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miR-142-3p inhibits LPS-induced activation of NF-κB by targeting IRAK1 in colorectal cancer

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Abstract

Inflammatory signals originating from colorectal cancer cells have a vital role in the development of colorectal cancer (CRC). microRNAs (miRNAs) have been demonstrated to be involved in the development and progression of CRC. miRNA-142-3p (miR-142-3p) has been reported to be a modulator of inflammatory signals. It has also been shown that miR-142-3p is downregulated in CRC and it acts to be a tumor suppressor. In the present study, interleukin-1 receptor-associated kinase 1 (IRAK1), a key mediator of NF-κB pathway, was identified as a direct target of miR-142-3p. It was also found that miR-142-3p repressed the activation of NF-κB signal induced by LPS in colorectal cancer cells, therefore reducing the expression of inflammatory cytokines, such as IL-6, IL-8, MCP-1, CCL5 and CSF-1. Thus, it is believed that miR-142-3p is a key component in the regulation of NF-κB activity and its anti-inflammatory role may contribute to its suppression of carcinogenesis of CRC.

Key words: *colorectal cancer, non-coding RNA, inflammatory factors.*

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Introduction

Colorectal cancer (CRC) is the third most common cancer and one leading cause of cancer-related death [1]. Although genetic predisposition and environmental factors have been reported to be involved in the development of CRC, the molecular mechanisms underlying the pathogenesis of the disease are still poorly understood [2-4]. Epidemiological data suggests that inflammatory bowel disease (IBD) raises more risks for the development of CRC [5-7]. In mouse model, the combination of a single hit of azoxymethane (AOM) with the exposure to the inflammatory agent dextran sodium sulphate (DSS) has been demonstrated to dramatically shorten the latency time for induction of CRC [8]. These clinical and experimental data clearly pinpoint that inflammation plays an important role in the carcinogenesis of CRC.

The transcription factor NF-κB and the signaling pathways are the central coordinators in inflammatory responses. Recently, roles of NF-κB signaling axis in the tumorgenesis of CRC have been extensively investigated [9, 10]. The activation of NF-κB pathways may be involved in the growth and metastasis of colorectal cancer cells, and also aggravate inflammatory responses, consequently promoting carcinogenesis.

MicroRNA (miRNA) is a class of small non-coding RNAs, which regulate the expression of genes through sequence-specific bindings [11]. Evidence suggests that miRNAs are involved in the development and progression of CRC [12-14]. miRNAs can function as tumor suppressors or oncogenes, thereby regulating the carcinogenesis of CRC [12]. miR-142-3p has been primarily reported to regulate immune responses [15]. Previous studies have shown that it is involved in the regulation of LPS-induced interleukin-6 (IL-6) expression [15]. miR-142-3p can also affect T cell development, regulatory T cell function and inflammatory responses [16]. Recently, miR-142-3p has been proven to be downregulated in human CRC and repress the malignancy of CRC by targeting several oncogenes, such as CD133, ABCG2, and Lgr5 [17]. However, the effects of miR-142-3p on regulation of inflammatory responses in colorectal cancer cells are still not known.

In this study, we identified interleukin-1 receptor-associated kinase 1 (IRAK1) as a new target of miR-142-3p. We also provided the evidence that miR-142-3p inhibits LPS-induced NF-κB activation and reduces LPS-induced expression of tumor-promoting cytokines in colorectal cancer cells. These data indicated that miR-142-3p may suppress inflammatory responses by inhibiting IRAK1-mediated activation of NF-κB, therefore inhibiting the development and metastasis of CRC.

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Material and methods

miRNA target prediction tools

The putative targets of miRNA were predicted using the TargetScan, PicTar and miRBase Targets algorithms.

Cell culture and transfection

HCT116 cells and HEK293 cells were both grown in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA, USA), 100 U/ml of penicillin and 100 μg/ml of streptomycin (Sigma Aldrich, Saint Louis, MI, USA). Transfection was performed with a Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol.

RNA isolation and real-time quantitative RT-PCR

Total RNA was isolated from the transfected cells with Trizol (Invitrogen, Carlsbad, CA, USA). And cDNA was synthesized from 1 μg of total RNA by means of the reverse reaction kit, according to the manufacturer's instructions (Promega, Madison, WI, USA). Real-time quantitative RT-PCR was conducted using IQ5 (BioRad, Hercules, CA, USA). The experiments were performed with 20 μl reaction volumes containing 10 μl 2 × SYBR® Premix Ex Taq (TaKaRa Biotech, Dalian, China), 0.4 μM of each primer, 1 μl of cDNA template, and 8.2 μl deionized water. PCR amplifications were done using the following parameters: 95°C for 15 s, 40 cycles through 95°C for 5 s, 60°C for 30 s. Melting curve analyses were also performed to exclude non-specific PCR products. For each biological sample technical triplicates were made. Primers used were as follows: GAPDH forward, AGAAGGCTGGG GCTCATTTG; GAPDH reverse, AGGGGCCATCCACAGTCTTC; IL-6 forward, GTACAT CCTCGACGCATC; IL-6 reverse, TTTCACCAGGCAAGTCTCC; IL-8 forward, ACTCCA AACCTTTCCACC; IL-8 reverse, AACTTCTCCACAACCCT; MCP-1 forward, CATTGT GGCCAAGGAGATCTG; MCP-1 reverse, CTTCGGAGTTTGGGTTTGCTT; CCL5 forward, TACATTGCCCGCCCACTGCC; CCL5 reverse, GGGTTGGCACACACTTGGCG; CSF-1 forward, AGGGCAGCCCCCTGACTCAG; CSF-1 reverse, GGAGGATGGCCAGGG AGGGG.

Western blotting

HCT116 cells were transfected with miR-142-3p mimics or negative control and the cells were harvested 72h post-transfection. Proteins were separated on polyacrylamide gels, transferred to a PVDF membrane, incubated with antibodies against IRAK1 (Cell Signaling Technology, Danvers, MA, USA) or GAPDH (Millipore, Billerica, MA, USA). GAPDH was used as loading control.

3'UTR luciferase assay

The IRAK1 3'UTR fragment containing a potential miR-142-3p binding site was amplified using PCR, and then cloned into a pMIR-REPORT Luciferase miR Expression Reporter Vector (Applied Biosystems). miR-142-3p seed sites were mutated using a QuickChange Site-Directed Mutagenesis Kit (Agilent Technologies, Palo Alto, CA, USA), thereby changing the sequence from ACACUACA to TCTCAAGA. The luciferase pMIR-REPORT plasmids were transfected to the HEK293 cells together with Renilla vector (Promega, Madison, WI, USA) and miR-142-3p mimics or negative control. 24 h post-transfection the cells were harvested and subjected to a luciferase assay using the Luciferase reporter assay reagents (Promega, Madison, WI, USA).

NF-κB activity assay

HCT116 cells were plated at 1×10^5 cells/well in 24-well plates. The NF-κB luciferase reporter plasmid, Renilla vector and miR-142-3p mimics, negative control or siRNA against IRAK1 were co-transfected to the cells. 24 h post-transfection the cells were stimulated with 20 μM LPS (Sigma Aldrich, Saint Louis, MI, USA). 24 h after stimulation the luciferase assay was performed.

Statistical analysis

The results are presented as the means ± standard deviation from three independent experiments performed in triplicate, and they were analyzed by parametric *t* tests. Analysis was performed using SPSS 16.0 and a level of $p < 0.05$ was considered significant.

Results

miR-142-3p targets IRAK1, a critical critical signaling mediator of NF-κB pathway

To identify the target genes of miR-142-3p, three algorithmm programs (TargetScan, PicTar, and miRBase) were used to screen for candidate targets. And it was worth noting that a putative binding site for miR-142-3p was found in the 3'UTR of IRAK1 (Fig. 1A). To investigate whether miR-142-3p was able to directly bind to the 3'UTR of IRAK1 and repress it, we constructed luciferase reporters with a wild-type 3'UTR (WT) of IRAK1 and a mutant-type 3'UTR (MUT) containing a mutant miR-142-3p binding site (Fig. 1A). These luciferase reporters were transfected with miR-142-3p mimics or negative control into HEK293 cells. Rellina vector was also co-transfected for normalization. As a result, miR-142-3p mimics significantly reduced the luciferase activity of the IRAK1 wild-type reporter, whereas it had no effect on the luciferase activity in cells transfected with the mutant-type 3'UTR vector (Fig. 1B). These data demonstrated that IRAK1 is a direct target of

miR-142-3p. Furthermore, to determine whether miR-142-3p could also suppress the expression of IRAK1, miR-142-3p mimics were transfected into HCT116 cells and IRAK1 expression levels were analyzed by qRT-PCR and western blot. As shown in Fig. 1C and Fig. 1D, in HCT116 cells transfected with miR-142-3p mimics, the IRAK1 mRNA and protein levels were significantly reduced.

miR-142-3p inhibits LPS-induced NF-κB activity by targeting IRAK1

IRAK1 is a key mediator in the signaling pathways of TLRs/IL-1Rs [18]. By means of its kinase and adaptor functions, IRAK1 initiates a cascade of signaling events eventually leading to NF-κB activation and induction of inflammatory target genes expression [18]. Our results showed that miR-142-3p targets IRAK1 and represses the expression of IRAK1. Herein, we wanted to establish its role in NF-κB signal transduction in colorectal cancer cells. For this purpose, we used LPS, a known activator of TLRs-mediated NF-κB-activation pathway. NF-κB luciferase reporter plasmid and Rellina vector were co-transfected to HCT116 cells together with miR-142-3p mimics, negative control or siRNA against IRAK1. 24 h post-transfection the cells were stimulated with LPS (20 μM). 24 h after stimulation the luciferase assay was performed. As expected, LPS-stiumlation significantly increased NF-κB activity in our luciferase reporter system, whereas miR-142-3p transfection or siRNA knockdown of IRAK1 inhibited LPS-induced activation of NF-κB in HCT116 cells (Fig. 2).

miR-142-3p reduces LPS-induced expression of inflammatory factors

Inflammation has a clear role in the initiation and development of CRC. Inflammatory factors can induce posi-

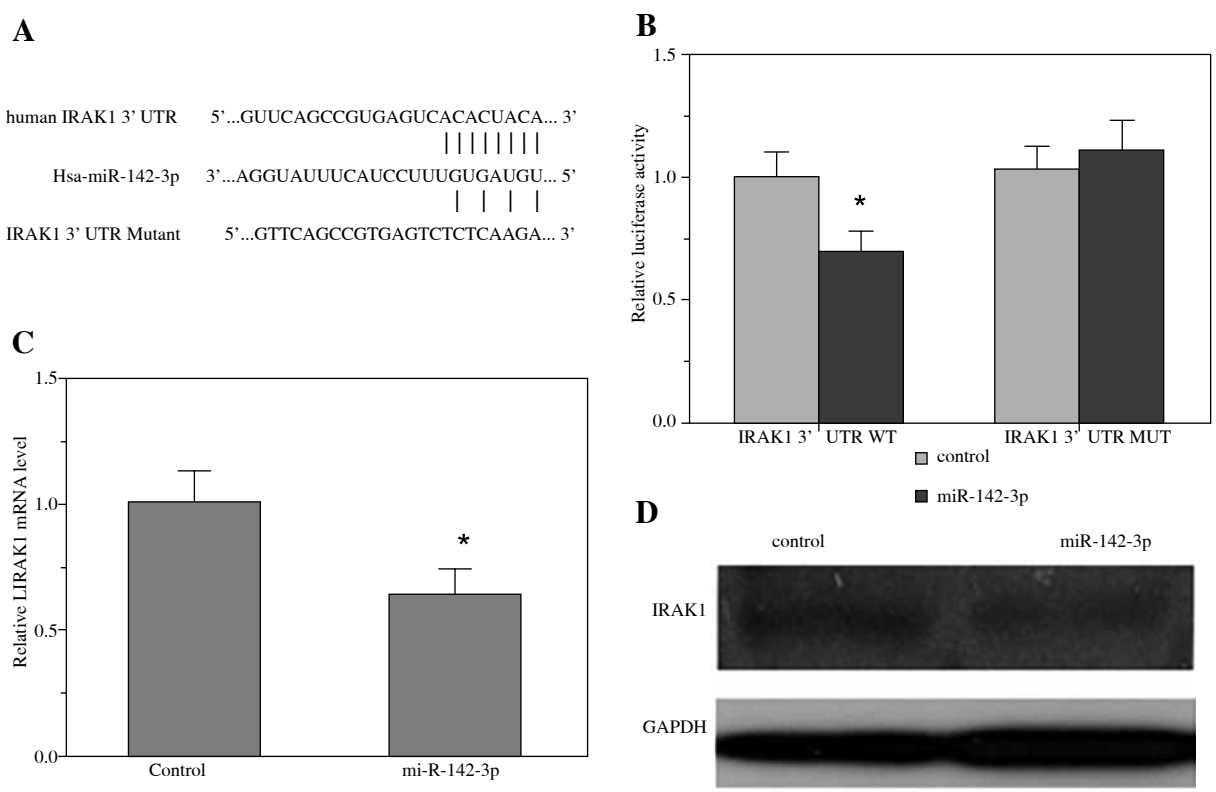

Fig. 1. IRAK1 is a direct target of miR-142-3p. (A) The IRAK1 3'UTR has a putative miR-142-3p binding site. The predicted duplex of miR-142-3p (mid) and its target site in the 3'UTR of IRAK1 (up) are shown. The 3'UTR mutation of IRAK1 is shown (bottom). (B) miR-142-3p reduces the luciferase activity of IRAK1 wild-type (WT) reporter. Reporter constructs containing either wild-type or mutant-type (MUT) 3'UTRs were transfected with miR-142-3p mimics or negative control to HEK293 cells. Rellina vector was also co-transfected for normalization. *$p < 0.05$. (C) IRAK1 expression is downregulated following miR-142-3p overexpression. The mRNA expression level of IRAK1 was determined by qRT-PCR in control- and miR-142-3p-transfected HCT116 cells. The expression of IRAK1 is presented relative to the expression in control-transfected cells. *$p < 0.05$. (D) miR-142-3p transfection also reduces the protein levels of IRAK1 in miR-142-3p-transfected HCT116 cells compared with control-transfected cells. GAPDH is used as loading control

tive signaling loops that increase cytokines and recruitment of inflammatory cells in the tumor microenvironment. Furthermore, these components of cancer-associated inflammation can promote colorectal carcinogenesis by regulating angiogenesis, cell proliferation and apoptosis, leading to tumor progression and metastasis. Therefore, we investigated whether miR-142-3p modulates the expression of inflammatory factors induced by extracelluar signals such as LPS in colorectal cancer cells. The results showed that LPS-stimulation significantly increased the expression levels of IL-6, IL-8, MCP-1, CCL5 and CSF-1 in HCT116 cells, whereas overexpression of miR-142-3p could inhibit the expression of these inflammatory factors (Fig. 3).

Discussion

Inflammation plays a vital role in the carcinogenesis of CRC. CRC tumors display constitutive activation of multiple inflammatory pathways, such as NF-κB pathway and exhibit increased expression of inflammatory cytokines, such as IL-6, IL-8, MCP-1, CCL5 and CSF-1 [5, 7, 9]. NF-κB is involved in tumorigenesis by promoting tumor cell proliferation, regulating tumor angiogenesis and invasiveness [9]. Furthermore, constitutive NF-κB activation can promote the secretion of major inflammatory factors. Such cytokines are the potent activators for NF-κB pathway [9]. Thus, it is believed that NF-κB pathway and inflammatory factors constitute a positive feedback loop to promote the carcinogenesis of CRC. In this study, we demonstrated that miR-142-3p negatively regulates LPS-induced activation of NF-κB pathway by directly targeting IRAK1, a key mediator of NF-κB pathway.

miR-142-3p has been primarily studied in the immune system [16]. Recent studies showed that expression levels of miR-142-3p are downregulated in hepatocellular carcinoma and esophageal squamous cell carcinoma [19, 20]. miR-142-3p can act as a tumor suppressor and suppress tumorgenesis [17, 19, 20]. miR-142-3p was also found to be decreased in colorectal cancer tissue and its downregulation is involved in the development of colorectal cancer [17]. Wang *et al.* reported that miR-142-3p is upregulated in colorectal cancer cells overexpressing NGX6, a tumor suppressor gene [21]. It was also demonstrated that miR-142-3p inhibits the proliferation of colorectal cancer cells by targeting CD133, ABCG2, and Lrg5 [17]. Recently, Sonda *et al.* reported that miR-42-3p could prevent macrophage differentiation and suppress tumor-released cytokines signaling by targeting gp130 and C/EBPβ LAP* [22]. The use of an oligo to enforce miR-142-3p expression could be a feasible option to modify tumor environment and favor antitumor immunity [22]. Therefore, the anti-inflammatory effect of miR-142-3p is worthy of fur-

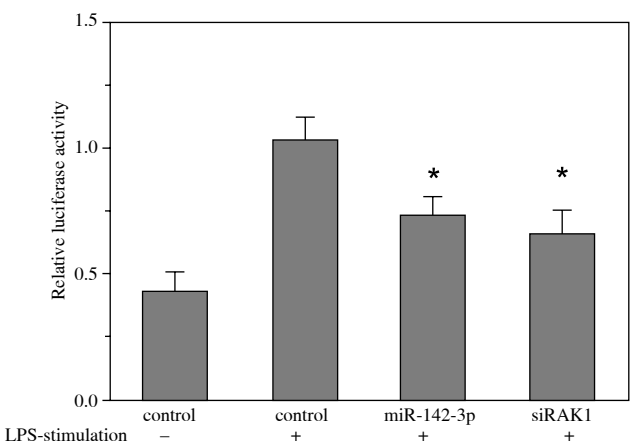

Fig. 2. miR-142-3p targets IRAK1-mediated NF-κB activation. HCT116 cells were co-transfected with NF-κB luciferase reporter plasmid and Renilla vector along with negative control, miR-142-3p mimics or siRNA against IRAK1. Cells were stimulated with 20 μM LPS, and then luciferase activity was determined. The luciferase activity is shown relative to activity in control transfected LPS-stimulated cells. LPS-stimulated NF-κB activation was repressed both by miR-142-3p transfection and IRAK1 knockdown. Data are shown as the mean ± SD of three biological replicates; *$p < 0.05$

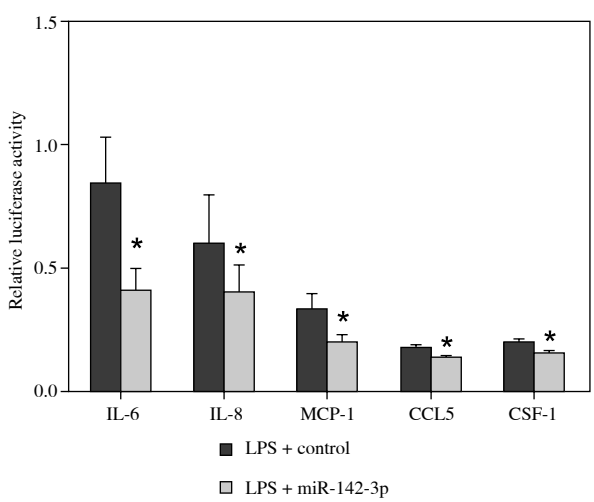

Fig. 3. miR-142-3p reduces LPS-induced inflammatory cytokine expression. HCT116 cells were transfected with miR-142-3p mimics or negative control, followed by stimulation of 20 μM LPS. LPS significantly enhanced the mRNA expression levels of IL-6, IL-8, MCP-1, CCL5 and CSF-1, which were measured using qRT-PCR and given relative to the expression level in untreated control cells. Data are shown as mean ± SD of three biological replicates, *$p < 0.05$

ther investigation. In this study, we showed that miR-142-3p suppresses the activation of NF-κB induced by LPS in colorectal cancer cells, thus decreasing the expression of inflammatory cytokins, such as IL-6, IL-8, MCP-1, CCL5 and CSF-1. As constitutive NF-κB activation and secretion of inflammatory factors in tumor microenvironment promote the development of CRC, we assumed that the miR-142-3p may function as a tumor suppressor partially by inhibiting NF-κB activation and the resulting expression of tumor-promoting cytokines.

To investigate the mechanism of anti-inflammatory role of miR-142-3p, we used three algorithmm programs (TargetScan, PicTar and miRBase) to search for its potential target genes. Surprisingly, it was found that IRAK1 may be a candidate target gene of miR-142-3p. IRAK1 is a member of interleukin-1 receptor activated kinases (IRAKs) family and a key component of the IL-1R/TLR signaling transduction [18]. Upon ligand binding to IL-1R/TLR, myeloid differentiation factor 88 (MyD88) is rapidly recruited to the receptor. IRAK1 is also recruited to the receptor complex through its interaction with MyD88, thus initiating a cascade of downstream signaling events, eventually leading to NF-κB activation and induction of inflammatory target genes expression [18]. Therefore we focused on characterizing IRAK1 as a direct miR-142-3p target in colorectal cancer cells. Our results indicated that miR-142-3p targets directly IRAK1, therefore suppressing its expression both in mRNA and protein levels.

In summary, we have identified a new target of miR-142-3p, IRAK1 that is involved in IL-1R/TLR-mediated activation of NF-κB pathway. And we have found that miR-142-3p inhibits LPS-induced NF-κB activation, thus decreasing the expression of inflammatory cytokines. Hence, these data suggest that miR-142-3p may function as a tumor suppressor by inhibiting NF-κB activity and the consequently expression of tumor-promoting cytokines.

Authors declare no conflict of interests.

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