Inhibition of miR-21 Induces Biological and Behavioral Alterations in Diffuse Large B-Cell Lymphoma

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Behavioral alterations · Diffuse large B-cell lymphoma · MicroRNA · miR-21 · PDCD4 · PTEN

Abstract
MicroRNA-21 (miR-21) has been ascribed a key role in many cellular processes, e.g. tumorigenesis via inhibition of target gene expression. However, its role in diffuse large B-cell lymphoma (DLBCL) is still unclear, and there are no in-depth studies on the relationship between miR-21 and the cellular phenotype of DLBCL. In this study, we investigated the expression and role of miR-21 in the regulation of cell biological processes in DLBCL. Firstly, miR-21 expression was evaluated in three DLBCL cell lines by real-time quantitative reverse-transcription (qRT) polymerase chain reaction (PCR). Then, to determine the possible role of miR-21 in the biological and behavioral characteristics of DLBCL, we performed miR-21 knockdown by transfection with anti-miR-21. In addition, PDCD4 and PTEN were assessed by luciferase reporter assay, qRT-PCR, and Western blot. Our study revealed that miR-21 was significantly upregulated in activated B-cell-like DLBCL cells compared to germinal center-like DLBCL cells. We demonstrated that inhibition of miR-21 induced suppression of proliferation and invasion, as well as increased apoptosis in DLBCL. Moreover, knockdown of miR-21 increased PDCD4 and PTEN expression at the protein level but not at the mRNA level. In conclusion, miR-21 can regulate proliferation, invasion, and apoptosis, and thus it has a potential therapeutic application in DLBCL.

Introduction
MicroRNAs (miRNAs) are endogenously expressed single-stranded noncoding RNAs (21–25 nucleotides in length) that orchestrate various cellular functions and gene expression [1–3]. miRNAs regulate gene expression at the posttranscriptional level, resulting in repression of translation and/or degradation of the mRNA [2–4]. About 3% of genes in the human genome encode for miRNAs, and up to 30% of human protein-coding genes are targets of miRNA regulation [5]. miRNAs play a critical role in multiple biological processes, including cell differentiation, apoptosis, proliferation, and cancer develop-
Diffuse large B-cell lymphoma (DLBCL) is a malignancy of large transformed B lymphocytes. It is the most common non-Hodgkin’s lymphoma, accounting for approximately 40% of all non-Hodgkin’s lymphomas [16]. Using gene expression array technology and immunohistochemical studies, DLBCL was successfully differentiated into at least two distinct molecular subgroups with postulated cell of origin and different prognoses, namely germinal center (GC)-like and activated B-cell (ABC)-like DLBCL [17–19]. Previous studies show that the expression of most miRNAs appears to be lower in malignant tissue compared with the corresponding nonmalignant tissues. However, the expression of some miRNAs is found to be increased in cancer, e.g. a set of miRNAs are upregulated in DLBCL [20], including miR-21 [21]. Indeed, miR-21 has emerged as a key oncomiR since it is the most consistently upregulated miRNA in a wide range of cancers [22–25]. Medina et al. [26] demonstrated that overexpression of miR-21 led to a pre-B malignant lymphoid-like phenotype in vivo. Although functional studies clearly implicate miR-21 as a key molecule in several cancers, the phenotypic alterations caused by miR-21 knockdown in DLBCL remain unclear. In this study, we identified PDCDC4 (programmed cell death 4) and PTEN (phosphatase and tensin homolog) as target genes of miR-21. By suppressing miR-21 in OCI-LY3 and OCI-LY10 cells, we discovered alterations in the biological behavior of DLBCL cells, such as cell proliferation, apoptosis, and invasion. These results may help us to improve the understanding of the biological function of miR-21 in DLBCL and provide promising candidate targets for the treatment of human DLBCL.

Materials and Methods

Cell Culture

Two ABC-type DLBCL (OCI-LY3 and OCI-LY10) cell lines and one GC B-cell (GCB)-type (DB) cell line were routinely cultured in RPMI 1640 containing 10% fetal bovine serum (Gibco, Carlsbad, Calif., USA), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco). OCI-LY3 and OCI-LY10 cells were provided by the Chinese Academy of Sciences (Guangzhou, China), and DB cells were donated by the Rejin Hospital (Shanghai, China). All cell lines were grown at 37°C in humidified cell culture incubators with CO₂ maintained at 5%.

miR-21 Quantification by Real-Time Polymerase Chain Reaction

Total RNA was extracted by TRIzol (Invitrogen, Carlsbad, Calif., USA). The relative expression levels of miR-21 were determined by real-time quantitative reverse transcription (qRT) polymerase chain reaction (PCR) using TaqMan miRNA assays (Applied Biosystems, Foster City, Calif., USA) according to the manufacturer’s instructions. In the RT step, cDNA was reverse transcribed from total RNA (10-ng) samples using a small RNA-specific, stem-loop primer. The cycling conditions were as follows: 30 min at 16°C, 30 min at 42°C, 5 min at 85°C, held at 4°C. In the PCR step, PCR products were amplified from cDNA samples and cycling conditions were as follows: 1 activation cycle (10 min at 95°C) and 40 cycles of PCR (15 s at 95°C, 1 min at 60°C) in the ABI Prism 7500 real-time PCR apparatus (Applied Biosystems). Consistent with the manufacturer’s recommendations, an miRNA was considered to be undetected if CT was >36 in all the three biological replicates. The relative expression was calculated using the 2–ΔΔCT method and normalized to the expression of U6 RNA.

Transfection

For miR-21 knockdown, miR-21 inhibitor (Applied Biosystems) and inhibitor control (Genepharma, Shanghai, China) were delivered at a final concentration of 50 nM in OCI-LY3 and OCI-LY10 using Entranster™-R transfection reagent (Engreen Biosystem, Beijing, China) according to the manufacturer’s protocol.

Cell Proliferation Assay

Cell proliferation in vitro was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT); 1 × 10⁴ cells were seeded per well in three 96-well microplates. After 24, 48, 72, and 96 h of transfection, cells were stained with 50 μl of MTT dye (5 mg/ml Sigma-Aldrich) in each well, and then the plates were returned to the incubator for an additional 4 h. Before supernatant removal, cells were centrifuged and then solubilized in 150 μl of dimethyl sulfoxide thoroughly for 15 min. Colorimetric analysis was performed by a microplate reader (Bio-Rad, Hercules, Calif., USA) at 490 nm. Each experiment was performed in triplicate.

In vitro Invasion Assay

Forty-eight hours after transfection, cells were seeded on a Matrigel-coated membrane matrix (BD Bioscience, San Jose, Calif., USA) in a 24-well cell culture insert companion plate. Fetal bovine serum was added to the lower chamber as a chemoattractant. After 24 h, noninvading cells were still in the upper chamber while the cells migrating into the lower chamber were stained with 0.1% crystal violet and counted under a microscope.

Flow-Cytometric Analysis

Cells were transfected with 50 nM anti-miR-21 or control miRNA for 48 h. After collection and resuspension of the cells with 500 μl of binding buffer, the cell suspension was supplemented with 5 μl annexin V and propidium iodide at room tem-
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The stained cells were analyzed with fluorescent-activated cell sorting using BD LSR II flow cytometry (BD Biosciences).

Dual Luciferase Reporter Assay
To confirm that PDCD4 and PTEN were targeted by miR-21, we cloned the 3′-untranslated region (UTR) fragment of PDCD4 and PTEN mRNA or region mutants (fig. 1a) into the XhoI and NotI sites (fig. 1b) directly downstream of renilla luciferase reporter gene in psi-CHECK™-2 vector (Promega, Madison, Wisc., USA). Primers for PTEN and PDCD4 are shown in table 1. Mutations in the mRNA sequences were created using the QuickChange site-directed mutagenesis kit (Stratagene, Foster City, Calif., USA). For reporter assays, 293T cells were seeded onto 12-well plates the day before transfection. Wild- or mutant-type reporter constructs were cotransfected with the miR-21-mimic/miR-control/luciferase reporters (psi-CHECK-2-3′-PDCD4, psi-CHECK-2-3′-PTEN). Relative luciferase expression from constructs bearing PDCD4 3′-UTR sequences were differentially downregulated by miR-21-mimic compared with those with mutated seed sequences. These experiments were performed in triplicate, and were shown as means ± SD, * p < 0.05. wt = Wild type; mt = mutant.

Western Blot Assay
Cells were collected and lysed, and then protein concentration was measured using Pierce BCA protein assay reagent (Thermo-Scientific, Rockford, Ill., USA). Cell lysates (50 μg) were electrophoresed through 10% polyacrylamide gels and transblotted onto PVDF membrane (Pall Corporation). After blocking with 5% nonfat dry milk in TBST buffer (10 mM Tris, pH 7.5, 150 mM NaCl, and 0.05% Tween-20) for 2 h at room temperature, the membrane was probed with rabbit polyclonal antibody to PDCD4 (1:5,000; Abcam, Cambridge, UK), rabbit monoclonal antibody to PTEN (1:500; Abcam), and mouse monoclonal antibody to β-actin using Lipofectamine 2000 (Invitrogen). After 48 h of transfection, firefly and renilla luciferase activities were measured by a dual luciferase assay (Promega). Renilla luciferase values were normalized to firefly luciferase activity and the ratio of renilla/firefly was presented.
The antibodies were detected using 1:5,000 goat anti-rabbit (Bio-synthesis, Beijing, China) and 1:2,000 goat anti-mouse (Santa Cruz) horseradish peroxidase-conjugated antibodies for 1 h at room temperature.

mRNA Quantification by Real-Time PCR
To determine the mRNA levels of PDCD4 and PTEN, we performed qRT-PCR using the two-step M-MLV platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) according to the manufacturer’s instructions, and normalized to the β-actin mRNA. The primer sequences used were: PDCD4 (sense) 5′-GCAAAAAGGC-GACTAAGGAAAAA-3′ and (antisense) 5′-TAAGGGCGTCAC-TCCCATG-3′; PTEN (sense) 5′-TGGATTGACATTAGACTTGGC-3′ and (antisense) 5′-TGCGGGTGTCAATGTCCTTCT-3′, and β-actin (sense) 5′-CTGGACGCTGAGGTGACA-3′ and (antisense) 5′-AAGGGGACTTCTGTAACAACCGGA-3′. Amplification was monitored on an ABI Prism 7500 real-time PCR apparatus. All reactions were performed in triplicate.

Statistical Analysis
Data were expressed as means ± SE from at least 3 separate experiments performed in triplicate, unless stated otherwise. Student’s t test (two tailed) was performed to compare two groups, and one-way analysis of variance and Pearson’s correlation were performed to determine significant differences in multiple comparisons using SPSS 18.0 software. Values of p < 0.05 were considered statistically significant.

### Results

**Expression of miR-21 in DLBCL Cell Lines**

miR-21 was selected for this study because it was the most overexpressed miRNA in several cancers, such as breast, colon, and hepatocellular cancer. Using miRNA real-time PCR quantitation to measure miR-21 levels in three DLBCL cell lines (OCI-LY3, OCI-LY10, and DB), expression differed between OCI-LY3 and OCI-LY10 and DB cell lines (fig. 2a), which was consistent with previous findings [27]. After transfection with anti-miR-21, the expression levels of miR-21, normalized to U6RNA, were significantly suppressed in the OCI-LY10 (77%) and OCI-LY3 cells (62%) compared with the control miR (fig. 2b).

**miR-21 Influences the Cell Biological Behavior of DLBCL Cells**

To characterize the effect of miR-21 on cell biological and behavioral alterations, we performed MTT, Transwell, and fluorescent-activated cell sorting analyses. Silencing of miR-21 reduced proliferation of DLBCL cells. Compared with the OCI-LY3/miR-control and OCI-LY10/miR-control, the proliferation of the OCI-LY3/anti-miR-21 and OCI-LY10/anti-miR-21 were inhibited down to 87 (p < 0.01), 85 (p < 0.01), 77 (p < 0.01) and 71 (p < 0.01), 73 (p < 0.01), and 67% (p < 0.01) of the pre-transfected levels at 2, 3, and 4 days, respectively (fig. 3a). To examine whether the downregulation of miR-21 in the OCI-LY3 and OCI-LY10 cells affected the invasive ability, we performed an in vitro Matrigel Transwell analysis. The results showed that the number of OCI-LY3/anti-miR-21 and OCI-LY10/anti-miR-21 cells passing through the Matrigel were markedly lower than the numbers of OCI-LY3/miR-21-control and OCI-LY10/miR-21-control cells.
Fluorescent-activated cell sorting was used to measure the percentage of early apoptosis in DLBCL cells after transfection with anti-miR-21. An increase in early apoptosis was detected in both the OCI-LY3/anti-miR-21 and OCI-LY10/anti-miR-21 cells (fig. 3c, d). These data demonstrate the tumorigenic properties of miR-21 in regulating cell proliferation, invasion, and apoptosis.

**PTEN and PDCD4 Are Direct Targets of miR-21**

We found that the relative luciferase activity of the reporter that contained wild-type 3′-UTR of PDCD4 and PTEN was significantly suppressed in the miR-21-mimic group as compared with the miR-21-control group. However, the relative luciferase activity of the mutant PDCD4 and PTEN 3′-UTR reporter construct with 8 nucleotide mutations within the putative seed sequence was almost at the same level as the control psi-CHECK-2 group and failed to respond to miR-21, respectively (fig. 1c, d).

**miR-21 Regulates PDCD4 and PTEN at the Translational Level**

The OCI-LY3 and OCI-LY10 cell lines were selected to investigate miR-21 functions and targets by sequence-specific functional inhibition of miR-21, because both cell
lines express higher levels of miR-21 compared with the DB cells. To investigate the regulation of endogenous target proteins by miR-21, we measured the expression levels of PDCD4 and PTEN by Western blot (fig. 4a, b) and qRT-PCR (fig. 4c) in ABC-type DLBCL. Upon transfection with anti-miR-21, endogenous protein levels were all significantly increased compared with the effect of the miR-control. However, we did not find any significant inhibition of PDCD4 and PTEN at the mRNA level, as measured by qRT-PCR. These results suggest that miR-21 targets PDCD4 and PTEN by functioning at the level of translational regulation.

**Discussion**

Dramatic upregulation of miR-21 has also been reported in most of the tumor types, such as neuroblastoma, lung, breast, colorectal and pancreas tumors, and lymphoma [22–25]. miR-21 has been ascribed a significant role in tumor cell behavior and malignant transformation. Despite the proposed role of miR-21 in several cancers, phenotypic alternations induced by miR-21 in DLBCL have not been determined. In our study, we demonstrated the relationship between miR-21 and the cellular phenotype of DLBCL, and identified the putative binding sites of human PDCD4 and PTEN for miR-21 using a luciferase reporter system. Therefore, we investigated not only the function of miR-21 as a potent oncomiR but also the relationship between miR-21, its targets, and phenotypic alterations.

The fundamental differences between ABC and GCB-like DLBCL are as follows. Firstly, DLBCL cell lines were classified as ABC-like or GCB-like and segregated on the basis of their different immunophenotype, which was based on the algorithm proposed by Hans et al. [19]. Secondly, GCB-like and ABC-like cell lines could be distinguished based on their miRNA expression profiles, and miR-21 was one of the miRNAs with highly increased expression in ABC-type cell lines compared with GCB-type cell lines [27]. Lastly, survival was significantly better in the GCB-like group than in the ABC-like group. miR-21 was reported to be associated with longer relapse-free survival [19]. These findings suggested that differences between the GCB-like and ABC-like DLBCL subtypes were not only based on their distinct cellular origin but also reflected differences in tumor biology.

Medina et al. [26] developed a mouse model to achieve tissue-specific and doxycycline-controlled expression of miR-21; they demonstrated that miR-21 was involved in both tumor initiation and in its maintenance in pre-B-cell
lymphoma. Previous reports showed that miR-21 was not only associated with tumor size and a positive lymph node status, but also could change cell growth and apoptosis in breast cancer [28, 29]. Moreover, miR-21 was demonstrated to regulate cell migration and invasion in hepatocellular cancer [30], and inhibition of miR-21 increased radiosensitivity of esophageal cancer cells [31].

First of all, miR-21 was significantly upregulated in ABC-type compared with GC-type DLBCL, so OCI-LY3 and OCI-LY10 cells were selected for further studies. Through gain or loss of function of the specific miRNA, its function can be clearly explained. On the basis of the miRNA knockdown method according to previous studies, anti-miR-21 was transfected to OCI-LY3 and OCI-LY10. We showed that inhibition of miR-21 not only suppressed cell proliferation of DLBCL cell lines by MTT, but also suppressed their invasive ability by Matrigel Transwell analysis, and the percentage of early apoptosis was markedly elevated. Hence, our study revealed that inhibition of miR-21 is sufficient to modulate tumor cell proliferation, invasion, and apoptosis in DLBCL.

PDCD4 has been identified as a transcript upregulated under apoptosis-inducing conditions [32]. At the molecular level, PDCD4 binds and inhibits the eukaryotic translation initiation factor 4A, thereby impacting on specific mRNA translation and suppressing tumorigenesis. The expression of PDCD4 is lost or suppressed in some tumors, but induced or stimulated in the others [33, 34]. PTEN, a well-known tumor suppressor in multiple cancers, affects the Akt and ERK signaling pathways [35, 36]. These pathways are linked to cell survival, proliferation, differentiation, cell migration, and invasion. PTEN regulates cell invasiveness and apoptosis in hepatocellular carcinoma [30]. In addition, PTEN was lost in 38–48% of advanced breast cancers, suggesting that low expression of PTEN may lead to resistance to trastuzumab [37, 38]. In this study, we presented PDCD4 and PTEN to be direct targets for miR-21. miR-21 and protein levels of PDCD4 and PTEN were inversely expressed in DLBCL, though there was no significant association between miR-21 and mRNA levels of PDCD4 and PTEN after anti-miR-21 transfection. In contrast to previous reports, the rate change in protein expression presented differences, and one possibility could be differences in transfection efficiency.

A previous report suggested that miRNA expression profiling could distinguish cancers according to the diagnosis and developmental stage of the tumor with greater accuracy than traditional gene expression analysis [39]. Several studies revealed that miR-21 expression could predict the prognostic outcome of gliomas and may be uniquely promising as a plasma biomarker for colorectal cancer [40, 41]. Considering miRNAs to be promising markers for cancer diagnosis [42], we presumed that miR-21 might be important in determining diagnosis and clinical outcome in DLBCL patients. If miRNAs could distinguish between different subtypes, they could provide additional prognostic information for DLBCL.

In conclusion, we summarized that inhibition of miR-21 induced biological and behavioral alterations in DLBCL, including proliferation, invasion, and apoptosis. It is likely that this miRNA involves associated changes in multiple targets, suggesting that suppression of miR-21 may be a novel approach for the treatment of DLBCL. Although the network of miRNA-mediated cellular signal transduction is complicated and the discovered mechanism may not be enough to explain the effect of miR-21 in DLBCL cells, the results of our experiment may help us to further understand the biological function of miR-21 in DLBCL and provide promising candidates for the treatment of human DLBCL.

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Disclosure Statement

The authors declare that they have no conflicts of interest.

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