Original Article
The expression and clinical significance of microRNA-34a in diffuse lager B-cell lymphoma

Xiaomin Zhong1, Feng Zhu2, Xiaojin Xu3, Jingdong He4, Xiaojuan Yu1, Xiaofei Chen1*, Kailin Xu2,4*

1Department of Medical Oncology, Huai’an First People’s Hospital, Nanjing Medical University, Huai’an 223300, China; 2Department of Hematology, The Affiliated Hospital of Xuzhou Medical College, Xuzhou 221002, China; 3Department of Hematology, Huai’an First People’s Hospital, Nanjing Medical University, Huai’an 223300, China; 4The First Clinical Medicine School of Nanjing Medical University, Nanjing 210029, China. *Equal contributors.

Received December 21, 2015; Accepted March 21, 2016; Epub June 15, 2016; Published June 30, 2016

Abstract: This study aims to investigate the role and mechanism of miR-34a in diffuse large B-cell lymphoma (DLBCL). Totally 30 DLBCL patients and 30 reactive lymph node hyperplasia patients were selected as DLBCL group and control group respectively. Lymph node tissues were collected. The miR-34a inhibitors or mimics were transfected into OCI-LY10 cells. RT-qPCR was performed to detect levels of miR-34a and AXL receptor tyrosine kinase (AXL) mRNA. Western Blot was used to detect protein levels of AXL. Cell migration and invasion was detected by transwell and matrigel assay. Cell proliferation was measured by MTT assay. Cell apoptosis was assessed by flow cytometry. The results showed that miR-34a level was significantly decreased in DLBCL lymph node tissue compared with that in control lymph tissue. However, the mRNA and protein levels of AXL were significantly higher in lymph tissue of DLBCL group than that in control group. In transfected OCI-LY10 cells, the over-expression of miR-34a could inhibit the mRNA and protein levels of AXL. However, the reduction of miR-34a in OCI-LY10 cells elevated the expressions of AXL mRNA and protein. Besides, the proliferation, metastasis and infiltration of OCI-LY10 cells were increased significantly while the apoptosis was decreased significantly when miR-34a level was reduced. MiR-34a may involve in the pathogenesis of DLBCL via its regulation on AXL, and miR-34a may be used as a new therapeutic target of DLBCL.

Keywords: MicroRNA-34a (miR-34a), diffuse large B-cell lymphoma (DLBCL), AXL

Introduction
Lymphoma is a kind of malignant cancer derived from lymph nodes or extranodal lymphoid tissues. Diffuse large B cell lymphoma (DLBCL), which is the most common type and accounts for 30% to 40% of adult non-Hodgkin lymphoma, is caused by multiple genes, and exhibits heterogeneity in many aspects, including genomics, clinical presentation, morphology and prognosis [1, 2]. Studies have shown that various factors may lead to the high incidence of lymphoma, such as infectious factors (EBV, HIV, measles virus, human T-cell lymphoma leukemia virus, and hepatitis C virus) [3-5], immune-suppression (congenital immune-deficiency, acquired and drug-treatment induced immunosuppression) [6], genes [7], environment pollution [8], and so on. The pathogenesis of DLBCL involves multiple steps. As reported, B-cell-specific chromosomal translocations [9], somatic hyper-mutation, and the amplification, deletion and mutation of common tumor genes [10, 11] are related with DLBCL tumorigenesis. More and more evidence finds that epigenetic mechanism plays important roles in tumorigenesis, and promotes the pathogenesis of DLBCL [12, 13].

Multiple signaling pathways participated in DLBCL may act independently or in forms of synergistic signaling networks [14, 15]. It has already been pointed out that the lymph node metastasis in breast cancer is related with AXL receptor tyrosine kinase (AXL) and its ligand Gas6 [16, 17]. AXL belongs to receptor tyrosine kinases (RTKs) family, and plays important role in regulation of gene transcription. It is involved
miR-34a in DLBCL

in regulation of cell proliferation, adhesion, migration and anti-apoptosis [18-20]. However, studies focusing on effects of RTKs on lymphoma pathogenesis are still rare until now.

Levels of miRNAs are found to be closely associated with DLBCL occurrence, type, invasiveness and prognosis recently [21, 22]. And miRNAs levels may be used as indicators to evaluate the prognosis and drug response of DLBCL [23, 24]. Cancer-specific miRNAs expression disorders can be considered as new tumor molecular signatures [25]. It has been found that levels of miR-155, miR-34a, miR-17, miR-125b, miR-223, miR-150, miR-181, miR-221, miR-21 and miR-29a were abnormal in DLBCL using miRNA chip technology [26-28]. Mark Mackiewicz et al. suggested that AXL was the target gene of miR-34a in breast cancer [29], and other studies have also shown that miR-34a can regulate tumor cell proliferation, invasion, metastasis and sensitivity to radiation in some cancers [30, 31]. Our study mainly investigated the levels of miR-34a and AXL in DLBCL, as well as their interactions and effects on cell function in DLBCL.

Materials and methods

Sample collection

From May 2014 to May 2015, the lymphoma tissues of 30 DLBCL patients (from the Department of Hematology, Affiliated Hospital of Xuzhou Medical College, and from the Department of Oncology, Affiliated Huai'an First Hospital of Nanjing Medical University) were collected; meanwhile, surgical species of 30 patients with reactive lymphoid hyperplasia were collected as control lymph tissues. All the patients accepted no treatment before sample collection, and were in line with the morphological diagnostic criteria for lymphoma pathological classification published by World Health Organization (WHO) on 2008 [32]. There were no significant differences in age and sex of patients in both groups. Samples were stored at -80°C for further use. All the sample collections were approved by ethics committee of Affiliated Hospital of Xuzhou Medical College, and the informed consents were signed by all the patients.

Reagents

DLBCL cell line OCI-LY10 was purchased from American Type Culture Collection (ATCC) (Rockefeller, Maryland, USA). Trizol and Lipofectamine 2000 were all purchased from Invitrogen (Carlsbad, California, USA). miR-34a mimics/inhibitors were purchased from Gene Pharma (Shanghai, China). Rabbit anti-human AXL polyclonal antibody and anti-GAPDH primary antibody were purchased from Abcam (Boston, MA, USA). Reverse Transcription System, SYBR® PrimeScript™ miRNA RT-PCR Kit and mRNA SYBR® Green RT-PCR Kit were supplied by Takara (Tokyo, Japan). BCA Protein Assay Kit was purchased from Thermo Scientific (Waltham, MA, USA). HRP-conjugated goat anti rabbit secondary antibody, MTT, Annexin V-FITC and propidium iodide were all purchased from Beyotime (Shanghai, China). Growth-factor-depleted Matrigel invasion chambers were purchased from BD Company (New York, NJ, USA). Transwell chambers were purchased from Corning (New York, NYS, USA). Image J software 1.49 was purchased from National Institutes of Health (Bethesda, MD, USA).

Cell culture and transfection

OCI-LY10 cells frozen at -80°C previously were recovered at 37°C quickly, seeded in a complete RPMI1640 medium containing 10% FBS and cultured in an incubator containing 5% CO₂ at 37°C. The medium was changed 12 h after the primary seeding firstly, and then was changed every 24 h. The cell passage was conducted at a ratio of 1:3 firstly when cells covered more than 85% of the flask bottom, and later once every 2 to 3 days.

Certain amount of OCI-LY10 cells were plated into 6-well plates when cells grew into the plateau period. For transient transfection, cells at 70% confluence were transfected with has-miR-34a mimics, has-miR-34a inhibitors or control mimics using Lipofectamine 2000 reagents according to manufacturer’s instructions. Briefly, small RNA fragments and Lipofectamine liposome were added to DMEM media respectively and incubated at room temperature for 5 min. The mixture was added to 6-wells and the culture was continued for 48 h before cells were collected.

Real-time fluorescence quantitative-PCR (RT-qPCR)

Total RNA from lymph tissues and cells were extracted by Trizol reagent, and the RNA quality
were detected using RNA molecular electrophoresis and 260/280 ratio calculated by UV spectrometer. And, 1 µg of total RNA were reverse transcribed into cDNA using Poly A tailing method.

RT-qPCR was performed using SYBR® Premix Ex Taq™. For miR-34a, the total 25 µl miRNA RT-qPCR system included 12.5 µl SYBR Premix Ex Taq, 1 µl forward primer and 1 µl Uni-miR RT-qPCR primer, 2 µl template and 8.5 µl double distilled water (ddH₂O). Each sample was set up three parallel wells. MiR-34a was amplified at 95°C for 30 s for denaturation followed by 40 cycles of 5 s at 95°C and 20 s at 60°C. The relative expression of miRNA was normalized against U6 snRNA. For AXL, the 20 µl mRNA RT-qPCR system included 10 µl SYBR Premix Ex Taq, 0.5 µl forward primer, 0.5 µl reverse primer and 8 µl ddH₂O. AXL mRNA was amplified at 95°C for 10 min for denaturation followed by 40 cycles of 1 min at 95°C, 40 s at 60°C, 30 s at 72°C, then AXL mRNA was extended at 72°C for 1 min. The relative expression of AXL was normalized against GAPDH. Each reaction was performed at least 3 times. Fold expression change was calculated using 2^ΔΔt method. Primers used here were presented in Table 1.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-34a</td>
<td>Forward: 5’TGG CAG TGT CCT TGG TGG T3’&lt;br&gt;Backward: Uni-miR qPCR Primer (Takara, Inc)</td>
</tr>
<tr>
<td>U6</td>
<td>Forward: 5’CTCGCTTCGGCAGAC A3’&lt;br&gt;Backward: 5’AACGCTTCAGAATTGCGT3’</td>
</tr>
<tr>
<td>AXL</td>
<td>Forward: 5’CACCCCCAGAAGTGCATAGG3’&lt;br&gt;Backward: 5’GAAGTTCCTTCACGTGGCG3’</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward: 5’GCAGGGCAAGTTGAGGAGT3’&lt;br&gt;Backward: 5’TGCAGGTGCATGCTGAC3’</td>
</tr>
</tbody>
</table>

Western blot

Total protein of lymph tissues and cells were extracted using RIPA lysates, and the protein concentration was detected BCA Protein Assay Kit. Protein samples were separated by SDS-PAGE and then transferred onto PVDF membrane. The membrane was blocked with 50 g/L skim milk for 1 h at room temperate. Then the membrane was incubated with primary antibody (AXL 1:1000; GAPDH 1:2000) at 4°C overnight. After the removal of primary antibody, the membrane was washed using PBST for 15 min and repeated for 3 times. The blots were incubated with HRP-conjugated goat anti rabbit secondary antibody with a dilution of 1:1000 for 1 h at room temperate, washed as previously described, and developed in the ECL system. The protein signal bands were acquired and analyzed by Image J software. The relative expression of targeted protein was presented as the ratio of its gray value against GAPDH gray value.

Cell migration assay

The effect of miR-34a on OCI-LY10 cell migration was detected using Transwell Chambers. Cell transfection was performed as above described. Transfected cells were digested using trypsin and re-suspended by DMEM media containing 0.1% bovine serum albumin (BSA) to the final concentration of 5×10⁵ cells/ml. Totally 200 µl cell suspension were added to the upper chambers while 700 µl DMEM containing 20% FBS was added to the lower chambers. After an incubation of 24 h, cells inside of upper chambers were wiped using a cotton swab, and cells penetrated to the other side of chambers were fixed using 100% methanol, and then stained with 0.1% crystal violet. Numbers of migrated cells were counted under a microscopy.

MTT assay

Cells were seeded in 96-well plate with 3000 cells in each well. Cell transfection was performed as above described. MTT (20 µl) were added at 24 h, 48 h, 72 h and 96 h after transfection, respectively. After a 4 h incubation, the supernatant was removed while 150 µl MTT was added, and the absorbance value at 492 nm wavelength was detected. The above experiments were repeated for 3 times and 5 wells were set in each group.

Cell invasion assay

The analysis of OCI-LY10 cell invasion was conducted using growth-factor depleted Matrigel invasion chambers. DMEM media (500 µl) were added to Matrigel chambers and incubated for 1 h at room temperature for chambers hydration. After removing DMEM medium, 750 µl DMEM medium containing 20% FBS were added to the lower chambers. Cell transfection
miR-34a in DLBCL

was performed as above described. Transfected OCI-LY10 cells were digested and collected at 48 h after transfection, and then re-suspended using DMEM medium containing 0.1% BSA to concentration of 4×10⁵ cells/ml. Cell suspension (500 µl) was added to champer. After incubation for 18 h at 37°C, cells inside champer were wiped using cotton swab. Meanwhile, cells invaded into the other side of champer were fixed with 100% methanol, stained with 0.1% crystal violet, and then counted using a microscopy.

Cell apoptosis

After the 48 h transfection, OCI-LY10 cells were re-suspended using 500 µl binding buffer.

Figure 1. MiR-34a and AXL mRNA level in DLBCL. To determine miR-34a and AXL mRNA level in DLBCL, RT-qPCR was conducted. A. Decreased miR-34a level in DLBCL compared with control group. B. Increased AXL mRNA level in DLBCL compared with control group. *P<0.05 compared with control group.

Figure 2. AXL protein level in DLBCL and its relation with miR-34a. To detect AXL protein level in DLBCL and control group, Western Blot was performed. Targetscan software was used to analyze the relation between miR-34a and AXL. A. Representative image of increased AXL protein level of each group in triplicate. B. Quantitative analysis of increased AXL protein level in each group. Values are presented as mean ± SD of three determinations. C. AXL was one of target genes of miR-34a. *P<0.05 compared with control group.
Next, 5 µl Annexin V-FITC were added to cell suspension and mixed well, and then another 5 µl propidium iodide were added and mixed well. The system incubated in the dark for 15 min at room temperature, and detected using flow cytometry.

**Statistical analysis**

All the data were presented as mean ± standard deviation. SPSS 16.0 was performed for statistical analyze. ANOVA analysis was used for comparison between multiple groups and t-Test was used for comparison between two groups, respectively. P<0.05 was considered statistical difference.

**Results**

**MiR-34a and AXL mRNA levels in DLBCL**

To detect miR-34a and AXL mRNA levels in different lymph tissues collected from DLBCL and reactive lymphoid hyperplasia, RT-qPCR was performed. As presented in Figure 1A, miR-34a level in DLBCL was significantly lower than that in reactive lymphoid hyperplasia (P<0.05), while AXL mRNA levels in DLBCL was significantly higher compared to reactive lymphoid hyperplasia reversely (Figure 1B) (P<0.05).

**AXL protein level in DLBCL and its relation with miR-34a**

To identify AXL protein level in DLBCL, Western Blot was conducted. The results showed that AXL protein level was significantly increased in DLBCL compared with that in reactive lymphoid hyperplasia, and this was in consistent with AXL mRNA expression (Figure 2A and 2B) (P<0.05).

Since decreased miR-34a level was observed in DLBCL accompanied by increased AXL levels, we speculated miR-34a might have regula-
miR-34a in DLBCL

To figure this out, Targetscan software was used to predict targeted genes of hsa-miR-34a. It was predicted there was a targeted regulatory relationship between miR-34a and AXL (Figure 2C).

**AXL levels in OCI-LY10 cells transfected with mimics or inhibitors of has-miR-34a**

To verify the miR-34a regulatory effects on AXL, OCI-LY10 cells were transfected with has-miR-34a mimics or inhibitors for 48 h using liposome transfection method. RT-qPCR and Western Blot were used for detection of RNA and protein, respectively. In OCI-LY10 cells transfected with has-miR-34a mimics, miR-34a level was increased significantly with an increase more than 70% compared with control group (Figure 3A) (P<0.05), however, the mRNA and protein levels of AXL in the same kind cells were significantly deceased compared with control group (Figure 3C, 3E and 3F) (P<0.05). In addition, when OCI-LY10 cells were transfected with has-miR-34a inhibitors, miR-34a level was significantly decreased with a decrease up to 30% compared with control group (Figure 3B) (P<0.05), meanwhile, mRNA and protein levels of AXL in the same kind cells were increased significantly compared to the control group (Figure 3D-F) (P<0.05). The results indicated that in OCI-LY10 cells of DLBCL, the high expression of miR-34a could inhibit gene transcription and translation of AXL.
MiR-34a suppressed OCI-LY10 cell motility

For further study, the effects of miR-34a on migration and invasion of OCI-LY10 cells were explored in vitro. It was found that high expression of miR-34a could lower numbers of migrated or invaded OCI-LY 10 cells significantly compared with control group (Figure 4A and 4C) (P<0.05). Conversely, numbers of migrated or invaded OCI-LY 10 cells were increased significantly compared with control when miR-34a expression was inhibited (Figure 4B and 4D) (P<0.05). The results showed that miR-34a could suppress OCI-LY10 cell motility in DLBCL.

MiR-34a suppressed proliferation of OCI-LY10 cells

To determine the effects of miR-34a on OCI-LY10 cells proliferation, MTT was used to detect cell proliferation at 24 h, 48 h, 72 h and 96 h after transfection, respectively. OCI-LY10 cell
proliferation was suppressed significantly since 48 h after transfection with has-miR-34a mimics transfection inducing high-expressed miR-34a (Figure 5A) (P<0.05), while the proliferation was increased since 48 h after transfection with has-miR-34a inhibitor induced miR-34a expression inhibition (Figure 5B) (P<0.05). The results indicated that miR-34a could suppress proliferation of OCI-LY10 cells in DLBCL.

**MiR-34a promoted OCI-LY10 cell apoptosis**

To identify the effects of miR-34a on apoptosis of OCI-LY10 cell, a flow cytometry was conducted to detect cell apoptosis in transfected cells. Compared to control group, number of apoptotic OCI-LY10 cells was significantly increased when miR-34a was highly expressed through has-miR-34a mimics transfection (Figure 6A) (P<0.05). However, number of apoptotic OCI-LY10 cells was significantly decreased compared with control group when miR-34a expression was inhibited by has-miR-34a inhibitor transfection (Figure 6B) (P<0.05). The results suggested that miR-34a could promote OCI-LY10 cell apoptosis at the early stage of DLBCL.

**Discussion**

MiRNAs are a family of small non-coding RNAs with a length of 21 to 25 nt, and they not only participate in various physiological processes of human body, but are involved in the pathogenesis and progression of tumors through abnormal expression [33]. Although the abnormal expression of miRNAs are closely related with tumors occurrence and development, the biological functions and mechanisms of miRNAs are not the same in different tissues and cells, which is probably due to the short seed sequence and not exactly match when miRNA binding mRNA [33]. MiR-34a is one of the miRNAs which are widespread and found early in human tissues and cells. The inactivation of miR-34a may be associated with a variety of tumors genesis and development [34]. In studies of Yan et al. and Li et al., it was found that miR-34a could inhibit cell metastasis via down-regulation of c-Met in hepatocellular carcinoma and uveal melanoma, respectively [35, 36]. However, role of miR-34a in DLBCL is still unclear.

In this study, we firstly found that miRNA level in DLBCL tissues was significantly lower than that in reactive lymphoid hyperplasia. Additionally, uncontrolled cell growth, proliferation, metastasis and infiltration are main features of malignant tumors [37]. Considering of this point, MTT and Transwell method was used to detect OCI-LY10 cell proliferation, metastasis and infiltration *in vitro*, respectively. The results suggested that inhibited miR-34a could enhance cell proliferation, metastasis and infiltration. Besides, flow cytometry results indicated that inhibited miR-34a reduce cell apoptosis in early stage of DLBCL, and high-expressed miR-34a increase cell apoptosis reversely. Thus, our research proves that miR-34a plays important roles in regulation of cell proliferation, metastasis, infiltration and apoptosis in DLBCL.

**AXL** is a transmembrane protein with a size of about 140 kD locating in chromosome 19, q13.1, and is coded by AXL proto-oncogene and characterized by both properties of adhesion molecules and tyrosine kinase [38]. AXL express in a variety of normal tissues including thyroid and colon mucosa, and in CD34+ cells and stromal cells of bone marrow as well, but AXL level is very low in normal lymphoid [39, 40]. It has already been reported that activated AXL can resist cell apoptosis [41], and abnormal high expression of AXL was found in many kinds of cancers including lung cancer, breast cancer, stomach cancer and ovarian cancer [42-45]. The high-expressed AXL were proved to participate in cell metastasis and infiltration in malignant tumors [29, 46-48]. Moreover, it was confirmed using luciferase reporter gene system that AXL was the target gene of miR-34a, and miR-34a overexpression in MDA-MB-231 breast cancer cells could inhibit AXL expression and reduce cell metastasis [29]. In this study, a negative correlation between miR-34a and AXL was found in DLBCL cell line, indicating that miR-34a can regulate AXL expression on transcription and translation levels and suggesting miR-34a may involve in DLBAL via its regulation on AXL. These findings referring to miR-34a and AXL expression in DLBCL are important complement to molecular mechanism research of DLBCL, and provide a basis for further study.

**Acknowledgements**

The authors want to thank Dr. Xiuying Pan (Department of Hematology, Affiliated Hospital...
of Xuzhou Medical College), Dr. Haiying Sun (Department of Hematology, Affiliated Hospital of Xuzhou Medical College), Dr. Depeng Li (Department of Hematology, Affiliated Hospital of Xuzhou Medical College), Dr. Wei Sang (Department of Hematology, Affiliated Hospital of Xuzhou Medical College) and Dr. Jiang Cao (Department of Hematology, Affiliated Hospital of Xuzhou Medical College) for their valuable help during the preparation of the manuscript.

Disclosure of conflict of interest

None.

Address correspondence to: Kailin Xu, Department of Hematology, Affiliated Hospital of Xuzhou Medical College, No. 99 West Huaihai Road, Xuzhou 22-1002, Jiangsu Province, China; The First Clinical Medicine School of Nanjing Medical University, Nanjing 210029, China. Tel: 13912074971; E-mail: xkl080@126.com; Xiaofei Chen, Department of Medical Oncology, Hua'ai First People's Hospital, Nanjing Medical University, Hua'ai 223300, China. Tel: 13912074971; E-mail: cxf003835@163.com

References


miR-34a in DLBCL


