Effects of miRNA-4804 on proliferation and cytokine secretion function by targeting chemokine-like receptor 1 in human monocytic cell line

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Abstract: Objective: This study aimed to investigate the effect of miRNA-4804 on the proliferation and cytokine secretion function of human monocytes by targeting chemokine-like receptor 1 (CMKLR1). Methods: The expression levels of miRNA-4804 and cytokine mRNA in human monocytic cell line (THP1) were detected by real-time quantitative PCR. THP1 cell proliferation and surface molecules were detected by CCK8 and flow cytometry. The expression of cytokines in the cell culture supernatant was detected by enzyme-linked immunosorbent assay. The regulatory effect of miRNA-4804 on the candidate target gene CMKLR1 was determined by dual-luciferase reporter assay. Western blot was used to detect the expression of CMKLR1 protein. Results: Compared with the control group, the proliferation and expression of HLA-DR and CD86 molecules of THP1 cell induced with IFN-γ were significantly inhibited by miRNA-4804 mimics and significantly enhanced by miRNA-4804 inhibitors. The expression of IL-6, TNF-α, and IL-12 significantly increased, whereas IL-10 expression decreased in the culture supernatant of THP-1 cells upon IFN-γ stimulation. This alteration in cytokine expression could be antagonistic and promoted after treatment with miRNA-4804 mimics and miRNA-4804 inhibitors. Dual-luciferase reporter assay and Western blot showed that miRNA-4804 inhibited CMKLR1 expression. The expression of CMKLR1 mRNA was inhibited by miRNA-4804 in the THP1 cell line. Conclusion: MiRNA-4804 could regulate the proliferation and cytokine secretion of monocytes by targeting CMKLR1, and it may be involved in the regulation of immune homeostasis in chronic hepatitis B.

Keywords: MicroRNA-4804, monocyte, cell proliferation, cytokine, chemokine-like receptor1

Introduction

Hepatitis B virus (HBV) infection is one of the major public health problems worldwide, despite the availability of modern vaccination and antiviral treatment modalities. About 240 million people have chronic HBV infection, and approximately 650,000 people die annually from complications of this viral infection [1]. Chronic inflammatory and fibrous changes in the liver during HBV infection are mainly propelled by complex interactions between the virus and the host immune system [2]. The host immunological response, particularly the T cell response, plays a key role in the pathogenesis and progression of the disease rather than from direct viral cytopathic effect [3]. Chronic hepatitis B (CHB) has become a treatable and controllable disease but it is not as curable as hepatitis C virus infection. Therefore, new immunotherapeutic and antiviral approaches to control persistent HBV infection are of urgent need.

MicroRNAs (miRNAs) are endogenous non-coding RNAs that regulate gene expression at the post-transcriptional level [4]. MiRNAs have been demonstrated to be involved in cell differentiation, development, metabolism, and development of immune and inflammatory responses [5]. Abnormalities in miRNA have been observed in many human diseases, such as cancer, viral infection, and CHB. Recent studies have revealed that miRNAs play significant roles in the pathogenesis of HBV infection [6, 7]. HBV infection can alter expression patterns of cellular miRNA through the products of HBV replication and expression [8]. Cellular miRNA then regulates the expression and replication of HBV [9]. Some miRNAs (miRNA-146 and miRNA-548) might modulate the immune response of CHB infection and contribute to the persistent infection of HBV [10, 11].
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Table 1. The primer sequences of IL-6, TNF-α, IL-12, IL-10 and GAPDH

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primer sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6 sense:</td>
<td>5' AAGCCAGAGCTGTGCAGATG3'</td>
</tr>
<tr>
<td>IL-6 antisense:</td>
<td>5' TTCCCCCACACCAAGTTGAG3'</td>
</tr>
<tr>
<td>TNF-α sense:</td>
<td>5' GCTGCACTTTGGAGTGATC3'</td>
</tr>
<tr>
<td>TNF-α antisense:</td>
<td>5' TCACCTCAGGTGAAAG3'</td>
</tr>
<tr>
<td>IL-12 sense:</td>
<td>5' GGAAGGCTTGTACCTAGTG3'</td>
</tr>
<tr>
<td>IL-12 antisense:</td>
<td>5' CTCCCATGTTAGAAGG3'</td>
</tr>
<tr>
<td>IL-10 sense:</td>
<td>5' CGAGATGCGCCAGTGAG3'</td>
</tr>
<tr>
<td>IL-10 antisense:</td>
<td>5' CTCCAGACAGGCCTGGAAC3'</td>
</tr>
<tr>
<td>GAPDH sense:</td>
<td>5' AAAGCTCTGCCGGTACTA3'</td>
</tr>
<tr>
<td>GAPDH antisense:</td>
<td>5' TAGGAAAGCATCACCAG3'</td>
</tr>
</tbody>
</table>

MiRNA-4804 is a highly expressed miRNA in the peripheral blood mononuclear cells (PB-MCs) of CHB patients that were screened using miRNA microarrays in a previous study [12]. The functions and molecular mechanisms of miRNA-4804 have not yet been reported. The expression gene of hsa-miR-4804-3p is located in 5q13.2 (72174418-72174490) of the human chromosome [13]. In the present study, THP-1 cell lines were selected to investigate the effect of miRNA-4804 on cell proliferation and secretion of cytokine by flow cytometry, real-time quantitative PCR and enzyme-linked immunosorbent assay (ELISA). The target genes of miRNA-4804 were predicted using bioinformatics software. The regulatory effect of miRNA-4804 on the candidate target gene chemokine-like receptor 1 (CMKLR1) was determined by dual-luciferase reporter assay and Western blot.

Materials and methods

Culture and transfection of THP-1 cell line

THP1 is a human monocytic cell line derived from an acute monocytic leukemia patient. THP-1 cell lines (Shanghai Institute for Biological Science, Shanghai, China) were cultured in RPMI 1640 with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C with 5% carbon dioxide. The miRNA-4804 mimic, miRNA-4804 inhibitor, and unrelated sequence positive and negative controls were purchased from GenePharma (Shanghai GenePharma Co., Ltd., Shanghai, China). Recombinant human IFN-γ (PeproTech Inc., Rocky Hill, NJ, USA) was used at a final concentration of 10 ng/mL to induce the THP-1 cells for 48 h. Cells were transfected using lipofectamine transfection reagent (Life Technology, Carlsbad, CA, USA) and harvested 48 h later.

RNA extraction and quantitative detection of miRNA-4804 and CMKLR1

Total RNA was isolated using TRIzol and miRNAeasy mini kits (QIAGEN, Duesseldorf, Germany) according to the manufacturer’s instructions. MiRNA-4804 and CMKLR1 in THP1 were detected by quantitative RT-PCR. The primers were synthesized by Shanghai Biotechnology Co., Ltd. The primer sequences of U6 and hsa-miR-4804-3p were F: 5'-ATTGGAGACGATACAGAAGATT-3', R: 5'-GGAACGCTTACGAGATTTG3'; and GSP: 5'-GCCCTGCTTACGACACG-3', R: 5'-TGCAGGGTCCGAGGT-3', respectively. In brief, 1 μL of cDNA, 10 μL of SYBR Green I, 1 μL of 20 μM specific PCR primer F, and 1 μL of 20 μM specific PCR primer R were mixed with water to a total volume of 20 μL. The mixture was carefully glued using paraffilm and briefly centrifuged. U6 small nuclear RNA mol-
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Table 2. Effect of miRNA-4804 on proliferation of THP1 cell

<table>
<thead>
<tr>
<th>Groups</th>
<th>A450</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>0.875±0.054</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>1.695±0.057*</td>
</tr>
<tr>
<td>Mimcs NC+ IFN-γ</td>
<td>1.621±0.026*</td>
</tr>
<tr>
<td>MiR-4804- mimics + IFN-γ</td>
<td>0.788±0.077*</td>
</tr>
<tr>
<td>Inhibitor NC+ IFN-γ</td>
<td>1.619±0.042*</td>
</tr>
<tr>
<td>MiR-4804- inhibitor + IFN-γ</td>
<td>1.985±0.008*</td>
</tr>
</tbody>
</table>

Note: *P<0.05 vs. Blank groups; #P<0.05 vs. IFN-γ groups.

Expression of HLA-DR and CD antigens on the surface of THP-1 cells detected by flow cytometry

THP1 cells were cultured for 48 h, washed with cold PBS, and centrifuged at 1500 rpm for 10 min. CD14 mAb labeled with FITC and CD86, CD80, HLA-DR, and IgG labeled with PE (BD Comp., Franklin Lakes, NJ, USA) were added, gently mixed, and incubated at 4°C for 30 min in the dark. The cells were washed twice with cold PBS. The expression levels of CD80, CD86, and HLA-DR were detected by flow cytometry with CD14 gating.

Detection of cytokines in the culture supernatant of THP1 cells

The expression levels of IL-6, TNF-α, IL-12, and IL-10 in the culture supernatant of THP1 cells were detected using an ELISA kit (Wuhan Boshide Biological Engineering Co. Ltd., Wuhan, China) according to the manufacturer’s instructions.

Detection of IL-6, TNF-α, IL-12, and IL-10 gene expression in THP1 cells

The mRNA expression levels of IL-6, TNF-α, IL-12, and IL-10 in THP1 cells were detected by quantitative fluorescence PCR. The primers were designed and synthesized by Shanghai Biological Engineering Co., Ltd. (Table 1). In brief, 1 μL of cDNA, 10 μL of SYBR Green I, 0.4 μL of 20 μM specific PCR primer F, and 0.4 μL of 10 μM specific PCR primer R were mixed with water to a total volume of 20 μL. GAPDH was used as an internal control. The reactions were performed as follows: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 34 s.

Prediction and analysis of miRNA-4804 target genes through GO and KEGG

The possible target genes of miRNA-4804 were predicted by Target Scan (http://www.targetscan.org) and miRDB (http://www.mirdb.org/). The intersection of these two databases was obtained. GO and KEGG analyses of possible target genes of miRNA-4804 were performed as previously described [14].

Construction and transfection of luciferase reporter plasmids

The 3′-untranslated region (UTR) fragment of CMKLR1 (1775-2037 nt, GenBank accession number NM_0001142343), which contains putative miRNA-4804 binding sequences (1906-2006).
Figure 3. Expression of HLA-DR, CD80 and CD86 in THP-1 cells by flow cytometry.
The expression levels of miRNA-4804 and CMKLR1 in THP1 cell lines were detected by quantitative fluorescence PCR. The results showed that miRNA-4804 expression in THP1 cells did not change significantly after stimulation with IFN-γ. MiRNA-4804 expression significantly increased after transfection with miRNA-4804 mimics but significantly decreased after transfection with miRNA-4804 inhibitors ($t = 6.523, P = 0.003; t = 4.387, P = 0.012$) (Figure 1). Compared with the control group, the expression of CMKLR1 significantly increased in THP1 cells induced with IFN-γ. The expression of CMKLR1 significantly decreased after transfection with miRNA-4804 mimics. CMKLR1 expression significantly increased after transfection with miRNA-4804 inhibitors ($F = 46.25, P < 0.001$) (Figure 2).

**Effect of miRNA-4804 on the proliferation of THP1 cell lines**

The effect of miRNA-4804 on the proliferation of THP1 cells was detected by CCK. The results showed that the proliferation of THP1 cells was significantly enhanced after stimulation with IFN-γ compared with that of the blank control. The proliferation of THP1 cells was significantly inhibited and enhanced after transfection with miRNA-4804 mimics and inhibitors, respectively, compared with that of the control group ($F = 285.3, P < 0.001$) (Table 2).

**Effect of miRNA-4804 on the expression of HLA-DR and CD in THP-1 cells induced by IFN-γ**

The expression of HLA-DR and CD antigens in THP1 cells was detected by flow cytometry (Figure 3). The expression of HLA-DR and CD86 in THP1 cells was significantly enhanced compared with that in the control group, whereas the expression of CD80 was not changed after induction with IFN-γ. Compared with the control group, the expression levels of HLA-DR and CD86 were significantly inhibited, and CD80 expression demonstrated no obvious change after transfection with miRNA-4804 mimics. The expression of HLA-DR, CD86, and CD80 significantly increased after transfection with miRNA-4804 inhibitors ($F = 6.28, P = 0.017$) (Table 3).
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**Table 4. Effect of miRNA-4804 on expression of cytokines in THP1 cell**

<table>
<thead>
<tr>
<th>Groups</th>
<th>IL-6 (pg/ml)</th>
<th>TNF-α (pg/ml)</th>
<th>IL-12 (pg/ml)</th>
<th>IL-10 (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>5.68±0.09</td>
<td>46.82±1.52</td>
<td>7.06±0.67</td>
<td>38.43±1.40</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>86.65±2.72</td>
<td>242.69±18.80*</td>
<td>206.65±14.93*</td>
<td>8.81±0.77*</td>
</tr>
<tr>
<td>mimcs NC+ IFN-γ</td>
<td>80.25±2.42</td>
<td>225.62±7.60</td>
<td>199.80±16.33*</td>
<td>9.42±0.96*</td>
</tr>
<tr>
<td>MiR-4804 mimics+IFN-γ</td>
<td>6.68±0.35*</td>
<td>50.78±2.40*</td>
<td>15.78±0.56*</td>
<td>148.52±15.47*</td>
</tr>
<tr>
<td>inhibitor NC+ IFN-γ</td>
<td>91.62±3.79</td>
<td>254.56±8.01*</td>
<td>217.42±17.23*</td>
<td>7.67±0.46*</td>
</tr>
<tr>
<td>MiR-4804 inhibitor+IFN-γ</td>
<td>181.98±9.74*</td>
<td>466.98±20.31*</td>
<td>561.38±20.49*</td>
<td>5.18±1.09*</td>
</tr>
<tr>
<td>F</td>
<td>627.20</td>
<td>486.77</td>
<td>602.79</td>
<td>233.82</td>
</tr>
<tr>
<td>P</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Note: *P<0.05 vs. Blank groups; **P<0.05 vs. IFN-γ groups.

**Figure 4. Effect of miRNA-4804 on expression of cytokines in THP1 cell.** A: IL-6; B: TNF-α; C: IL-12; D: IL-10.

**Effect of miRNA-4804 on the expression of cytokines in the culture supernatant of THP-1 cells**

ELISA was used to detect the expression of IL-10, TNF-α, IL-12, and IL-6 in the culture supernatant of THP-1 cells. The expression of IL-6, TNF-α, and IL-12 in THP-1 cells significantly increased, whereas the expression of IL-10 significantly decreased after stimulation with IFN-γ. The role of secreted cytokines in THP-1 cells induced with IFN-γ was antagonistic after transfection with miRNA-4804 mimics, in which the expression of IL-6, TNF-α, and IL-12 was low,
Effects of miRNA-4804 on function in THP1 cells

The mRNA expression of IL-6, TNF-α, and IL-12 was significantly enhanced, whereas the expression of IL-10 mRNA significantly decreased in THP-1 cells after stimulation with IFN-γ. Treatment with miRNA-4804 mimics inhibited the expression levels of IL-6, TNF-α, and IL-12, and promoted IL-10 expression in THP-1 cells. However, treatment with miRNA-4804 inhibitors promoted the expression of IL-6, TNF-α, and IL-12, and inhibited the expression of IL-10 in THP-1 cells induced with IFN-γ ($F = 124.18, 115.89, 215.03, 267.26$, respectively; $P < 0.001$) (Figure 4).

**Effect of miRNA-4804 on the expression of the cytokine genes in THP-1 cells**

Quantitative fluorescence PCR was used to detect the mRNA expression of IL-10, TNF-α, IL-12, and IL-6 in THP1 cells stimulated by IFN-γ. The mRNA expression of IL-6, TNF-α, and IL-12 was significantly enhanced, whereas the expression of IL-10 mRNA significantly decreased in THP-1 cells after stimulation with IFN-γ. Treatment with miRNA-4804 mimics inhibited the expression levels of IL-6, TNF-α, and IL-12, and promoted IL-10 expression in THP-1 cells. However, treatment with miRNA-4804 inhibitors promoted the expression of IL-6, TNF-α, and IL-12, and inhibited the expression of IL-10 in THP-1 cells induced with IFN-γ ($F = 124.18, 115.89, 215.03, 267.26$, respectively; $P < 0.001$) (Figure 4).
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Figure 6. The regulatory effect of miR-4804 on target gene CMKLR1 in 293T cells.

Figure 7. The effect of miR-4804 on the expression of CMKLR1 protein in THP1 cells.

Prediction and analysis of miRNA-4804 target genes

The target genes of miRNA-4804 were analyzed using TargetScan and miRDB database. The number of target genes of miRNA-4804 was 2206 and 143 in TargetScan and miRDB, respectively. Their intersection was 110. CMKLR1 is one of the potential target genes. Sequence analysis showed one binding site of miRNA-4804 in the 3'-UTR sequence of the CMKLR1 gene. GO analysis showed that miRNA-4804 was involved in voltage-gated sodium channel activity, lipoprotein particle receptor binding activity, and ubiquitin-specific protease activity. Pathway analysis demonstrated that miRNA-4804 was involved in cancer, extracellular matrix-receptor interaction, mTOR signal transduction, and focal adhesion (Figure 5).

Regulation of miR-4804 on the target gene CMKLR1

The regulatory effect of miR-4804 on the 3'-UTR of the target gene CMKLR1 was detected by dual-luciferase reporter gene assay. The results showed that the ratios of hsa-miR-4804-3p/NC in wild-type CMKLR1 and the control group were 53%±9.1% and 100%±6.4%, respectively ($t = 7.42$, $P = 0.0019$). The ratios of hsa-miR-4804-3p/NC in the CMKLR1 mutant strain and the control group were 96%±1.5% and 97%±8.8% ($t = 0.19$, $P = 0.878$) (Figure 6).

Effects of miR-4804 on the expression of CMKLR1 protein

The effect of miR-4804 on the expression of CMKLR1 protein was detected by Western blot. The values of CMKLR1/GAPDH in inhibitor NC, mimic NC, miRNA-4804 inhibitor, and miRNA-4804 mimic were 0.50, 0.51, 0.67 and 0.36, respectively (Figure 7).

Discussion

Monocytes are one of the most active cells in vivo, and they play an important role in innate immune response. Macrophages and dendritic cells are developed from monocytes under the condition of inflammation. HLA-DR, CD80, and CD86 are abundantly expressed during monocyte maturation. Some studies have showed that macrophages and dendritic cells play a defective role in CHB patients [15, 16]. However, Tavakoli et al reported that peripheral blood dendritic cells are phenotypically and functionally intact in chronic hepatitis B virus infection [17]. The present study found that THP1 cell proliferation was significantly enhanced after IFN-γ stimulation, and miRNA-4804 could inhibit the proliferation of monocytes induced with IFN-γ. The expression of HLA-DR and CD86 on the surface of THP1 cells was significantly enhanced after IFN-γ stimulation. The expression of HLA-DR and CD86 was significantly inhibited by miRNA-4804 agonist and significantly increased by miRNA-4804 inhibitors. These results suggested that miRNA-4804 may inhibit the proliferation and expression of...
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surface molecules of monocytes. Previous studies have found that miRNA-4804 is elevated in PBMCs of CHB patients. MiRNA-4804 might lead to the differentiation and maturation of monocytes by inhibiting the proliferation and surface molecular expression of monocytes, thereby affecting the innate immune response to HBV infection in patients.

Monocytes are stimulated by different stimuli, such as lipopolysaccharide and IFN-γ, which can secrete various cytokines. Studies have shown that alterations in miRNA expression can regulate the innate immune response in HBV infection through Toll-like receptor and the NF-KB pathway [18]. The present study demonstrated that the expression of IL-12, TNF-a, and IL-6 significantly increased, whereas the expression of IL-10 significantly decreased after stimulation with IFN-γ in THP-1 cells. MiRNA-4804 could inhibit the expression of IL-6, TNF-a, and IL-12 and promote the expression of IL-10 in THP1 cells induced by IFN-γ. The results of these genes were similar to those of the proteins. IL-6, TNF-a, and IL-12 are important contributing factors in the inflammatory and immune response, whereas IL-10 is an anti-inflammatory and immune inhibitory factor [19]. The elevation of miRNA-4804 in CHB patients may affect the immune inflammatory reaction by regulating monocyte secretory function and play an important role in the immune homeostasis mechanism. However, the specific regulatory mechanism remains unclear and requires further study.

CMKLR1 is a highly conserved protein that is mainly expressed on the surface of plasmacytoid dendritic cells, macrophages, and monocyte cells in humans. Its ligand is the chemerin, and it is mainly expressed in liver and lung tissues [20]. Previous studies have shown that chemerin can raise CMKLR1 expression in macrophages through an adjacent secretory pathway and accumulates in liver tissues; chemerin is also involved in the liver’s inflammatory response and insulin resistance [21, 22]. In the current study, the expression of CMKLR1 in monocytes was upregulated with IFN-γ. This result was similar to the findings reported by Wittamer [23]. Dual-luciferase reporter assay and Western blot showed that miRNA-4804 could inhibit the expression of CMKLR1 mRNA and protein. The expression of CMKLR1 mRNA was inhibited by miRNA-4804 in the THP1 cell line. These results indicated that CMKLR1 could be the potential target gene of miRNA-4804. Activated CMKLR1 may be directly involved in the regulation of inflammatory responses [24]. Thus, miRNA-4804 might regulate the inflammatory response in CHB by targeting CMKLR1. A recent report showed that the expression levels of chemerin and CMKLR1 significantly increase in the liver of patients with chronic hepatitis C [25]. However, the expression of chemerin and CMKLR1, as well as the effects of their interaction, in CHB patients must be explored in future investigations.

In conclusion, the results suggested that miRNA-4804 influenced the proliferation of monocytes and the secretion of cytokines by regulating the expression of CMKLR1, which may play a role in the immune homeostasis of CHB infection.

Acknowledgements

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Disclosure of conflict of interest

None.

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