ACTA ABP BIOCHIMICA POLONICA

Vol. 70, No 2/2023
247–252
https://doi.org/10.18388/abp.2020_6214

Regular paper

Overexpression of miR-483-5p predicts venous thromboembolism onset in patients with lung cancer especially in high BMI cases

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Background: Venous thromboembolism (VTE) is a common complication in patients with lung cancer. The important roles of microRNAs (miRNAs) in VTE have emerged, however, studies about the roles of miRNAs in VTE remain scarce. This study aimed to measure the expression of miR-483-5p in lung cancer patients with VTE, evaluate whether miR-483-5p could predict VTE onset in patients, and further evaluate its predictive value in patients with different BMI values. **Methods:** A total of 170 patients with lung cancer were recruited in this study, including 70 patients with VTE, and 110 patients with non-VTE. The expression of miR-483-5p was detected by quantitative real time PCR. Receiver operating characteristic analysis was used to screen VTE patients from non-VTE patients. Whether miR-483-5p was independently associated with VTE onset in lung cancer patients was evaluated by univariate and multivariate logistic regression analyses. **Results:** miR-483-5p was higher in VTE patients than that in non-VTE patients. miR-483-5p was correlated with body mass index (BMI), hypertension, C-reactive protein (CRP), and platelet count in VTE patients. In addition, miR-483-5p had high diagnostic value to differentiate between VTE patients and non-VTE patients and served as an independent biomarker in predicting the VTE onset in lung cancer patients. Moreover, miR-483-5p had the highest diagnostic accuracy to screen VTE patients from non-VTE patients in patients with high BMI values. **Conclusion:** miR-483-5p, increased in VTE patients, can independently predict VTE onset in lung cancer patients especially in patients with high BMI values.

Keywords: miR-483-5p; Predict; Venous thromboembolism; Lung cancer; BMI

Received: 15 February, 2022; revised: 11 May, 2022; accepted: 07 July, 2022; available on-line: 16 May, 2023

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Abbreviations: AUC, area under the curve; BMI, body mass index; CDFI, color Doppler ultrasound imaging; CRP, C-reactive protein; CTPA, CT pulmonary angiography; miRNAs, microRNAs; PCSK9, proprotein convertase subtilisin/kexin type 9; qRT-PCR, Quantitative real time PCR; RBM5, RNA binding motif protein 5; ROC, receiver operating characteristic; 3′-UTR, 3′-untranslated region; VET, Venous thromboembolism

INTRODUCTION

Venous thromboembolism (VTE) is a common vascular disease in which blood clots occur within a vessel. The pathogenesis of VTE formation is endothelial injury, hypercoagulability or vessel walls injury, and blood stasis, known as Virchow's triad (Kruger *et al.*, 2019). It is known that cancer was one of the most common risk factors for VTE (Falanga & Zacharski, 2005), and the risk of VTE occurrence among cancer patients was estimated to be 4 to 7 times that of the general population (Heit *et al.*, 2000; Walker *et al.*, 2013; Blom *et al.*, 2005). In cancer patients, VTE is not only associated with a worse prognosis, but also with increased medical costs and worse quality of life (Lopez-Nunez *et al.*, 2018). The prevalence of VTE in patients with lung cancer is one of the highest among all cancer patients (Corrales-Rodriguez & Blais, 2012). The risk factors for VTE in lung cancer patients are diverse, such as obesity and high body mass index (BMI), trauma, leukocyte, complications and platelet elevation (Du & Chen, 2018). Notably, obesity has been found to function as an independent and moderate risk factor for VTE (Hotoleanu, 2020). A previous study has found that the survival rate in lung cancer patients with VTE is lower than that in lung cancer patients without VTE (Chen *et al.*, 2015). Thus, early and accurate assessment to identify the patients who at high risk for VTE is clinically necessary for the right treatment option. However, to date, reliable VTE risk assessment tools for lung cancer patients treated with anticancer therapies remain an unmet medical need. Therefore, exploring the key molecules involved in the VTE development may provide targets for treating VTE in lung cancer patients.

The microRNAs (miRNAs) are a group of endogenous, small (about 22 nucleotides) non-coding RNAs that can regulate gene expression by binding to the 3'-untranslated region (3'-UTR) of their target mRNAs (Li *et al.*, 2016). Many studies have demonstrated that miRNAs are associated with VTE development and can be used as markers for the prediction of VTE. For example, miR-134 may be a novel biomarker for predicting VTE (Xiang *et al.*, 2019). miR-374b-5p promotes the formation of VTE and may be used as a promising diagnostic marker for VTE (Zhang, Miao, *et al.*, 2020). However, there are only a few studies on the relationship between miRNAs and VTE disease. miR-483-5p has been found to be significantly associated with obesity and BMI (Gallo *et al.*, 2018), and serves as an angiogenesis-regulating factor (Qiao *et al.*, 2011). In addition, a study has shown that miR-483-5p can regulate the function of pulmonary microvascular endothelial cells (Leng *et al.*,

2020). Thus, we speculated that miR-483-5p may be associated with VTE development.

Therefore, the purpose of this study was to measure the expression of miR-483-5p in VTE and non-VTE patients, and to explore its clinical value for predicting VTE occurrence in patients and in patient groups with different BMI values. This study will provide new biomarker and insight for early predicting VTE occurrence in lung cancer patients.

MATERIALS AND METHODS

Patient recruitment

This study was approved by the Ethics Committee of Qingdao Jiaozhou Central Hospital and all patients had signed the informed consent before this study. A total of 170 patients with lung cancer who received therapy in Qingdao Jiaozhou Central Hospital from 2017 to 2020 were enrolled in the case-control study. Sixty of the lung cancer patients developed VTE after surgery (VTE group), and 110 patients with lung cancer did not develop VTE after surgery (non-VTE group). The inclusion criteria were as follows: (a) age $\geq$ 18 years, (b) patients received surgical therapy, (c) patients were diagnosed with lung cancer pathologically, and (d) VTE was diagnosed by CT pulmonary angiography (CTPA) and/or vascular color Doppler ultrasound imaging (CDFI). Patients were excluded if they: (a) had other malignant tumors, (b) had other hematologic diseases, (c) had combined VTE before surgery or had previous history of VTE, (d) got anticoagulant therapy preoperatively and postoperatively because of other diseases, (e) were in TNM stage IV, and (f) were in gestation and lactation.

Collection of serum samples

Venous blood samples were collected from all the lung cancer patients on admission. Then, serum was obtained by centrifuging blood samples at 1500×*g* for 10 min at 4°C and stored at –80°C for further use.

RNA isolation

Total RNA was isolated from the serum using TRIzol Reagent (Invitrogen; Thermo Fisher Scientific, Inc.). To prevent contamination of genomic DNA, samples were treated with RNase free DNase I (Fermentas, Thermo Fisher Scientific). Then, the optical density (OD) at 260 nm and 280 nm (OD 260/OD 280) was measured using a NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA) to evaluate the purity of total RNA, and the concentration of total RNA was evaluated by the OD at 260 nm. All the protocols were performed following the instructions of manufacturers.

Table 1. Baseline characteristics of all the patients

Characteristics	VTE patients (n=60)	Non-VTE patients (n=110)	*P* value
Age (years)	63.39±12.46	61.93±8.96	0.380
Gender			
Female	27	42	0.387
Male	33	68	
BMI (kg/m^2)	22.36±3.17	20.67±3.05	0.001
Histological type			
SCLC	23	38	0.623
NSCLC	37	72	
TNM stage			
I-II	24	35	0.284
III	36	75	
Hypertension			
Yes	41	57	0.037
No	19	53	
Diabetes			
Yes	26	41	0.440
No	34	69	
Coronary heart disease			
Yes	31	50	0.438
No	29	60	
CRP (mg/L)	17.07±8.84	14.55±5.35	0.022
Hemoglobin (g/L)	130.45±14.10	135.40±19.14	0.080
Platelet count (10^9/L)	244.16±33.25	234.70±20.54	0.023
Leukocyte count (10^9/L)	9.19±2.28	8.49±2.02	0.041

Abbreviations: VTE, venous thromboembolism; BMI, body mass index; SCLC, small cell lung cancer; NSCLC, non-small cell lung cancer; CRP, C-reactive protein

Quantitative real time PCR (qRT-PCR)

At first, a reverse transcription step was performed to synthesize the cDNA of miRNA with the PrimeScript RT reagent kit (Takara Bio Inc.). The SYBR Green PCR Master Mix kit (Invitrogen; Thermo Fisher Scientific, Inc.) was used for the qRT-PCR analysis of miR-483-5p expression in a 7300 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). All procedures were performed according to the manufacturer's instructions. The thermocycling conditions were as follows: an initial denaturation step at 95°C for 10 min, followed by 40 cycles at 95°C for 30 s, 60°C for 30 s and 72°C for 10 s. Primer sequences were as follows (5'-3'): miR-483-5p, forward: GCCGAGAAGACGGGAGGAAA and reverse: CTCAACTGGTGTCGTGGA; U6, forward: CTCGCTTCGGCAGCACA and reverse: AACGCTTCACGAATTTGCGT. The data of the relative miR-483-5p expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001). U6 was used as an internal control for miR-483-5p expression.

Statistical analysis

SPSS 21.0 software (SPSS, Inc., Chicago, USA) and GraphPad Prism 7.0 software (GraphPad Software, Inc.) were used to perform all statistical analyses in this study. All data were shown as mean ± standard deviation (S.D.). Differences between two groups of continuous variables and categorical variables were compared using Student's *t*-test and Chi-square test, respectively. Pearson correlation analysis was used to analyze the correlation of miR-483-5p with continuous variables, and Chi-square test was used to analyze the association between miR-483-5p and categorical variables. The ability of miR-483-5p to differentiate between VTE patients and non-VTE patients in all the patients and in patient groups with different BMI values was evaluated by receiver operating characteristic (ROC) analysis. Univariate and multivariate logistic regression analyses were used to evaluate the ability of miR-483-5p to predict VTE onset in lung cancer patients. $P<0.05$ was considered as statistically significant.

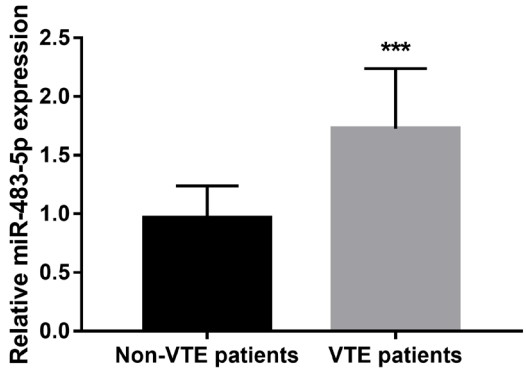

Figure 1. The expression level of miR-483-5p in VTE patients and non-VTE patients.
***$P<0.001$ *vs.* Non-VTE patients. VTE, venous thromboembolism.

RESULTS

Baseline characteristics of lung cancer patients

The baseline characteristics of all patients with lung cancer were presented in Table 1. There were no significant differences in age, gender, histological type, TNM stage, diabetes, coronary heart disease, and hemoglobin between VTE patients and non-VTE patients (all $P>0.05$). Additionally, compared to non-VTE patients, VTE patients had significantly higher BMI ($P=0.001$), C-reactive protein (CRP, $P=0.022$), platelet count ($P=0.023$), leukocyte count ($P=0.041$), and a higher proportion of population with hypertension ($P=0.037$).

High miR-483-5p expression in VTE patients

The expression level of miR-483-5p was presented in Fig. 1. miR-483-5p expression was significantly upregulated in VTE patients compared with that in non-VTE patients ($P < 0.001$).

Correlation between miR-483-5p expression and VTE patients' clinicopathological characteristics

As shown in Table 2, miR-483-5p expression was found to be correlated with BMI (r=0.416, $P=0.001$), hy-

Table 2. Correlation between miR-483-5p expression and the clinicopathological characteristics of VTE patients

Characteristics	Mean±S.D./Number (n=60)	miR-483-5p	
		χ^2/r value	*P* value
Age (years)	63.39±12.46	0.104	0.429
Male	33	1.684	0.194
BMI (kg/m^2)	22.36±3.17	0.416	0.001
Histological type (NSCLC)	37	0.071	0.791
TNM stage (III)	36	2.500	0.114
Hypertension	41	6.239	0.012
Diabetes	26	2.443	0.118
Coronary heart disease	31	1.669	0.196
CRP (mg/L)	17.07±8.84	0.301	0.020
Hemoglobin (g/L)	130.45±14.10	-0.116	0.379
Platelet count (10^9/L)	244.16±33.25	0.291	0.024
Leukocyte count (10^9/L)	9.19±2.28	0.276	0.038

VTE, venous thromboembolism; BMI, body mass index; NSCLC, non-small cell lung cancer; CRP, C-reactive protein; S.D., standard deviation

Table 3. Logistic analysis of factors influencing the risk of VTE in lung cancer patients

Characteristics	Univariate analysis		Multivariate analysis	
	OR (95% CI)	*P*	OR (95% CI)	*P*
Age	1.190 (0.616–2.300)	0.605		
Gender	1.325 (0.700–2.506)	0.387		
BMI	2.939 (1.338–6.456)	0.007	3.587 (1.411–9.119)	0.009
Histological type	1.178 (0.614–2.261)	0.623		
TNM stage	1.429 (0.743–2.748)	0.285		
Hypertension	2.006 (1.037–3.883)	0.039	1.901 (0.874–4.134)	0.112
Diabetes	1.287 (0.678–2.442)	0.440		
Coronary heart disease	1.283 (0.683–2.409)	0.439		
CRP	1.937 (1.022–3.672)	0.043	1.401 (0.612–3.207)	0.389
Hemoglobin	1.414 (0.752–2.660)	0.283		
Platelet count	2.231 (1.169–4.258)	0.015	2.023 (0.921–4.443)	0.081
Leukocyte count	1.323 (1.049–1.668)	0.048	1.304 (0.899–1.891)	0.093
miR–483–5p	7.747 (3.720–16.135)	<0.001	8.075 (3.723–17.514)	<0.001

VTE, venous thromboembolism; BMI, body mass index; CRP, C-reactive protein; OR odds ratio; CI, confidence interval

pertension (*P*=0.012), CRP (r=0.301, *P*=0.020), platelet count (r=0.291, *P*=0.024), and leukocyte count (r=0.276, *P*=0.024) in VTE patients. In addition, no correlation was found between miR-483-5p expression and age, male, histological type, TNM stage, diabetes, coronary heart disease and hemoglobin (all *P*>0.05).

The significance of miR-483-5p for predicting VTE onset in lung cancer patients

The clinical characteristics that might predict the onset of VTE in lung cancer patients were included in the logistic regression analysis, and the analysis results were shown in Table 3. The results after multivariate correction demonstrated that BMI [odds ratio (OR)=3.731; 95% confidence interval (CI)=1.414-9.848; *P*=0.008] and miR-483-5p (OR=8.441; 95% CI=3.732–19.094; *P*<0.001) were independently associated with VTE onset in lung cancer patients. In addition, by ROC analysis (Fig. 2), miR-483-5p was demonstrated to have high ability to screen VTE patients from non-VTE patients with an area under the ROC curve (AUC) of 0.907. At the cutoff value of 1.32, the sensitivity and specificity were 71.67% and 92.73%, respectively.

Comparison of the predictive value of miR-483-5p in patients with different BMI values

As shown in Fig. 3, the percentage referred to the ratio of the number of VTE patients in different BMI groups to the total VTE patients, and the ratio of the number of non-VTE patients in different BMI groups to the total non-VTE patients. Patients with BMI<18.5, 18.5≤BMI<24, and BMI≥24 were defined as underweight, normal BMI, and overweight groups, respectively. The percentage of VTE patients and non-VTE patients was the highest in the normal BMI group. The percentage of VTE patients was higher and the percentage of non-VTE patients was lower in the overweight group than that in the underweight group. In addition, in the underweight group, the percentage of non-VTE patients was higher than the percentage of VTE patients. In the overweight group, the percentage of VTE patients was higher than the percentage of non-VTE patients. miR-485-3p had a

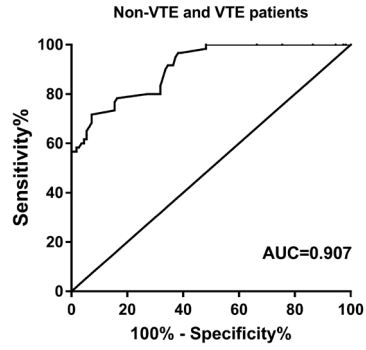

Figure 2. ROC analysis results showed that miR-483-5p had high diagnostic value in screening VTE patients from non-VTE patients (AUC=0.907).
AUC, area under the ROC curve; ROC, receiver operating characteristic. VTE, venous thromboembolism.

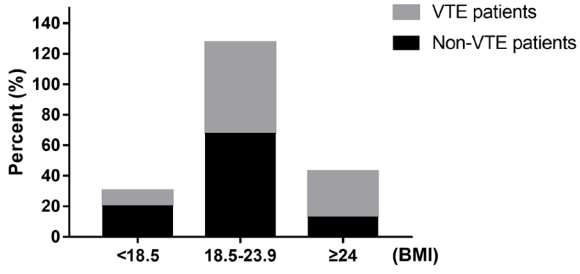

Figure 3. The percent of VTE patients and non-VTE patients in patients with different BMI values.
VTE, venous thromboembolism.

certain role in differentiating between VTE patients (n=6) and non-VTE patients (n=22) in the underweight group (AUC=0780, Fig. 4A). Figure 4B revealed that miR-485-3p had the ability to discriminate between VTE patients (n=36) and non-VTE patients (n=74) in the normal BMI group with an AUC of 0.906 (sensitivity of 80.56%, specificity of 85.14% and cutoff value of 1.21). Figure 4C showed the high ability of miR-485-3p in screening VTE

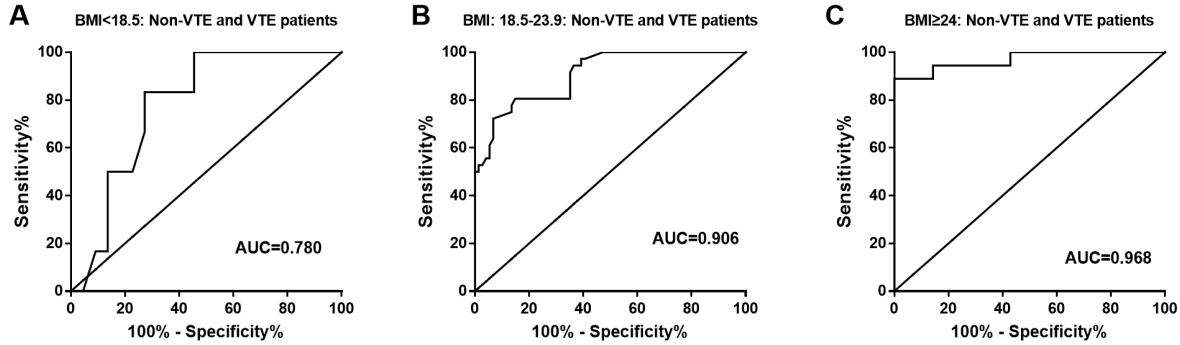

Figure 4. ROC analysis for miR-483-5p for screening VTE patients in different BMI level group.
The ability of miR-483-5p in differentiating between VTE patients and non-VTE patients in BMI<18.5 group (Fig. 4A, AUC=0.780), in normal BMI group (Fig. 4B, AUC=0.906), and in BMI≥24 group (Fig. 4C, AUC=0.968). AUC, area under the ROC curve; ROC, receiver operating characteristic. VTE, venous thromboembolism.

patients (n=18) from non-VTE patients (n=14) in the overweight group (AUC=0.968, sensitivity=88.89%, specificity=100% and cutoff value=1.705).

DISCUSSION

Increasing evidence has indicated the correlation of miRNAs with VTE. For example, Wang and others have shown the role of miR-195-5p and miR-205-5p as potential biomarkers in cervical cancer patients treated for VTE (Wang *et al.*, 2020). Zhang and others have reported that miR-338-5p is downregulated in VTE and that it suppresses VTE (Zhang *et al.*, 2020). In addition, miR-483-5p has been found to play an inhibitory effect on angiogenesis (Qiao *et al.*, 2011). A study has shown that miR-483-5p can regulate the function of pulmonary microvascular endothelial cells (Leng *et al.*, 2020). Therefore, we concluded that miR-483-5p may also be correlated with the progression of VTE. This study revealed a higher miR-483-5p expression in VTE patients than that in non-VTE patients. In addition, the correlation of miR-483-5p with BMI, hypertension, CRP, and platelet count in VTE patients was found. Thus, miR-483-5p may be correlated with VTE progression in lung cancer patients.

miRNAs may be ideal biomarkers with the advantages of low detection limits and strong stability. Many studies have shown the prognostic value of miRNAs for various diseases. For instance, miR-892a may act as a prognostic marker for patients with gastric cancer (Lv *et al.*, 2020). Serum miR-22 may be used as a new prognostic biomarker for acute myeloid leukemia (Qu *et al.*, 2020). miR-211-5p can serve as an independent prognostic factor for the poor prognosis of atherosclerosis (Zhang *et al.*, 2021). In addition, many studies have found the potential of miRNAs in predicting the VTE occurrence, such as miR-296-5p (Pan *et al.*, 2021), miR-195-5p (Jin *et al.*, 2019), and miR-374b-5p (Zhang *et al.*, 2020). In this study, Cox analysis results indicated that BMI and miR-483-5p were independently correlated with the occurrence of VTE in lung cancer patients. Additionally, ROC analysis results indicated that miR-483-5p has a high ability to differentiate between VTE patients and non-VTE patients. Thus, our study results demonstrated that miR-483-5p can be used as an independent predictor of VTE in lung cancer patients. Notably, aberrant miR-483-5p has been reported to be a biomarker for other diseases, such as adrenocortical cancer (Oreglia *et al.*, 2020), coronary plaque rupture (Li *et al.*, 2017), and esophageal squamous cell carcinoma (Xue *et al.*, 2017). Thus, serum miR-483-5p may function as an independent biomarker to predict the occurrence of VTE in lung cancer patients.

Considering that miR-483-5p and BMI were two independent risk factors for VTE occurrence and that they were significantly correlated, we analyzed the predictive value of miR-483-5p for VTE occurrence in three groups of patients with different BMI values. Notably, it has been found that BMI is an independent risk factor for VTE (Hotoleanu, 2020), and a study has demonstrated that miR-483-5p is significantly correlated with obesity and BMI (Gallo *et al.*, 2018). The results of this study indicated that miR-483-5p had the highest ability in differentiating between VTE patients and non-VTE patients in the overweight group. Thus, in the group with high BMI values, referencing miR-483-5p levels would enable a more accurate screening of patients at high risk of VTE. Therefore, it can be speculated that miR-483-5p may be related to obesity and BMI, thereby participating in the occurrence and development of BMI-related diseases. In clinical practice, patients with higher BMI should pay more attention to their miR-483-5p levels, because patients with significantly increased miR-483-5p levels have a higher risk of developing VTE.

Further validation analyses are important, so we estimate the statistical power of the study due to the time limitation. A total of 170 lung cancer patients treated at our hospital were enrolled in this study, including 60 VTE patients and 110 non-VTE patients. Where an included sample has been identified, the statistical power has a high probability value of meeting the statistical data variation of VTE patients being different from non-VTE patients, suggesting that the included VTE patients indeed differ from non-VTE patients in a statistical sense and that the findings were reliable. However, there were some limitations. First, of the 170 lung cancer patients recruited who met the inclusion criteria, there were only 32 (18.82%) overweight lung cancer patients, including 18 (30.00%) overweight lung cancer patients who developed VTE after surgery, and 14 (12.73%) overweight lung cancer patients did not develop VTE after surgery. Whether it concerns the overall included lung cancer patients or the overweight lung cancer patients, the sample size of this study is small and further research is needed on a larger scale. Second, this study has not elucidated how miR-483-5p functions in VTE, and the mechanism is urgently needed to be explored by further experimental analysis. Previous studies have shown that miR-483-5p targeted RNA binding motif protein 5 (RBM5) (Wang *et al.*, 2018) and proprotein convertase subtilisin/kexin type 9 (PCSK9) (Dong *et al.*, 2020) and was involved in the disease progression. The results

of the present study revealed that miR-483-5p was increased in VTE patients and could independently predict VTE onset in lung cancer patients, eliciting a potential indirect link of VTE to RBM5 and PCSK9 via miR-483-5p. Therefore, we speculated that the mechanism might be that miR-483-5p was associated with VTE by targeting RBM5 or PCSK9. However, this speculation needs to be verified by further experimental analysis, which is also our next research direction.

In conclusion, the findings of this study indicate that miR-483-5p is higher in VTE patients than that in non-VTE patients, is associated with BMI in VTE patients, and can serve as an independent predictor of VTE in lung cancer patients. Additionally, miR-483-5p had the highest value in differentiating patients with and without VTE in patients with high BMI. Thus, miR-483-5p overexpression can independently predict VTE onset in patients with lung cancer, especially in patients with high BMI. This study may provide new targets and ideas for screening patients with high VTE risk among lung cancer patients.

Declaration

Ethics approval and consent to participate. The experimental procedures were all in accordance with the guideline of the Ethics Committee of Qingdao Jiaozhou Central Hospital and has approved by the Ethics Committee of Qingdao Jiaozhou Central Hospital. This study complies with the Declaration of Helsinki.

A signed written informed consent was obtained from each patient.

Consent for publication. Not applicable.

Availability of data and materials. The data used and analyzed can be obtained from the corresponding author under a reasonable request.

Competing interests. The authors declare that they have no competing interests.

Funding. None.

Acknowledgements. Not applicable.

Authors' contributions. MZ carried out the research design and conception; JL and CL analyzed and interpreted the data regarding; GT performed the examination of sample; JL and GT contributed essential reagents or tools. MZ and HW authors wrote and revised the manuscript. All authors read and approved the final manuscript.

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