

MiR-214 regulates CD3 ζ expression in T cells

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Abstract

Introduction: T-cell activation requires the T-cell receptor (TCR)-CD3 complex, which integrates and transduces signals. CD3 ζ plays a vital role in TCR signalling by mediating T-cell activation. Abnormal CD3 ζ expression is a common characteristic of haematological malignancies with T-cell immune dysfunction or autoimmune diseases. Targeted regulation of CD3 ζ expression by either direct or indirect approaches is important for regulating T-cell activation.

Aim of the study: In this study, we focused on identifying miRNAs that may regulate CD3 ζ expression.

Material and methods: Three microRNA target search algorithms (TargetScan, PicTar, and micro-rna.org) were used to identify hypothetical miRNAs that target CD3 ζ in T cells. Of the predicted miRNAs, miR-214 was chosen and validated to determine whether miR-214 directly binds to the CD3 ζ 3'-UTR and regulates CD3 ζ expression by luciferase reporter assays, real-time PCR, and Western blotting.

Results: The results indicate that miR-214 specifically binds the CD3 ζ 3'-UTR, and miR-214 mimics remarkably reduce the expression of CD3 ζ in MOLT-4 cells.

Conclusions: We identify for the first time that miR-214 targets expression in MOLT-4 cells, suggesting that miR-214 might negatively regulate T-cell activation by targeting CD3 ζ .

Key words: T cell, CD3 ζ , miR-214.

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Introduction

The activation of antigen-specific T cells is key for developing adaptive immunity [1]. The T-cell receptor (TCR)-CD3 complex, which is expressed on T-cell membranes, is capable of integrating and transducing signals engaged by peptide-major histocompatibility complex (MHC) complexes expressed on antigen-presenting cells (APCs) [2]. Each TCR-CD3 complex contains one CD3 γ and CD3 δ chain and two CD3 ϵ and CD3 ζ chains [3].

Recent evidence has demonstrated that CD3 ζ plays a vital role in multiple autoimmune, inflammatory, and malignant diseases [4-6]. The CD3 ζ gene is regulated at the transcriptional, posttranscriptional, and posttranslational levels [7].

We previously demonstrated that different CD3 ζ 3' untranslated region (3'-UTR) alternatively spliced isoforms regulate the CD3 ζ mRNA expression level in T cells in patients with aplastic anaemia (AA) and chronic myeloid leukaemia (CML) [8, 9]. These results revealed that a posttranslational mechanism was involved in regulating CD3 ζ expression.

MicroRNAs (miRNAs) are endogenous, small non-coding RNA molecules that act as posttranscriptional regulators. miRNAs specifically pair with complementa-

ry sites in 3'-UTRs of target mRNAs to block translation or increase mRNA degradation [10]. Recently, strong evidence has suggested the important role of miRNAs in T-cell immunity [11]. miR-202-3p plays a role in post-transcriptional regulation of CD3 ζ in pancreas-infiltrating T cells of non-obese diabetic mice [12]. It is well known that each gene is target regulated by many miRNA; thus, we explored whether there are other miRNAs involved in the regulation of CD3 ζ .

In this study, miRNA target gene databases were used to predict potential miRNAs that target CD3 ζ mRNA. The predicted miRNA miR-214, which regulates CD3 ζ gene expression, was verified in the MOLT-4 cell line, demonstrating that miR-214 may be a key regulator of CD3 ζ .

Material and methods

Cell cultures

Acute lymphoblastic leukaemia MOLT-4 cells were maintained in RPMI-1640 medium (Gibco, USA) supplemented with 10% foetal bovine serum (Gibco, USA) and antibiotics (100 U/ml penicillin and 100 U/ml streptomycin).

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Human embryonic kidney (HEK 293T) cells were maintained in GlutaMAX High Glucose DMEM medium (Gibco, USA) supplemented with 10% foetal bovine serum (Gibco, USA) and antibiotics (100 U/ml penicillin and 100 U/ml streptomycin).

All cell lines were incubated at 37°C in a humidified atmosphere of 5% CO₂.

Construction of CD3ζ 3'-UTR luciferase reporter vectors

The wild type CD3ζ 3'-UTR, which includes a conserved putative miR-214 binding site (1050-1056 bp), was amplified by polymerase chain reaction (PCR). The PCR products were cloned into the pGEM-T Easy Vector (Promega, USA).

After cloning, a Fast Site-Directed Mutagenesis Kit (TIANGEN, China) was used to generate a mutation in the CD3ζ 3'-UTR, according to the manufacturer's protocol. The site-directed mutagenesis primers were as follows: forward: 5'-TCGAGTGTGTCTGAGTGGCTTCACTCAGACGTAATTTGGCTTCTGTTG TCGTTT-3' and reverse: 5'-AAACGACAACAGAAGCCAAATTTACGTCGTG AGTGAAGCCACTCAGACACAC-3' (mutations are underlined). The wild type and mutant CD3ζ 3'-UTR were subcloned from the pGEM-T Easy Vector into the psiCHECK-2 plasmid (Promega, USA) using the following primers: forward: 5'-CCGCTCGAGCAGC-CAGGGGATTTCACTCAAG-3' (*Xho* I), and reverse: 5'-AGCTTTGTTTAAACCCCTAGTACATTGACGGGTTTT TCC TG-3' (*Pme* I). Successful construction of the psiCHECK-2 plasmids including the wildtype, and mutant CD3ζ 3'-UTRs (CHK2-CD3Z-WT and CHK2-CD3Z-MU) was verified by sequencing.

Luciferase assays

HEK293T cells were cultured in 12-well plates (2.5 × 10⁵/well) to approximately 80% confluence after 24 hours. The cells were then cotransfected with the CHK2-CD3Z-WT or CHK2-CD3Z-MU plasmids plus a miR-214 mimic or negative control (NC) for 48 hours. The miR-214 mimics or negative control were synthesised by Guangzhou RiboBio (RiboBio, China). The Dual-Glo Luciferase Assay System (Promega, USA) was used to evaluate the relative activity of Firefly and Renilla luciferase according to the manufacturer's protocol. Transfections were performed in duplicate and repeated three times.

Transfection and Western blotting

The miR-214 mimic or negative control (RiboBio, China) were transfected into MOLT-4 cells using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's protocol. Transfections were performed in duplicate and repeated three times.

The miR-214 mimic or negative control were transfected into MOLT-4 cells for 72 hours, and the protein levels were analysed by Western blotting. Briefly, total cellular protein was extracted with RIPA lysis buffer (Cell Signaling Technology, USA), and protein concentrations were determined using the BCA assay. Protein aliquots (100 µg) were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and the separated proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane. The PVDF membranes were blocked and incubated with antibodies directed against CD3ζ (1 : 500) or β-actin (1 : 1,000) followed by mouse anti-human secondary antibodies. All antibody reagents were from Cell Signal Technology with the exception of the CD3ζ antibody, which was from Abcam.

Real-time relative quantitative PCR (qRT-PCR) for CD3ζ gene expression

A miR-214 mimic or negative control was transfected into MOLT-4 cells. After 48 hours, RNA extraction and cDNA synthesis were performed according to the manufacturer's instructions. qRT-PCR using the SYBR Green I method was then used to examine the *CD3ζ* gene expression level with β₂M serving as an internal control. The CD3ζ and β₂M primer sequences and PCR conditions were described previously [13]. The 2^(-ΔCT) method was used to analyse the *CD3ζ* gene expression level relative to control.

Statistical analysis

The two-tailed Student's *t*-test was performed to compare the relative luciferase activities of the *CD3ζ* gene expression levels in the different groups. *p* < 0.05 was considered to be statistically significant.

Results

Predicting a potential miRNA that targets CD3ζ using bioinformatics databases

In this study, we predicted a potential miRNA that targets CD3ζ mRNA using bioinformatics databases. First, miR-214 was identified by three bioinformatics tools: TargetScan (<http://www.targetscan.org>), PicTar (<http://www.pictar.mdc-berlin.de>), and microRNA (<http://www.microRNA.org>). By coincidence, miR-214 was also predicted and concluded to target CD3ζ using a bioinformatics analysis tool and reviewing reports [14]. Therefore, miR-214 was chosen for experimental validation.

TargetScan predicted a binding site for miR-214 in the CD3ζ 3'-UTR (1050-1056 bp, NM_198,053.2). To determine whether miR-214 could specifically pair with its putative complementary site in the CD3ζ 3'-UTR, a psiCHECK-2 luciferase reporter expressing the wild type CD3ζ 3'-UTR (CHK2-CD3Z-WT) or a mutant CD3ζ

receptor tyrosine-based activation motifs (ITAMs), which are essential for TCR signal transduction and T-cell activation [15, 16]. Each CD3 ζ chain contains three ITAMs. The structural characteristics of CD3 ζ suggest that CD3 ζ plays a unique role in the TCR signalling pathway. Up- and down-regulation of CD3 ζ was found in different diseases with abnormal T-cell immune statuses, including autoimmune diseases such as aplastic anaemia, which overexpress CD3 ζ in T cells, and cancer patients with T-cell immunosuppression, who have significantly decreased CD3 ζ expression [8, 9, 17]. Reasons for the abnormal CD3 ζ expression may be the transcription factor E-74-like factor (Elf-1), ubiquitination, granzyme-B, and caspase-mediated degradation [18-21]. Several studies have indicated that the CD3 ζ 3'-UTR has regulatory elements that affect CD3 ζ expression in T cells in patients with systemic lupus erythematosus (SLE) [22, 23]. Our previous studies have found that different CD3 ζ 3'-UTR alternative splicing isoforms might regulate the CD3 ζ mRNA expression level in T cells in patients with AA and CML [8, 9]. However, the mechanism involved in regulating the CD3 ζ 3'-UTR remains unclear. It has been shown that miRNAs play an important role in T-cell immune homeostasis by directly targeting the 3'-UTRs of mRNAs that mediate gene expression [24]. A recent study has shown that miRNAs can modulate TCR signalling molecules [25]. Meanwhile, miR-202-3p was identified regulating CD3 ζ in pancreas-infiltrating T cells of non-obese diabetic mice [12]. Taking into account the important function of miRNAs in T-cell immunity, in this work, we sought to ascertain whether other miRNAs were involved in regulation of CD3 ζ .

In this study, luciferase activity assays revealed that miR-214 directly binds the CD3 ζ 3'-UTR to downregulate luciferase activity. miR-214 mimic down-regulated CD3 ζ expression in mRNA and protein level in T-cell line. These results revealed that miR-214 regulates CD3 ζ .

Targets of miRNA-214 are complex and mediate diverse processes such as differentiation, senescence, angiogenesis, cell migration, and virus replication; it also acts as a tumour suppressor or oncogene in different cancers [26, 27]. In this study, we found that miR-214 might have an additional function involving the regulation of T-cell activation. However, it has also been reported that there is increased miR-214 expression in activated T cells. In this case, it was thought that the role of miR-214 might be to promote T-cell activation in C57BL/6 mouse T cells by targeting PTEN [28]. Therefore, it is thought that the contrasting effects of miR-214 on T-cell activation might be related to different cell types and disease statuses. This hypothesis is similar to findings with miR-155, which was demonstrated to possess multiple opposing functions in various cell types, including promoting regulatory T cells and T-effector function [29]. However, further investigation is needed to determine the function of miR-214 in

different T-cell subsets with different T-cell immune statuses and diseases, and whether miR-214 regulates different genes in T cells through its complex target networks.

Conclusions

In conclusion, we identified for the first time that miR-214 targets CD3 ζ expression in MOLT-4 cells, implying that miR-214 might negatively regulate T-cell activation by targeting CD3 ζ . This finding might provide a novel approach for targeted regulation of the CD3 ζ expression level to control the T-cell activation status. Further investigation will focus on the regulatory mechanisms of miR-214 in haematological diseases with different T-cell immune statuses.

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The authors declare no conflict of interest.

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