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Effect of rituximab combined with chemotherapy on the expression of serum exosome miR-451a in patients with diffuse large b-cell lymphoma

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Abstract. – OBJECTIVE: The aim of this study was to investigate the influence of rituximab combined with chemotherapy on the expression of serum exosome micro ribonucleic acid 451a (miR-451a) in patients with diffuse large B-cell lymphoma (DLBCL), and to explore the possible underlying mechanism.

PATIENTS AND METHODS: 89 DLBCL patients (DLBCL group) receiving rituximab combined with chemotherapy were enrolled in this study. Meanwhile, 48 healthy controls (control group) were enrolled as well. Serum samples were collected from all patients before and after treatment, respectively. At the same time, blood samples of healthy people were collected, and serum exosome was extracted. Real-Time fluorescence-quantitative Polymerase Chain Reaction (qRT-PCR) was applied to measure the expression level of serum exosome miR-451a. Receiver operating characteristics (ROC) curve was used to evaluate the diagnostic efficiency of miR-451a. Statistical Product and Service Solutions (SPSS) 22.0 was employed for statistical analysis. Two-sided 95% confidence interval (CI) was used for all tests, and $p<0.05$ was considered statistically significant.

RESULTS: The expression level of miR-451a in the DLBCL group was significantly lower than that of the control group. The area under the ROC curve (AUC) for the diagnostic efficacy of serum exosome miR-451a for DCBCL was 0.7147. After treatment, the level of serum exosome miR-451a in patients was significantly increased, whereas was still lower than the normal level. The AUC of ROC for evaluating the effect of serum exosome miR-451a in DCBCL was 0.8038.

CONCLUSIONS: Serum exosome miR-451a has moderate diagnostic efficiency for DLBCL. Moreover, miR-451a can act as an indicator for evaluating the efficacy of rituximab combined with chemotherapy in the DLBCL treatment.

Key Words:
Serum exosome, MiR-451a, DLBCL, Rituximab, Chemotherapy.

Introduction

As an aggressive malignant lymphoma, diffuse large B-cell lymphoma (DLBCL) accounts for 35-40% of all non-Hodgkin's lymphomas. Based on the classification by World Health Organization (WHO) in 2008, DLBCL is classified into the following subtypes according to gene expression profiles, including activated B-cell-like subtype, germinal center B-cell-like subtype and unassigned subtype[1]. Recently, researchers have identified four gene subtypes in DLBCL, namely MCD (based on MYD88L265P and CD79B mutations), BN2 (based on BCL6 fusion and NOTCH2 mutation), N1 (based on NOTCH1 mutation) and EZB (based on EZH2 mutation and BCL2 translocation)[2]. As for the DLBCL treatment, the standard treatment regimen for affected patients is the R-CHOP regimen (cyclophosphamide, doxorubicin, vincristine, prednisone, anti-CD20 monoclonal antibody-rituximab). Over 50% of patients with DLBCL can be cured by this regimen. However, around one-third of patients suffer from relapsed/refractory disease due to chemotherapy resistance[3]. International prognostic index (IPI), an existing prognostic assessment method, can only predict the survival time. However, IPI cannot forecast the therapeutic effect[4]. Therefore, discovering new targets and treatments to increase the chemical sensitivity of DLBCL is urgently needed.

Micro-ribonucleic acids (miRNAs) are one of the most promising biomarkers for the diagnosis and prediction of prognosis[5]. MiRNAs are a type of non-coding RNAs with about 20-24 nt in length. Previous studies have shown that miRNAs play roles by regulating the expression of downstream target genes. By directly cleaving mRNA

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or inhibiting protein synthesis, miRNAs target proteins to encode messenger RNAs (mRNAs) at the post-transcriptional level. It is reported that their dysregulation is involved in the occurrence and development of malignant tumors, including cell growth, invasion, metastasis and apoptosis. These findings suggest that miRNAs can serve as molecular markers for cancer diagnosis and prognosis prediction. Recent evidence has manifested that miRNAs are identified in plasma and serum (plasma/serum), which are important minimally-invasive liquid biomarkers for cancer patients[6,7]. Moreover, miRNAs protected by degradation of endogenous RNase[8] have been identified in some plasma/serum in a more stable form *in vitro*. The exosomes are extracellular vesicles (30-150 nm) secreted by cells, which mostly exist in circulating body fluid[9,10]. Serum exosomes contain a kind of tissue-specific protein and selectively-packaged RNAs (mRNA and miRNA). Meanwhile, they are disordered in numerous human malignancies, including renal cell carcinoma (RCC)[11,12]. Previously, Wang et al[13] have revealed that miR-451a significantly inhibits the proliferation and migration of non-small cell lung cancer cells *in vitro*. In contrast, Su et al[14] and Guo et al[15] have shown that miR-451a promotes the proliferation and migration of RCC and pancreatic cancer cells. These findings suggest that miR-451a plays an important role in the occurrence of malignant tumors. It is well known that miRNAs exert different functions in different types of cancer cells. Furthermore, miR-451a may play different roles in the regulation of tumor type-specific invasion and metastasis.

In this study, the expression level of serum exosome miR-451a in DLBCL was measured. Furthermore, its relationship with the treatment response of rituximab in combination with chemotherapy was analyzed.

Patients and Methods

Clinical Data

From January 2016 to January 2018, a total of 89 patients diagnosed with DLBCL *via* pathology in our hospital were enrolled as DLBCL group. All patients were treated based on the R-CHOP regimen. The general data of patients were shown in Table I. Normal serum samples were collected from 48 subjects who received a physical examination in our hospital during the same period. These 48 patients were collected as a control group. This investigation was approved by the Ethics Committee of The Second Affiliated Hospital of Zhejiang University School of Medicine. Signed informed consent was obtained from each subject before the study.

Table I. General data of patients.

Clinical feature		n	p
Gender	Male	43	0.816
	Female	46	
Age	<60 years old	45	0.466
	≥60 years old	44	
IPI	Low risk	23	
	Low-medium risk	19	0.494
	Medium-high risk	26	
	High risk	21	
B symptom	No	41	0.864
	Yes	48	
LDH	Normal	36	0.311
	Increased	53	
Stage	I-II	34	0.054
	III-IV	55	

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the instructions. 2 μg RNA was synthesized into cDNA using PrimeScript® RT (TaKaRa, Dalian, China). ReverTra Ace quantitative PCR (qPCR) RT Kits (article number: FSQ-101, Toyobo, Tokyo, Japan) were used to measure the expression levels of mRNAs. Primers of all genes were synthesized by Sangon Biotechnology (Shanghai, China). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal reference. The relative mRNA expression level of genes was calculated by the $2^{-\Delta Ct}$ method [Δ cycle threshold (Ct) = Ct (target gene) - Ct (GAPDH)]. Primer sequences used in this study were as follows: miR-451a, F: 5'-CGGCATTTGAGCAGGACAA-3', R: 5'-CGAATCGTGAGCGACAGTTCT-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

Extraction of Serum Exosome

Exosome was extracted in strict accordance with an exosome kit. Whole blood (10 mL) was collected from DLBCL patients and controls, and serum was separated. Then, the exosome was isolated from the serum using the ExoQuick Kit (EXOQ20A-1, System Biosciences, Palo Alto, CA, USA) in accordance with the instructions. Subsequently, the expression level of specific

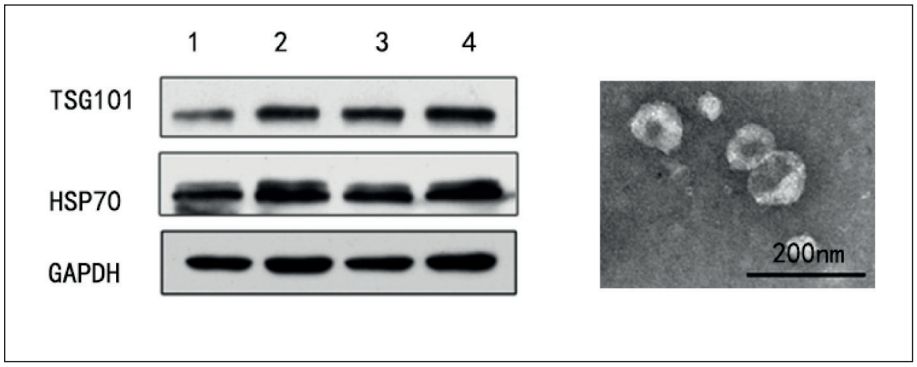

Figure 1. Identification of exosome extracted from serum. **A**, Expression of exosome specific markers (TSG101 and HSP70) in 4 different samples detected *via* Western blotting. **B**, Size and shape observed under the electron microscope. The vesicle's diameter is about 110 nm.

markers in exosomes was detected by Western blotting analysis [TSG101 (Abcam, Cambridge, MA, USA) ab133586, diluted at 1:1000 and HSP70 (Abcam, Cambridge, MA, USA) ab2787, diluted at 1:1000)] according to a previous study[14]. Finally, the exosomes were identified *via* dynamic light scattering (DLS; Malvern Instruments Ltd., Worcestershire, UK) and transmission electron microscopy (TEM).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 (IBM, Armonk, NY, USA) was utilized for all statistical analyses. The χ^2-test was used for the comparison of enumeration data between the two groups. The relative quantitative level of miR-451a was expressed as median (interquartile range). Kruskal-Wallis test and Mann-Whitney U test were performed to compare the difference between the two groups. Spearman analysis was conducted for the correlation between the two groups. Receiver operating characteristic (ROC) curve was used to evaluate the diagnostic efficiency. For all tests, two-sided 95% confidence interval (CI) was used, and $p<0.05$ was considered statistically significant.

Results

Identification of Exosome

Western blotting analysis showed that the specific markers (TSG101 and HSP70) of exosomes were expressed in the extract. The size of the extract was about 116 nm under an electron microscope (Figure 1).

Expression Level and Diagnostic Efficacy of Serum Exosome MiR-451a

The expression of miR-451a in the DLBCL group and control group was analyzed. The results manifested that the expression level of miR-451a in the DLBCL group was significantly lower than that of the control group, showing a statistically significant difference ($p<0.01$) (Figure 2). Meanwhile, the diagnostic efficacy of serum exosome miR-451a in DCBCL was analyzed. It was found that AUC of ROC was 0.7147 (95% CI 0.6654-0.8531) ($p<0.05$) (Figure 3), indicating that the serum exosome miR-451a had medium diagnostic efficacy for DLBCL.

Changes of Serum Exosome MiR-451a in DCBCL Group During Treatment

After treatment, the level of serum exosome miR-451a in DCBCL group was markedly elevated, whereas was still lower than the normal level ($p<0.01$) (Figure 4).

Among 89 patients treated with the R-CHOP regimen, 49 exhibited complete response (CR), 24 partial response (PR), and 25 no response (NR, including stable and progressive diseases). The

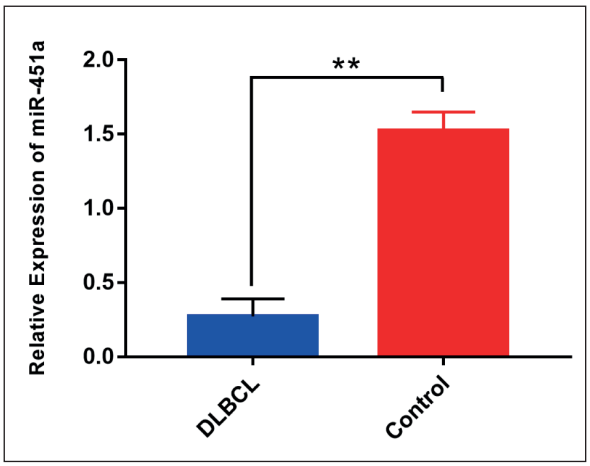

Figure 2. Relative expression level of serum exosome miR-451a in DCBCL and control groups. The expression level of miR-451a in DLBCL group is lower than that in the control group ($p<0.01$).

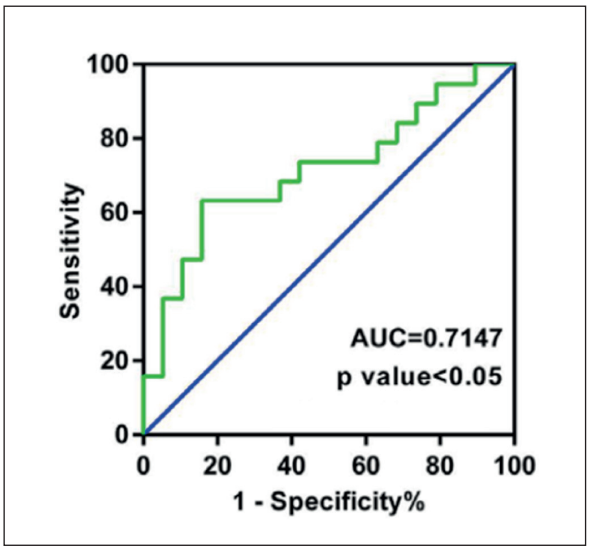

Figure 3. ROC curve of serum exosome miR-451a in diagnosing DCBCL. The AUC of ROC is 0.7147 (95% CI 0.6654-0.8531) (p<0.05).

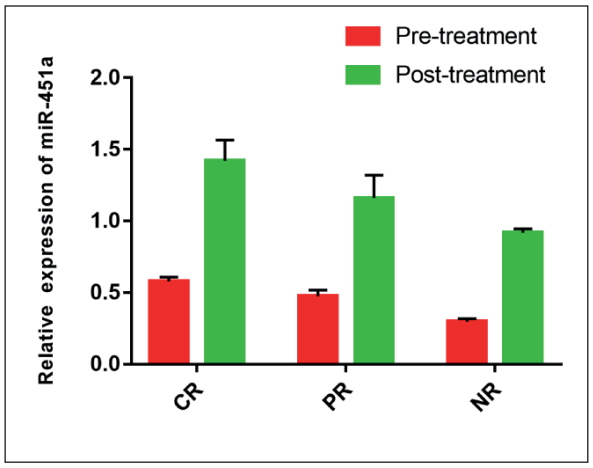

Figure 5. Changes of serum exosome miR-451a in different groups of DCBCL patients. The level of serum exosome miR-451a is gradually elevated (p<0.01) in the three groups. Meanwhile, miR-451a level in the CR group is almost close to the normal level after treatment.

results revealed that the levels of serum exosome miR-451a increased gradually in the three groups (p<0.01). Meanwhile, the level of serum exosome miR-451a in the CR group was mostly close to the normal level after treatment (Figure 5).

ROC Curve for Exosome MiR-451a in Evaluating Therapeutic Effects

The expression level of serum exosome miR-451a was used as the test variable, and the curative effect (response group and non-response group) after chemotherapy was used as the state variable. The ROC curve was plotted, and the critical value was calculated. The results demonstrated that the AUC of ROC was 0.8038 (95% CI 0.7964-0.8491) (p<0.01) (Figure 6), implying that the serum exosome miR-451a performed well in evaluating the efficacy of treatment in patients.

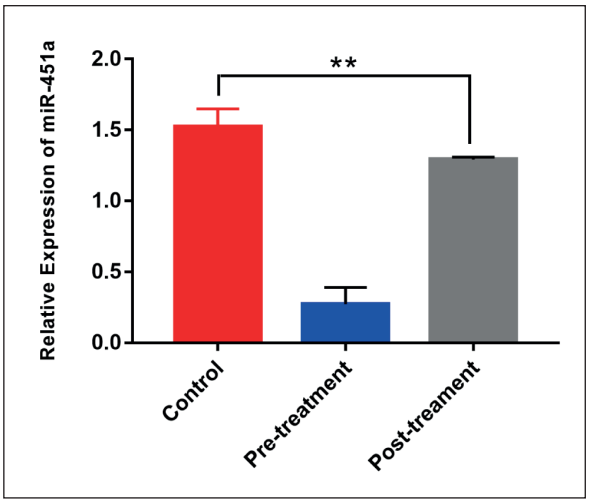

Figure 4. Level of serum exosome miR-451a before and after treatment. After treatment, the level of serum exosome miR-451a is increased, but still lower than normal level (p<0.01).

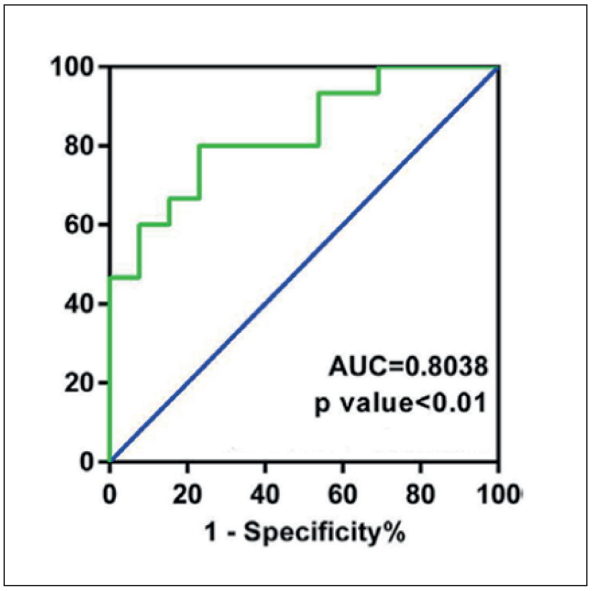

Figure 6. ROC curve for exosome miR-451a in evaluating therapeutic effects. The AUC of ROC is 0.8038 (95% CI 0.7964-0.8491) (p<0.01).

Discussion

DLBCL is an aggressive non-Hodgkin's lymphoma. Chemotherapy resistance and low therapeutic response are major obstacles to its treatment. The exosomes are vesicles secreted by almost all types of cells, which play key roles in tumor therapy. For example, they can mediate drug efflux, direct tumor cells to the paraventricular pathway in case of closure of the primary pathway, and reduce the penetration of anti-tumor drugs[16]. MiRNAs are non-coding RNAs participating in various biological processes. Abnormal expressions of miRNAs are associated with multiple diseases, including cancer. Many miRNAs are detected in malignant tumors. In DLBCL, some miRNAs, such as miR-155 and miR-17-92 clusters, have expression patterns distinguishing DLBCL from non-malignant B-cells[17]. In addition, the expression of miR-21 in tumor cells and serum is closely correlated with the prognosis of DLBCL patients[18]. Exosomes, containing subsets of functional miRNAs, can be delivered to cells and lead to chemical resistance of tumors. Studies have shown that exosomal miRNAs circulate in body fluids in a highly-stable form. Therefore, they can be used as biomarkers to predict the clinical outcome of chemotherapy and the risk of tolerance to chemotherapy in patients.

Previous studies have reported that miRNA-451a serves as a tumor suppressor in various cancers. However, conventional mechanisms or targets are tissue-specific. Wang et al[19] have reported that miRNA-451a plays a key role in the head and neck squamous cell carcinoma (HNSCC). The expression of miRNA-451a is significantly down-regulated in two kinds of HNSCC tissues and cell lines. The up-regulated expression of miRNA-451a leads to the decrease of c-Myc oncogene, which is considered conducive to its antitumor effect in HNSCC. In glioma cells, miRNA-451a modulates transport proteins such as glutamate on the cell membrane, thereby downregulating glucose metabolism and blocking energy supply for cancer cells[21]. MiRNA-451a inhibits the PI3K/Akt pathway in glioma cells by down-regulating cab39. Moreover, a comprehensive study on the mechanism of miRNA-451a action in the HCC progression and metastasis was conducted by Huang *et al*[20] in 2015. They have clearly demonstrated that miR-451a can induce G0/G1 arrest and depress the growth of HCC cells in functional loss and gain experiment. Importantly, miR-451a suppresses epithelial-mesenchymal transition process, suggesting that it may be an inhibitor for HCC metastasis. The antitumor effect of miR-451a in HCC is at least partially achieved by targeting MYC (190080). Previous studies[21] have indicated that MYC is identical with the regulation of targets proposed in HNSCC. Similarly, the antitumor effect of miRNA-451a was observed in this study. Moreover, according to this work, miR-451a was lowly expressed in DLBCL patients. This indicates that miR-451a also functioned as a tumor suppressor in patients with DLBCL. However, its specific mechanism of action needs to be further investigated.

Existing studies have manifested that circulating miRNAs can be used as predictive biomarkers for therapeutic response of R-CHOP. A previous work analyzed 736 miRNAs. The results have found that the up-regulation of miR-455-3p and miR-33a is related to chemo-sensitivity. Meanwhile, the increases in miR-224, miR-1236 and mir-520-3p are associated with tolerance to chemotherapy. Another study analyzing 8 miRNAs has discovered that the up-regulation of miR-125b and miR-130a is correlated with R-CHOP chemical resistance. In this work, the value of serum exosome miR-451a in predicting the efficacy of R-CHOP was discovered for the first time. However, due to the small sample size of this study, large sample size prospective studies are required to more clearly understand the value of exosome miR-451a. Furthermore, miRNAs can bind to the 3'-untranslated region of specific mRNAs, thus regulating gene expression at the post-transcriptional level[8]. However, no target genes of miR-451a were detected in this work. Therefore, further in-depth experiments are required for elaboration.

Conclusions

We showed that the serum exosome miR-451a has moderate diagnostic efficiency for DLBCL and can act as an indicator evaluating the efficacy of rituximab combined with chemotherapy in the DLBCL treatment.

Conflict of Interests

The authors declare that they have no conflict of interest.

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