MiR-223 suppresses proliferation and promotes apoptosis of diffuse large B-cell lymphoma cells through Lmo2 and MAPK signaling pathway

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Summary

Purpose: The purpose of this study was to explore the effects of micro ribonucleic acid (miR)-223 on the proliferation and apoptosis of diffuse large B-cell lymphoma (DLBCL) cells, and its associations with the LIM domain only 2 (Lmo2) gene and mitogen-activated protein kinase (MAPK) signaling pathway.

Methods: The DLBCL samples were obtained to analyze the expression levels of miR-223 and Lmo2, with para-carcinoma normal tissues as controls. The DLBCL OCI-LY8 cell lines were cultured in suspension in vitro, and transfected with miR-223 mimics or miR-negative control (NC) and Lmo2-siRNA or Lmo2-NC, respectively, with cells normally cultured as controls. Moreover, the target gene Lmo2 of miR-223 was predicted online, and the Lmo2 luciferase reporter vectors containing miR-223 target site were constructed.

Results: In 8 out of 9 cases of DLBCL tissues, the expression of miR-223 was significantly lower (p<0.01), while the mRNA expression of Lmo2 was significantly higher than those in para-carcinoma normal tissues (p<0.01). After overexpression of miR-223 or inhibition on Lmo2, the proliferation ability of OCI-LY8 cells was obviously weakened (p<0.01), the apoptosis rate was obviously raised (p<0.01), and the protein expressions of phosphorylated (p)-p38 and p-c-Jun N-terminal kinase (JNK) were obviously up-regulated (p<0.01). Moreover, the results of luciferase reporter assay revealed that the expression of Lmo2 was remarkably inhibited by miR-223 mimics (p<0.01).

Conclusions: MiR-223 may suppress the proliferation and promote the apoptosis of DLBCL cells through targeting the Lmo2 gene and activating the MAPK signaling pathway.

Key words: diffuse large B-cell lymphoma, miR-223, Lmo2, MAPK, cell proliferation, apoptosis

Introduction

As the most common non-Hodgkin’s lymphoma in adults, diffuse large B-cell lymphoma (DLBCL) is recognized as heterogeneous solid malignancy, and only less than half of patients can be cured by currently available therapies [1,2]. In an important study in 2000, DLBCL was divided into two molecularly different subtypes using gene expression profile analysis: germinal center B-cell (GCB)-like DLBCL and activated B-cell (ABC)-like DLBCL [3].

Micro ribonucleic acids (miRs) are a class of small non-coding RNAs with 18-25 nucleotides in length, which can regulate the gene expression through degrading target mRNAs or inhibiting their translation [4]. Research results have shown that miRs play important roles in a variety of biological processes, including cell cycle, proliferation, differentiation, apoptosis and metabolism, and they also exert crucial effects in
the occurrence and development of cancer [5,6]. The role of miR-223 in hematopoiesis has been widely studied, and it serves as a regulator of granulocyte differentiation, maturation and function, and can restore granulocyte differentiation in leukemia cells [7,8]. Another study has demonstrated that miR-223 is also involved in the hematopoiesis and incidence of malignant tumors, such as ovarian cancer [9]. However, there are few studies on the biological function of miR-223 in DLBCL.

LIM domain only 2 (Lmo2), also known as RBTN2 and TTG2, is a cysteine-rich LIM domain-containing transcription factor of 156 amino acids, which indirectly mediates the gene expression through regulating the interaction between proteins and other transcription factors, thereby promoting the formation of DNA-binding complex. Moreover, Lmo2 plays an important role in angiogenesis and erythropoiesis, and it is also required for hematopoiesis during embryogenesis [10,11]. In human T-cell acute lymphoblastic lymphoma/leukemia (T-ALL), it is activated at t (P13; Q11) or t (Q35; P13) chromosomal translocations [12]. In the B-cell lineage, Lmo2 has been proved to be highly expressed in GC lymphocytes and GC-derived non-Hodgkin’s lymphoma, and it is a potent predictive index for the survival of DLBCL patients [13,14].

In the present study, the mechanism of miR-223 in regulating the proliferation and apoptosis of DLBCL cells was explored, so as to better understand the roles of miR-223, Lmo2 and mitogen-activated protein kinase (MAPK) signaling pathway in the pathogenesis of DLBCL.

**Methods**

**Acquisition of human DLBCL samples**

A total of 9 DLBCL samples were isolated from 9 DLBCL patients, and written informed consent was obtained from all patients. After resection, the tumor samples were stored in sterile solution at 4℃ and immediately sent to the laboratory. Then, the normal tissues and tumor tissues were separated on a super clean bench for later use. This study was approved by the Ethics Committee of the Third People’s Hospital of Dalian. Signed written informed consents were obtained from all participants before the study entry.

**OCI-LY8 cell culture in vitro**

After resuscitation, OCI-LY8 cell lines were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS) in an incubator with 5% CO₂ at 37°C. When the cell mass grew larger, the culture flask was shaken to disperse the cells, and the medium was added for culture in separate flasks.

**Cell transfection with miR-223**

The cells were transfected with miR-223 mimics or miR-negative control (NC) as follows: 5 μL of miR-223 mimics (concentration of mother solution: 20 μM) or miR-NC was added and mixed evenly with 250 μL of α-MEM, followed by incubation at room temperature for 5 min. Then, the transfection solution mixed was added into a culture dish and shaken evenly, followed by culture in the incubator with 5% CO₂ at 37°C. At 48 h after transfection, the expression of miR-223 in cells was assessed.

**Lmo2 mRNA interference**

A total of 5 μL of Lmo2-small-interfering RNA (siRNA) (concentration of mother solution: 20 μM) or Lmo2-NC were added into 83 μL of serum-free OPTI-MEM. The mixture was added and mixed evenly with 12 μL of HiPerFect transfection reagent for cell incubation at room temperature for 10 min. Then, the transfection complex was added dropwise into a culture dish and mixed evenly, and the cells were cultured in the incubator with 5% CO₂ at 37°C. After 48 h, the expression of Lmo2 gene was detected.

**Total RNA extraction and quantitative RT-polymerase chain reaction (qRT-PCR)**

The tissue samples were ground with liquid nitrogen for later use, and the cultured cells were collected after centrifugation, from which the total RNA was extracted using TRizol reagent. The RNA purity and concentration were measured using a NanoDrop spectrophotometer. The primers were designed by Primer Premier 6.0 and synthesized by Sangon (Shanghai, China), and the primer sequences of miR-223, Lmo2 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) are shown in Table 1. Subsequently, the total RNA was synthesized into complementary DNA (cDNA) using the qScript microRNA cDNA synthesis kit for miR-223, while the total RNA was synthesized into cDNA using the random primers of RT Master Mix for Lmo2 and GAPDH. Then qRT-PCR was performed using SYBR Green Real-Time PCR Master Mix and ABI 7500 sequence detection system according to the manufacturer’s instructions. Finally, the transcription level was evaluated using the cycle threshold (Ct), and the gene expression normalized to the endogenous reference was determined using the 2⁻ΔΔCt method.

**Table 1. Primer sequences of miR-223, Lmo2 and GAPDH**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-223 F</td>
<td>5'-TGTCAGTTTGTCAAATACCCCA-3'</td>
</tr>
<tr>
<td>miR-223 R</td>
<td>5'-CACAGCCTGAGAACAGGAGG-3'</td>
</tr>
<tr>
<td>Lmo2 F</td>
<td>5'-ACTCAAACCTGGCCGGAAG-3'</td>
</tr>
<tr>
<td>Lmo2 R</td>
<td>5'-TCTCATAGGCAGAATCCCG-3'</td>
</tr>
<tr>
<td>GAPDH F</td>
<td>5'-GTAAGGCTGAGAACGAGAA-3'</td>
</tr>
<tr>
<td>GAPDH R</td>
<td>5'-AAATGAGCCCCAGCTTCCT-3'</td>
</tr>
</tbody>
</table>

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Flow cytometry

The cells in each group were digested with 0.25% trypsin+0.02% EDTA into single cell suspension, and washed with phosphate buffered saline (PBS) for 3 times. Then, the cells were counted and the cell density was adjusted to 5×10^5/mL, followed by incubation with Annexin V and propidium iodide (PI) in the dark for 30 min. After that, the cells were washed with PBS for 3 times, and the cell density was adjusted to 10^6/mL, followed by flow cytometry. Finally, the data were analyzed using CFlow Plus.

CCK8 assay

The cells were cultured in 96-well plates (3,500 cells/well) in the incubator with 5% CO_2 at 37°C, with 3 replicates in each group. At 24 and 48 h after culture, the medium was discarded, and the cells were collected and added with freshly-prepared medium containing 10 μL of CCK8 solution for another 1 h of culture in the incubator. Finally, the optical density (OD) value was measured at 450 nm using a microplate reader. The assay was repeated for 5 times, and the average value was taken as the final result.

Luciferase reporter assay

The cells in each group were transfected with miR-223 mimics or miR-NC and wild-type Lmo2 (Lmo2-WT) or mutant Lmo2 (Lmo2-MUT) for 48 h. After the medium was discarded, the cells were collected and washed with PBS for 3 times, and lysed with 50 μL of newly-prepared lysis buffer for 30 min. Then, 10 μL of lysate was taken and added with 100 μL of luciferase assay reagent prepared already. Finally, the luciferase activity was measured using the luciferase reporter assay system and bioluminescent plate reader. The assay was repeated independently for 3 times, with 3 parallel controls in each group.

Western blotting

After centrifugation, the cells in each group were lysed with an appropriate amount of lysis buffer at 4°C overnight. The total protein was extracted via centrifugation at 13,000 rpm, and its concentration was measured using the bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA). After separation via 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis, the protein was transferred onto a polyvinylidene fluoride membrane, sealed with 5% skim milk powder and 0.1% tris-buffered saline with Tween-20, and incubated with Lmo2, p58, p-p58, JNK, p-JNK and β-actin primary antibodies at 4°C overnight. After that, the protein was incubated again with horse radish peroxidase (HRP)-labeled secondary antibodies, followed by exposure with electrochemiluminescence (ECL) reagent.

Statistics

SPSS 20.0 software (IBM, Armonk, NY, USA) was used for data processing. The data in each group were expressed as mean ± standard deviation, and independent-samples t-test was performed for the intergroup comparison. P<0.05 suggested that the difference was statistically significant.

Results

Expressions of miR-223 and Lmo2 gene in DLBCL

The expression levels of miR-223 and Lmo2 were determined via qRT-PCR in 9 cases of DLBCL tissues and para-carcinoma normal tissues. As shown in Figure 1, in 8 out of 9 cases of DLBCL tissues, the expression of miR-223 was significantly lower (p<0.01), while the mRNA expression of Lmo2 was significantly higher than those in para-carcinoma normal tissues (p<0.01).

Effects of miR-223 on the proliferation and apoptosis of OCI-LY8 cells

The DLBCL OCI-LY8 cell lines were cultured in vitro and transfected with miR-NC or miR-223 mimics, respectively, with cells normally cultured as controls. The results of qRT-PCR revealed that the expression of miR-223 obviously rose after transfection with miR-223 mimics (p<0.01), while it showed no changes after transfection with miR-NC (p>0.05) (Figure 2A). The results of CCK8 assay showed that the proliferation of cells transfected with miR-223 mimics was obviously weakened at

Figure 1. Expressions of miR-223 and Lmo2 gene detected using qRT-PCR. A: Expression of miR-223. B: mRNA expression of Lmo2 (**p<0.01).
24 h (p<0.05) and 48 h (p<0.01), while miR-NC had no significant influence on the cell proliferation (p>0.05) (Figure 2B). Besides, it was found via flow cytometry that the apoptosis rate was increased from 3.6±0.6% to 20.5±2.4% after transfection with miR-223 mimics (p<0.01), while miR-NC had no significant influence on the apoptosis rate (p>0.05) (Figure 2C).

Relation between miR-223 and Lmo2

The target relation between miR-223 and Lmo2 was predicted online via TargetScan, and the target gene sequences are shown in Figure 3A. According to the target sequences, the Lmo2 luciferase reporter vector containing the miR-223 target site (Lmo2-WT) and the luciferase reporter vector containing no target site (Lmo2-MUT) were constructed. The cells were transfected with Lmo2-WT or Lmo2-MUT and miR-223 mimics or miR-NC, respectively. The results of luciferase reporter assay manifested that the Lmo2-WT luciferase activity was evidently inhibited by miR-223 mimics (p<0.01), while Lmo2-WT luciferase activity had no evident changes (p>0.05) (Figure 3B).

![Figure 2](image2.png)

**Figure 2.** Effects of miR-223 on proliferation and apoptosis of OCI-LY8 cells. A: Expression of miR-223 after transfection of OCI-LY8 cells with miR-NC and miR-223 mimics detected using qRT-PCR. B: Cell proliferation level at 0, 24 and 48 h detected using CCK8 assay. C: Apoptosis rate detected using flow cytometry. (*p<0.05; **p<0.01; ***p<0.001).

![Figure 3](image3.png)

**Figure 3.** Lmo2-WT and Lmo2-MUT luciferase activity detected via luciferase reporter assay. A: Comparison of target gene sequences of miR-223 and Lmo2. B: Relative luciferase activity. (**p<0.01).

![Figure 4](image4.png)

**Figure 4.** Effects of Lmo2 on proliferation and apoptosis of OCI-LY8 cells. A: mRNA expression of Lmo2 after transfection of OCI-LY8 cells with Lmo2-NC and Lmo2-siRNA detected using qRT-PCR. B: Cell proliferation level at 0, 24 and 48 h detected using CCK8 assay. C: Apoptosis rate detected using flow cytometry. **p<0.01: A significant difference compared with other groups. (**p<0.01; ***p<0.001).
Effects of Lmo2 on the proliferation and apoptosis of OCI-LY8 cells

The DLBCL OCI-LY8 cell lines were cultured in vitro and transfected with Lmo2-NC or Lmo2-siRNA, respectively, with cells normally cultured as controls. The results of qRT-PCR revealed that the mRNA expression of Lmo2 markedly declined after transfection with Lmo2-siRNA (p<0.01), while it had no changes after transfection with Lmo2-NC (p>0.05) (Figure 4A). The results of CCK8 assay showed that the proliferation of cells transfected with Lmo2-siRNA was markedly weakened at 24 h and 48 h (p<0.01), while Lmo2-NC had no significant influence on the cell proliferation (p>0.05) (Figure 4B). Besides, it was confirmed via flow cytometry that the apoptosis rate was increased from 3.6±0.6% to 25.5±3.1% after transfection with Lmo2-siRNA (p<0.01), while Lmo2-NC had no significant influence on the apoptosis rate (p>0.05) (Figure 4C).

Associations of miR-223 with protein expressions of Lmo2 and MAPK signaling pathway

To detect the effects of miR-223 on the protein expressions of Lmo2 and MAPK signaling pathway in OCI-LY8 cells, OCI-LY8 cells were transfected with miR-223 mimics and Lmo2-siRNA. The results of Western blotting manifested that after transfection with miR-223 mimics in OCI-LY8 cells, the protein expression of Lmo2 was remarkably down-regulated (p<0.01) (Figures 5A and 5B), while the protein expressions of p-p38 and p-JNK were remarkably up-regulated (p<0.01) (Figure 5A, 5C, and 5D), but the protein expressions of p38 and JNK had no remarkable changes (p>0.05) (Figure 5A, 5C and 5D). The same results as above were obtained after transfection with Lmo2-siRNA in OCI-LY8 cells.

Discussion

MiRs are small non-coding RNAs able to affect the gene expression at the post-transcriptional level through promoting the degradation of target mRNAs and inhibiting their translation [4,15]. Studies have shown that miR-223 can reduce the gene and protein expressions of Lmo2. Moreover, during erythroid differentiation, the expression of miR-223 declines in CD34+ cells in umbilical cord blood, while the gene and protein expressions of Lmo2 rise. Furthermore, it has been found that

![Figure 5. Associations of miR-223 with protein expressions of Lmo2 and MAPK signaling pathway. A: Protein expressions of Lmo2, p-p38, p38, p-JNK, JNK and β-actin determined using Western blotting. B,C and D: Relative quantification of Lmo2, p-p38, p38, p-JNK and JNK expressions. (**p<0.01).](image-url)
miR-223 interacts with the 3’-untranslated region of Lmo2 mRNA, and its overexpression reduces the mRNA and protein expressions of Lmo2, and inhibits erythroid differentiation [16]. Malumbres et al [17] characterized the miR expression profile in GCB-like and ABC-like DLBCL using microarrays, and found that the expression of miR-223 was down-regulated. The above findings demonstrate that the malignant biological behaviors of cells are inherited from their untransformed progenitor cells, but the expression of miRs becomes abnormal after cell transformation, and it may play an important role in the occurrence of lymphoma. It has also been confirmed that miR-223 is able to down-regulate the expression of Lmo2 in normal B cells [17]. In this study, the expressions of miR-223 and Lmo2 mRNA were analyzed in 9 cases of DLBCL tissues. In 8 cases of DLBCL tissues, the expression of miR-223 was distinctly down-regulated, while the mRNA expression of Lmo2 was distinctly up-regulated, similar to previous research results. Besides, there was a negative regulatory relation between expressions of miR-223 and Lmo2. After overexpression of miR-223 in DLBCL cells, the cell proliferation was greatly suppressed, while the cell apoptosis was greatly enhanced.

Natkunam et al [18] proved the significance of Lmo2 protein expression for the prognosis of DLBCL. Lmo2 also plays an important role in leukemic transformation, and the incidence rate of chromosomal translocation involving Lmo2 is 8-10% in T-ALL [19]. Lmo2 has a synergistic effect with other transcription factors (HOX11, TAL1/SCL, LYL1, Lmo1 and Lmo2), thus inducing oncogenic transformation [20,21]. The mRNA expression of Lmo2 is also high in DLBCL [12]. Lmo2 regulates the gene expression via promoting the formation of DNA-binding complex. In B cells, Lmo2 is specifically up-regulated in germinal center (GC) and also expressed in GC-derived non-Hodgkin’s lymphoma. In addition, Lmo2 is one of the most potent prognostic indexes for DLBCL patients [14]. In this study, it was found that the mRNA and protein of Lmo2 were highly expressed in DLBCL cells. After the Lmo2 expression was inhibited, DLBCL cells had significantly weaker proliferation and stronger apoptosis.

The MAPK family is an important player in complex cellular processes, such as proliferation, differentiation, development, transformation and apoptosis. At least three MAPK family members have been characterized, namely extracellular signal-regulated kinase (ERK), JNK/SAPK and p38 MAPK [22]. JNK and p38 MAPK are crucial for regulating the signal transduction mechanism of cells in many types of stress, and also control the proliferation, differentiation, survival and migration of specific types of cells [22,23]. The activation of JNK is related to the transformation mediated by many oncogenes and growth factors, as well as the cell apoptosis and survival. According to a related study, activated JNK is required for the ultraviolet ray-induced apoptosis of fibroblasts [24]. P38 can be activated in apoptosis and other stress responses [22]. In this study, it was confirmed that miR-223 mimics or Lmo2-siRNA inhibited the proliferation and promoted apoptosis of DLBCL cells, and also activated the phosphorylation of JNK and p38.

Conclusions

In conclusion, miR-223 may suppress the proliferation and promote apoptosis of DLBCL cells through targeting the Lmo2 gene and activating the MAPK signaling pathway.

Conflict of interests

The authors declare no conflict of interests.

References

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