. The correlation between circ-MTHFD2 level and the clinicopathologic staging of NSCLC patients was analyzed by χ^2-test. The survival analysis was performed *via* Kaplan-Meier method, and the influencing factors of survival rate were analyzed with Cox proportional hazards model. $p<0.05$ was considered as significantly different.

Results

Expression of Circ-MTHFD2 In Cancer Tissues and Adjacent Tissues of Patients With NSCLC

qRT-PCR was used to detect the expression of circ-MTHFD2 in cancer tissues and adjacent tissues of NSCLC patients. The results were shown in Figure 1. Compared with that in adjacent tissues (1.016±0.028), the expression of circ-MTHFD2 in cancer tissues was higher (9.355±0.116), with a significant difference ($p<0.001$).

Circ-MTHFD2 Was Resistant to RNase R Digestion

The special circular structure of circRNAs explains their resistance to RNase R, while linear RNAs are easily digested by it. To detect the circRNA characteristics of circ-MTHFD2, we detected relative levels of circ-MTHFD2 and GAPDH in NSCLC tissues under the induction of RNase R. As results shown in Figure 2, the expression of circ-MTHFD2 did not change, while GAPDH was markedly downregulated ($p<0.05$), indicating that circ-MTHFD2 was a circRNA.

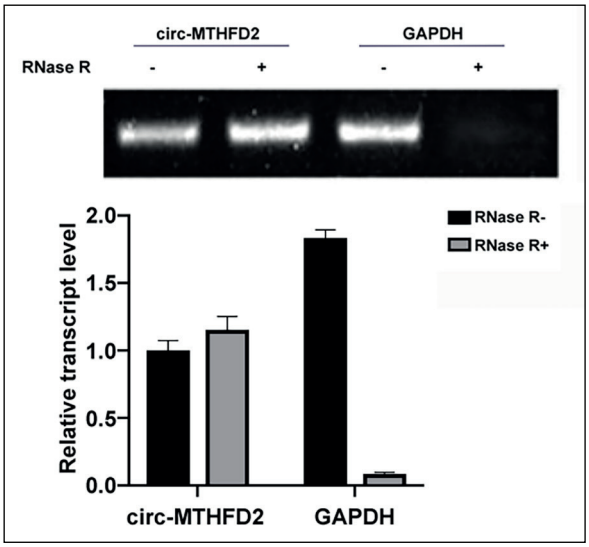

Figure 2. Circ-MTHFD2 was resistant to RNase R digestion.

Diagnostic Potential of Circ-MTHFD2

The ROC curve was depicted to evaluate the role of circ-MTHFD2 in predicting the overall survival rate of NSCLC patients. The ROC curve presented that the cut-off value was 3.534, with 90% sensitivity and 71% specificity. Therefore, the cut-off value of circ-MTHFD2 was set to 3, and the cases with a relative expression level below 9 were classified as low expression group, while those with a relative expression level above 9 were classified as high expression group (Figure 3).

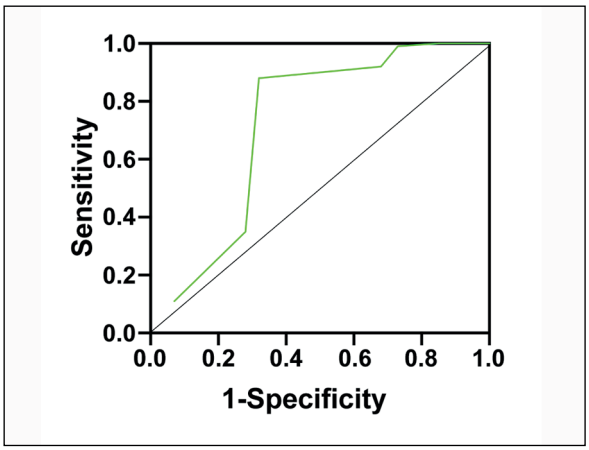

Figure 3. ROC curves of circ-MTHFD2.

Relationship Between the Expression of Circ-MTHFD2 and Pathological Features of NSCLC Patients

Recruited NSCLC patients were thereafter assigned into high expression group (n=42) and low expression group (n=58). According to the analysis by χ^2-test, the expression of circ-MTHFD2 was not related to gender, age, pathological type, and differentiated degree in NSCLC patients (p>0.05). Notably, circ-MTHFD2 expression was correlated with smoking history, tumor size, TNM stage, lymph node metastasis, and recurrence (p<0.05). Among them, the expression of circ-MTHFD2 in NSCLC patients with smoking history, tumor size ≥ 3 cm, TNM stage ≥ stage II, lymph node metastasis, and recurrence were markedly alleviated (Table I).

Table I. Relationship between the expression of circ-MTHFD2 and clinical parameters in 100 NSCLC patients.

Clinicopathologyparameter	Cases (n =100)	Low expression (n = 58)	High expression (n = 42)	*p*-value
Gender				0.7480
Male	59	35 (60.3%)	24 (57.1%)	
Female	41	23 (39.7%)	18 (42.9%)	
Age (years old)				0.4272
< 60	43	23 (39.6%)	20 (47.6%)	
≥ 60	57	35 (60.4%)	22 (52.4%)	
Smoking history				< 0.0001
No	36	31 (53.5%)	5 (11.9%)	
Yes	64	27 (46.5%)	37 (88.1%)	
Tumor size (cm)				0.0002
< 3	66	47 (81.0%)	19 (45.2%)	
≥ 3	34	11 (19.0%)	23 (54.8%)	
Pathological type				0.3641
Adenocarcinoma	58	37 (63.8%)	21 (50%)	
Squamous cell carcinoma	35	19 (32.8%)	16 (38.1%)	
Others	7	2 (3.4%)	5 (11.9%)	
Degree of differentiation				0.0824
High and medium	60	39 (67.2%)	21 (50.0%)	
Low	40	19 (32.8%)	21 (50.0%)	
TNM stage				0.0352
Stage I	25	19 (32.8%)	6 (14.3%)	
≥ Stage II	75	39 (67.2%)	36 (85.7%)	
Lymph node metastasis				0.0196
No	61	41 (70.7%)	20 (47.6%)	
Yes	39	17 (29.3%)	22 (52.4%)	
Recurrence				0.0055
No	52	37 (63.8%)	15 (35.7%)	
Yes	48	21 (36.2%)	27 (64.3%)	

Association Between the Expression of Circ-MTHFD2 and Overall Survival Rate

Association between the expression of circ-MTHFD2 and overall survival rate was analyzed based on Kaplan-Meier curves. The results were demonstrated in Figure 4. The 5-year survival rate in high expression group (67.2%) was remarkably lower than that in low expression group (90.0%, $p<0.05$).

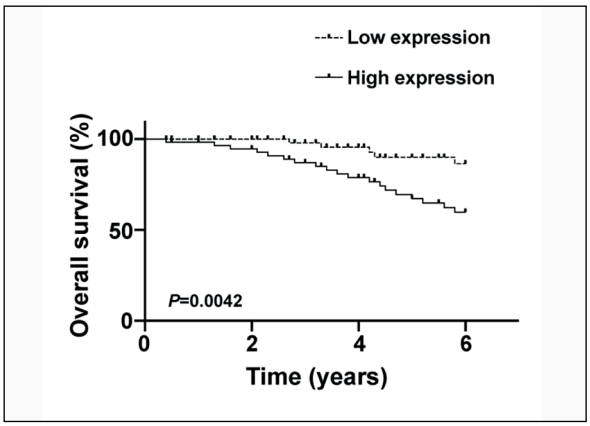

Figure 4. Survival rate of NSCLC patients in high expression group and low expression group.

Single Factor Analysis and Multi-Factor Analysis of Total Survival Rate of Patients

Single factor analysis based on Cox proportional hazards model revealed that tumor size, pathological type, degree of differentiation, TNM stage, lymph node metastasis, recurrence, and the expression of circ-MTHFD2 were risk factors for overall survival in NSCLC patients. Multi-factor analysis suggested that lymph node metastasis, recurrence, and the expression of circ-MTHFD2 were independent risk factors for overall survival in NSCLC patients (Table II).

Table II. Single factor analysis and multi-factor analysis of total survival rate.

Item	Single factor analysis		Multi-factor analysis	
	HR (95% CI)	*p*-value	HR (95% CI)	*p*-value
Gender		0.172		0.197
Male	1			
Female	1.018 (0.992, 1.045)		0.698 (0.404, 1.205)	
Age (years old)		0.133		0.891
< 60	1			
≥ 60	2.007 (0.809, 4.976)		1.034 (0.640, 1.673)	
Smoking history		0.086		0.241
No	1			
Yes	0.549 (0.277, 1.090)		1.575 (0.737, 3.366)	
Tumor size (cm)		0.012		0.107
< 3	1			
≥ 3	2.265 (1.196, 4.289)		0.636 (0.367, 1.103)	
Pathological type		0.036		0.334
Adenocarcinoma	1			
Squamous cell carcinoma	0.41 (0.185, 0.917)		1.328 (0.682, 2.512)	
Others	0.874 (0.433, 1.765)	0.707	0.954 (0.893, 1.064)	0.398
Degree of differentiation		0.025		0.529
High and medium	1			
Low	3.423 (1.834, 6.983)		1.538 (0.403, 5.867)	
TNM stage		0.008		0.138
Phase I	1			
≥ Phase II	4.843 (2.512, 9.245)		1.008 (0.970, 1.044)	
Lymph node metastasis		0.004		0.031
No	1			
Yes	3.925 (1.974, 7.412)		1.595 (0.837, 3.124)	
Recurrence		0.001		0.002
No	1			
Yes	3.225 (1.594, 6.532)		4.223 (1.592, 10.023)	
Circ-MTHFD2 expression		0.0014		0.022
Low expression	1			
High expression	8.543 (4.712, 16.60)		2.231 (1.002, 4.998)	

Discussion

Although great progress has been made in medical technology in the past several decades, the morbidity rate and prognosis of NSCLC are still unsatisfactory. It is very critical to explore new diagnostic biomarkers and therapeutic targets to improve the overall survival rate of NSCLC patients[12]. In this study, diagnostic biomarkers relevant to the occurrence and development of NSCLC were investigated. CircRNAs are a new type of lncRNAs with unclear biological functions in mammalian cells, which are featured by tissue and time specificities. CircRNAs are highly conserved and stable, which make them become the most potential cancer biomarkers[13-15]; they have a strong correlation with the occurrence and development of Alzheimer's disease, type 2 diabetes, cancers, and other diseases[16,17]. Hang et al[18] found that 185 circRNAs are specifically expressed in 10 pairs of NSCLC tissues and adjacent tissue samples, of which circ-FARSA is evidently upregulated in NSCLC patients. Therefore, circ-FARSA can be used as a non-invasive potential biomarker of NSCLC. Li et al[10] discovered that hsa-circ-007950 is significantly upregulated in 92 pairs of NSCLC tissues than adjacent ones. The area under ROC curve (0.756) indicated the great importance of hsa-circ-007950 in NSCLC diagnosis. In addition, the study on the relationship between circRNAs and TNM staging of NSCLC pointed out that the expression difference of circRNAs is closely related to the rate of lymph node metastasis. Jiang et al[19] identified 7 upregulated circRNAs and 2 downregulated ones in patients with NSCLC accompanied by lymph node metastasis, with significant statistical significance. In the study of the relationship between circRNAs and the prognosis of NSCLC, Liu et al[20] found that the survival rate of patients with high expression of hsa-circ-103809 is significantly lower than those with low expression. CircRNAs are expected to be potential biomarkers for predicting the prognosis.

This study mainly explored the clinical significance of circ-MTHFD2 in NSCLC. The results showed that the expression of circ-MTHFD2 was upregulated in NSCLC tissues than adjacent ones. Subsequently, the resistance of circ-MTHFD2 to RNase R confirmed that circ-MTHFD2 was a circRNA. The ROC curve of circ-MTHFD2 demonstrated that circ-MTHFD2 had a certain significance in the diagnosis of NSCLC. We further explored the relationship between circ-MTHFD2 level and clinical features of NSCLC patients. The data showed that circ-MTHFD2 was closely related to smoking history, tumor size, TNM stage, lymph node metastasis, and recurrence in NSCLC patients. Moreover, highly expressed circ-MTHFD2 was unfavorable to the prognosis in NSCLC as Kaplan-Meier curves depicted. In addition, single factor and multi-factor analyses on factors affecting the prognosis of NSCLC patients were conducted by introducing the Cox proportional hazards model. It is discovered that the high expression of circ-MTHFD2 was an independent risk factor for NSCLC.

Conclusions

Circ-MTHFD2 has great clinical significances in the diagnosis, pathological staging, and prognosis of NSCLC, which is expected to become a specific biomarker for NSCLC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Acknowledgements

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References

1) Siegel RL, Miller KD, Jemal A. Cancer statistics, 2018. CA Cancer J Clin 2018; 68: 7-30.
2) Herbst RS, Morgensztern D, Boshoff C. The biology and management of non-small cell lung cancer. Nature 2018; 553: 446-454.
3) Gridelli C, Rossi A, Carbone DP, Guarize J, Karachaliou N, Mok T, Petrella F, Spaggiari L, Rosell R. Non-small-cell lung cancer. Nat Rev Dis Primers 2015; 1: 15009.
4) Lasda E, Parker R. Circular RNAs: diversity of form and function. RNA 2014; 20: 1829-1842.
5) Chen I, Chen CY, Chuang TJ. Biogenesis, identification, and function of exonic circular RNAs. Wiley Interdiscip Rev RNA 2015; 6: 563-579.
6) Li CH, Wang YB, Chen KB. Circ_0017247 accelerates epithelial-mesenchymal transition in non-small cell lung cancer. Eur Rev Med Pharmacol Sci 2019; 23: 256-263.
7) Glazar P, Papavasileiou P, Rajewsky N. CircBase: a database for circular RNAs. RNA 2014; 20: 1666-1670.

8) Zhang HD, Jiang LH, Sun DW, Hou JC, Ji ZL. CircRNA: a novel type of biomarker for cancer. Breast Cancer-Tokyo 2018; 25: 1-7.

9) Meng S, Zhou H, Feng Z, Xu Z, Tang Y, Li P, Wu M. CircRNA: functions and properties of a novel potential biomarker for cancer. Mol Cancer 2017; 16: 94.

10) Li J, Wang J, Chen Z, Chen Y, Jin M. Hsa_circ_0079530 promotes cell proliferation and invasion in non-small cell lung cancer. Gene 2018; 665: 1-5.

11) Osmani L, Askin F, Gabrielson E, Li QK. Current WHO guidelines and the critical role of immunohistochemical markers in the subclassification of non-small cell lung carcinoma (NSCLC): Moving from targeted therapy to immunotherapy. Semin Cancer Biol 2018; 52: 103-109.

12) Osmani L, Askin F, Gabrielson E, Li QK. Current WHO guidelines and the critical role of immunohistochemical markers in the subclassification of non-small cell lung carcinoma (NSCLC): Moving from targeted therapy to immunotherapy. Semin Cancer Biol 2018; 52: 103-109.

13) Ashwal-Fluss R, Meyer M, Pamudurti NR, Ivanov A, Bartok O, Hanan M, Evantal N, Memczak S, Rajewsky N, Kadener S. CircRNA biogenesis competes with pre-mRNA splicing. Mol Cell 2014; 56: 55-66.

14) Shao Y, Li J, Lu R, Li T, Yang Y, Xiao B, Guo J. Global circular RNA expression profile of human gastric cancer and its clinical significance. Cancer Med 2017; 6: 1173-1180.

15) Qu S, Yang X, Li X, Wang J, Gao Y, Shang R, Sun W, Dou K, Li H. Circular RNA: a new star of noncoding RNAs. Cancer Lett 2015; 365: 141-148.

16) Haque S, Harries LW. Circular RNAs (circRNAs) in health and disease. Genes (Basel) 2017; 8: 353.

17) Zhao Z, Li X, Jian D, Hao P, Rao L, Li M. Hsa_circ_0054633 in peripheral blood can be used as a diagnostic biomarker of pre-diabetes and type 2 diabetes mellitus. Acta Diabetol 2017; 54: 237-245.

18) Hang D, Zhou J, Qin N, Zhou W, Ma H, Jin G, Hu Z, Dai J, Shen H. A novel plasma circular RNA circFARSA is a potential biomarker for non-small cell lung cancer. Cancer Med 2018; 7: 2783-2791.

19) Jiang MM, Mai ZT, Wan SZ, Chi YM, Zhang X, Sun BH, Di QG. Microarray profiles reveal that circular RNA hsa_circ_0007385 functions as an oncogene in non-small cell lung cancer tumorigenesis. J Cancer Res Clin Oncol 2018; 144: 667-674.

20) Liu W, Ma W, Yuan Y, Zhang Y, Sun S. Circular RNA hsa_circRNA_103809 promotes lung cancer progression via facilitating ZNF121-dependent MYC expression by sequestering miR-4302. Biochem Biophys Res Commun 2018; 500: 846-851.