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miR-17-92 promotes leukemogenesis in chronic myeloid leukemia via targeting A20 and activation of NF-κB signaling

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ABSTRACT

miR-17-92 cluster are overexpressed in hematological malignancies including chronic myeloid leukemia (CML). However, their roles and mechanisms that regulate BCR-ABL induced leukemogenesis remain unclear. In this study, we demonstrated that genomic depletion of miR-17-92 inhibited the BCR-ABL induced leukemogenesis by using a mouse model of transplantation of BCR-ABL transduced hematopoietic stem cells. Furthermore, we identified that miR-19b targeted A20 (TNFAIP3). A20 overexpression results in inactivation of NF-κB activity including decrease of phosphorylation of P65 and IκBα, leads to induce apoptosis and inhibit proliferation and cycle in CML CD34 $^{+}$ cells. Thus we proved that miR-17-92 is a critical contributor to CML leukemogenesis via targeting A20 and activation of NF-κB signaling. These findings indicate that miR-17-92 will be important resources for developing novel treatment strategies of CML and better understanding long-term disease control.

1. Introduction

Chronic myelogenous leukemia (CML) is a hematological malignancy derived from the hematopoietic stem cell malignant transformation caused by BCR-ABL fusion oncogene. BCR-ABL protein with the tyrosine kinase activity, which activates various signaling pathways that robust hematopoietic cell proliferation, block cell differentiation and reduce apoptosis, results in leukemogenesis [1]. Currently, targeting therapy with tyrosine kinase inhibitors (TKIs), such as Imatinib and Dasatinib, has fundamentally improved the survival rate of CML patients [2]. However, the insensitivity and resistance of leukemia stem cells to TKI are still the major relapse roots of CML [3]. Thus it is necessary to extensively elucidate the mechanism favoring initiation and progression of CML.

MicroRNAs (miRNAs) are the highly conserved post-transcriptional regulators that regulate target gene expression by binding to their 3′ untranslated region (UTR). Numerous microRNAs such as miR-142, miR-155, miR-181, miR-221 and miR-17-92 cluster have identified as major regulators in the processes of occurrence, progression, and drug resistance of CML [4–7]. miR-17-92 cluster, which maps on human chromosome 13q31, consists of six individual miRNAs (miR-17, miR-18a, miR-19a, miR-20a, miR-19b and miR-92a) and highly expresses in various human cancer onset. Its overexpression occurs in a broad spectrum of human cancers, including lymphoma, chronic myeloid leukemia and solid tissue cancers [8,9]. miR-17-92 is recognized as an important CML-associated oncogene and highly expressed in primary CML CD34 $^{+}$ cells [10]. However, the oncogenic mechanisms of miR-17-92 in CML are not completely understood.

Constitutive activation of nuclear factor-κB (NF-κB) is typical of most malignancies and plays a major role in CML leukemogenesis [11,12]. In CML, BCR-ABL-induced NF-κB is in an IKK independent manner and regulated by multiple inhibitory signal pathways [13]. A20, named as tumor necrosis factor α-induced protein 3 (TNFAIP3), was identified as a TNF-inducible zinc-finger protein in

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endothelial cells. It works as a central and inducible inhibitory regulator of NF-κB and mediates the outer negative feedback response by attenuating the catalytic activity of the IKK complex [14]. Although NF-κB signaling is well documented in CML, limited data are available regarding the involvement of A20-mediated downregulation of NF-κB in CML leukemogenesis.

A20 is inactivated in various haematological malignancies and results in constitutive NF-κB activation in tumour cells. Bioinformatic analysis revealed that A20 was the predicted targets for miR-17-92 cluster [15,16]. In T cells of asthma, miR-19 promotes TH2 cytokine production and amplifies NF-κB signaling by direct targeting of A20 [17]. BCR/ABL1 was found to positively regulate several genes including A20 involved in negative feedback regulation of cellular signaling pathways [18]. However, the roles of miR-17-92 cluster and its targets in CML leukemogenesis remain unclear. In this study, by using a mouse model of transplantation of BCR-ABL transduced hematopoietic stem cells with genomic depletion of miR-17-92, we clarified that miR-17-92 contributes to CML leukemogenesis by suppression of A20 and activation of NF-κB signal pathway.

2. Materials and methods

2.1. Cell lines, patient samples and CD34$^+$ cell isolation

Human embryonic kidney 293 cells were maintained in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FCS (HyClone Laboratories, Logan, UT, USA). Human leukemia cell lines, K562 and KCL22 from our laboratory were grown in RPMI-1640 (Gibco, USA) containing 10% FBS (Gibco, USA). Human bone marrow (BM) samples from CML patients or donors were obtained under protocols approved by the Institutional Review. The samples of patients diagnosed with CML and healthy donors were obtained after informed consent. Briefly, the mononuclear cells were isolated through Ficoll/Hypaque gradient centrifugation and then processed for CD34$^+$ sorting by using EasySep human CD34 Selection Kit (StemCell Technologies, USA) according to the manufacturer's instructions.

2.2. RNA isolation, quantitative real-time PCR analysis and Taqman miRNA assay

Total RNA was extracted from cells by Trizol Reagent and cDNA synthesis was performed with Taqman@ MicroRNA Reverse Transcription Kit (Thermo, USA). hsa-miR-17-5p, hsa-miR-18a-5p, hsa-miR-19a-3p, hsa-miR-20a-5p, hsa-miR-19b-1-5p, has-miR-92a-3p and U6 (internal control) specific cDNAs were amplified according to Taqman MicroRNA assay protocol (Applied Biosystems, USA). Total RNA were reversed to cDNA by reverse transcription with Oligo dT primers (Thermo, USA). Primer sequences were listed as Table 1. Samples were tested in triplicate on the Applied Biosystems 7500HT Fast Real-Time PCR System (Life Technology, USA). Relative quantitative evaluation of target genes levels in triple reactions were performed by comparing $\triangle$Ct.

2.3. Generation of conditional miR-17-92 knockout mice

miR-17-92 mutant mice (STOCK Mir17-92tm1.1Tyj/J)and Mx1-cre transgenic mice (B6.Cg-Tg (Mx1-cre)1Cgn/J)were purchased from The Jackson Laboratory. Expression of Mx1-Cre/miR-17-92$^{loxP/loxP}$ in the resulting offspring of the former cross was induced by intraperitoneal injection of 500 μg pIpC (Sigma, USA) on 3 alternate days. The knockout efficiency of miR-17-92 expression was confirmed by Taqman Q-PCR analysis.

2.4. Transplantation of BCR-ABL transduced hematopoietic stem cells for a mouse chronic myeloid leukemia model

All animal experiments were approved by the ethics committee in Academy of Military Medical Science, China. Wild C57BL/6 mice and miR-17-92 knockout mice from 8 week of age as male donor mice were primed by intravenous injection with 200 mg/kg 5-fluorouracil (5-FU) at 4 days before harvest. BM cells were harvested and prestimulated in medium of IMDM, 5% FCS, 1% penicillin/streptomycin, 6 ng/ml recombinant murine IL-3, 10 ng/ml recombinant murine IL-6, 100 ng/ml recombinant murine stem cell factor (SCF). B cells in the BM of donor mice was stained B220 and CD3, and checked by flow cytometry (BD, USA) according to protocols. After prestimulation for 24 h at 37 °C, viable cells were transduced twice with BCR-ABL retrovirus in the same medium containing 2 μg/ml polybrene, and cosedimented with virus at 3000 rpm for 2 h to increase transduction efficiency. The cells were collected at the second day, 5×10^5 cells transduced cells were transplanted and injected into per lethally irradiated (900 cGy) female C57 recipients via tail vein. After transplant, recipient mice were evaluated daily for signs of morbidity, weight loss and splenomegaly. The clinical features and histopathology of BCR-ABL-induced CML-like disease was checked.

2.5. Lentiviral, miR-19b inhibitor and Imatinib transduction of K562, KCL22 and CML CD34 $^+$ cells

CML CD34 $^+$ cells prestimulated with hematopoietic growth factors were transfected with A20 and NEG in the presence of 8 μg/ml polybrene (Sigma, USA). Transfection was repeated twice and the cells were cultured for 48 h. GFP-positive cells were sorted by FACS Aria II (BD, USA) for further analysis. K562 and KCL22 cells were transduced with A20/NEG, miR-17-92/pCDH lentiviral and miR-19b inhibitor (RIBOBIO, China) and treated with Imatinib (5 μM) for 48 h according to the manufacturer's instructions, and then subjected to qRT-PCR and Western blot analyses.

2.6. Luciferase assay

To create a luciferase reporter construct, 3′-UTR segments of A20 that contained the putative binding sites for miR-18a and miR-19a/b were synthesized by annealing oligos using the following primers:

miR − 18: forward : 5′ − CTAGCTTTATAATATGCACCTTTTAAAAAA − 3′,
reverse : 5′ − TATTTTTTAAAAGGTGCATATTATAAAG − 3′;
miR − 19: forward : 5′ − CTAGAGTATTTGAAATTTGCACATTTAATTG − 3′,
reverse : 5′ − TACAATTAAATGTGCAAATTTCAAATACT − 3′.

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