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Overexpression of microRNA-21 in peripheral blood mononuclear cells of patients with B-cell non-Hodgkin's lymphoma is associated with disease stage and treatment outcome

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Abstract. – OBJECTIVE: We wished to assess the association between microRNA-21 (miR-21) and disease stage and treatment outcome in patients with B-cell non-Hodgkin's Lymphoma (B-NHL).

PATIENTS AND METHODS: A total of consecutive 128 patients with B-NHL were enrolled; 30 healthy individuals served as controls. qPCR assay was utilized to quantify expression levels of miR-21 in peripheral blood mononuclear cells (PBMC; Ficoll isolation protocol). Expression of the miR-21 target, phosphatase and tensin homolog (PTEN), was assessed by Western blot analysis.

RESULTS: miR-21 was overexpressed in PBMC of patients with B-NHL ($p < 0.05$ vs. healthy individuals). Furthermore, miR-21 expression levels were significantly higher in patients with the stage III/IV B-NHL ($p < 0.05$ vs. stage I/II B-NHL). After chemotherapy, miR-21 expression levels were significantly decreased in patients in complete remission and became comparable to those of healthy individuals. Also, miR-21 expression levels were lower in patients treated with chemotherapy combined with rituximab. There was a negative association between miR-21 overexpression and post-chemotherapy survival rates of the patients. Expression of PTEN was significantly lower in patients with B-NHL ($p < 0.05$ vs. healthy individuals).

CONCLUSIONS: Overexpression of miR-21 is associated with disease stage and treatment outcome of B-NHL. This potentially involves negative modulation of PTEN.

Key Words:
B-cell non-Hodgkin's lymphoma, microRNA-21, Progression-free survival curve.

Introduction

The B-cell non-Hodgkin's lymphoma (B-NHL) is a common malignant tumor with a high degree of heterogeneity, mainly occurring in the lymph node tissues and extranodal lymphoid tissues. According to epidemiological studies, mortality from B-NHL is the tenth highest among other malignancies. The prognosis of B-NHL patients is relatively poor[1]. The pathogenesis of B-NHL is complex and involves aberrant gene expression, antigen receptor gene rearrangement, and chromosome aberrations[2,3]. Due to diverse clinical and pathological manifestations, clinical diagnosis and prognosis estimate of B-NHL is difficult. With development of molecular markers, diagnosis and treatment for B-NHL have been greatly improved. However, long-term survival rates are still low, mainly caused by resistance to chemotherapy drugs and disease relapse. Therefore, it is important to find new molecular targets for B-NHL.

MicroRNA (miRNA) are a type of small endogenic non-coding RNA (18-22 nt) which regulate gene expressions through binding to the 3'-untranslated region of the target mRNA. Many miRNA are associated with proliferation and migration of tumour cells, such as was demonstrated for miRNA-25, miRNA-141, and miRNA-1. Abnormal expression of these miRNA plays an important role in the development of stomach and colon cancers[4-8]. miRNA-21 (miR-21) is a recently discovered tumour miRNA. Its expression is positively associated with malignancy grade[9]. miR-21 regulates the phosphatase and tensin homolog (PTEN) pathway. Activation of the PTEN pathway suppresses tumour genes, leading to proliferation and migration of tumour cells[10]. Up until now, the involvement of miR-21 in B-NHL was unknown. In this study, we tested expression levels of miR-21 in peripheral blood mononuclear cells of patients with B-NHL. Furthermore, we quantified expression levels of PTEN to assess the relationship between miR-21

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and PTEN. In addition, we studied an association of miR-21 expression with disease stage and treatment outcome of B-NHL.

Patients and Methods

Reagents

The Trizol reagent (Life Technologies, Carlsbad, CA, USA) was used for RNA extraction. The Taqman MicroRNA Reverse Transcript Kit and Taqman Real-Time PCR Master Mixes were purchased from Applied Biosystems (Foster City, CA, USA). The rabbit anti-human PTEN polyclonal antibody was purchased from Abcam (Cambridge, MA, USA), whereas the mouse anti-human GAPDH monoclonal antibody was from BioWorld Products Inc. (Dublin, OH, USA). The Ficoll PM 400 Lymphocyte Separation Buffer was purchased from Sigma-Aldrich (St Louis, MO, USA).

Patients

A total of consecutive 128 patients with epidural spinal cord compression were enrolled in this study. The patients included 77 cases of diffuse large B-cell lymphoma, 31 cases of follicular lymphoma, and 20 cases of mantle cell lymphoma. According to Ann Arbor staging system, there were 28 cases at stage I, 32 cases at stage II, 37 cases at stage III, and 31 cases at stage IV of the disease. As control individuals, we enrolled 30 healthy individuals.

Chemotherapy Regimen

The CHOP chemotherapy regimen was used to treat 72 patients with B-NHL. The regimen consisted of 750 mg/m^2 cyclophosphamide (day 1), 40 mg/m^2 hydroxydaunorubicin (day 1), 1.4 mg/m^2 oncovin (day 1), and 60 mg/m^2 prednisone (day 1-5). Three weeks comprised a course of treatment. After 4-6 courses, the efficacy of the treatment was assessed. Another chemotherapy regimen was used in the treatment of the remaining 68 patients with B-NHL: CHOP regimen combined with 375 mg/m^2 monoclonal antibody rituximab. Intravenous infusion of rituximab was performed 1 day prior to each course of chemotherapy treatment. Before infusion of rituximab, 5 mg dexamethasone and 25 mg promethazine were infused.

Evaluation and Follow-up

Peripheral blood was collected at the time of diagnosis and after the chemotherapy. Patients were followed-up after the chemotherapy. The size of lymph nodes was evaluated by the ultrasound, CT or MRI examination. According to the international evaluation criteria of B-NHL, patients were classified as follows: (1) complete remission (palpable lymph node biopsy was negative, diameter of lymph node was not longer than 1.5 cm, and morphological examination of bone marrow biopsy was normal), (2) partial remission (all of the lesions were reduced by about 50% compared with before treatment), (3) stable disease (lesions were enlarged by not more than 25%, or reduced by not more than 50%), (4) progressive disease (lesions were enlarged by more than 25%, and new lesions emerged). Patients who died of causes other that primary disease, or those lost to follow-up at the time of last contact or before the study termination were excluded. Written informed consents were obtained from every patient. The study protocol was approved by the Institutional Ethics Committee of Yuhuangding Hospital.

qPCR Assay

Total RNA of intervertebral disc tissue, peripheral blood and cerebrospinal fluid were extracted with Trizol reagent. Quality of RNA was checked by RNA electrophoresis and spectrophotometrically at optical density of 260/280. RNA was reversely transcribed into cDNA using the Taqman MicroRNA Reverse Transcript Kit. qPCR assay was conducted using Taqman Real-Time PCR Master Mix in accordance with the manufacturer's instructions. U6 was used as an internal control for miR-21. The forward primer for miR-21 was 5'-TAGCTTATCAGACTGATG-3', and reverse primer was the universal type primer provided by the kit. The primers for U6 were, respectively, 5'-CTCGCTTCGGCAGCACA-3' and 5'-AACGCTTCACGAATTTGCGT-3'. The primers for miR-21 and U6 were synthesized by Shenggong Company (Shanghai, China). Each sample was quantified in triplicate.

Western Blotting

Blood peripheral mononuclear cells were isolated using Ficoll PM 400 Lymphocyte Separation Buffer and homogenized in RIPA buffer (50 mM Tris-base, 1 mM EDTA, 150 mM NaCl, 0.1% SDS, 1% Triton X100, 1% sodium deoxycholate). Proteins were separated on a 12% SDS-PAGE gel and analyzed by immunoblotting, with

Glyceraldheyde-3-phosphate Dehydrogenase (GAPDH) used as a gel-loading control. Primary antibodies were rabbit anti-human PTEN polyclonal antibodies (1:1000 dilution) and mouse anti-human anti-GAPDH monoclonal antibodies (1:2000 antibody). The goat anti-rabbit secondary antibody (HRP-conjugate) was used at a 1:10,000 dilution. The blots were detected using ECL chemiluminescence reagent. Band analyses were performed using the Image Lab software version 4.1 (Bio-Rad Laboratories, Hercules, CA, USA). Experiments were repeated at least 3 times.

Statistical Analysis

Data were expressed as mean ± SEM. Statistical analyses were performed with SPSS version 17.0 for Windows (SPSS Inc., Chicago, IL, USA). The paired *t*-test was used for comparisons between groups and analysis of paired data. Progression-free survival analysis for patients with B-NHL was performed to investigate the relationship between miR-21 expression and survival rate of B-NHL. The p value of < 0.05 was considered to indicate significant differences.

Results

miR-21 is Overexpressed in Peripheral Blood Mononuclear Cells of Patients with B-NHL

As shown in Figure 1A, expression levels of miR-21 in peripheral blood mononuclear cells of patients with B-NHL were significantly higher compared with those of healthy individuals. Specifically, expression levels of miR-21 in cells from patients with diffuse large B-cell lymphoma, follicular lymphoma, and mantle cell lymphoma were, respectively, 3.17 ± 0.10, 4.68 ± 0.15, and 2.98 ± 0.05 folds higher than those in healthy cells.

Expression levels of miR-21 in different stages of B-NHL are presented in Figure 1B. It is evident that miR-21 expression in stage III/IV B-NHL is markedly higher than stage I/II B-NHL.

Overall, these findings indicate that miR-21 is overexpressed in peripheral blood mononuclear cells of patients with B-NHL, and the expression levels is further increased with progression of B-NHL.

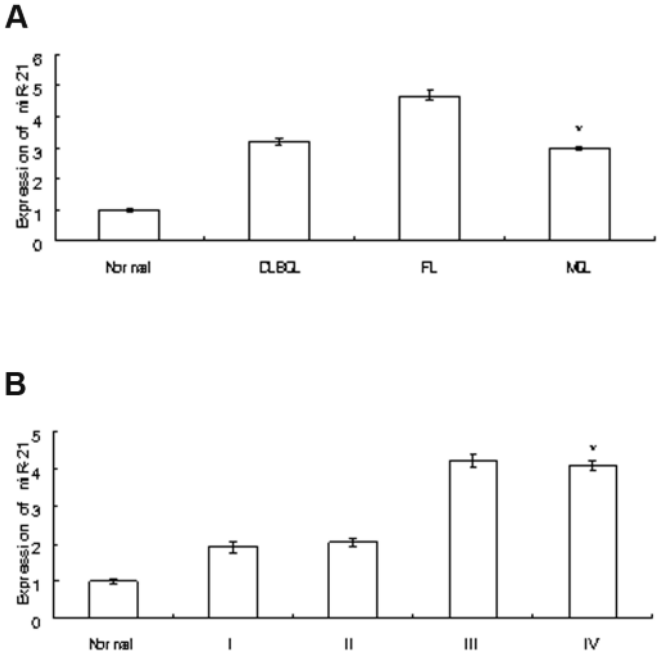

Figure 1. Expression levels of miR-21 in peripheral blood mononuclear cells of patients with B-NHL. Expression levels of miR-21 were detected by qPCR. ***(A)*** Mean ± SEM of normalized expression levels of miR-21 in patients with different types of B-NHL. (B) Mean ± SEM of normalized expression levels of miR-21 in patients with different stages of B-NHL. $^{*}p < 0.05$ vs. healthy ("normal") individuals. DLBCL, diffuse large B-cell lymphoma; FL, follicular lymphoma; MCL, mantle cell lymphoma.

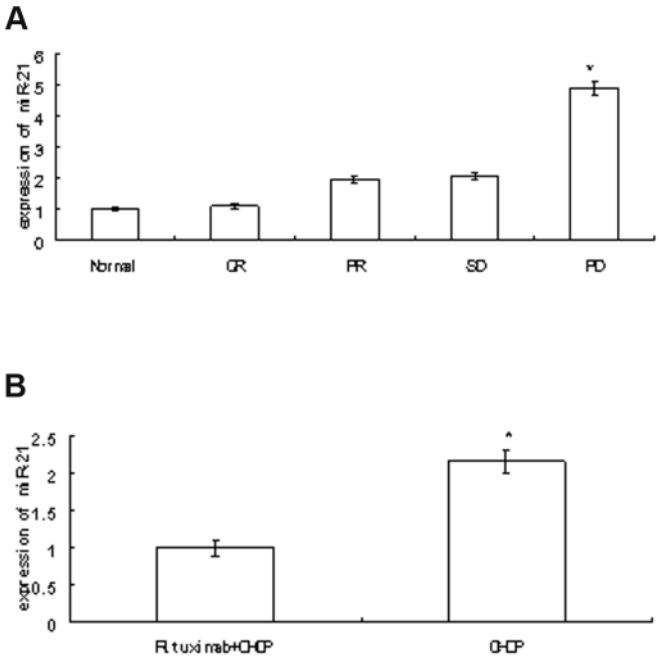

Figure 2. Expression levels of miR-21 in peripheral blood mononuclear cells of patients with B-NHL after the chemotherapy. Expression levels of miR-21 were detected by qPCR. ***(A)*** Mean ± SEM of mean normalized expression levels of miR-21 in patients with B-NHL after the chemotherapy. $^{*}p < 0.05$ vs. healthy ("normal") group ***(B)*** Mean ± SEM of normalized expression levels of miR-21 in patients with B-NHL treated with CHOP or CHOP combined with rituximab chemotherapy. $^{*}p < 0.05$ vs. CHOP combined with ritumixab chemotherapy.

Expression Levels of miR-21 are Associated with Treatment Outcome in B-NHL

We next quantified expression levels of miR-21 in patients with B-NHL after the chemotherapy. As shown in Figure 2A, expression levels of miR-21 decreased after chemotherapy treatment. Furthermore, in patients with complete remission, expression levels of this miR were comparable to those in healthy individuals, whereas in patients with partial remission, stable disease, and progressive disease expression of miR-21 continuously increased (Figure 2B). These observations indicate that expression levels of miR-21 are associated with treatment outcome.

Lower Survival rate of Patients with B-NHL who Express high Levels of miR-21

Subsequently, we performed the progression-free survival analysis. A total of 36 patients were followed up for 6 months after the chemotherapy. These included 16 cases with the stage I/II B-NHL and 20 cases with the stage III/IV disease. Expression levels of miR-21 in peripheral blood mononuclear cells were 2.36 ± 0.17 folds higher in patients with the stage I/II B-NHL compared with those in healthy individuals. However, expression levels in patients with the stage III/IV B-NHL were increased even further (3.89 ± 0.21 folds over healthy levels). Based on the 2.4-fold miR-21 expression level, progression-free survival curve was calculated (Figure 3). The survival rate of patients expressing >2.4-fold elevated miR-21 levels was significantly lower than in those with a lesser degree of overexpression ($p = 0.02$). Thus, the survival rate is lower in patients with B-NHL with high miR-21 expression levels.

miR-21 is Associated to B-NHL Potentially Through Regulation of PTEN

To study whether overexpression of miR-21 modulates the levels of PTEN, a Western blotting analysis was carried out. As shown in Figure 4A, expression levels of PTEN in peripheral blood mononuclear cells of patients with B-NHL were significantly lower compared with those in healthy cells ($p < 0.05$). However, in patients with complete remission after the chemotherapy, expression levels of PTEN were significantly increased when ($p < 0.05$; Figure 4B). These results suggest that miR-21 is associated with B-NHL potentially through regulation of PTEN.

Discussion

In recent years, the incidence of B-NHL has increased. Due to complex pathogenesis, fast process, and high invasiveness, the diagnosis, treatment, and prognosis of B-NHL are difficult. Some cell surface markers (CD20, CD3, and CD43) are used in the diagnosis and classification of B-NHL[11,12]. Currently, traditional CHOP chemotherapy is mainly used for the treatment of B-NHL. With introduction of rituximab and other monoclonal antibody drugs, the prognosis B-NHL has improved. However, reliable molecular markers are still needed for reliable assessment of disease and treatment outcome.

Many miRNA play an important role in the development, invasion, and metastasization of tumours[4-8]. For example, it was shown that expression of miR-7 plays an important role in the differentiation and activation of B cells by regulating the PTEN pathway. Furthermore, activation of T and B cells can be regulated by miR-146, miR-2909, and miR-155[10,13-15]. miR-21 functions, as a proto-oncogene and thereby contributes to cancerogenesis. In our study, expression levels of miR-21 were found to be overexpressed in peripheral blood mononuclear cells of patients with B-NHL. miR-21 expression levels positively correlated to the clinical stage of B-NHL and treatment outcome. Therefore, our findings suggest that miR-21 is clinically significant for assessments of disease stage and treatment outcome in patients with B-NHL.

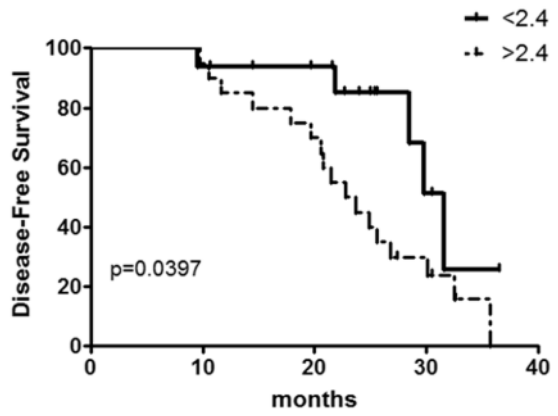

Figure 3. Progression-free survival analysis of patients with B-NHL. A total of 36 patients were followed up 6 months after the chemotherapy. Based on miR-21 expression level increased less or more than 2.4-fold over healthy levels, progression-free survival curve were built. Dotted line shows survival rates of post-chemotherapy patients with more than 2.4-fold increased miR-21 expression levels. Solid line shows survival rates of post-chemotherapy patients with less than 2.4 fold miR-21 expression levels.

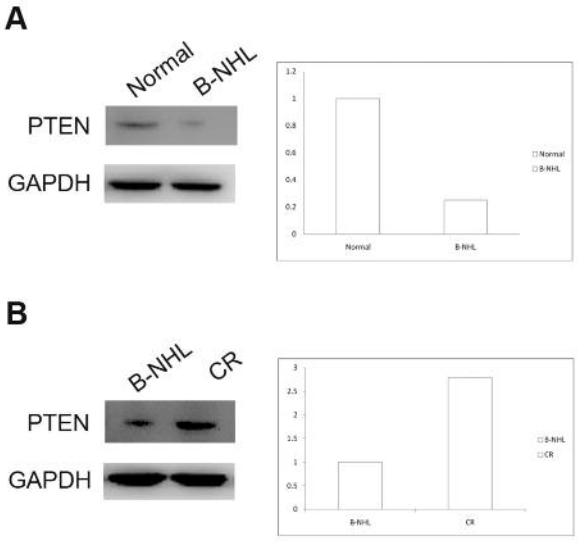

Figure 4. Expression levels of PTEN in peripheral blood mononuclear cells of patients with B-NHL. Protein lysates were separated on a 12% SDS-PAGE gel. GAPDH was used as a gel-loading control. ***(A)*** Expression levels of PTEN in patients with B-NHL and healthy individuals. Left panel: representative Western blot. Right panel: densitometric analysis. $^{*}p < 0.05$ vs. control group. ***(B)*** Expression levels of PTEN in patients with complete remission after the chemotherapy treatment and patients before the chemotherapy. Left panel: representative Western blot. Right panel: densitometric analys. $^{*}p < 0.05$ vs. before the chemotherapy.

We further observed that overexpression of miR-21 was negatively associated with PTEN expression levels. This could indicate the mechanism by which miR-21 contributes to disease progression and resistance to treatment in these patients.

Conclusions

miR-21 is overexpressed in peripheral blood mononuclear cells of patients with B-NHL and is positively associated with the clinical stage and treatment outcome in B-NHL. This may possibly involve negative regulation of PTEN.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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