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Review Article

Expression of miR-135a in cerebrospinal fluid of patients with tuberculous meningitis and its association with clinicopathological features

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Abstract: Objective: This study aimed to investigate the expression of miR-135a in cerebrospinal fluid (CSF) of patients with tuberculous meningitis (TBM) and its association with clinicopathological features. Methods: Forty-three patients with TBM admitted to Jiujiang the third People's Hospital from September 2016 to December 2018 were enrolled as observation group and 34 healthy individuals as control group. The expression level of miR-135a in CSF of all subjects was quantified by quantitative real-time polymerase chain reaction (qRT-PCR), and the prognostic significance of miR-135a and its association with clinicopathological features of TBM were analyzed. Results: The expression of miR-135a in observation group was remarkably lower than that in the control group ($P<0.05$), and was associated with age and neurological symptoms of patients with TBM ($P<0.05$). Receiver operating characteristic (ROC) curve exhibited that the area under the curve (AUC), Cut-off, sensitivity, and specificity of CSF miR-135a for diagnosing TBM was 0.761 (95% CI: 0.659~0.872), 1.176 ng/mL, 64.71%, and 79.07%, respectively. Logistic regression demonstrated that CSF pressure, protein, chlorine levels, course of disease, and miR-135a were associated with the prognosis of patients with TBM ($P<0.05$). Conclusion: MiR-135a may be involved in TBM progression and related to poor prognosis of patients with TBM.

Keywords: miR-135a, tuberculous meningitis, clinicopathological features, prognosis, diagnostic value

Introduction

Due to the prevalence of human immunodeficiency virus (HIV) and the increase of drug resistance of mycobacterium tuberculosis (MTB), tuberculosis (TB) has become one of the most dangerous infectious diseases in the world [1, 2]. Tuberculous meningitis (TBM), a non-purulent inflammation of meninges and spinal cord membranes induced by MTB, is the most severe form of TB with extremely high mortality and morbidity, and more than 100,000 new cases are reported every year [3]. In 2015, 1.8 million of 10.4 million patients with TBM died worldwide, and for those complicated with HIV-1 infection, the mortality was close to 50% [4, 5].

MicroRNAs (miRNAs) are a kind of ~22 nucleotides long, endogenous, single-stranded, non-coding RNAs widely distributed in eukaryotes [6, 7], which regulate gene expression at post-transcriptional level and participate in cell differentiation, growth, proliferation, as well as apoptosis [8, 9]. Besides, miRNAs play a regulatory role in the development and progression of TB [10]. Ma et al. pointed out that up-regulated miR-579 mediates MTB infection and leads to death of human macrophages [11]. Pan et al. found that the expression of miR-29 in peripheral blood mononuclear cells (PBMCs) and cerebrospinal fluid (CSF) of TBM children was significantly higher than that in healthy controls [12]. However, there were few reports on the differential expression of miRNAs in plasma and CSF of patients with TBM. MiR-135a has been proved to be abnormally expressed in non-small cell lung cancer [13] and Alzheimer's disease [14], but its expression in CSF of TBM patients remains unclear.

Table 1. Primer of miR-135a and U6

Genes	Forward primer	Reverse primer
miR-135a	5'-ACACTCCAGCTGGGTATGGCTTTTTATTCCT-3'	5'-GGTGTCGTGGAGTCGGCAA-3'
U6	5'-CTCGCTTCGGCAGCACA-3'	5'-AACGCTTCACGAATTTGCGT-3'

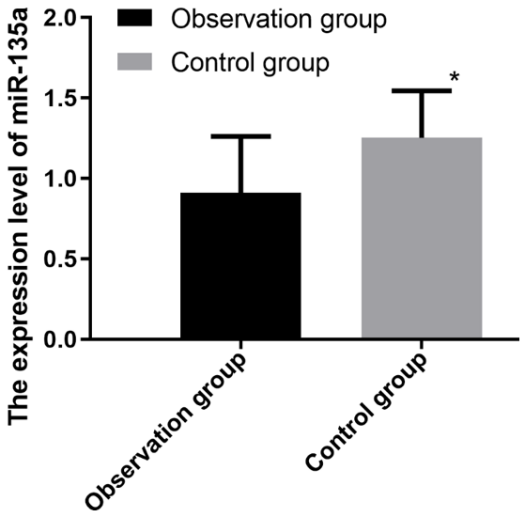

Figure 1. Expression of miR-135a. Note: *indicates that compared with the observation group, P<0.05.

No previous studies have tested the expression of miR-135a in CSF of patients with TBM, and the prognostic factors of TBM are mostly limited to clinicopathological indicators, generally lack of accuracy [15, 16]. MiRNAs are expected to be potential molecular targets for evaluating the prognosis of TBM, so we monitored the expression of miR-135a in patients with TBM and analyzed its association with clinicopathological features and prognosis, so as to provide reference for clinical practice.

Materials and methods

General data

Forty-three patients (19 males and 24 females, aged 16-51 years) with TBM admitted to Jiujiang the third People's Hospital from September 2016 to December 2018 were enrolled as observation group and 34 healthy individuals (15 males and 19 females, aged 17-54 years) as control group. The average age of subjects in the two groups was 37.27±11.48 years and 35.55±10.68 years, respectively. The study was approved by the ethics committee, and all subjects signed the informed consent form. Inclusion criteria: Patients in the observation group developed tuberculous toxic symptoms. CSF cells were collected by slide centrifugal precipitation and stained with acid-fast staining to capture acid-fast bacilli. Purified Protein Derivative of Tuberculin (PPD) test was strongly positive. Antibodies to TB in blood and CSF were tested positive, and head CT was positive as well. Combining these outcomes with clinical symptoms, the patients were diagnosed with TBM. The patients in the control group received physical examinations in Jiujiang the third People's Hospital and had no close contact with TB patients; patients with negative head CT and PPD; patients without TB and other diseases.

Exclusion criteria: patients with other serious infections, severe visceral organ diseases, autoimmune deficiency, other infectious diseases (HIV, typhoid), cryptococcus neoformans meningitis, purulent meningitis, viral meningitis, or meningeal carcinomatosis; patients with contraindications to lumbar puncture or those cannot undergo lumbar puncture within 48 hours; pregnant or lactating women.

Instruments and reagents

Table-top high speed centrifuge (Hangzhou Allsheng Instrument Co., Ltd., AS-08120-00), polymerase chain reaction (PCR) instrument (ABI, USA, 7500), total RNA extraction kit (Shanghai Hengfei Biotechnology Co., Ltd., Solarbio R1200), PCR+Reverse Transcription Kit TransScript® Green miRNA Two-Step qRT-PCR SuperMix (Beijing Bioneeds Biotechnology Co., Ltd., abx 098036), ultraviolet spectrophotometer (Peking Care Industrial Park, uv-1100), primers of miR-135a and internal reference U6 (Shanghai Haling Biotechnology Co., Ltd.). See **Table 1**.

Detection methods

MiR-135a was quantified by quantitative real-time PCR (qRT-PCR): Total RNA in CSF was isolated according to the instructions of total RNA extraction kit. And the concentration was measured using an ultraviolet spectrophotometer. Two microliters of total RNA were reverse-transcribed into cDNA with the reaction system of 42°C for 60 min and 95°C for 5 min. Taking the cDNA as template, amplification was per-

Table 2. Association between miR-135a and clinicopathological features ($\bar{x}$ ±sd)

Clinicopathological features	n	miR-135a (n=53)	t	P
Age			2.164	0.0351
<60	32	0.954±0.368		
≥60	21	0.723±0.398		
Sex			0.165	0.8695
Male	34	0.921±0.322		
Female	19	0.905±0.366		
Concomitant TB at other sites			0.403	0.6889
Yes	22	0.896±0.303		
No	31	0.933±0.347		
Cerebral infarction			0.285	0.777
Yes	18	0.894±0.297		
No	35	0.921±0.341		
Early intrathecal injection			0.772	0.4429
Yes	30	0.943±0.364		
No	35	0.874±0.355		
Meningeal irritation sign			0.198	0.8437
Yes	48	0.937±0.381		
No	5	0.902±0.307		
Epileptic seizure			0.192	0.8487
Yes	7	0.899±0.276		
No	46	0.924±0.327		
Clinical symptoms				
Neurological symptoms			2.025	0.048
Yes	46	0.774±0.264		
No	8	0.985±0.319		
Fever			0.484	0.6303
Yes	37	0.893±0.378		
No	16	0.927±0.384		
Night sweat			0.482	0.6319
Yes	39	0.878±0.332		
No	14	0.927±0.309		

formed on a qRT-PCR instrument. Reaction system (20 μL): 10 μL PCR Premix, 2 μL upstream primer (10×), 2 μL downstream primer (10×), 6 μL ddH_2O (RNase-free and DNase-free). PCR conditions: 90°C for 5 min, 90°C for 5 s, 60°C for 30 s, 72°C for 5 s, for a total of 40 cycles. U6 was taken as the internal reference, and $2^{-\Delta\Delta ct}$ was employed for data analysis.

Prognosis evaluation

The patients were classified into good prognosis group and poor prognosis group at discharge by Glasgow Outcome Scale (GOS) [17]: patients with a score of 4 or 5 were considered to have a good prognosis, and those with a score of 1 to 3 poor prognosis. GOS classification: 5, good recovery: patients resume normal life, but still suffer from minor defects; 4, moderated disability: patients are disabled but independent, and able to work under protection; 3, severe disability: patients are conscious but disabled, and incapable of living independently; 2, vegetative state: patients exhibit only minimal response (e.g. open eyes with sleep/wakefulness cycle); 1, dead.

Statistical methods

SPSS22 statistical software was employed to process the data. The measurement data were expressed by mean ± standard deviation ($\bar{x}$ ±sd), and comparison between groups was

Table 3. Diagnostic value of CSF miR-135a

Diagnostic index	AUC	95% CI	Standard Error	Cut-off	Sensitivity (%)	Specificity (%)
miR-135a	0.761	0.659-0.872	0.054	1.176	64.71	79.07

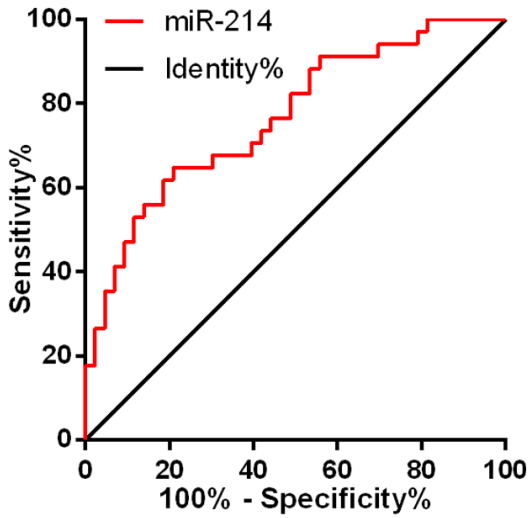

Figure 2. ROC curve of CSF miR-135a in diagnosis of TBM. The AUC of CSF miR-135a for diagnosis of TBM is 0.761, the cut-off is 1.176 ng/ml, the sensitivity is 64.71%, and the specificity is 79.07%.

conducted by *t* test, while comparison of counting data was conducted by χ^2 test. Receiver operating characteristic (ROC) curve assessed the effects of miR-135a on the diagnosis of TBM. Multivariate Logistic regression identified the influencing factors on the prognosis of TBM. Difference was statistically significant as P<0.05.

Results

Expression of miR-135a

According to RT-PCR, CSF miR-135a in the observation group (0.911±0.35) was remarkably lower than that in the control group (1.252±0.293) (P<0.05). See **Figure 1**.

Association between miR-135a and clinicopathological features

The expression of miR-135a in TBM was not related to sex, concomitant TB at other sites, cerebral infarction, early intrathecal injection, meningeal irritation sign, epileptic seizure, fever, or night sweat (P>0.05), but associated with age and neurological symptoms (P<0.05). See **Table 2**.

Diagnostic value of CSF miR-135a for TBM

ROC curve demonstrated that the area under the curve (AUC), cut-off, sensitivity, and specificity of CSF miR-135a for diagnosing TBM was 0.761 (95% CI: 0.659~0.872), 1.176 ng/mL, 64.71%, and 79.07%, respectively. See **Table 3** and **Figure 2**.

Univariate analysis on prognosis of patients with TBM

Univariate analysis showed that age, early intrathecal injection, miR-35a, GOS score, CSF sugar, pressure, protein levels, and course of disease were related to the prognosis of TBM patients (P<0.05), as shown in **Table 4**.

Multivariate analysis on prognosis of patients with TBM

Multivariate analysis illustrated that CSF pressure, protein, and chlorine levels, as well as course of disease, and miR-135a were factors influencing the prognosis of patients with TBM (P<0.05), as shown in **Tables 5** and **6**.

Discussion

TBM, a non-suppurative inflammation of meninges caused by MTB, is the most harmful extrapulmonary TB, and patients may develop permanent sequelae of the central nervous system [18, 19]. Due to its hidden onset and long course of disease, clinical manifestations and laboratory tests lack specificity [20]. The incidence of TBM has been on the rise over years because of the growing AIDS cases and the increase in drug resistance of tubercle bacillus induced by drug abuse and excessive use of immunosuppressants. At present, the diagnosis of TBM mainly relies on pathogen detection, which has the limitations of long cycle, poor sensitivity, and low specificity, thus delaying the diagnosis and treatment timing and resulting in high fatality and high morbidity of TBM [21, 22]. Therefore, it is of great significance to explore methods for the early diagnosis of TBM [23].

Table 4. Univariate analysis on prognosis of patients with TBM ($\bar{x}\pm sd$)

Factor	Good prognosis group (n=29)	Poor prognosis group (n=24)	t/χ^2	P
Sex				
Male	18 (62.07)	16 (66.67)	0.121	0.728
Female	11 (37.93)	8 (33.33)		
Age (years)			9.596	0.002
≤60	23 (79.31)	9 (37.5)		
>60	6 (20.69)	15 (62.5)		
GOS score (points)	12.12±1.86	10.73±2.09	2.561	0.013
CSF sugar (mmol/L)	2.62±0.23	2.12±0.82	3.143	0.003
CSF pressure (mmH_2O)	217.41±48.67	269.38±64.33	3.347	0.002
CSF chlorine (mmol/L)	121.34±8.41	112.91±9.52	0.53	0.001
Concomitant TB at other sites			2.894	0.089
Yes	9 (31.03)	13 (54.17)		
no	20 (68.97)	11 (45.83)		
Cerebral infarction			2.756	0.097
Yes	7 (24.14)	11 (45.83)		
No	22 (75.86)	13 (54.17)		
Early intrathecal injection			5.731	<0.01
Yes	12 (41.38)	4 (16.67)		
No	17 (58.62)	20 (83.33)		
Meningeal irritation sign			1.424	0.233
Yes	25 (86.21)	23 (95.83)		
No	4 (13.79)	1 (4.17)		
CSF protein (g/L)	4317.23±1573.65	6127.54±3023.12	2.802	0.007
miR-135a	0.976±0.312	0.756±0.214	2.929	0.005
Epileptic seizure			0.676	0.4986
Yes	3 (10.34)	4 (16.67)		
No	26 (89.66)	20 (83.33)		
Course of disease (d)	27.42±4.31	35.28±5.67	5.731	<0.01

Table 5. Assignment table

Factor	Assignment
Age	≤60=1, >60=0
Early intrathecal injection	Yes =1, No =0
miR-35a	Continuous variable, analysis is conducted on raw data.
GOS score	Continuous variable, analysis is conducted on raw data.
CSF sugar	Continuous variable, analysis is conducted on raw data.
CSF pressure	Continuous variable, analysis is conducted on raw data.
CSF protein	Continuous variable, analysis is conducted on raw data.
Course of disease (d)	Continuous variable, analysis is conducted on raw data.

Table 6. Multivariate analysis on prognosis of patients with TBM

Variable	β	Standard Error	Wald χ^2	OR (95% CI)	P
Course of disease	-0.364	0.112	11.33	0.671 (0.543-0.824)	0.005
CSF pressure	-0.025	0.009	7.821	0.923 (0.943-0.972)	0.007
CSF chlorine	0.107	0.053	4.415	1.117 (1.103-1.275)	<0.01
Cerebrospinal fluid protein	-0.004	0.321	7.452	0.823 (0.972-1.054)	<0.01
miR-35a	6.632	5.984	1.277	0.754 (0.643-0.987)	<0.01

MiRNA, a single-stranded non-coding endogenous RNA, degrades or inhibits target mRNA translation by binding to the 3' untranslated region, thus negatively regulating gene expression and mediating biological functions [24, 25]. MiRNA molecules play an essential regulatory role in the immune response induced by pathogen infection [26] Dong et al found that miR-135a affects the growth of astrocytes derived from bacterial meningitis via downregulating hypoxia inducible factor 1α (HIF-1α) [27]. CSF miR-135a in the observation group (0.911±0.35) was remarkably lower than that in the control group (1.252±0.293), and it was related to age and neurological symptoms, indicating the abnormal expression of miR-135a in patients with TBM and its involvement in TBM progression. This involvement may be related to the regulation of target proteins, but its specific mechanisms need further investigation. Several miRNAs have been previously reported to be abnormally expressed in TB patients. Singh PK et al. summarized that miR-29a, miR-125b, and miR-155 may be novel diagnostic markers for TB [28]. However, the role of miR-135a expression in TB remains largely unknown. Therefore, we plotted ROC curves and found the AUC, cut-off, sensitivity, and specificity of CSF miR-135a for diagnosing TBM was 0.761 (95% CI: 0.659~0.872), 1.176 ng/mL, 64.71%, and 79.07%, respectively, indicating that miR-135a may be a biological indicator with high diagnostic value in TBM.

GOS score, CSF pressure and protein levels often affect the prognosis of patients with TBM [29, 30]. In recent years, miRNAs have gained a prominent position for their high prognostic value in various diseases [31]. For example, Shi et al. [32] pointed out that the exosomal level of CSF miRNA-21 is related to the poor prognosis and recurrence of malignant glioma. Therefore, we analyzed the factors affecting TBM prognosis. Univariate analysis demonstrated that age, early intrathecal injection, miR-35a, GOS score, CSF pressure, protein, chlorine levels, course of disease were associated with the prognosis of patients with TBM. Further multivariate analysis showed that CSF pressure, protein, and chlorine levels, as well as course of disease, and miR-135a were independent risk factors affecting the prognosis of patients with TBM, which indicated that miR-135a could be used as an indicator for clinical evaluation of prognosis of TBM. Therefore, miR-135a exerts a vital effect on the development, progression, as well as prognosis of TBM.

Although the diagnostic value of miR-135a for TBM and its association with prognosis have been confirmed, there are still several limitations. For example, the specific mechanism of miR-135a in TBM is unclear yet. Therefore, we will perform relevant basic experiments to verify the conclusion of our study.

To sum up, miR-135a may be involved in TBM progression and related to poor prognosis of patients with TBM.

Disclosure of conflict of interest

None.

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