Expressions of miR-29a, TNF-A and Vascular Endothelial Growth Factor in Peripheral Blood of Pulmonary Tuberculosis Patients and Their Clinical Significance

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Abstract

Background: To investigate the expression levels of miRNA-29a (miR-29a), tumor necrosis factor-α (TNF-α) and vascular endothelial growth factor (VEGF) in peripheral blood of pulmonary tuberculosis patients and their correlation with clinical and pathological features.

Methods: A prospective analysis was performed on 192 pulmonary tuberculosis patients (pulmonary tuberculosis group) and 186 healthy patients (control group) who were admitted to Beijing Chest Hospital, Capital Medical University, Beijing, China from Jun 2015 to Jun 2019. Real-time quantitative PCR was used to detect the expression levels of miR-29a, and ELISA to detect the concentrations of TNF-α and VEGF in serum. The diagnostic value of miR-29a, TNF-α and VEGF in tuberculosis was analyzed using receiver operating characteristic curve (ROC). The correlation of the expression levels of miR-29a, TNF-α and VEGF with gender, age, low-grade fever, expectoration, hemoptysis and pulmonary tuberculosis classification was analyzed.

Results: The expression levels of miR-29a, TNF-α and VEGF in pulmonary tuberculosis group were significantly higher than those in control group ($P<0.001$). The area under curves of miR-29a, TNF-α, and VEGF were 0.818, 0.743, and 0.805, respectively. miR-29a was closely correlated with low-grade fever, expectoration, hemoptysis and pulmonary tuberculosis classification ($P<0.050$). TNF-α and VEGF were closely correlated with patient age, low-grade fever, expectoration, hemoptysis and pulmonary tuberculosis classification ($P<0.050$).

Conclusion: Highly expressed in pulmonary tuberculosis patients, TNF-α and VEGF are closely correlated with the disease progression of patients, expected to become targets for the diagnosis and treatment of pulmonary tuberculosis in the future.

Keywords: miR-29a; Tumor necrosis factor-α (TNF-α); Pulmonary tuberculosis; Clinical pathology; Correlation

Introduction

Pulmonary tuberculosis is a chronic infectious disease caused by *Mycobacterium tuberculosis* infection that seriously endangers human health (1). Up to 10.4 million new cases of tuberculosis occur worldwide each yr, resulting in 1.7 million deaths (2). However, the traditional detection method of sputum smear examination for ob-

serving pathogens by microscopy has a lower diagnostic coincidence rate (3). Due to its higher susceptibility and fatality rate, if the patient is not treated in time after the onset of pulmonary tuberculosis, the patient's quality of life will be seriously affected (4). Having no obvious specific clinical symptoms, pulmonary tuberculosis is easy

to be confused with other respiratory diseases, which makes the patient miss the best treatment time, leading to difficulties in the clinical diagnosis and treatment of it currently (5). Pulmonary tuberculosis should be prevented through "early detection and early treatment". Its low detection rate, pathogenic diagnostic rate and screening rate are important challenges in the prevention and treatment of it at present (6).

At present, clinically pulmonary tuberculosis-related biological markers are monocyte chemotactic protein and inflammatory markers, the expression levels of which are susceptible to interference by drugs and inflammatory reactions (7). miRNAs, a group of non-coding RNA that consists of approximately 19-25 nucleotides in length, can horizontally regulate the expressions of multiple genes after transcription, thus functioning as oncogenes or tumor suppressor genes (8-10). Tumor necrosis factor-α (TNF-α) is an immune regulatory factor. If highly expressed in the body's serum, it can reduce the effect of killing tumor cells and mediate various diseases, resulting in inflammatory reactions in the body (11). Vascular endothelial growth factor (VEGF) is a permeability factor that promotes neovascularization (12). The elevated level of VEGF in serum may be an indicator of active pulmonary tuberculosis, playing an important role in the pathogenesis and progression of pulmonary tuberculosis lesion (13).

In this study, the expression levels of miR-29a, TNF-α and VEGF in peripheral blood of pulmonary tuberculosis patients were analyzed, and their correlation with the clinical and pathological features of pulmonary tuberculosis was explored, in order to provide clinical value in the occurrence and development, prediction and prognosis, diagnosis and treatment of pulmonary tuberculosis.

Materials and Methods

Patient data

A prospective analysis was performed on 192 pulmonary tuberculosis patients and 186 healthy patients who were admitted to Beijing Chest Hospital, Capital Medical University, Beijing, China from Jun 2015 to Jun 2019. Overall 192 pulmonary tuberculosis patients were in pulmonary tuberculosis group, including 119 males and 73 females, aged 18-73 yr old, with an average age of (47.24±5.38) yr old. Overall, 186 healthy patients were in control group, including 101 males and 85 females, aged 19-70 yr old, with an average age of (45.63±4.16) yr old.

All subjects and their families signed informed consent form and cooperated with medical staff to complete relevant medical treatment.

Inclusion criteria were referring to 2013 diagnostic guidelines for pulmonary tuberculosis (14), patients diagnosed as in the progressive stage of pulmonary tuberculosis by imaging, sputum examination and T-point positive diagnosis; patients with complete cases; patients >18 yr old; patients who had not received anti-tuberculosis treatment in other hospitals.

Exclusion criteria: patients with other pulmonary diseases; patients with acute gastrointestinal bleeding or other severe diseases; patients with tumors; patients with a history of blood and respiratory diseases.

Four mL of peripheral venous blood was extracted from two groups of patients on an empty stomach. After anticoagulation treatment, the blood was centrifuged at 3000 r/min for 20 min. The upper serum was taken and stored in a refrigerator at -80 °C for testing.

Main instruments and reagents

TRIzol kit (Wuhan Purity Biotechnology Co., Ltd., Item No.: CD-13433-ML), miRNA reverse transcription kit (Beijing Baiao Laibo Technology Co., Ltd., Item No.: ALH266-PTO), SYBR Premix Ex Taq™ II kit (Beijing Zhijie Fangyuan Technology Co., Ltd., Item No.: DRR041A). All primer sequences were designed and synthesized by Shanghai Sangon Biotech Co., Ltd., (Table 1). GeneAmp 7300 PCR system amplification instrument (American ABI Company), human TNF-α ELISA kit (Shanghai Walan Biotechnology Co., Ltd., Item No.: E03680), human VEGF ELISA kit (Shanghai Jitai Yikesai Biotechnology Co., Ltd., Item No.: EH015).

Table 1: MiR-29a primer and internal reference sequences

Primer sequence	Upstream primer	Downstream primer
miR-29a	5'-GGGTAGCACCATCTGAAA-3'	5'-CAGTGCGTGTCCTGGAGT-3'
U6	5'-CTCGCTTCGGCAGCACA-3'	5'-AACGCTTCACGAATTTGCGT-3'

Detection of miR-29a expression level

Real-time quantitative PCR was used to detect miR-29a, and TRIzol kit to extract whole blood total RNA, with specific steps in strict accordance with the instructions. The concentration and purity of extracted RNA were determined by UV spectrophotometer and protein electrophoresis. After the detection of nucleic acid concentration, the instructions of miRNA reverse transcription kit were used for reverse transcription to generate cDNA in strict accordance with the instructions. The primer sequence was designed and synthesized by Shanghai Sangon Biotech Co., Ltd., with U6 as an internal reference gene in this experiment. Operations were carried out based on the instructions of PCR kit. Then PCR amplification experiment was carried out. The PCR reaction system was: 2 µL of cDNA template, each 0.5 µL of upstream and downstream primers, 10 µL of SYBR Premix, and ddH2O added to 20 µL. An amplification instrument was used for amplification. PCR reaction conditions: pre-denaturation at 94 °C for 30 s, denaturation at 94 °C for 5 s, annealing at 60 °C for 30 s, a total of 40 cycles. Three replicate wells were set for each sample and the experiment was performed 3 times. We used U6 as an internal reference, and 2-△Ct method was used to analyze the expression level of miR-29a in the specimen. See Table 1 for primer sequences.

Detection of TNF-α and VEGF expression levels

ELISA was used to detect the concentrations of TNF-α and VEGF in serum, with specific steps in strict accordance with the instructions of human TNF-α ELISA kit and human VEGF ELISA kit. The kits and samples to be tested were removed from the refrigerator 30 min in advance to balance room temperature, with a sample well to be tested, a standard well and a blank well set. The standard sample was accurately loaded with 50 µL. The sample well to be tested was added with 40 µL of sample dilution and 10 µL of sample to be tested, shaken gently and mixed uniformly, sealed with a microplate sealer and then incubated at 36 °C for 90 min, with the liquid of each well discard and dried. Each well was added with 100 µL of biotinylated antibody working solution, covered with membrane and incubated at 37 °C for 1 h, with the liquid of each well discard and dried, and the plate washed 3 times. Each well was added with 100 µL of enzyme-binding working solution, covered with membrane and incubated at 36 °C for 1 h, with the liquid of each well discard and dried, and the plate was washed 5 times. Each well was added with 100 µL of chromogenic substrate, shaken gently and mixed uniformly, covered with a microplate sealer and then coloring at 37 °C for 30 min in the dark. Each well was added with 100 µL of stop solution to terminate their reactions. Immediately, a microplate reader was used to measure the OD value of each well at a wavelength of 450 nm to calculate the concentrations of TNF-α and VEGF.

Statistical methods

SPSS19.0 (Boyi Zhixun (Beijing) Information Technology Co., Ltd.) statistical software was used for analyzing and processing the data. The patient basic count data were expressed as percentage [n(%)], and the expression levels of miR-29a, TNF-α and VEGF as mean ± standard deviation. T test was used for comparison between two groups, and F test for comparison among four groups. When $P<0.05$, there was a statistically significant difference.

Results

Comparison of patient clinical data

Patients were compared between two groups in terms of gender, age, body weight, smoking and

drinking, with no statistically significant difference, which proves that the two groups of patients are comparable (Table 2).

Changes in expression levels of miR-29a, TNF-α and VEGF in pulmonary tuberculosis group and control group of patients

The expression level of miR-29a was significantly lower in control group (1.01±0.24) than that in pulmonary tuberculosis group (2.85±0.76), (t=31.530, P<0.001) (Fig. 1A).

The expression level of TNF-α was significantly lower in control group (2.19±0.53 pg/ml) than that of pulmonary tuberculosis group (17.23±4.86 pg/ml), (t=41.960, P<0.001) (Fig. 1B). The expression level of VEGF was significantly lower in control group (71.52±6.28 pg/ml) than that of pulmonary tuberculosis group (117.26±12.82 pg/ml), (t=43.830, P<0.001) (Fig. 1c).

Diagnostic value of miR-29a, TNF-α and VEGF in tuberculosis

The area under curve of miR-29a was 0.818, 95% CI: 0.772~0.853, specificity was 70.83%, sensitivity was 89.78%, cut-off value was 1.842. The area under curve of TNF-α was 0.743, 95% CI: 0.693~0.794, specificity was 54.17%, sensitivity was 89.78% cut-off was 10.277. The area under curve of VEGF was 0.805, 95% CI: 0.761~0.850, the specificity was 70.83%, the sensitivity was 78.49%, and the Cut-off was 3.883, (Table 3, Fig. 2).

Correlation of miR-29a expression level with clinical and pathological features of pulmonary tuberculosis

The expression level of miR-29a was higher in patients with low-grade fever symptoms than that in patients without low-grade fever symptoms (P=0.014).

Table 2: Patient basic data of pulmonary tuberculosis group and control group [n(%)]

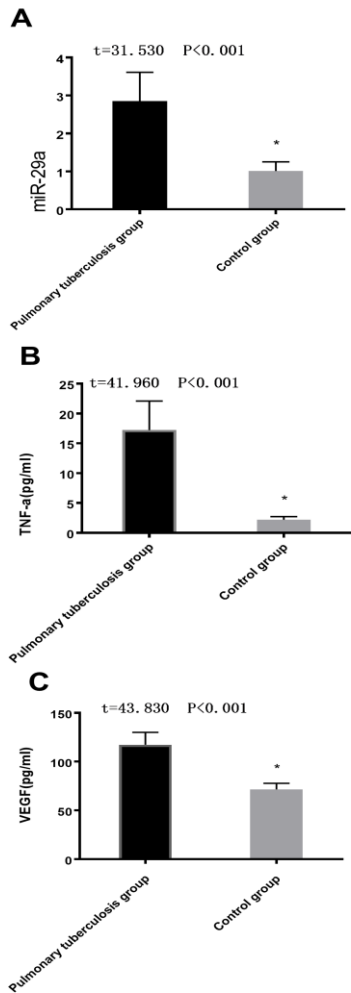| Variable	Pulmonary tuberculosis group (n=192)	Control group (n=186)	X^2	P
Gender			2.289	0.130
Male	119(61.98)	101(54.30)		
Female	73(38.02)	85(45.70)		
Age (yr)			1.399	0.237
≤45	89(46.35)	65(34.95)		
>45	103(53.65)	121(65.05)		
Body weight (kg)			0.827	0.363
≤60	86(44.79)	92(49.46)		
>60	106(55.21)	94(50.54)		
Smoking			1.738	0.187
Yes	123(64.06)	131(70.43)		
No	69(35.94)	55(29.57)		
Drinking			0.695	0.404
Yes	134(69.79)	137(73.66)		
No	58(30.21)	49(26.34)		
Pulmonary tuberculosis classification		-	-	-
Primary pulmonary tuberculosis	93(48.44)	-		
Hematogenous disseminated pulmonary tuberculosis	45(23.44)	-		
Secondary pulmonary tuberculosis	29(15.10)	-		
Tuberculous pleurisy	25(13.02)	-		

Fig. 1: Changes in expression levels of miR-29a, TNF-α and VEGF in pulmonary tuberculosis group and control group of patients

It was higher in patients with expectoration symptoms than that of patients without expectoration symptoms ($P=0.028$). Table 4 shows other variables.

Correlation of TNF-α expression level with clinical and pathological features of pulmonary tuberculosis

The expression level of TNF-α was not correlated with gender ($P>0.05$). It was higher in patients >45 yr old than that in patients ≤45 yr old ($P<0.05$), higher in patients with low-grade fever symptoms than that in patients without low-

grade fever symptoms ($P<0.05$), higher in patients with expectoration symptoms than that in patients without expectoration symptoms ($P<0.05$).
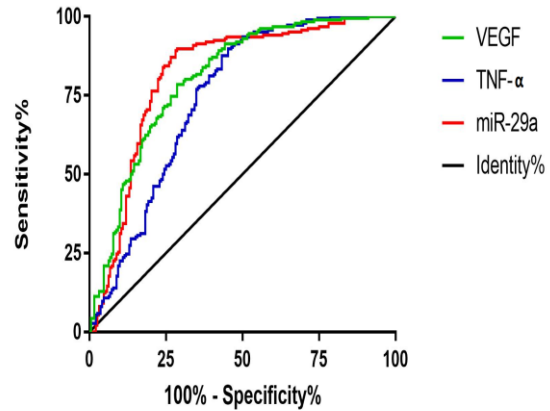

Fig. 2: The ROC curve of miR-29a, TNF-α, and VEGF in the diagnosis of tuberculosis

The area under curve of miR-29a was 0.818. The optimal cut-off point was 1.842, the Yoden index is 60.61% when the specificity was 70.83% and the sensitivity was 89.78%. The area under curve of TNF-α was 0.743. The optimal cut-off point was 10.277, the Yoden index was 43.95% when the sensitivity was 89.78% and the sensitivity was 89.78%. The area under curve of VEGF was 0.805. The optimal cut-off point was 99.107, the Yoden index was 49.32% when the specificity was 70.83% and the sensitivity was 78.49%.

Besides, it was higher in patients with hemoptysis symptoms than that in patients without hemoptysis symptoms ($P<0.05$), higher in patients with chest pain symptoms than that in patients without chest pain symptoms ($P<0.05$) and was statistically significant in pulmonary tuberculosis classification ($P<0.05$) (Table 5).

Correlation of VEGF expression level with clinical and pathological features of pulmonary tuberculosis

The expression level of VEGF was not correlated with gender. It was higher in patients >45 yr old than that in patients ≤45 yr old ($P<0.05$). Other variables are demostarted in Table 6.

Table 3: ROC curves data

Indicator	AUC	95%CI	specificity	sensitivity	Youden index	Cut-off
miR-29a	0.818	0.772~0.853	70.83%	89.78%	60.61%	<1.842
TNF-α	0.743	0.693~0.794	54.17%	89.78%	43.95%	< 10.277
VEGF	0.805	0.761~0.850	70.83%	78.49%	49.32%	< 99.107

Note: AUC: area under the curve, Cut-off: cut-off point

Table 4: Correlation of miR-29a expression level with clinical and pathological features of pulmonary tuberculosis

Variable		n=192	miR-29a	F/t	P
Gender				1.642	0.102
	Male	119	2.91±0.27		
	Female	73	2.79±0.72		
Age (yr)				1.558	0.121
	≤45	89	2.78±0.81		
	>45	103	2.92±0.39		
Low-grade fever				2.492	0.014
	Yes	143	2.92±0.54		
	No	49	2.74±0.25		
Expectoration				2.216	0.028
	Yes	167	2.94±0.38		
	No	25	2.73±0.74		
Hemoptysis				5.229	<0.001
	Yes	110	2.98±0.41		
	No	82	2.71±0.26		
Chest pain				3.336	0.001
	Yes	158	2.96±0.29		
	No	34	2.76±0.35		
Pulmonary tuberculosis classification				4.397	0.005
	Primary pulmonary tuberculosis	93	2.71±0.28		
	Hematogenous disseminated pulmonary tuberculosis	45	2.82±0.31*		
	Secondary pulmonary tuberculosis	29	2.75±0.24		
	Tuberculous pleurisy	25	2.92±0.25*#		

Note: * indicates that compared with primary pulmonary tuberculosis, there is a statistically significant difference (*P*<0.05). # indicates that compared with secondary pulmonary tuberculosis, there is a statistically significant difference (*P*<0.05)

Table 5: Correlation of TNF-α expression level with clinical and pathological features of pulmonary tuberculosis (pg/mL)

Variable		n=192	TNF-α (pg/mL)	F/t	P
Gender				1.953	0.052
	Male	119	18.06±3.27		
	Female	73	16.83±2.51		
Age (yr)				2.696	0.008
	≤45	89	16.83±3.18		
	>45	103	17.98±2.73		
Low-grade fever				3.193	0.002
	Yes	143	18.63±3.59		
	No	49	16.81±2.97		
Expectoration				4.620	<0.001
	Yes	167	18.98±3.41		
	No	25	15.73±2.18		
Hemoptysis				7.270	<0.001
	Yes	110	19.36±3.28		
	No	82	16.03±2.94		
Chest pain				5.991	<0.001
	Yes	158	19.01±3.18		
	No	34	15.52±2.56		
Pulmonary tuberculosis classification				17.150	<0.001
	Primary pulmonary tuberculosis	93	16.60±2.14		
	Hematogenous disseminated pulmonary tuberculosis	45	19.47±3.18*#		
	Secondary pulmonary tuberculosis	29	17.25±2.73		
	Tuberculous pleurisy	25	20.25±4.34*#		

Note: * indicates that compared with primary pulmonary tuberculosis, there is a statistically significant difference ($P<0.05$). # indicates that compared with secondary pulmonary tuberculosis, there is a statistically significant difference ($P<0.05$)

Table 6: Correlation of VEGF expression level with clinical and pathological features of pulmonary tuberculosis

Variable		n=192	VEGF(pg/mL)	F/t	P
Gender				0.747	0.456
	Male	119	116.81±11.64		
	Female	73	118.12±12.06		
Age (yr)				3.129	0.002
	≤45	89	114.37±12.29		
	>45	103	120.46±14.37		
Low-grade fever				3.418	0.001
	Yes	143	122.57±11.48		
	No	49	115.18±10.73		
Expectoration				4.002	<0.001
	Yes	167	123.51±13.07		
	No	25	112.38±12.24		
Hemoptysis				7.478	<0.001
	Yes	110	126.17±15.49		
	No	82	111.48±10.12		
Chest pain				2.436	<0.001
	Yes	158	120.19±13.36		
	No	34	114.21±11.03		
Pulmonary tuberculosis classification				9.743	<0.001
	Primary pulmonary tuberculosis	93	113.48±11.24		
	Hematogenous disseminated pulmonary tuberculosis	45	121.57±12.15*#		
	Secondary pulmonary tuberculosis	29	115.42±11.32		
	Tuberculous pleurisy	25	125.71±13.43*#		

Note: * indicates that compared with primary pulmonary tuberculosis, there is a statistically significant difference ($P<0.05$). # indicates that compared with secondary pulmonary tuberculosis, there is a statistically significant difference ($P<0.05$)

Discussion

Pulmonary tuberculosis is a chronic pulmonary infection disease caused by *M. tuberculosis*, in which inflammatory reactions and allergic reactions coexist, with its main cause as the involvement of various cytokines in the process of immune reactions (15). The strength of autoimmunity and the dynamic equilibrium of cytokines secreted by immune cells are also extremely important (16). At present, pulmonary tuberculosis has high incidence and poor prognosis, with the high risk of infection. Clinical research on the diagnosis, treatment and prevention of it are extremely important (17, 18).

In this study, miR-29a was significantly highly expressed in peripheral blood of patients in pulmonary tuberculosis group, suggesting that it is involved in the occurrence and development of pulmonary tuberculosis. The area under curve of miR-29a, TNF-α, and VEGF were 0.818, 0.743, and 0.805, respectively, suggesting that miR-29a, TNF-α, and VEGF may be diagnostic indicators for tuberculosis. It was closely correlated with patient low-grade fever, expectoration, hemoptysis and pulmonary tuberculosis classification. With the deepening of researches, more and more studies have shown that miRNAs may play a decisive role in pulmonary diseases. Currently, the most common abnormal expression of miRNA is tumor-like diseases, and there are few studies on pulmonary tuberculosis (19, 20). The inter-individual differences in mR-29a expression were significant (especially in pulmonary tuberculosis group of patients) (21). Both TNF-α and VEGF were closely correlated with patient age, low-grade fever, expectoration, hemoptysis and pulmonary tuberculosis classification. TNF-α is an inflammatory cytokine secreted by lymphocytes and mononuclear macrophages. It can promote the inflammatory reaction by up-regulating the expression of adhesion molecule (22). VEGF is a well-recognized vascular permeability factor, large amounts of which can be produced by a variety of cells in the human body when stimulated (23).

Due to limited experimental conditions, there were still some shortcomings. Firstly, we have drawn ROC curves separately for each indicator. Although the area under the curve was high, the specificity was still relatively low. Secondly, we only collected patients with advanced tuberculosis, and did not further observe the indicators of patients in remission period. Thirdly, because of the short time of this study, the patients were not followed up for a long time, and it is unclear whether the expression of each index is related to the prognosis of patients. Therefore, we hope to add more test indicators and joint predictions in future research, and collect more samples to follow up patients to supplement our experiments.

Conclusion

Highly expressed in pulmonary tuberculosis patients, TNF-α and VEGF are closely correlated with the disease progression of patients, expected to become targets for the diagnosis and treatment of pulmonary tuberculosis in the future.

Ethical considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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Conflicts of interest

The authors declare that there is no conflict of interest.

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