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Original Article

miR-21 expression predicts prognosis in diffuse large B-cell lymphoma

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Abstract: Background: Expression patterns of microRNAs in serum are involved in potentially biomarkers for various diseases. The aim of the study was to investigate the expression level of *miR-21* in diffuse large B cell lymphoma (DLBCL) and its prognostic value. Methods: Real-time quantitative polymerase chain reaction (qRT-PCR) was used to measure *miR-21* levels in serum samples from 112 patients with DLBCL as well as in serum samples from 45 healthy controls. The associations between *miR-21* expression and clinicopathologic parameters and overall survival of the patients, were analyzed by chi-square test and Kaplan-Meier method. The Cox proportional hazards regression analyses were performed to estimate the prognostic values for patient survival prediction. Results: We found that serum *miR-21* expression was markedly upregulated in patients with DLBCL than healthy controls. Increased *miR-21* expression was significantly correlated with B symptoms, IPI score, CHOP-like treatment and Rituximab (all *P*s<0.05). Moreover, DLBCL patients with *miR-21* higher expression have shown significantly worse overall survival than those with lower *miR-21* expression. And *miR-21* expression was an independent prognostic marker of overall survival in a multivariate analysis (*P*=0.001, HR: 4.404, 95% CI: 1.770-10.956). Conclusion: The results of the present study suggested *miR-21* expression level could be a novel potential biomarker for DLBCL prognosis.

Keywords: *MicroRNA-21*, diffuse large B-cell lymphoma, prognosis

Introduction

Diffuse large B-cell lymphoma (DLBCL) is one of the most lethal malignancies and is becoming one of most deadly threat to human health and life worldwide [1]. DLBCL is the most common lymphoma worldwide, accounting for nearly 30 to 40% of non-Hodgkin's lymphoma cases and is highly heterogeneous from both morphological and clinical standpoints [2]. The pathogenic mechanism contributing to the malignant biological characteristics in DLBCL urgently remains to be clarified by the reason of lacking of specific clinical manifestations and responding poor to existing treatment [3, 4]. Although there are latest advancements in diagnostic and therapeutic techniques, a large number of DLBCL patients still have an unfavorable prognosis every year. In addition, despite several biomarkers emerged to better classify and predict outcome at diagnosis, there are not yet routinely used in clinical practice [5]. Thus, exploring more molecular biomarkers involved in DLBCL pathogenesis may novel provide effective therapeutic opportunity.

MicroRNAs (miRNAs) are a class of small, naturally occurring, noncoding and single-stranded RNA molecules (18, 22 nucleotides) that function as post-transcriptional regulators by directly cleaving target messenger RNA (mRNA) or translational repression [6]. A growing number of both direct and indirect evidence suggests a relationship between differential miRNA expression and cancer [7, 8]. However, some miRNAs were found to act as tumor suppressors, whereas others acted as oncogenes, depending on the targets of the miRNAs, which may provide insights into the functional detection of human malignancies [9].

It is reported that specific miRNAs may be associated with outcome in patients with DLBCL [10]. The *microRNA-21* gene (*miR-21*) is the

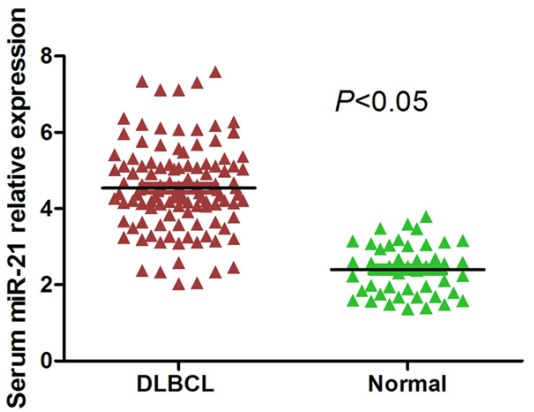

Figure 1. The relative expression level of *miR-21* in serum samples from DLBCL patients and healthy individuals. Serum *miR-21* expression was significantly higher in DLBCL patients compared with healthy individuals ($P<0.05$).

most commonly over-expressed miRNA in cancers. It has been identified as the only miRNA commonly over-expressed in various solid tumors, including lung, breast, stomach, prostate, colon, brain, head and neck, esophagus, and pancreas, as well as in chronic lymphocytic leukemia, uterine leiomyomas, and malignant hepatocytes [9, 11-15]. In addition, a correlation between *miR-21* expression and the carcinogenesis of DLBCL has also been reported [16]. However, the underlying mechanism is not completely clear. Thus, further analyses are needed to clarify the role of *miR-21* in DLBCL prognosis based on clinicopathologic stage.

In the present study, serum *miR-21* expression levels in DLBCL were examined, and the clinicopathologic significance and potential prognostic value for DLBCL were assessed.

Methods and materials

Patients and serum samples

Serum samples were obtained from 112 patients who were diagnosed with DLBCL enrolled at the Tianjin Medical University General Hospital at the time of diagnosis. All of these patients have undergone molecular and phenotypic classification with available clinical data. 45 serum samples from healthy individuals. Blood samples of all patients and healthy control patients were collected. The samples were allowed to stand at room temperature for 30 min and then centrifuged at 3,000 rpm for 15 min at 4°C. To remove cellular contaminants, serum samples were subjected to additional centrifugation at 12,000 rpm for 10 min. The supernatants to be used for RNA extraction were snap-frozen and then stored at -80°C. The study was approved by the Ethics Committee of Tianjin Medical University General Hospital, and ethical permission and informed consent were obtained from all participants.

Isolation of total RNA and real-time quantitative PCR analysis (qRT-PCR)

MiR-21 expression in serum samples from 112 patients with DLBCL and 45 healthy controls was measured by reverse transcription and real-time PCR (RT-PCR). Total RNA was isolated from frozen samples using Trizol reagent (Invitrogen, CA, USA) according to the manufacturer's protocol. The TaqMan microRNA assay and TaqMan universal PCR master mix were used to detect the expression of *miR-21*, and the *U6* gene was used as an internal control to normalize variances. Relative quantification of target miRNA expression was evaluated using the comparative cycle threshold (CT) method. Each sample was examined in triplicate and the raw data were presented as the relative quantity of target miRNA, normalized with respect to U6. RT-PCR primers: *miR-21*: F: 5'-GCGGGTAGCTTATCAGACTG-3'; R: 5'-GTGCAGGGTCCGAGGT-3'; *U6*: F: 5'-GCGCGTCGTGAAGCGTTC-3'; R: 5'-GTGCAGGGTCCGAGGT-3.

Statistical analysis

All statistical calculations were performed using SPSS 18.0 for Windows (SPSS Inc, IL, USA) and GraphPad Prism 5 (GraphPad Software Inc., CA, USA). *miR-21* expression levels in serum samples were shown by mean and standard deviation (mean ± SD) and compared using Student's t-test. The two-tailed Chi-squared test was employed to explore the correlation between *miR-21* expression and clinical pathological features. Survival rates were calculated according to the Kaplan-Meier method and survival curves were plotted; statistical differences were analyzed using the log-rank test. Multivariate analysis of the prognostic factors was performed with Cox regression model. $P<0.05$ was considered statistically significant.

Table 1. *miR-21* expression and clinicopathological features

Characteristics	No. (n=112)	*miR-21* expression levels		*P* values
		Low (n=53)	High (n=59)	
Age (years)				
<60	55	27	28	0.713
≥60	57	26	31	
Gender				
Male	58	28	30	0.834
Female	54	25	29	
Ann Arbor stages				
I-II	47	25	22	0.290
III-IV	65	28	37	
B symptoms				
Absent	67	37	30	0.041
Present	45	16	29	
Extra nodal status				
<2	41	21	20	0.530
≥2	71	32	39	
Serum CRP				
Normal	52	26	26	0.597
High	60	27	33	
IPI score				
0-2	42	25	17	0.045
3-5	70	28	42	
CHOP-like treatment				
No	65	25	40	0.027
Yes	47	28	19	
Rituximab				
No	55	20	35	0.023
Yes	57	33	24	

IPI: International Prognostic Index; CHOP-like refers to CHOP (cyclophosphamide, adriamycin, vincristine and prednisone) or a CHOP-like regimen.

Results

Serum miR-21 is significantly up-regulated in patients with DLBCL

We analyzed the expression levels of *miR-21* in serum samples from 112 DLBCL patients and 45 healthy individuals. As revealed by qRT-PCR analysis, *miR-21* expression was significantly higher in serum samples from DLBCL patients than that from healthy controls ($P<0.05$, **Figure 1**).

Association between miR-21 expression and the clinicopathological features of DLBCL

For better understanding of the clinical relevance of *miR-21* expression in DLBCL, we divided the 112 DLBCL patients into a high expression group (n=53) and a low expression group (n=59), according to the expression level of *miR-21* in all samples. And the relationship of the *miR-21* with various clinical features of DLBCL was analyzed and is summarized in **Table 1**. As shown in the results, *miR-21* expression was closely associated with B symptoms, IPI score, CHOP-like treatment and Rituximab (all *P*s<0.05). However, but there was no relationship with other characteristics, such as age, gender, Ann Arbor stages, extra nodal status, and serum CRP (all *P*s>0.05).

Prognostic values of miR-21 expression in serum samples from DLBCL patients

The correlation between *miR-21* level and survival time of the patients with DLBCL was evaluated by Kaplan-Meier survival analysis. As determined by the log-rank test in **Figure 2**, the survival rates of the patients with high *miR-21* level were significantly lower than those with low *miR-21* level ($P=0.000$). Multivariate analysis using the Cox proportional hazards model for all variables suggested that high *miR-21* expression was an independent prognostic factor for patients with DLBCL (**Table 2**) ($P=0.001$, HR: 4.404, 95% CI: 1.770-10.956).

Discussion

Recently, miRNAs have been demonstrated to play a key role in tumorigenesis. miRNAs may offer a new regulatory model of gene expression, and miRNA expression levels correlate closely with specific clinical features of cancer, so that they can be used to classify normal and cancerous tissues, as well as for prognosis [17-19]. It has been reported that *miR-21* plays a role in the development of tumor via regulating the expression of the tumor suppressor, such as PDCD4, PTEN, and TPM1. Suppression of

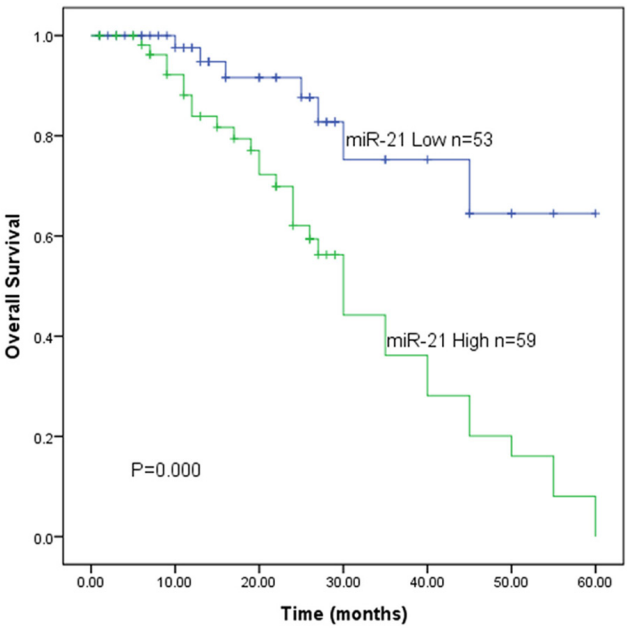

Figure 2. Overexpression of *miR-21* is associated with poor overall survival in patients with DLBCL. Kaplan-Meier analysis of overall survival was analyzed according to *miR-21* expression levels (P=0.000).

Table 2. Multivariate analyses of prognostic variables of overall survival in DLBCL patients

Variables	HR	95% CI	*P* Values
MiR-21 expression	4.404	1.770-10.956	0.001
B symptoms	1.137	0.551-2.348	0.729
IPI score	1.128	0.498-2.556	0.773
CHOP-like treatment	1.036	0.484-2.217	0.928
Rituximab	1.709	0.831-3.514	0.145

miR-21 can inhibit tumor growth, which could indicate *miR-21* functions as an oncogene [20-22]. It has been reported that the dysregulation of *miR-21* performed an important function in different types of cancer [9, 11-15]. However, few studies are available on its expression and functions in DLBCL.

In this study, we investigated the expression of *miR-21* in serum samples from DLBCL patients and healthy controls by qRT-PCR for the first time. Based on the relative expression level analysis, it was investigated that the association of *miR-21* with clinicopathological factors and prognosis of patients with DLBCL. Results showed that the serum level of *miR-21* was up-regulation in DLBCL patients compared with that in healthy controls (P<0.05). The expression pattern of *miR-21* found in our study is in line with previous findings that the expression of *miR-21* was increased in DLBCL cells and was used as an independent prognostic indicator for DLBCL patients by Lawrie's studies [23]. Fang *et al.* found that *miR-21* were significantly elevated in DLBCL serum when compared with normal controls [24]. Wang *et al.* reported that *miR-21* expression was significantly higher in hepatocellular carcinoma tissues compared with normal adjacent liver tissues [25]. Alexander *et al.* showed that *miR-21* showed significantly increased levels in the cerebrospinal fluid of patients with primary central nervous system lymphoma compared with the cerebrospinal fluid of control patients [26]. Charles *et al.* revealed that *miR-21* levels were higher in DLBCL patient than healthy control serum [27]. Chen *et al.* reported that expression of *miR-21* was increased in DLBCL cell lines (OCI-Ly1, OCI-Ly3, OCI-Ly4, OCI-Ly7, OCI-Ly8, OCI-Ly10, OCI-Ly18, OCI-Ly19, and HBL) [28].

In addition, it was also proved that the relative expression level of *miR-21* was closely associated with B symptoms, IPI score, CHOP-like treatment and Rituximab. However, *miR-21* expression was not associated with age, gender, Ann Arbor stages, extra nodal status, and serum CRP.

More importantly, we proved that *miR-21* expression was significantly associated with overall survival by Kaplan-Meier analysis and log-rank test. Patients with high levels of *miR-21* expression had worse overall survival compared with those with low levels of *miR-21* expression. By a Cox proportional hazards model adjusted for factors related to survival of DLBCL, *miR-21* up-regulation could be an independent prognostic marker in patients with DLBCL. These data indicated that *miR-21* expression play a crucial role in tumorigenesis, and progression of DLBCL. Mao *et al.* revealed that serum *miR-21* was used as an independent and powerful predictor of overall survival for primary central nervous system lymphoma [29]. Go *et al.* reported that overexpression of *miR-21* was significantly associated with shorter progression-free survival and overall survival and was an independent prognostic factor in DLBCL patients treated with rituximab-com-

bined chemotherapy [16]. MG Narducci *et al.* reported that *miR-21* was upregulated in cutaneous T-cell lymphoma and could discriminate patients with unfavorable and favorable outcome [30].

In conclusion, the results suggest that *miR-21* is a novel biomarker and a prognostic target for DLBCL in future. Because there are only a few studies on the relationship between *miR-21* expression and the prognosis of DLBCL, further study is required to elucidate the exact molecular mechanisms to verify our conclusions.

Disclosure of conflict of interest

None.

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